Neonatal Glucocorticoid Treatment and Predisposition to Cardiovascular Disease in Rats

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The publication of this thesis has been kindly supported by St. Jude Medical Nederland B.V. and Merck Sharp & Dohme B.V.

ISBN-10: 90-8559-246-1
ISBN-13: 978-90-8559-246-4

Cover image by: Paul Broekman (Catch India "detail ratten" 40x40)

Printed by: Optima Grafische Communicatie te Rotterdam, The Netherlands

Neonatal Glucocorticoid Treatment and Predisposition to Cardiovascular Disease in Rats

Neonatale glucocorticosteroïd behandeling en gevoeligheid voor cardiovasculaire ziekten in ratten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 8 december 2006 des namiddags te 2.30 uur

door

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The study described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF-B2001-081). Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Tough Times Never Last, But Tough People Do
(Robert H. Schuller)

Ter nagedachtenis aan Petronella Klein-Bal

Dankzij mijn ouders

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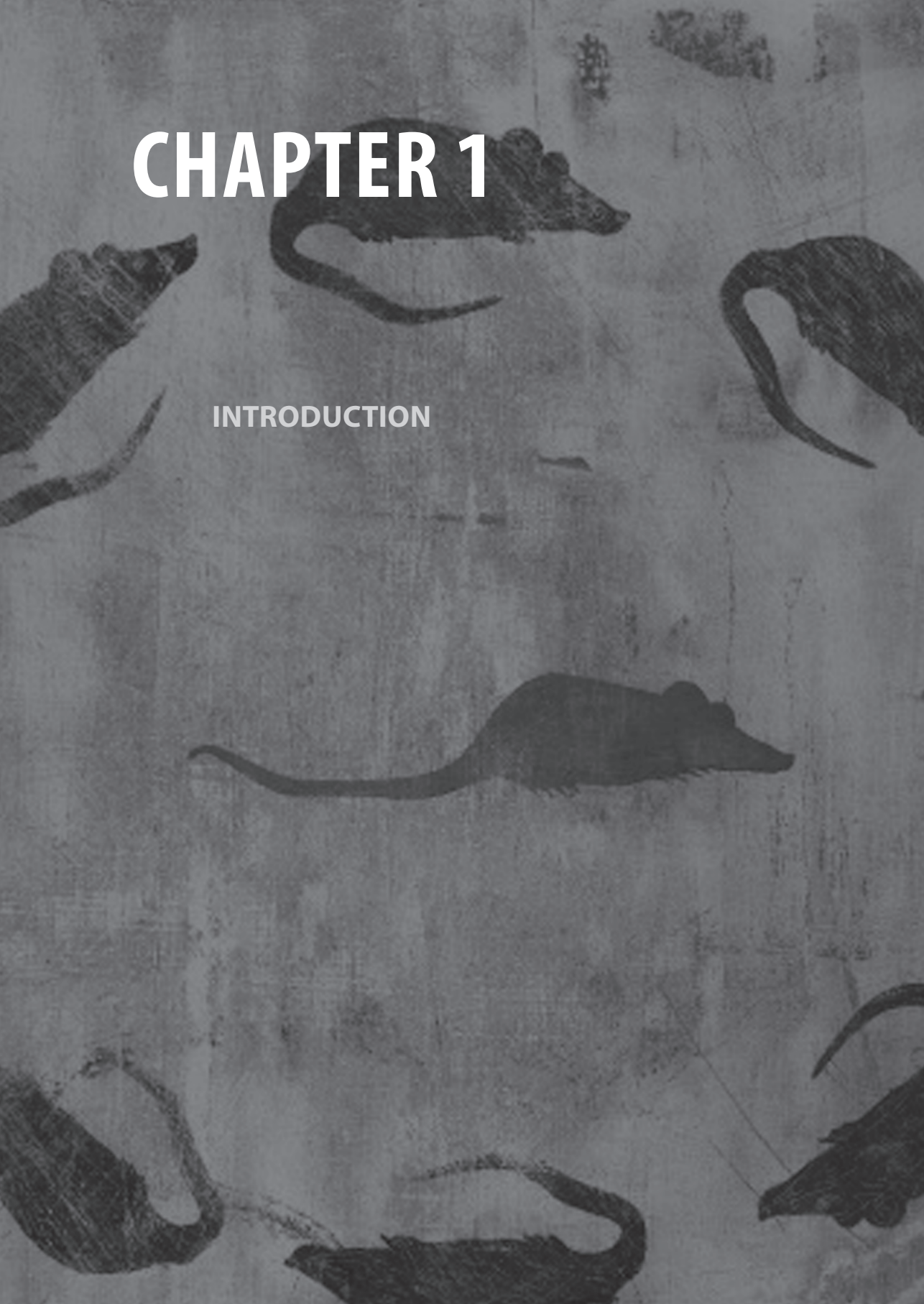

CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Bronchopulmonary dysplasia (BPD), a term first introduced by Northway and colleagues [86], is a serious chronic lung disease in premature infants, which usually develops after severe infant respiratory distress syndrome (IRDS), and requires mechanical ventilation and prolonged oxygen therapy [38,66]. The first diagnostic criteria used for BPD included positive pressure ventilation during the first week of life for a minimum of 3 days, clinical signs of respiratory insufficiency, supplemental oxygen for more than 28 days and a chest radiograph demonstrating persistent densities in both lungs, alternating with areas of normal or increased lucency [10]. Nowadays these definitions are not considered appropriate for the smallest preterm infants, who may require supplemental oxygen because of pulmonary immaturity beyond 28 days without clinical and radiographic signs of BPD [93]. The requirement for supplemental oxygen at 36 weeks postconceptional age may be a more accurate criterium and this complication is now usually referred to as chronic lung disease (CLD) [102]. The incidence of CLD has not changed over the past decade but various treatment strategies in clinical practice have changed the severity of the disease [66]. The aetiology of CLD is multifactorial and has been attributed to a number of factors: oxygen toxicity and barotrauma from mechanical ventilation, infection, primary surfactant deficiency or nutritional factors. Regardless of the aetiologic causes, early and prolonged postnatal inflammation in the lungs of infants with CLD have been demonstrated [89]. The inflammation reaction seen in the alveoli is already evident shortly after birth and this supports the hypothesis that the inflammatory process may start in utero [127]. Nearly 40 years after BPD or CLD was first described, it remains a major complication of premature birth. New ways of preventing or modulating CLD are still warranted and may lead to improvement of long-term outcome in preterm infants [66].

Prevention of premature birth, especially extreme prematurity, would reduce the occurrence of CLD. Enhancement of lung maturity via induction of surfactant production and induction of antioxidant enzymes represents a major advancements in reducing the incidence and severity of CLD and neonatal mortality [9,28]. The most common antenatal strategy used to mitigate the adverse effects of preterm delivery, particularly the respiratory outcome of premature infants, is the use of antenatal glucocorticoid (GC) therapy. However, despite the widespread use of antenatal GC therapy, CLD is still prevalent. There is no evidence that multiple courses (>3 courses) of antenatal GC therapy are more beneficial than 1 or 2 complete courses. Prolonged oxygen exposure and mechanical ventilation in the immature baby cause lung injury [91]. Avoidance of excessive oxygen exposure and careful attention to blood gas tensions and oxygen saturations may help to reduce injury to the lung related to oxygen exposure. In addition, the duration of ventilation before surfactant therapy is started should be minimized and especially in the most immature babies, preventive surfactant therapy has proven its value in preventing or reducing CLD [40]. Another strategic goal to prevent CLD is

to avoid lung over-inflammation by using the smallest possible tidal volume [27]. The rate of death due to CLD is reduced, but not prevented by exogenous surfactant therapy [62]. This could be due to the survival of extremely low-birth weight premature infants, who are at high risk for CLD [66]. Preliminary reports suggest the benefits of inhaled nitrous oxide (iNO) therapy to reduce CLD [4,11]. Deficiencies in antioxidant nutrients have been implicated in the pathogenesis of CLD, therefore replacement and administration of those supplements may reduce CLD. Fluid restriction [113] and diuretic therapy are additional possible means to reduce severity and mortality related to CLD [16].

Chronic lung disease and Glucocorticoids

Because of the anti-inflammatory properties of glucocorticoids (GCs), GC therapy is considered one of the best alternatives to reduce the inflammatory process involved in the pathogenesis of severe CLD. GCs, besides stimulating surfactant producing pneumocytes, appear to have additional effects on pulmonary maturation. Several mechanisms have been proposed to explain the effects of GCs on pulmonary function. The effects of GCs on structural lung growth and development [100,133], on antioxidant enzymes [98], on lung tissue growth factors [61,98], on inflammatory mediators [124] and the regulation of pulmonary fluid absorption [138] have been implicated to play a role in pulmonary maturation, in conjunction with stimulation of surfactant synthesis [51]. The rapid clinical improvement seen in infants with CLD after the initiation of postnatal Dexamethasone (Dex) therapy is due in part also to improvement in the integrity of the alveolar-capillary barrier and the potential for GCs to limit pulmonary fibrosis [128].

GCs for ventilator-dependent infants facilitate extubation and reduce the rate of CLD whether they are given early, moderately early or later [54-56,116]. There have been a plethora of studies evaluating the use of postnatal GCS, the most popular of which is Dex [20,52].

Liggins and Howie published the first study demonstrating the value of maternal *antenatal* GCs (ACS) in 1972 [74]. They reported decreased mortality and morbidity rates in preterm infants and a significantly decreased incidence of respiratory distress syndrome. Since then antenatal steroid therapy has become a proven beneficial intervention [2,28,29] when preterm birth occurs. The consensus statement of the American National Institutes of Health (NIH) recommends administration of ACS to all patients at risk of preterm delivery prior to 34 weeks gestation [2]. The benefits of ACS outweigh any harmful effects, although multiple courses of ACS should be avoided because they do not offer an advantage in promoting alveolar development compared to single courses. To the contrary, neurological adverse effects (decreased head circumference, delayed psychomotor development) have been described especially after multiple courses of ACS [46].

The first randomized trial of *postnatal* GC treatment was performed by Baden et al. in 1972 [8] with the administration of hydrocortisone and reported no significant beneficial effects. However, the surviving babies of this trial treated with hydrocortisone showed increased risk

of severe intraventricular hemorrhages [112] and neurological problems [44]. In the 1980s, several investigators [6,77] reported results of controlled trials of Dex treatment in CLD. The study performed by Cummings et al. [30] is important since it addresses the issue of duration of therapy and demonstrates the long-term benefits (reduced duration of mechanical ventilation, oxygen supplementation and hospitalization). From the mid-1980s throughout the 1990s', postnatal GCs, in particular Dex has been used increasingly for prevention or treatment of CLD, supported by evidence of benefit on some short-term outcomes, including earlier weaning from mechanical ventilation and extra oxygen [38].

GCs and adverse effects

Although GCs show beneficial effects, most studies reported serious and sometimes fatal side effects in a large proportion of the treated infants (see below), strongly suggesting that Dex treatment should be used only restrictively [15,38,53,85,103]. Several acute side effects have been described such as: suppressed function of the hypothalamo-pituitary-adrenal (HPA) axis [92], increased risk of infection [109], bacteriaemia and gastrointestinal perforation/hemorrhage [108], hypertriglyceridemia and increased free fatty acid levels [5], poor glucose tolerance with hyperglycaemia [90], adrenal suppression [110], immune suppression [131], neutrophilia, increased diuresis, severe catabolism with protein wasting [117,121], muscle breakdown [22], increased plasma amino acid concentrations [134], reduced growth/slower weight gain [48], bone demineralization [129] and seizures [1,54-56]. Although these effects are thought to be resolved after discontinuation of the therapy, long-term follow-up studies are lacking. Few studies have addressed the possible long-term consequences of systemic GC therapy during a stage of rapid organ growth and development. Animal experiments have revealed significant negative effects of GCs on cell multiplication in different organs such as the lung and the central nervous system [42,58,115,118]. The most prominent adverse effect of GC treatment on lung growth is a decrease in the final numbers of alveoli, in addition to a decrease in lung growth [78,100,118,119,124]. Recent concern about adverse long-term effects on the brain has led to a decrease in the use of GC. The American Academy of Pediatrics [3] made recommendations regarding the use of corticosteroids in preterm infants. Because high doses of GCs started only a few years ago, little is known about the long-term side effects of these treatments in humans. Therefore studies performed in animals have tried to mimic the human situation and investigated the long-term side effects. Studies in mammals show that exposure to chronic high levels of exogenous GCs during early development results in morphological, physiological and behavioral modifications in later life. The precise nature of these effects is dependent on the dose of the steroid, the developmental stage at which the exposure occurs [25,70] and the species studied [79,97]. In species that give birth to immature pups (rats, mice and rabbits) much neurodevelopment occurs in the postnatal period [97] and thus Dex treatment can have a deleterious effect in that period [39]. Animal experiments have shown that GC administration during critical periods of brain development

either prenatally or postnatally may impair myelinization, brain cell division and longer-term behavioral effects [18,63,68,120,122]. Moreover, postnatal Dex treatment appeared to be associated with subsequent impairment of spatial learning, deficits in motor coordination and reduced cerebellar weights [18,50].

In humans magnetic resonance imaging has shown that the volume of grey matter in the brains of preterm babies is reduced by one-third after postnatal Dex treatment and periventricular leucomalacia has been reported [84,108]. These findings are consistent with the approximately 2-fold increased risk of abnormal neurological findings, including cerebral palsy, in babies given steroids soon after birth [14,15,38,55,87,104,108,135,136]. Also late treatment, beyond 3 weeks of age, is associated with an increased risk of abnormal neurological findings upon clinical examination but the effect is less pronounced than with early treatment [54]. Neuromotor-dysfunction, reduced head growth and adverse effects on cognition have been revealed in GC (Dex)-treated children [14,15,84,114,135,136].

Adverse effects of *antenatal* GCs on the heart

Experimental studies in rats and fetal lambs showed that antenatal GC exposure induce lifetime changes in cardiovascular function [36,71]. Although in the acute phase antenatal GC treatment seems to have no effect on cardiac wall thickness, systolic function and the ratio between left interventricular septum/left ventricular posterior wall thickness reportedly are affected [123].

The mechanisms behind the long-term effects of antenatal GCs may be similar to those involved in the Barker hypothesis [12,13]. This theory suggests an association between low birth weight and increased risk of adult onset-cardiovascular disease. Maternal stress causes high concentrations of fetal GCs and alters the number of steroid receptors in the hypothalamic-pituitary-adrenal (HPA)-axis. It affects the renin-angiotensin system and ultimately leads to pathological cardiovascular changes in adults [12,37]. Benediktsson [17] reported that GC exposure in utero leads to hypertension in adult life, due to GC-induced reprogramming of the HPA-axis during perinatal life. Rudolph [96] found that perinatal administration of GCs in lambs changes the pattern of neonatal myocardial growth. Myocardial growth during fetal life in rats and humans is accomplished predominantly by proliferation of the number of cardiomyocytes (hyperplasia). In contrast, during the transition period after birth, growth is increasingly characterized by increase in cell size (hypertrophy) as evidenced by an increase in the percentage of binucleated myocytes [26,59,72,73,76,80,137]. As a consequence, in a period where cells are undergoing cell division, GCs could have deleterious side effects [96].

Short-term effects of *neonatal* GCs on the heart

After the use of GCs and in particular Dex became widespread, several reports of infants developing cardiac changes consistent with hypertrophic cardiomyopathy (HCM) began to appear [19,21,60,88,107,130]. Also in animal models (mostly rats) it was shown that HCM

developed during neonatal treatment with Dex [33,69,83,105,106,132]. HCM is characterized by left ventricular hypertrophy and concomitant alterations in systolic and diastolic function. Hypertrophy alone is not sufficient to make the diagnosis of HCM. Usually an asymmetric increase in septal thickness is present, which can lead to the development of left ventricular outflow tract obstruction. Another acute effect of treatment with Dex in the neonatal period is hypertension [19,90]. These effects are thought to be transient and to disappear after cessation of Dex treatment, although there is some concern that Dex induced interruption of growth during fetal life may induce long-term negative effects on somatic development (see below) [135]. The mechanism of the effects of Dex on the heart is unknown albeit that the concomitant hypertension is known to cause hypertrophy through a direct anabolic effect on cardiac muscle [81].

Long-term effects of *neonatal* GCs on the heart

Less is known about the possible long-term effects of neonatal Dex treatment on the cardiovascular system. It appears that GC treatment in rat pups results in hypertension in adult life due to reprogramming of the HPA-axis [17]. Moreover in a recent study performed by our group [33] the heart weights of neonatally Dex-treated rats at adult age were lower and showed a tendency for lower DNA-content than control animals. This was accompanied by increased collagen content at adult age, presumably indicating early degeneration of myocytes in those rats neonatally treated with GCs. In a survival study of rats treated with Dex in the neonatal period it appeared that those rats had a reduced life expectancy [64].

Perinatally administered cortisol [95,96] inhibits myocyte proliferation and accelerates the change in myocardial growth pattern normally occurring after birth. In a recent study performed by our group [32] it was shown that also early postnatal administration of Dex can alter myocardial growth in the neonatal period. This study showed a permanent inhibition of normal physiological myocardial growth after neonatal treatment with Dex in rat pups. These acute short-term adverse effects could possibly result in long-term negative cardiovascular side effects. Since the hearts of Dex-treated rats contain a lesser number of cardiomyocytes at adult age, these cells will have to hypertrophy over time as compensation to fulfill hemodynamic demands.

Model and methods used in this thesis

The animal model and the methodologies applied in this thesis to determine the functional, histopathological and biochemical characteristics of the heart after neonatal Dex treatment are briefly summarized in the next few sections.

The animal model

The studies in this thesis were performed using a rat model. The neonatal rat is an adequate model to represent the very preterm infant, because the developmental stage of a preterm

infant in the third trimester of pregnancy is comparable with that of a 1- to 10-day-old rat pup [34,35,45], at least for the brain. Furthermore, many of the GC-induced cardiovascular changes observed in rats are similar to those found in humans [33,69,83,105,106,132]. Given these correlations, we used the neonatal rat pup to investigate effects of tapering courses of neonatal Dex treatment on the developing cardiovascular system. Rat pups were injected intraperitoneally with Dex on day 1, 2 and 3 of life.

Long-term clinical follow-up studies are not yet available because very few neonatally GC-treated humans have reached adulthood at this point. Therefore the experimental studies described in this thesis may serve as a basis for early detection of long-term consequences and treatment of possible cardiovascular dysfunction in a large number of individuals treated in the neonatal period with GCs.

In this thesis we focused on the chronic effects of neonatal Dex treatment on the cardiovascular system by hemodynamic, histopathological, and biochemical/molecular studies. Studies were performed in 4-, 8-, 50- and 80-week-old rats, representing respectively the prepubertal, postpubertal/young adult, middle-aged, and elderly period during life span [45,94]. Normally the average life span of untreated Wistar rats is about two and a half year [64].

Assessment of myocardial performance, pressure-volume loops

The fundamental variables to describe the performance of the left ventricle as a pump are pressure and volume of the ventricular cavity. Pressure and volume can be plotted in a graph resulting in a pressure-volume loop, representing the cardiac cycle, as schematically illustrated in Figure 1. In the normal heart, four phases can be clearly distinguished: iso-volumic contraction, ejection, iso-volumic relaxation, and filling. Contraction of the left ventricle begins at end-diastole (**A**), where a new cardiac cycle starts. The ventricle is completely filled at this point and the electrical activation causes contraction to start. Moving upwards in the loop the iso-volumic contraction phase is represented and the increasing left ventricular pressure closes the mitral valve (left ventricular pressure exceeds left atrial pressure). At point **B** left ventricular pressure exceeds aortic pressure, the aortic valve opens and ejection starts. Ventricular volume decreases with little change in pressure, until the activation stops and the aortic valve closes. At point **C** relaxation begins and pressure drops rapidly during the iso-volumic relaxation phase. Finally at point **D**, left ventricular pressure falls below left atrial pressure, the mitral valve opens and filling of the left ventricle begins until the mitral valve closes and the next contraction starts (**A**).

Several parameters generally used to characterize performance of the left ventricle can be readily identified from the pressure-volume loop (Figure 1): the volume at maximal filling, end-diastolic volume (EDV), and the corresponding end-diastolic pressure (EDP). The minimal volume reached at end-ejection, end-systolic volume (ESV), and the pressure at end-systole (ESP). The distance between A/B and C/D represents the volume change during ejection and filling; stroke volume (SV). This is the volume ejected during systole and, in steady state

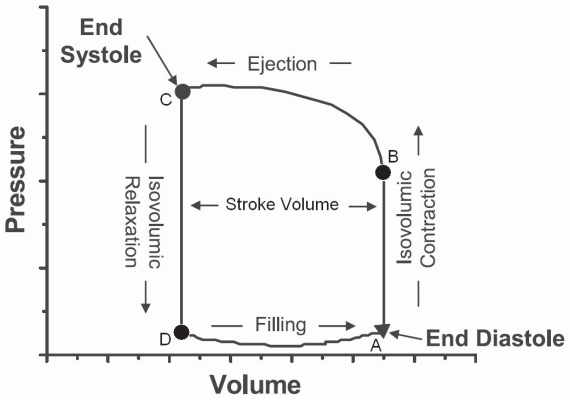

Figure 1: Figure showing a typical pressure-volume loop during one cardiac cycle. Four phases of the cardiac cycle can be distinguished in the loop: isovolumic contraction starts at **A** (closing of mitral valve); ejection starts at **B** (opening of aortic valve); isovolumic relaxation starts at **C** (closing of aortic valve); and filling starts at **D** (opening of mitral valve).The width of the loop represents stroke volume. The area enclosed by the loop represents stroke work.

conditions, equals the volume that subsequently enters the left ventricle during filling in diastole. SV multiplied by heart rate defines cardiac output (CO). Assuming that SV remains constant, CO is directly proportional to heart rate. The ratio of SV to EDV represents ejection fraction (EF).

The surface area of the pressure-volume loop represents stroke work (SW), which is the external work (mechanical energy) performed by the left ventricle during one heart beat. Other parameters are obtained by differentiating volume and pressure; by looking at changes in pressure, e.g. the maximal rate of pressure rise, dP/dt_{MAX}, is obtained while the time derivative of volume yields outflow and inflow.

Determinants of cardiac performance

Cardiac performance is dependent not only on the intrinsic properties of the heart (referred to as contractility) but also on other factors. The main factors that determine cardiac performance are contractility, loading conditions, and heart rate. Contractility is often considered as the intrinsic capacity of the myocardium to contract independent of preload or afterload conditions. Contractility of a cardiac muscle fiber may be represented by its (isometric) force-length relationship [65] (Figure 2).

Increased contractility is defined then as a greater force generated at a given length or as the same force generated at a decreased length, and vice versa for decreased contractility.

Preload is the degree to which myocardial fibers are stretched before the start of contraction. The most logical measure of preload for the left ventricle is end-diastolic volume. The famous Frank-Starling law [82] implies that with increased end-diastolic fiber length the contractile force increases. Through this mechanism, preload plays an important role in the regulation of CO. Afterload is the load on the ventricle during contraction. In a normally ejecting left ventricle arterial pressure is often used as a measure of afterload. Assuming that stroke volume remains constant, CO is directly proportional to heart rate.

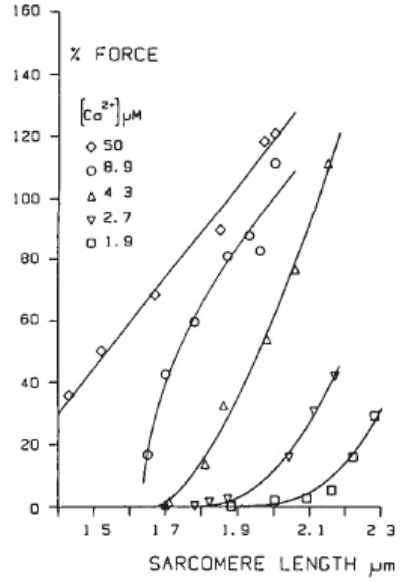

Figure 2: The relation between (isometric) force and sarcomeric length for skinned trabecular muscle. The position and shape of the force-length relation is dependent on the calcium concentration of the bathing solution which controls the calcium available for contraction (adapted from Kentish [65])

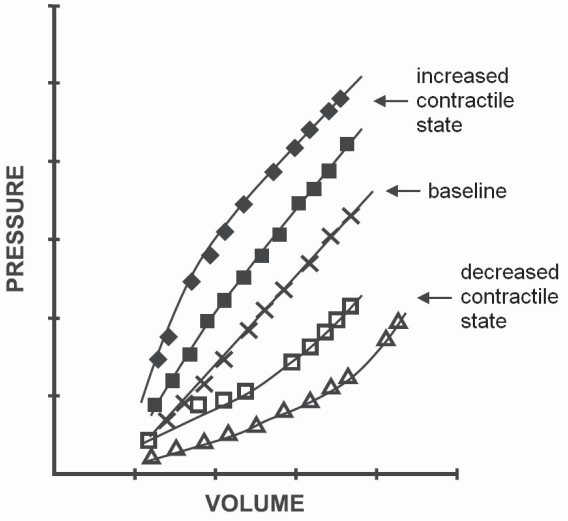

Figure 3: This picture shows that at enhanced levels of contractility the ESPVRs (isolated canine heart) displace leftward and become concave to the volume axis, whereas at lower levels of contractility the curves displace rightward and become convex to the volume axis. Contractility was modified pharmacologically or by ischemia (adapted from Burkhoff [23]). Note the similarity between Figures 3 and 4.

Assessment of contractile state: the End-Systolic Pressure-Volume Relation (ESPVR)

To study the intrinsic systolic left ventricular function, the relation between the end-systolic pressure and volume, the ESPVR, is often used [7,111]. It may be considered as the macroscopic equivalent of the (microscopic) force-length curve of the cardiac muscle fiber, with pressure equivalent to force and volume equivalent to length. With changes in contractility, the ESPVR behaves similar to the myocardial force-length relationship: a steeper slope (at least in the lower pressure range) and/or a displacement to the left and upwards represents increased contractility and vice versa (Figure 3). The ESPVR is obtained by continuous

recording of PV-loops during a change in preload (Figure 4). The slope of the ESPVR is the maximal stiffness of the ventricle and is often called end-systolic elastance (E_{ES}). Its position is determined either as the intersection with the volume axis (at zero end-systolic pressure, Vd) or, even better as the end-systolic volume intercept at a fixed end-systolic pressure within the operating range (e.g. ESV_{100}). The ESPVR is affected only by changes in the intrinsic properties of the myocardium and, in good approximation, not by changes in loading conditions. This is in contrast with more conventional parameters such as ESV, ESP, SV, EF, SW and dP/dt_{max}, which are strongly dependent on pre-and/or afterload. Similarly, the end-diastolic pressure-volume relation (EDPVR) is a load-independent measure of the diastolic compliance of the left ventricle. Increased chamber compliance is reflected by a downward shift and/or a shallower slope of the EDPVR. In addition to the ESPVR, two relations are frequently used to assess ventricular function: the preload-recruitable stroke work (PRSW) relation (relation between stroke work and EDV), which is a direct reflection of the Frank-Starling mechanism [49], and the relation between dP/dt_{MAX} and EDV [75]. By design these relations are essentially preload-independent, but also fairly independent of afterload and therefore, like the ESPVR, represent excellent tools to investigate the intrinsic function of the heart.

Assessment of pressure-volume relations and their derived relationships requires continuous registration of ventricular pressure (P) and volume (V) as well as PV loops during a preload intervention, usually realized by temporary inflow reduction To obtain these signals, the studies described in this thesis were performed using a miniature pressure-conductance catheter (Figure 5). This catheter was recently developed [43,47] for use in small animals, based on the conductance method developed originally in our laboratory for studies in large animals and humans. A detailed description of this methodology including its calibration has been published [7].

Histopathological investigations of the rat hearts

The rat-hearts of four different age groups as described before were investigated histopathologically. Hearts were arrested at end-diastole, using cadmium-chloride and then perfusion fixed with formalin. Subsequently the hearts were removed and the bi-ventricular weights were obtained after removal of both atria. Microscopic slices from a cross section of each heart were stained with Hematoxilin and Eosin (HE) staining for general histopathological assessment and for measurement of LV wall thickness. The Sirius Red staining was performed to determine collagen content. The Cadherin-Periodic Acid Schiff (PAS) staining was used for myocyte morphometric measurements. From these measurements cell volume could be calculated.

Biochemical investigations of the rat heart

The contractile proteins in cardiomyocytes include myosin, actin, tropomyosin and the troponins. Another group is formed by the true cytoskeletal proteins such as tubulin and

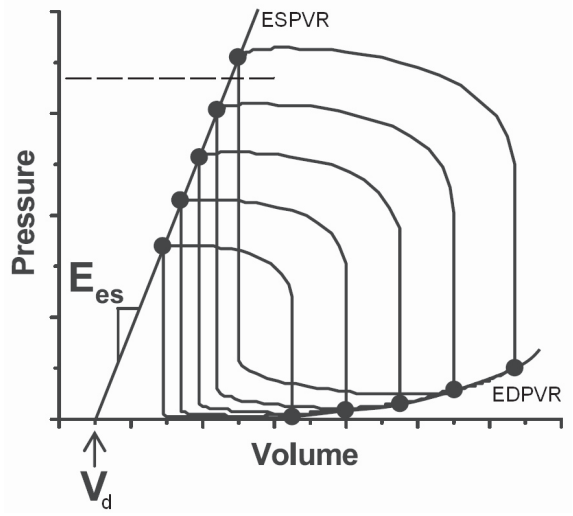

Figure 4: End-systolic pressure-volume relationship (ESPVR). A reduction in preload, obtained by transient occlusion of the vena cava, produces a narrowing and left- and downward shift of the pressure-volume loop in such a way that all end-systolic points of the loop can be connected by a line (ESPVR), which in first approximation, is straight. The slope of the ESPVR is the maximal stiffness of the ventricle and is called end-systolic elastance (E_{ES}). Its position is determined either as the intersection with the volume axis (at zero end-systolic pressure, Vd) or, even better, as the end-systolic volume intercept at a fixed end-systolic pressure level within the operating range (e.g. at 100 mmHg: ESV_{100}).

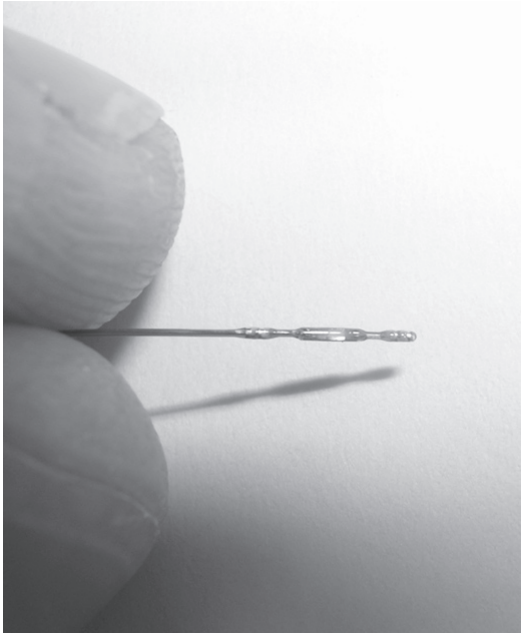

Figure 5: A 1.4F miniature pressure-conductance catheter containing four platinum electrodes and a solid-state pressure transducer used for measuring pressure-volume loops in small animals. The 2 outermost electrodes (proximal and distal), are used to apply a current to set up an electrical field inside the ventricle; the 2 innermost electrodes measure a continuous voltage which, together with the current, determine a continuous electrical conductance signal proportional to ventricular volume. The pressure transducer is located between the 2 innermost electrodes. In the studies presented in this thesis this catheter was introduced into the left ventricle of the rats via the right carotid artery.

desmin [67]. Myosin is the main component of the thick filament of the sarcomere of cardiac and skeletal muscle and is responsible for energy transduction and force development [31,41,57,101]. Alpha-actin is the main component of the thin filament of the sarcomere. It can be present as two isoforms in the cardiac muscle cell, the skeletal isoform and the cardiac isoform [101]. The cytoskeletal proteins tubulin and desmin are abundant in cardiomyocytes

and play an important role in maintaining the structural integrity of myocytes [126] together with transmitting mechanical and chemical stimuli within and between cells [24,99,125].

To determine the expression level of the various proteins, we used Western blotting techniques. Proteins were separated by gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. Specific proteins were visualized by incubation with specific monoclonal antibodies.

Specific bands were visualized using the enhanced chemiluminescence detection system and exposure to X-ray film. Autoradiographs were scanned using a GS-700 Imaging Densitometer.

Aim and outline of the thesis

The aim of the studies described in the present thesis was to investigate the long-term effects of neonatal Dex treatment on the heart. Data from the literature and from a previous study [33], suggest at least two phenomena: hypertension in later life and drastic alterations in cardiac myocyte structure and performance. The hypertension may be related to GC-induced perinatal programming of the hypothalamic-pituitary-adrenal axis that persists during adulthood. De Vries et al. [33] found that compared to age-matched controls, neonatally GC-treated rats had lower heart weight and DNA content in the adult period and signs of early degeneration of the myocytes. Moreover GC-treated rats showed a reduced life expectancy possibly due to cardiac failure [64]. Therefore, we hypothesized that neonatal GC treatment in rats induced long-term cardiac alterations. To test this hypothesis, we determined functional, histopathological and biochemical characteristics at four points in the life span of the rat: 4-, 8-, 50- and 80 weeks of age. At these time-points, reflecting pre-pubertal, post-pubertal (young adult), middle-aged, and elderly stages, respectively, differences between rats treated with Dex in the neonatal period and saline-treated control animals were investigated.

In **Chapter 2** we describe the early consequences of neonatal Dex treatment on normal physiological cardiomyocyte proliferation and the influence of the treatment later in life.

The experiment described in **Chapter 3** was performed to investigate the effects of neonatal Dex on functional capacity in 4-week-old animals by means of pressure-volume loops in the pre-pubertal period by comparing ESPVR's in treated versus untreated rats.

Subsequently we wanted to look at age-dependent effects using PV-relations and since it is not known how these parameters behave as a function of age, we first studied the physiological effects of aging on the ESPVR. Therefore, the study described in **Chapter 4** was designed to get insight into the intrinsic systolic function from the juvenile to the middle-aged period in normal Wistar rats. Furthermore we investigated the normalization of indexes derived from pressure-volume loops to establish if correction of pressure-volume indexes for body weight is a good substitute for heart weight correction in these animals.

The study described in **Chapter 5** was performed to assess hemodynamic data in rats treated neonatally with Dex during growth and senescence (8, 50 and 80-week-old rats).

To determine the exact histopathological changes during growth and development (4-, 8- and 50-week-old rats) after neonatal Dex treatment, we describe changes in wall thickness, collagen content and morphological myocyte measurements in **Chapter 6**.

To complete the overall picture of the long-term consequences of neonatal Dex treatment beside the functional and histopathological changes, we investigated the biochemical differences in treated versus non-treated animals during the course of time. We report the changes found in contractile and structural proteins in 4-, 8- and 50-week-old rats in **Chapter 7**.

The summary and concluding remarks are presented in **Chapter 8**

REFERENCE LIST

1. Dexamethasone for neonatal chronic lung disease. Lancet 1991;338:982-983
2. Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH Consensus Development Panel on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes. JAMA 1995;273:413-418
3. Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics 2002;109:330-338
4. Abman SH, Kinsella JP, Schaffer MS, Wilkening RB Inhaled nitric oxide in the management of a premature newborn with severe respiratory distress and pulmonary hypertension. Pediatrics 1993;92:606-609
5. Amin SB, Sinkin RA, McDermott MP, Kendig JW Lipid intolerance in neonates receiving dexamethasone for bronchopulmonary dysplasia. Arch Pediatr Adolesc Med 1999;153:795-800
6. Avery GB, Fletcher AB, Kaplan M, Brudno DS Controlled trial of dexamethasone in respirator-dependent infants with bronchopulmonary dysplasia. Pediatrics 1985;75:106-111
7. Baan J, van der Velde ET, de Bruin HG, Smeenk GJ, Koops J, van Dijk AD, Temmerman D, Senden J, Buis B Continuous measurement of left ventricular volume in animals and humans by conductance catheter. Circulation 1984;70:812-823
8. Baden M, Bauer CR, Colle E, Klein G, Taeusch HW, Jr., Stern L A controlled trial of hydrocortisone therapy in infants with respiratory distress syndrome. Pediatrics 1972;50:526-534
9. Bakker JM, Kavelaars A, Kamphuis PJ, Cobelens PM, van Vugt HH, van Bel F, Heijnen CJ Neonatal dexamethasone treatment increases susceptibility to experimental autoimmune disease in adult rats. J Immunol 2000;165:5932-5937
10. Bancalari E, Abdenour GE, Feller R, Gannon J Bronchopulmonary dysplasia: clinical presentation. J Pediatr 1979;95:819-823
11. Banks BA, Seri I, Ischiropoulos H, Merrill J, Rychik J, Ballard RA Changes in oxygenation with inhaled nitric oxide in severe bronchopulmonary dysplasia. Pediatrics 1999;103:610-618
12. Barker DJ Fetal nutrition and cardiovascular disease in later life. Br Med Bull 1997;53:96-108
13. Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. BMJ 1989;298:564-567
14. Barrington KJ Postnatal steroids and neurodevelopmental outcomes: a problem in the making. Pediatrics 2001;107:1425-1426
15. Barrington KJ The adverse neuro-developmental effects of postnatal steroids in the preterm infant: a systematic review of RCTs. BMC Pediatr 2001;1:1
16. Barrington KJ, Finer NN Treatment of bronchopulmonary dysplasia. A review. Clin Perinatol 1998;25:177-202
17. Benediktsson R, Lindsay RS, Noble J, Seckl JR, Edwards CR Glucocorticoid exposure in utero: new model for adult hypertension. Lancet 1993;341:339-341
18. Benesova O, Pavlik A Perinatal treatment with glucocorticoids and the risk of maldevelopment of the brain. Neuropharmacology 1989;28:89-97
19. Bensky AS, Kothadia JM, Covitz W Cardiac effects of dexamethasone in very low birth weight infants. Pediatrics 1996;97:818-821
20. Bhuta T, Ohlsson A Systematic review and meta-analysis of early postnatal dexamethasone for prevention of chronic lung disease. Arch Dis Child Fetal Neonatal Ed 1998;79:F26-F33
21. Brand PL, van Lingen RA, Brus F, Talsma MD, Elzenga NJ Hypertrophic obstructive cardiomyopathy as a side effect of dexamethasone treatment for bronchopulmonary dysplasia. Acta Paediatr 1993;82:614-617
22. Brownlee KG, Ng PC, Henderson MJ, Smith M, Green JH, Dear PR Catabolic effect of dexamethasone in the preterm baby. Arch Dis Child 1992;67:1-4

23. Burkhoff D, Mirsky I, Suga H Assessment of systolic and diastolic ventricular properties via pressure-volume analysis: a guide for clinical, translational, and basic researchers. Am J Physiol Heart Circ Physiol 2005;289:H501-H512
24. Choquet Y, Wollman FA Translational regulations as specific traits of chloroplast gene expression. FEBS Lett 2002;529:39-42
25. Clarke AS Social rearing effects on HPA axis activity over early development and in response to stress in rhesus monkeys. Dev Psychobiol 1993;26:433-446
26. Clubb FJ, Jr., Bishop SP Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. Lab Invest 1984;50:571-577
27. Cole CH, Fiascone JM Strategies for prevention of neonatal chronic lung disease. Semin Perinatol 2000;24:445-462
28. Crowley P Antenatal corticosteroids--current thinking. BJOG 2003;110 Suppl 20:77-78
29. Crowley P, Chalmers I, Keirse MJ The effects of corticosteroid administration before preterm delivery: an overview of the evidence from controlled trials. Br J Obstet Gynaecol 1990;97:11-25
30. Cummings JJ, D'Eugenio DB, Gross SJ A controlled trial of dexamethasone in preterm infants at high risk for bronchopulmonary dysplasia. N Engl J Med 1989;320:1505-1510
31. Czerwinski SM, Kurowski TT, McKee EE, Zak R, Hickson RC Myosin heavy chain turnover during cardiac mass changes by glucocorticoids. J Appl Physiol 1991;70:300-305
32. de Vries WB, Bal MP, Homoet-van der Kraak P, Kamphuis PJ, van der Leij FR, Baan J, Steendijk P, de Weger RA, van Bel F, van Oosterhout MF Suppression of physiological cardiomyocyte proliferation in the rat pup after neonatal glucocorticosteroid treatment. Basic Res Cardiol 2006;101:36-42
33. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr Res 2002;52:900-906
34. Dobbing J The later development of the brain and its vulnerability. Scientific Foundations of Paediatrics 1984;2:744-759
35. Dobbing J, Sands J Comparative aspects of the brain growth spurt. Early Hum Dev 1979;3:79-83
36. Dodic M, May CN, Wintour EM, Coghlan JP An early prenatal exposure to excess glucocorticoid leads to hypertensive offspring in sheep. Clin Sci (Lond) 1998;94:149-155
37. Dodic M, Peers A, Coghlan JP, Wintour M Can Excess Glucocorticoid, Predispose to Cardiovascular and Metabolic Disease in Middle Age? Trends Endocrinol Metab 1999;10:86-91
38. Doyle LW, Halliday HL, Ehrenkranz RA, Davis PG, Sinclair JC Impact of postnatal systemic corticosteroids on mortality and cerebral palsy in preterm infants: effect modification by risk for chronic lung disease. Pediatrics 2005;115:655-661
39. Edwards HE, Burnham WM The impact of corticosteroids on the developing animal. Pediatr Res 2001;50:433-440
40. Egberts J, Brand R, Walti H, Bevilacqua G, Breart G, Gardini F Mortality, severe respiratory distress syndrome, and chronic lung disease of the newborn are reduced more after prophylactic than after therapeutic administration of the surfactant Curosurf. Pediatrics 1997;100:E4
41. Eisenberg E, Greene LE The relation of muscle biochemistry to muscle physiology. Annu Rev Physiol 1980;42:293-309
42. Ellington B, McBride JT, Stokes DC Effects of corticosteroids on postnatal lung and airway growth in the ferret. J Appl Physiol 1990;68:2029-2033
43. Feldman MD, Mao Y, Valvano JW, Pearce JA, Freeman GL Development of a multifrequency conductance catheter-based system to determine LV function in mice. Am J Physiol Heart Circ Physiol 2000;279:H1411-H1420
44. Fitzhardinge PM, Eisen A, Lejtenyi C, Metrakos K, Ramsay M Sequelae of early steroid administration to the newborn infant. Pediatrics 1974;53:877-883

45. Flagel SB, Vazquez DM, Watson SJ, Jr., Neal CR, Jr. Effects of tapering neonatal dexamethasone on rat growth, neurodevelopment, and stress response. Am J Physiol Regul Integr Comp Physiol 2002;282:R55-R63
46. French NP, Hagan R, Evans SF, Godfrey M, Newnham JP Repeated antenatal corticosteroids: size at birth and subsequent development. Am J Obstet Gynecol 1999;180:114-121
47. Georgakopoulos D, Mitzner WA, Chen CH, Byrne BJ, Millar HD, Hare JM, Kass DA In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. Am J Physiol 1998;274:H1416-H1422
48. Gibson AT, Pearse RG, Wales JK Growth retardation after dexamethasone administration: assessment by knemometry. Arch Dis Child 1993;69:505-509
49. Glower DD, Spratt JA, Snow ND, Kabas JS, Davis JW, Olsen CO, Tyson GS, Sabiston DC, Jr., Rankin JS Linearity of the Frank-Starling relationship in the intact heart: the concept of preload recruitable stroke work. Circulation 1985;71:994-1009
50. Gramsbergen A, Mulder EJ The influence of betamethasone and dexamethasone on motor development in young rats. Pediatr Res 1998;44:105-110
51. Gross I Regulation of fetal lung maturation. Am J Physiol 1990;259:L337-L344
52. Halliday HL Clinical trials of postnatal corticosteroids: inhaled and systemic. Biol Neonate 1999;76 Suppl 1:29-40
53. Halliday HL The effect of postnatal steroids on growth and development. J Perinat Med 2001;29:281-285
54. Halliday HL, Ehrenkranz RA Delayed (>3 weeks) postnatal corticosteroids for chronic lung disease in preterm infants. Cochrane Database Syst Rev 2000CD001145
55. Halliday HL, Ehrenkranz RA Early postnatal (<96 hours) corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst Rev 2001CD001146
56. Halliday HL, Ehrenkranz RA Moderately early (7-14 days) postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst Rev 2001CD001144
57. Harrington WF, Rodgers ME Myosin. Annu Rev Biochem 1984;53:35-73
58. Howard E Reductions in size and total DNA of cerebrum and cerebellum in adult mice after corticosterone treatment in infancy. Exp Neurol 1968;22:191-208
59. Huttenbach Y, Ostrowski ML, Thaller D, Kim HS Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. Cardiovasc Pathol 2001;10:119-123
60. Israel BA, Sherman FS, Guthrie RD Hypertrophic cardiomyopathy associated with dexamethasone therapy for chronic lung disease in preterm infants. Am J Perinatol 1993;10:307-310
61. Jaskoll T, Choy HA, Melnick M The glucocorticoid-glucocorticoid receptor signal transduction pathway, transforming growth factor-beta, and embryonic mouse lung development in vivo. Pediatr Res 1996;39:749-759
62. Jobe AH Pulmonary surfactant therapy. N Engl J Med 1993;328:861-868
63. Kamphuis PJ, Croiset G, Bakker JM, van Bel F, Van Ree JM, Wiegant VM Neonatal dexamethasone treatment affects social behaviour of rats in later life. Neuropharmacology 2004;47:461-474
64. Kamphuis PJ, de Vries WB, Bakker JM, Kavelaars A, van Dijk JE, Schipper ME, van Oosterhout MF, Croiset G, Heijnen CJ, Van Bel F, Wiegant VM Reduced life expectancy in rats after neonatal dexamethasone treatment. Pediatr Res (in press)
65. Kentish JC, ter Keurs HE, Ricciardi L, Bucx JJ, Noble MI Comparison between the sarcomere length-force relations of intact and skinned trabeculae from rat right ventricle. Influence of calcium concentrations on these relations. Circ Res 1986;58:755-768
66. Kinsella JP, Greenough A, Abman SH Bronchopulmonary dysplasia. Lancet 2006;367:1421-1431
67. Kostin S, Hein S, Arnon E, Scholz D, Schaper J The cytoskeleton and related proteins in the human failing heart. Heart Fail Rev 2000;5:271-280
68. Kreider ML, Tate CA, Cousins MM, Oliver CA, Seidler FJ, Slotkin TA Lasting effects of developmental dexamethasone treatment on neural cell number and size, synaptic activity, and cell signaling:

critical periods of vulnerability, dose-effect relationships, regional targets, and sex selectivity. Neuropsychopharmacology 2006;31:12-35
69. La Mear NS, MacGilvray SS, Myers TF Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol Neonate 1997;72:175-180
70. Levine S, Johnson DF, Gonzalez CA Behavioral and hormonal responses to separation in infant rhesus monkeys and mothers. Behav Neurosci 1985;99:399-410
71. Levitt NS, Lindsay RS, Holmes MC, Seckl JR Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology 1996;64:412-418
72. Li F, Wang X, Capasso JM, Gerdes AM Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28:1737-1746
73. Li F, Wang X, Gerdes AM Formation of binucleated cardiac myocytes in rat heart: II. Cytoskeletal organisation. J Mol Cell Cardiol 1997;29:1553-1565
74. Liggins GC, Howie RN A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. Pediatrics 1972;50:515-525
75. Little WC, Cheng CP, Mumma M, Igarashi Y, Vinten-Johansen J, Johnston WE Comparison of measures of left ventricular contractile performance derived from pressure-volume loops in conscious dogs. Circulation 1989;80:1378-1387
76. Loud AV, Anversa P, Giacomelli F, Wiener J Absolute morphometric study of myocardial hypertrophy in experimental hypertension. I. Determination of myocyte size. Lab Invest 1978;38:586-596
77. Mammel MC, Green TP, Johnson DE, Thompson TR Controlled trial of dexamethasone therapy in infants with bronchopulmonary dysplasia. Lancet 1983;1:1356-1358
78. Massaro D, Massaro GD Dexamethasone accelerates postnatal alveolar wall thinning and alters wall composition. Am J Physiol 1986;251:R218-R224
79. Matthews SG Dynamic changes in glucocorticoid and mineralocorticoid receptor mRNA in the developing guinea pig brain. Brain Res Dev Brain Res 1998;107:123-132
80. Mayhew TM, Pharaoh A, Austin A, Fagan DG Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors. J Anat 1997;191 (Pt 1):107-115
81. Miranda-Mallea J, Perez-Verdu J, Gasco-Lacalle B, Saez-Palacios JM, Fernandez-Gilino C, Izquierdo-Macian I Hypertrophic cardiomyopathy in preterm infants treated with dexamethasone. Eur J Pediatr 1997;156:394-396
82. Moss RL, Fitzsimons DP Frank-Starling relationship: long on importance, short on mechanism. Circ Res 2002;90:11-13
83. Muangmingsuk S, Ingram P, Gupta MP, Arcilla RA, Gupta M Dexamethasone induced cardiac hypertrophy in newborn rats is accompanied by changes in myosin heavy chain phenotype and gene transcription. Mol Cell Biochem 2000;209:165-173
84. Murphy BP, Inder TE, Huppi PS, Warfield S, Zientara GP, Kikinis R, Jolesz FA, Volpe JJ Impaired cerebral cortical gray matter growth after treatment with dexamethasone for neonatal chronic lung disease. Pediatrics 2001;107:217-221
85. Ng PC The effectiveness and side effects of dexamethasone in preterm infants with bronchopulmonary dysplasia. Arch Dis Child 1993;68:330-336
86. Northway WH, Rosan RC, Porter DY Pulmonary disease following respirator therapy of hyaline membrane disease. New England Journal of Medicine 1976;276:357-368
87. O'Shea TM, Kothadia JM, Klinepeter KL, Goldstein DJ, Jackson BG, Weaver RG, III, Dillard RG Randomized placebo-controlled trial of a 42-day tapering course of dexamethasone to reduce the duration of ventilator dependency in very low birth weight infants: outcome of study participants at 1-year adjusted age. Pediatrics 1999;104:15-21
88. Ohning BL, Fyfe DA, Riedel PA Reversible obstructive hypertrophic cardiomyopathy after dexamethasone therapy for bronchopulmonary dysplasia. Am Heart J 1993;125:253-256

89. Ozdemir A, Brown MA, Morgan WJ Markers and mediators of inflammation in neonatal lung disease. Pediatr Pulmonol 1997;23:292-306
90. Papile LA, Tyson JE, Stoll BJ, Wright LL, Donovan EF, Bauer CR, Krause-Steinrauf H, Verter J, Korones SB, Lemons JA, Fanaroff AA, Stevenson DK A multicenter trial of two dexamethasone regimens in ventilator-dependent premature infants. N Engl J Med 1998;338:1112-1118
91. Pierce MR, Bancalari E The role of inflammation in the pathogenesis of bronchopulmonary dysplasia. Pediatr Pulmonol 1995;19:371-378
92. Rizvi ZB, Aniol HS, Myers TF, Zeller WP, Fisher SG, Anderson CL Effects of dexamethasone on the hypothalamic-pituitary-adrenal axis in preterm infants. J Pediatr 1992;120:961-965
93. Rojas MA, Gonzalez A, Bancalari E, Claure N, Poole C, Silva-Neto G Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. J Pediatr 1995;126:605-610
94. Romijn HJ, Hofman MA, Gramsbergen A At what age is the developing cerebral cortex of the rat comparable to that of the full-term newborn human baby? Early Hum Dev 1991;26:61-67
95. Rudolph AM Myocardial growth before and after birth: clinical implications. Acta Paediatr 2000;89:129-133
96. Rudolph AM, Roman C, Gournay V Perinatal myocardial DNA and protein changes in the lamb: effect of cortisol in the fetus. Pediatr Res 1999;46:141-146
97. Sapolsky RM, Meaney MJ Maturation of the adrenocortical stress response: neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Res 1986;396:64-76
98. Saugstad OD Chronic lung disease: the role of oxidative stress. Biol Neonate 1998;74 Suppl 1:21-28
99. Schaper J, Froede R, Hein S, Buck A, Hashizume H, Speiser B, Friedl A, Bleese N Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. Circulation 1991;83:504-514
100. Schittny JC, Djonov V, Fine A, Burri PH Programmed cell death contributes to postnatal lung development. Am J Respir Cell Mol Biol 1998;18:786-793
101. Schwartz K, Chassagne C, Boheler KR The molecular biology of heart failure. J Am Coll Cardiol 1993;22:30A-33A
102. Shennan AT, Dunn MS, Ohlsson A, Lennox K, Hoskins EM Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. Pediatrics 1988;82:527-532
103. Shinwell ES Current dilemmas in postnatal steroid therapy for chronic lung disease in preterm infants. Biol Neonate 2003;84:96-100
104. Shinwell ES, Karplus M, Reich D, Weintraub Z, Blazer S, Bader D, Yurman S, Dolfin T, Kogan A, Dollberg S, Arbel E, Goldberg M, Gur I, Naor N, Sirota L, Mogilner S, Zaritsky A, Barak M, Gottfried E Early postnatal dexamethasone treatment and increased incidence of cerebral palsy. Arch Dis Child Fetal Neonatal Ed 2000
105. Sicard RE, Werner JC Dexamethasone induces a transient relative cardiomegaly in neonatal rats. Pediatr Res 1992;31:359-363
106. Sicard RE, Werner JC Dexamethasone-induced histopathology of neonatal rat myocardium. In Vivo 1994;8:353-358
107. Skelton R, Gill AB, Parsons JM Cardiac effects of short course dexamethasone in preterm infants. Arch Dis Child Fetal Neonatal Ed 1998;78:F133-F137
108. Stark AR, Carlo WA, Tyson JE, Papile LA, Wright LL, Shankaran S, Donovan EF, Oh W, Bauer CR, Saha S, Poole WK, Stoll BJ Adverse effects of early dexamethasone in extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. N Engl J Med 2001;344:95-101
109. Stoll BJ, Temprosa M, Tyson JE, Papile LA, Wright LL, Bauer CR, Donovan EF, Korones SB, Lemons JA, Fanaroff AA, Stevenson DK, Oh W, Ehrenkranz RA, Shankaran S, Verter J Dexamethasone therapy increases infection in very low birth weight infants. Pediatrics 1999;104:e63

110. Strauss A, Brakin M, Norris K, Modanlou HD Adrenal responsiveness in very-low-birth-weight infants treated with dexamethasone. Dev Pharmacol Ther 1992;19:147-154
111. Suga H, Sagawa K Instantaneous pressure-volume relationships and their ratio in the excised, supported canine left ventricle. Circ Res 1974;35:117-126
112. Taeusch HW, Jr., Wang NS, Baden N, Bauer CR, Stern L A controlled trial of hydrocortisone therapy in infants with respiratory distress syndrome: II. Pathology. Pediatrics 1973;52:850-854
113. Tammela OK, Koivisto ME Fluid restriction for preventing bronchopulmonary dysplasia? Reduced fluid intake during the first weeks of life improves the outcome of low-birth-weight infants. Acta Paediatr 1992;81:207-212
114. Thebaud B, Lacaze-Masmonteil T, Watterberg K Postnatal glucocorticoids in very preterm infants: "the good, the bad, and the ugly"? Pediatrics 2001;107:413-415
115. Thibeault DW, Heimes B, Rezaiekhaligh M, Mabry S Chronic modifications of lung and heart development in glucocorticoid-treated newborn rats exposed to hyperoxia or room air. Pediatr Pulmonol 1993;16:81-88
116. Truffert P, Empana JP, Breart G, Saugstad OD, Goelz R, Halliday HL, Anceschi M Treatment strategies for bronchopulmonary dysplasia with postnatal corticosteroids in Europe: the EURAIL survey. Acta Paediatr 2003;92:948-951
117. Tsai FJ, Tsai CH, Wu SF, Liu YH, Yeh TF Catabolic effect in premature infants with early dexamethasone treatment. Acta Paediatr 1996;85:1487-1490
118. Tschanz SA, Damke BM, Burri PH Influence of postnatally administered glucocorticoids on rat lung growth. Biol Neonate 1995;68:229-245
119. Tschanz SA, Makanya AN, Haenni B, Burri PH Effects of neonatal high-dose short-term glucocorticoid treatment on the lung: a morphologic and morphometric study in the rat. Pediatr Res 2003;53:72-80
120. Uno H, Lohmiller L, Thieme C, Kemnitz JW, Engle MJ, Roecker EB, Farrell PM Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. Brain Res Dev Brain Res 1990;53:157-167
121. van Goudoever JB, Wattimena JD, Carnielli VP, Sulkers EJ, Degenhart HJ, Sauer PJ Effect of dexamethasone on protein metabolism in infants with bronchopulmonary dysplasia. J Pediatr 1994;124:112-118
122. Vicedomini JP, Nonneman AJ, DeKosky ST, Scheff SW Perinatal glucocorticoids disrupt learning: a sexually dimorphic response. Physiol Behav 1986;36:145-149
123. Vural M, Yilmaz I, Oztunc F, Ilikkan B, Erginoz E, Perk Y Cardiac effects of a single course of antenatal betamethasone in preterm infants. Arch Dis Child Fetal Neonatal Ed 2006;91:F118-F122
124. Vyas J, Kotecha S Effects of antenatal and postnatal corticosteroids on the preterm lung. Arch Dis Child Fetal Neonatal Ed 1997;77:F147-F150
125. Wang N, Ingber DE Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension. Biophys J 1994;66:2181-2189
126. Wang X, Osinska H, Gerdes AM, Robbins J Desmin filaments and cardiac disease: establishing causality. J Card Fail 2002;8:S287-S292
127. Watterberg KL, Demers LM, Scott SM, Murphy S Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics 1996;97:210-215
128. Watts CL, Bruce MC Effect of dexamethasone therapy on fibronectin and albumin levels in lung secretions of infants with bronchopulmonary dysplasia. J Pediatr 1992;121:597-607
129. Weiler HA, Wang Z, Atkinson SA Dexamethasone treatment impairs calcium regulation and reduces bone mineralization in infant pigs. Am J Clin Nutr 1995;61:805-811
130. Werner JC, Sicard RE, Hansen TW, Solomon E, Cowett RM, Oh W Hypertrophic cardiomyopathy associated with dexamethasone therapy for bronchopulmonary dysplasia. J Pediatr 1992;120: 286-291

131. Wheeler W, Kurachek S, McNamara J, Fugate J, Hoogenhous N Consequences of hypogammaglobulinemia and steroid therapy in severe bronchopulmonary dysplasia. Pediatr Pulmonol 1996;22:96-100
132. Whitehurst RM, Jr., Zhang M, Bhattacharjee A, Li M Dexamethasone-induced hypertrophy in rat neonatal cardiac myocytes involves an elevated L-type Ca(2+) current. J Mol Cell Cardiol 1999;31: 1551-1558
133. Whitsett JA, Stahlman MT Impact of advances in physiology, biochemistry, and molecular biology on pulmonary disease in neonates. Am J Respir Crit Care Med 1998;157:S67-S71
134. Williams AF, Jones M Dexamethasone increases plasma amino acid concentrations in bronchopulmonary dysplasia. Arch Dis Child 1992;67:5-9
135. Yeh TF, Lin YJ, Huang CC, Chen YJ, Lin CH, Lin HC, Hsieh WS, Lien YJ Early dexamethasone therapy in preterm infants: a follow-up study. Pediatrics 1998;101:E7
136. Yeh TF, Lin YJ, Lin HC, Huang CC, Hsieh WS, Lin CH, Tsai CH Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity. N Engl J Med 2004;350:1304-1313
137. Zak R Development and proliferative capacity of cardiac muscle cells. Circ Res 1974;35:suppl-26
138. Zhou H, Gao Y, Raj JU Antenatal betamethasone therapy augments nitric oxide-mediated relaxation of preterm ovine pulmonary veins. J Appl Physiol 1996;80:390-396

CHAPTER 2

SUPPRESSION OF PHYSIOLOGICAL CARDIOMYOCYTE PROLIFERATION IN THE RAT PUP AFTER NEONATAL GLUCOCORTICOID TREATMENT

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Basic Res Cardiol 2006; 101: 36-42

ABSTRACT

Glucocorticoids (mostly dexamethasone) are widely used to prevent chronic lung disease in premature infants. Neonatal rats treated with dexamethasone have been shown to have reduced cardiac mass and cardiomyocyte hypertrophy, suggesting a lower number of cardiomyocytes at adult age, and a severely reduced life expectancy. In the present study we tested the hypothesis that a lower number of cardiomyocytes in later life is caused by a reduced cardiomyocyte proliferation and/or by early cell death (apoptosis).

Rat pups received dexamethasone or saline control on day 1, 2 and 3 and were sacrificed at day 0, 2, 4, 7 and 21. The cardiomyocytes of dexamethasone-treated pups showed a reduced proliferation as indicated by a lower mitotic index and reduced number of Ki-67 positive cardiomyocytes on day 2 and 4 as compared to day 0 and day 7 and also as compared to the age matched saline pups. On day 7 and day 21 the mitotic index was not different between groups. From day 2 onward up to day 21 dexamethasone-treated pups showed a lower number of cardiomyocytes. The cardiomyocytes showed no signs (<<1%) of apoptosis (Caspase-3 and cleaved-PARP) in any group.

The temporary suppression of cardiomyocyte hyperplasia found in dexamethasone-treated pups eventually leads to a reduced number and hypertrophy of cardiomyocytes during adult life.

INTRODUCTION

Chronic lung disease is a frequently occurring complication with high morbidity and mortality in preterm infants who suffer from severe (infant) respiratory distress syndrome (RDS) and therefore represents a significant health problem [2]. Since an excessive pro-inflammatory reaction seems to play an important role in chronic lung disease, glucocorticoids, predominantly dexamethasone (Dex), are widely used for prevention and/or reduction of this complication because of their anti-inflammatory properties [8,11,14]. Increasing evidence emerges however, that Dex treatment in the preterm infant at risk for chronic lung disease has a wide range of adverse effects [1,3,15,17,34,37]. These adverse effects also involve the cardiovascular system [10,22], consisting of short-term phenomena such as transient hypertrophic cardiomyopathy and hypertension [21,36] observed in human premature babies. A recent study by our group in 50 week old rats which were neonatally treated with Dex showed a lower heart weight accompanied by cardiomyocyte hypertrophy as compared to saline (Sal)-treated controls, strongly suggesting that these Dex-treated rats ended up with a reduced number of cardiomyocytes as compared to Sal-treated control rats [9]. Furthermore, it turned out that the neonatally Dex-treated rats had a severely reduced life expectancy and showed signs of myocardial fibrosis [9,20].

Earlier studies have shown that elevation of fetal stress hormones, such as glucocorticoids, in the third trimester of ovine pregnancy, alters fetal growth and cardiovascular development and function [23,31]. Rudolph [27] found that perinatal administration of cortisol in fetal lambs inhibited cardiomyocyte replication, whereas Slotkin et al. [32] reported that late-gestationally administered Dex slowed the postnatal cell division in the rat heart as indicated by a decrease in DNA.

Long term effects of postnatal glucocorticoid treatment on cardiomyocyte replication and growth of the immature heart have not been studied yet, but the results of our study mentioned above suggested a lower number of cardiomyocytes in adult rats treated neonatally with Dex [9].

To investigate this process we performed histopathological and quantitative immunohistochemical studies on hearts of rat pups before, during and after Dex treatment. We hypothesized that neonatal Dex treatment alters the pattern of normal myocardial growth and cardiomyocyte proliferation. If so, this may be an important cause for the reported cardiac abnormalities in neonatally Dex-treated adult rats.

Since apoptosis is known to play an important role in cardiac failure after cardiac hypertrophy [13,24], albeit in adult life, we also investigated whether Dex-induced apoptosis, responsible for abnormal myocardial development, was present during the neonatal period.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Animal Research Committee of the University of Leiden. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No.85–23, revised 1996). Pregnant Wistar rats (270 – 300 g) were housed individually and kept under conventional housing conditions with free access to food and water. Pups were born on day 21-22 of gestation. On the day of birth (day 0) male pups were selected and randomly divided between treatment (day 2 n=10, day 4 n=8, day 7 n=6, day 21 n=6) and control (day 2 n=10, day 4 n=9, day 7 n=7, day 21 n=6) groups. A separate group was sacrificed on day 0 before treatment (n=8). Treatment and control animals were kept separately and placed with foster mothers in groups of 6 to 10 pups. Temperature and humidity were kept constant and the rats had free access to food and water. An artificial 12hrs light/12hrs dark cycle was used.

Experimental design

Rat pups in the treatment group were injected intraperitoneally with dexamethasone (Dex) (Dexamethasone Sodium Phosphate; BUFA, Uitgeest, The Netherlands) applying a 3-day tapering dose. The dose and duration of the treatment was based on and proportional to a 21-day tapering course of Dex (starting dose 0.5 µg/g) to prevent or reduce chronic lung disease in preterm infants. Consequently, the treated animals received 0.5, 0.3 and 0.1 µg/g body weight Dex on days 1, 2 and 3 of life respectively. The animals in the control group received equal volumes of sterile pyrogen-free saline (Sal**,**10 µl/g). The pre-treatment group was killed 12 hours after birth, the other groups at days 2, 4, 7 and 21 after birth. Body weight (Bw) was measured before sacrificing. The heart was harvested and wet weight (Hw) was measured. The heart was directly immersion-fixed in buffered formalin 4% for histopathological and immunohistochemical analyses.

Histopathology

The atria were dissected from the formalin-fixed heart, the ventricles were sliced in two parts parallel to the short axis at the mid-papillary level and embedded in paraffin after which 3 µm sections were cut.

Routine staining with haematoxylin and eosin (H&E) was performed for conventional histopathology and for counting of mitosis. The proliferation marker Ki-67 (Rabbit Monoclonal Antibody Clone SP6, Neomarkers) was used to evaluate cardiomyocyte proliferation. The tissue was treated with citrate buffer (10 mMol, pH 6.0, boiled 20 min). After incubation for 1 hr, the slides were incubated with a polymeric HRP-linker antibody (PowerVision Detection System, ImmunoVision Technologies, Brisbane, CA) for 30 minutes. Diaminobenzidine (DAB, DAKO) was used as chromogen. The number of positive cardiomyocyte nuclei and the total number of nuclei were counted.

To assess apoptosis, primary antibodies were used against active Caspase-3 (clone C92-605 purified rabbit anti-caspase-3 monoclonal antibody, BD Pharmingen), Poly-ADP-ribose polymerase (PARP, clone c2-10 mouse anti-PARP, monoclonal antibody, Biopharmingen) and cleaved PARP (Anti-PARP p85 fragment pAb polyclonal antibody, Promega) were applied in an assay performed in the same way as the proliferation marker.

Proliferative activity of cardiomyocytes was detected and quantified by two methods 1) the mitotic index (counting of mitotic spindles) and 2) the proliferation marker Ki-67 (immunohistochemistry).

Mitotic spindles are visible in the M phase of the cell cycle whereas the Ki-67 protein is expressed in all proliferating cells in late G1, S, G2 and M phases of the cell cycle [29]. Thus, the Ki-67 protein is positive in the cell cycle for a longer period than the mitotic spindle is visible and therefore a more sensitive marker for proliferation. The mitotic index of cardiomyocytes was determined by counting the mitotic spindles in a total area of 1.51 mm^2 for each ventricle (20 microscopic fields, magnification of 400x). Mitotic spindles were counted simultaneously by two observers aiming at consensus, taking care to use only longitudinally oriented cardiomyocytes located at the mid myocardial layer sampled equally from all parts of the left ventricle (septum, anterior, lateral and posterior wall).

Slides stained with the Ki-67 proliferation marker were photographed digitally at a final magnification of 400x (Nikon Eclipse E800 rendering an image field of $35.7 \cdot 10^{-3}$ mm^2).

In four fields (taken from the mid myocardial layer of septum, anterior, lateral and posterior left ventricular wall of each heart) the total number of cardiomyocyte nuclei and number of Ki-67 positive cardiomyocyte nuclei were counted. Only dark brown nuclear staining was counted as positive.

Statistics

All data are expressed as mean ± SD. The possible differences between and within the Sal and Dex groups were assessed by using a two-way ANOVA with a Bonferroni post-hoc test using the SPSS v 12.0 statistical package. Statistical significance was defined as $p < 0.05$.

RESULTS

Anatomical parameters and routine histopathology

As indicated in table 1 bodyweight was significantly lower at all stages in the Dex-treated rats, except for day 2. Their heart weight was until day 7 not significantly different (table 1). At day 21 the heart weight was significantly lower in the Dex-treated rats ($p<0.001$). With routine histopathology no signs of necrosis of cardiomyocytes or fibrosis of the interstitium were found in the left ventricles of any group (Figure 3A and B).

Table 1: Anatomical parameters

Age (days)	Treatment	Number	Body weight (g)	P	Heart weight (g)	P
0	none	8	4.99 ± 0.38	NS	0.036 ± 0.012	NS
2	Sal	10	7.69 ± 0.38	NS	0.048 ± 0.008	NS
	Dex	10	6.91 ± 0.45	NS	0.057 ± 0.008	NS
4	Sal	9	9.73 ± 0.38	NS	0.071 ± 0.015	NS
	Dex	8	6.43 ± 0.71	0.002	0.054 ± 0.009	NS
7	Sal	7	13.78 ± 1.08	NS	0.077 ± 0.010	NS
	Dex	6	10.66 ± 1.58	0.016	0.077 ± 0.019	NS
21	Sal	6	47.67 ± 3.54	NS	0.276 ± 0.014	NS
	Dex	6	42.72 ± 4.41	<0.001	0.228 ± 0.022	<0.001

Data are presented as mean ± SD (p-value Dex vs Sal)

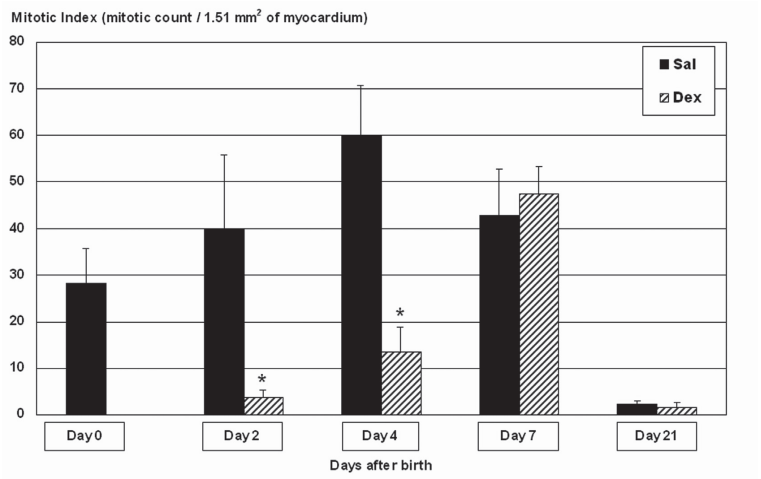

Figure 1: Proliferation of cardiomyocytes (Mitotic index) of dexamethasone- and saline-treated neonatal rats. The mitotic index is showing a significant decrease in proliferative activity of cardiomyocytes on day 2 and day 4 in the dexamethasone-treated rats (diagonal stripes), as compared to the saline (control) group (black). On day 7 and day 21 there is no difference in mitotic index between both groups. At day 21 there is a low mitotic activity in both groups. Data are presented as mean ± SD. * $p < 0.05$ vs saline. Mitotic index = number of mitotic spindles in a myocardial area of 1.51 mm^2/ventricle (see methods).

Proliferative activity

Proliferative activity of cardiomyocytes was determined by the mitotic index (number of mitotic spindles per 1.51 mm^2) and by the number of immunohistochemically stained Ki-67 positive cardiomyocyte nuclei. As shown in Figure 1 a significantly lower mitotic index in the cardiomyocytes of the Dex-treated rats was found on day 2 and day 4, compared to day 0 ($p<0.001$) and day 7 ($p<0.001$) and also compared to the age matched control group ($p<0.001$). On day 7 the mitotic index was no longer different between the two groups. On day 21 almost no more mitotic activity was present in any group (Figure 1).

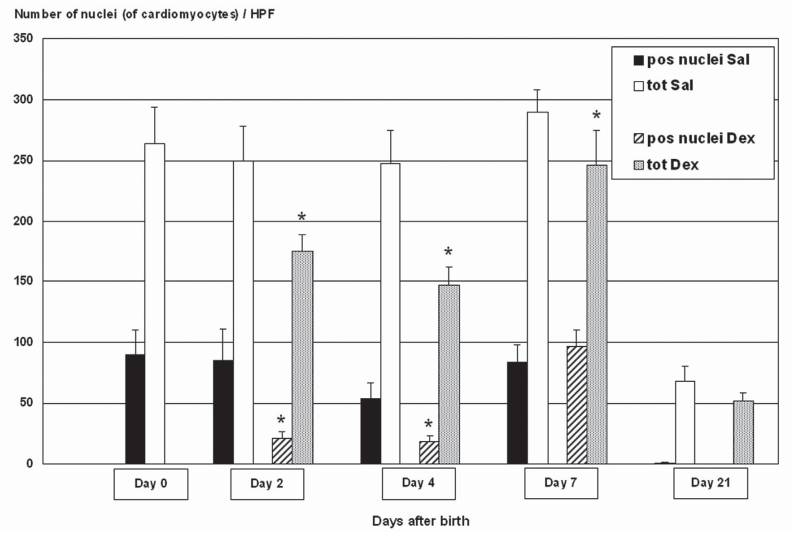

Figure 2: Proliferation of cardiomyocytes (Ki-67 staining) in dexamethasone- and saline-treated neonatal rats. The Ki-67 staining is showing a significant decrease in cardiomyocyte proliferation on day 2 and day 4 in dexamethasone-treated rats (diagonal stripes), as compared to the saline (control) group (black). At day 7 there is no difference in positive nuclei between both groups. From day 2 onward the total number of cardiomyocytes in the dexamethasone-treated rats is lower compared with the saline rats (although not significant at day 21). Data are presented as mean ± SD. * $p < 0.05$ vs saline. The total number of cardiomyocyte nuclei and number of cardiomyocyte nuclei that is positive with the proliferation marker Ki-67 as found in a myocardial area of $35.7*10^{-3}$ mm^2 (one high power field=HPF).

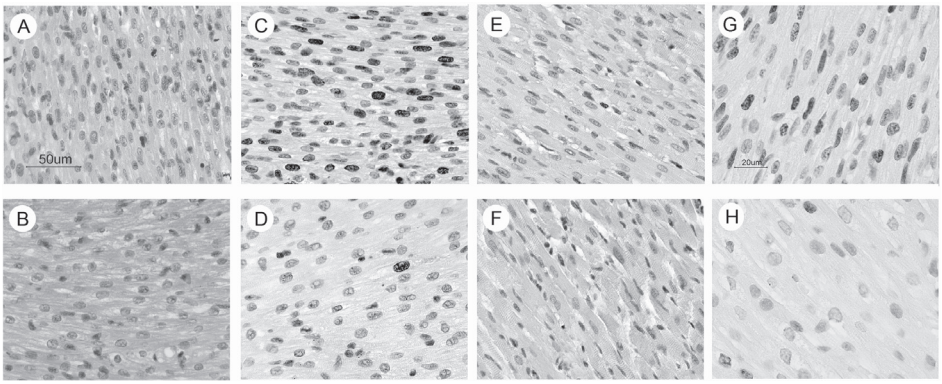

Figure 3: Histology and immunohistochemistry of 4 and 21 day old rats, neonatally treated with saline and dexamethasone. Left row: In the H&E-staining on day 4 mitotic spindles are more readily seen in the saline (control) (A) and hardly in the dexamethasone (B) group. Second row: On day 4 the Ki-67 staining shows clearly a reduction in proliferative activity in the dexamethasone-treated rat pups (D) compared with the saline-treated pups (C). The former picture shows significantly less myocyte nuclei that stain positive with the proliferation marker Ki-67. Note also the lower number of cardiomyocytes in the dexamethasone-treated group (D) compared with saline (C) photographed at the same magnification indicating cardiomyocyte hypertrophy. Third row: On day 21 the Ki-67 staining shows hardly any mitotic activity both in the saline (E) and the dexamethasone (F) group. There are less cardiomyocytes per field in the dexamethasone-treated rats, again indicating cardiomyocyte hypertrophy. Right row: Ki-67 staining on day 4 at a larger magnification showing the cardiomyocytes (saline (G) en dexamethasone (H)). Magnification is the same for the pictures A-F, the bar in picture A indicates 50 micrometer. The pictures G and H are at a larger magnification, the bar in picture G indicates 20 micrometer.

With the use of the proliferation marker Ki-67 the lower proliferative activity of cardiomyocytes in the Dex-treated rats was confirmed (Figure 2). On day 2 and day 4 significantly less cardiomyocyte nuclei of Dex-treated pups stained positive as compared to the age matched control group ($p<0.001$) (see for a typical example Figure 3C and D). At day 7 the total number of Ki-67 positive nuclei was no longer significantly different between Dex- and Sal-treated pups (Figure 2). However, Dex pups showed a significantly lower number of cardiomyocytes per myocardial area ($p=0.011$), indicating cardiomyocyte hypertrophy at that time. At the age of 21 days only a very low proliferative activity was present that did not differ between the Dex and the Sal group (also shown in Figures 3E and F). The total number of cardiomyocytes per myocardial area tended to be lower in Dex as compared to Sal-treated pups although not statistical significant.

Apoptosis

Only a small fraction of cardiomyocytes (<<1%) were Caspase-3 positive with no differences between Dex and Sal groups at any age. The DNA repair enzyme Poly (ADP-ribose) polymerase (PARP) was widely present in the nuclei of cardiomyocytes in both groups and at all ages. The cleaving product of PARP (indicating activity of PARP and thus apoptosis) showed only a small fraction of cardiomyocytes, comparable to the Caspase-3 staining.

DISCUSSION

Normal growth of the fetal heart occurs largely by an increase in the number of cardiomyocytes (hyperplasia), whereas postnatal growth of the heart, also during adult age after an increased afterload, is accomplished almost exclusively by an increase in size of the cardiomyocytes (hypertrophy) [33]. Although there is evidence for cardiomyocyte hyperplasia in the first month postnatally in rats and sheep [6,33], increase in myocardial mass thereafter will predominantly occur by hypertrophy. Possible factors responsible for this modified increase in cardiac mass are thought to be related to changes in the hormonal milieu around birth: for example plasma catecholamine concentrations increase during labor and are increased in the newborn [35], as are triiodothyronine concentrations during the first hours after birth [5]. In particular changes in endogenous glucocorticoid levels probably play an important role in this respect [23,31,32]. Fetal blood corticosteroid concentrations increase manifold shortly before birth in the lamb fetus [26]. In an elegant study in the perinatal lamb preparation, Rudolph et al. showed that selective exogenous cortisol infusion into the left coronary artery markedly decreased left ventricular DNA [28], indicating that exogenous cortisol inhibits cardiomyocyte mitosis and in fact mimics the modification of the myocardial growth pattern during the postnatal period. In view of these findings it seems reasonable to assume that glucocorticoid treatment during the ante- and perinatal period (mostly by

Dex or betamethasone) may have an important negative influence on myocardial growth by prematurely inhibiting cell division of (fetal) cardiomyocytes [27]. The present experimental study indeed showed an evidently negative effect of early postnatal Dex treatment on the proliferative activity of cardiomyocytes of rat pups in the first days after birth, as indicated by the significantly lower mitotic index and lower fraction of cardiomyocyte nuclei staining positively with the Ki-67 proliferation marker in the Dex-treated rat pups at days 2 and 4. On day 7 there were no longer differences in proliferative activity as there was no difference in mitotic index and number of proliferative Ki-67 positive cardiomyocyte nuclei between Dex- and Sal-treated pups.

At day 7 the total number of cardiomyocyte nuclei per unit area was lower in the Dex compared to Sal. This finding indicates not only that the cardiomyocytes were larger in the Dex than in the Sal group but, since heart weights were not different between the groups, this finding also indicates a decreased total number of cardiomyocytes in Dex-treated hearts. Because no signs of necrosis or increased apoptosis of cardiomyocytes were found in the Dex group, we believe that the reduced number of cardiomyocytes is caused by a suppression of proliferative activity of cardiomyocytes during the first days of life.

At day 21 there was only a very low number of proliferating cardiomyocytes, the number being not different between Sal and Dex. The total number of cardiomyocytes tended to be lower in Dex-treated hearts. However, with an about 20% reduced heart weight in the Dex group as compared to Sal, this finding indicates that there were less cardiomyocytes in the heart of the Dex-treated group and that there was no catch up of the suppressed proliferation. The latter finding stresses the notion that there is only a limited postnatal time frame in which cardiomyocytes can undergo cell division. Furthermore, because of this limited time frame, it illustrates that even if cardiomyocyte proliferation is suppressed for only a few postnatal days (within the first week) it already has negative repercussions on the total number of cardiomyocytes later in life.

The results of the present study strongly suggest that postnatal treatment with glucocorticoids such as Dex are at least in part responsible for the long term and eventually fatal changes of myocardial tissue which we reported in an earlier study [9]. It showed that neonatally Dex-treated rats during adulthood end up with a smaller number of (although hypertrophied) cardiomyocytes and a severe reduction in life span [9,20]. It has been postulated that cardiomyocyte hypertrophy is mandatory in Dex-treated rats to maintain normal cardiac function with less cardiomyocytes during adult life [9,27,28].

These results obtained from experimental studies put into question the safety of early postnatal glucocorticoid treatment for prevention of chronic lung disease in the human baby. Of course, we are fully aware of the fact that we cannot simply extrapolate the reported findings, obtained in the newborn and adult rat model, to the preterm infant and adult human. Two important differences are obvious when comparing rat studies with the clinical situation. First, we are dealing here with term rat pups whose myocardial tissue may have an affinity

to Dex different from the affinity of myocardial tissue in the preterm infant. Second, human fetal and neonatal development of the myocardium may differ from development of the rat or lamb myocardium. With respect to the first issue however, there is a large amount of data showing that the term rat heart responds to neonatal Dex treatment in a way comparable to the heart of preterm babies treated with Dex for prevention of chronic lung disease: in both situations transient systemic hypertension and hypertrophic cardiomyopathy of the left ventricle occurs [21,30,36]. With respect to the second issue regarding fetal and postnatal growth of myocardial tissue, Huttenbach et al. [18] investigated cardiomyocyte proliferation in autopsy material from hearts of preterm and term infants. They reported that cardiomyocyte proliferative activity in left ventricular myocardial tissue remained present and constant during the early preterm period and only decreased in late preterm and early postterm periods. Moreover, a recent study of Nadal-Ginard et al. [24], discussing regeneration of myocardial tissue during cardiac hypertrophy and failure, stated that cardiomyocytes that retain the ability to divide always remain present. Although the above stated arguments do not prove that the newborn rat or lamb heart is fully comparable to the (preterm) human heart with regard to cardiomyocyte proliferative activity and their reaction to Dex exposure, it is valid to consider the possibility of transient down regulation of cardiomyocyte proliferative activity in preterm infants neonatally treated with glucocorticoids such as Dex. In the routine care of the preterm fetus and infant at risk for chronic lung disease neonatal (but also antenatal) treatment with glucocorticoids has been established not so long ago yet [8,11]. Although nowadays controversy exists regarding the routine postnatal use of glucocorticoids for management of chronic lung disease in neonates [7,12] glucocorticoids (mainly Dex) are still widely used. With regard to the cardiovascular status of individuals treated pre- or neonatally with glucocorticoids no follow-up studies have been reported yet. However since neonatal treatment with glucocorticoids appears to have long lasting consequences as shown in the rat, a mandatory cardiovascular follow-up program should be seriously considered.

Although apoptotic activity is a physiological event during fetal and postnatal cardiac development [16] and plays an important role in loss of cardiomyocytes during the aging process especially in the failing heart [4,13,16,19,24,25], we did not find evidence in this study that neonatal Dex treatment in rat pups gave rise to an upregulation of apoptotic activity. Also no signs of early necrotic cardiomyocyte cell death in the rat pups could be found.

In summary, neonatal Dex treatment in rat pups, with a duration and dosage similar to those used in the preterm infant to prevent chronic lung disease, caused a temporary suppression of the proliferative activity of cardiomyocytes. This finding provides an explanation for the reduced number of cardiomyocytes and for the established hypertrophy in adult rats treated with Dex in the neonatal period as reported earlier and might give a clue as to the cause of the reduction in their life span.

Acknowledgement

The authors thank Dr. M. Schipper, statistician, Centre for Biostatistics, University of Utrecht for her advice regarding the statistical analysis.

REFERENCE LIST

1. Bakker JM, van Bel F, Heijnen CJ Neonatal glucocorticoids and the developing brain: short-term treatment with life-long consequences? Trends Neurosci 2001;24:649-653
2. Bancalari E, Claure N, Sosenko IR Bronchopulmonary dysplasia: changes in pathogenesis, epidemiology and definition. Semin Neonatol 2003;8:63-71
3. Barrington KJ Postnatal steroids and neurodevelopmental outcomes: a problem in the making. Pediatrics 2001;107:1425-1426
4. Blankenberg FG, Strauss HW Will imaging of apoptosis play a role in clinical care? A tale of mice and men. Apoptosis 2001;6:117-123
5. Breall JA, Rudolph AM, Heymann MA Role of thyroid hormone in postnatal circulatory and metabolic adjustments. J Clin Invest 1984;73:1418-1424
6. Chacko S DNA synthesis, mitosis, and differentiation in cardiac myogenesis. Dev Biol 1973;35:1-18
7. Committee on Fetus and Newborn Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics 2002;109:330-338
8. Cummings JJ, D'Eugenio DB, Gross SJ A controlled trial of dexamethasone in preterm infants at high risk for bronchopulmonary dysplasia. N Engl J Med 1989;320:1505-1510
9. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr Res 2002;52:900-906
10. Dodic M, Peers A, Coghlan JP, Wintour M Can Excess Glucocorticoid, Predispose to Cardiovascular and Metabolic Disease in Middle Age? Trends Endocrinol Metab 1999;10:86-91
11. Durand M, Sardesai S, McEvoy C Effects of early dexamethasone therapy on pulmonary mechanics and chronic lung disease in very low birth weight infants: a randomized, controlled trial. Pediatrics 1995;95:584-590
12. Finer NN, Craft A, Vaucher YE, Clark RH, Sola A Postnatal steroids: short-term gain, long-term pain? J Pediatr 2000;137:9-13
13. Freude B, Masters TN, Kostin S, Robicsek F, Schaper J Cardiomyocyte apoptosis in acute and chronic conditions. Basic Res Cardiol 1998;93:85-89
14. Halliday HL, Ehrenkranz RA., Doyle LW Postnatal corticosteroids for preventing chronic lung disease in preterm infants. Cochrane Database Syst RevCD001144-46 2003
15. Halliday HL The effect of postnatal steroids on growth and development. J Perinat Med 2001;29:281-285
16. Hammel JM, Caldarone CA, Van Natta TL, Wang LX, Welke KF, Li W, Niles S, Barner E, Scholz TD, Behrendt DM, Segar JL Myocardial apoptosis after cardioplegic arrest in the neonatal lamb. J Thorac Cardiovasc Surg 2003;125:1268-1275
17. Heide-Jalving M, Kamphuis PJ, van der Laan MJ, Bakker JM, Wiegant VM, Heijnen CJ, Veen S, van Bel F Short- and long-term effects of neonatal glucocorticoid therapy: is hydrocortisone an alternative to dexamethasone? Acta Paediatr 2003;92:827-835
18. Huttenbach Y, Ostrowski ML, Thaller D, Kim HS Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. Cardiovasc Pathol 2001;10:119-123
19. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. Lab Invest 1996;74:86-107
20. Kamphuis PJGH, Croiset G, Bakker JM, Wiegant VM, Van Bel F Neonatal dexamethasone treatment results in a reduction of life-span in rats. Pediatr Res 2001;49:299A (abstr)
21. La Mear NS, MacGilvray SS, Myers TF Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol Neonate 1997;72:175-180

22. le Cras TD, Markham NE, Morris KG, Ahrens CR, McMurtry IF, Abman SH Neonatal dexamethasone treatment increases the risk for pulmonary hypertension in adult rats. Am J Physiol Lung Cell Mol Physiol 2000;278:L822-L829
23. Moss TJ, Harding R, Newnham JP Lung function, arterial pressure and growth in sheep during early postnatal life following single and repeated prenatal corticosteroid treatments. Early Hum Dev 2002;66:11-24
24. Nadal-Ginard B, Kajstura J, Leri A, Anversa P Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ Res 2003;92:139-150
25. Narula J, Kolodgie FD, Virmani R Apoptosis and cardiomyopathy. Curr Opin Cardiol 2000;15:183-188
26. Nathanielsz PW A life before birth. Life before birth and a time to be born. Ithaca, NY:Prometheon Press 1992
27. Rudolph AM Myocardial growth before and after birth: clinical implications. Acta Paediatr 2000;89:129-133
28. Rudolph AM, Roman C, Gournay V Perinatal myocardial DNA and protein changes in the lamb: effect of cortisol in the fetus. Pediatr Res 1999;46:141-146
29. Scholzen T, Gerdes J The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311-322
30. Sicard RE, Werner JC Dexamethasone induces a transient relative cardiomegaly in neonatal rats. Pediatr Res 1992;31:359-363
31. Sloboda DM, Moss TJ, Gurrin LC, Newnham JP, Challis JR The effect of prenatal betamethasone administration on postnatal ovine hypothalamic-pituitary-adrenal function. J Endocrinol 2002;172:71-81
32. Slotkin TA, Seidler FJ, Kavlock RJ, Bartolome JV Fetal dexamethasone exposure impairs cellular development in neonatal rat heart and kidney: effects on DNA and protein in whole tissues. Teratology 1991;43:301-306
33. Smolich JJ, Walker AM, Campbell GR, Adamson TM Left and right ventricular myocardial morphometry in fetal, neonatal, and adult sheep. Am J Physiol 1989;257:H1-H9
34. Stark AR, Carlo WA, Tyson JE, Papile LA, Wright LL, Shankaran S, Donovan EF, Oh W, Bauer CR, Saha S, Poole WK, Stoll BJ Adverse effects of early dexamethasone in extremely-low-birth-weight infants. National Institute of Child Health and Human Development Neonatal Research Network. N Engl J Med 2001;344:95-101
35. van Bel F, Roman C, Iwamoto HS, Rudolph AM Sympathoadrenal, metabolic, and regional blood flow responses to cold in fetal sheep. Pediatr Res 1993;34:47-50
36. Werner JC, Sicard RE, Hansen TW, Solomon E, Cowett RM, Oh W Hypertrophic cardiomyopathy associated with dexamethasone therapy for bronchopulmonary dysplasia. J Pediatr 1992;120:286-291
37. Yeh TF, Lin YJ, Lin HC, Huang CC, Hsieh WS, Lin CH, Tsai CH Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity. N Engl J Med 2004;350:1304-1313

CHAPTER 3

NEONATAL GLUCOCORTICOID TREATMENT CAUSES SYSTOLIC DYSFUNCTION AND COMPENSATORY DILATATION IN EARLY-LIFE: STUDIES IN 4-WEEK-OLD PREPUBERTAL RATS

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Pediatr Res 2005; 58: 46-52

ABSTRACT

Glucocorticoid treatment is widely used to prevent chronic lung disease in premature infants. Recent studies in adult rats, treated with dexamethasone in the neonatal period, report negative long-term effects on the heart and severely reduced life expectancy. We treated neonatal rats with dexamethasone and studied cardiac function after 4 weeks (prepubertal age) to investigate whether the late effects as previously described are preceded by detectable alterations in cardiac function at a younger age.

Male rat pups (n=12) were injected intraperitoneally with dexamethasone on day 1, 2, and 3 (0.5, 0.3 and 0.1μg/g) of life. Control pups (n=10) received saline. At 4 weeks the animals were anesthetized, intubated and ventilated and a miniature pressure-conductance catheter was introduced into the left ventricle to measure pressure-volume loops. Cardiac function was measured and systolic and diastolic pressure-volume relations were determined to quantify intrinsic systolic and diastolic function. Subsequently, hearts were excised for histological examination. Compared to saline-treated animals, dexamethasone-treated rats had a reduced ventricular weight (270±40 vs. 371±23mg, $p<0.001$) and reduced systolic function (end-systolic elastance: 1.24±0.43 vs. 2.50±1.39mmHg/μL, $p=0.028$). Cardiac output was maintained and end-diastolic volume was increased (84±23 vs. 59±19μL, $p=0.012$) indicating a state of compensatory dilatation. Heart rate, diastolic function and systemic vascular resistance were unchanged.

Neonatal dexamethasone treatment causes cardiac alterations which can be detected in the prepubertal period and which may precede more severe cardiac dysfunction later in life. If our findings are confirmed in humans, this may have consequences for a potentially large patient population and cardiac screening at relatively young age may be indicated to enable secondary prevention.

INTRODUCTION

Glucocorticoids (GCs), in particular dexamethasone (Dex), are used widely to treat or prevent chronic lung disease in preterm infants [1]. Besides their anti-inflammatory action, GCs stimulate lung maturation including enhanced surfactant production [2,3]. Thus GC treatment improves pulmonary function and enables early weaning from the ventilator and consequently increased survival of extremely premature neonates [4].

In the past two decades Dex therapy in preterm infants has gained wide acceptance. However, despite the short-term beneficial effects, concerns have been raised about long lasting negative side effects of GCs [5-7]. Several studies describe neuro-developmental impairments in infants treated with GCs in the neonatal period [8-10].

With regard to the cardiovascular system hypertension and reversible myocardial hypertrophy have been reported [11-13], but possible long-term effects became apparent only recently. De Vries et al. [14] showed that rats treated with Dex in the neonatal period had decreased heart weights, as well as hypertrophy and signs of early degeneration of cardiomyocytes in adulthood. Moreover, preliminary findings indicate a significantly reduced life expectancy [15].

The above mentioned concerns and reports prompted us to investigate if these late cardiac side effects are preceded by detectable alterations in cardiac function at a younger age. Early detection would be of great importance for possible early intervention in the large number of individuals previously treated with Dex for prevention of chronic lung disease.

METHODS

Animals

The study protocol was approved by the Animal Research Committee of the University of Leiden. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (NIH Publication No.85-23, revised 1996).

Pregnant Wistar rats (270–300 g) were housed individually and kept under conventional housing conditions. Pups were born on day 21-22 of gestation. On the day of birth (day 0) male pups were selected and randomly divided between treatment and control groups. Treatment and control animals were kept separately and placed with foster mothers in groups of 4-6 pups. Rat pups in the treatment group were injected intraperitoneally (IP) with dexamethasone using a 3-day tapering dose mimicking the 21-days tapering course used in human preterm infants [14]. Consequently, the treated animals received 0.5, 0.3 and 0.1µg/g body weight Dex on, respectively, day 1, 2 and 3 of life. The animals in the control group received equal volumes (10µl/g) sterile pyrogen-free saline (Sal). Temperature and humidity were kept constant and the rats had free access to food and water. An artificial 12hrs-light/12hrs-dark

cycle was used. Body weight was measured daily during the first week and weekly from day 7 onward. The rats were weaned on day 21 and hemodynamic studies were performed on day 28 (4-weeks) as described below.

Animal preparation

Twenty-two male rats (12 Dex and 10 Sal) were studied. The animals were sedated by inhalation of a mixture of halothane (4%) and oxygen. Subsequently, general anesthesia was initiated by intraperitoneal injection (IP) of a fentanyl-fluanison-midazolam mixture (FFM). The FFM mixture consisted of 2 parts Hypnorm® (0.315mg/ml fentanyl + 10mg/ml fluanison), 1 part Dormicum® (5mg/ml midazolam) and 1 part water. This mixture was administered in a dose of 0.4ml/100g body weight. Supplemental IP injections (one-third of initial dose) were provided if necessary. The animals were placed on a regulated warming pad to keep body temperature constant, intubated using a 20G cannula and ventilated with air/oxygen mixture (FiO_2=0.5, 120strokes/minute) using a pressure-controlled ventilator.

Instrumentation

The animals were placed under a microscope (Zeiss, Germany) and the left jugular vein and the right carotid artery were exposed via a midline cervical incision. The jugular vein was cannulated for infusion of hypertonic saline to determine parallel conductance (see below). Via the carotid artery a combined pressure-conductance catheter (Millar Instruments, Houston, TX, USA) was introduced and positioned into the left ventricle (LV) guided by on-line pressure and volume signals. The abdomen was opened via a small incision just below the diaphragm to enable temporary preload reductions by directly compressing the inferior vena cava using a cotton-tipped stick. The pressure-conductance catheter was connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, The Netherlands) for on-line display and registration of LV pressure and volume signals. All data were acquired using Conduct-NT software (CD Leycom) at a sample rate of 2000Hz and analyzed off-line by custom-made software.

Conductance catheter method

The conductance catheter method to measure instantaneous LV volume has been developed by Baan et al. [16]. Recently, miniaturized 1.4F pressure-conductance catheters have been developed which enable pressure-volume studies in closed-chest small animals [17]. The catheter used in this study contains four platinum electrodes, each 0.25mm in width with interelectrode distances between electrodes 1-2, 2-3 and 3-4, respectively, 0.5mm, 4.5mm and 0.5mm. A high-fidelity pressure sensor is incorporated between electrodes 2 and 3. A 30μA, 10kHz current is applied between electrodes 1 and 4 to generate an intracavitary electric field and the voltage gradient between electrodes 2 and 3 is measured to determine the instantaneous electrical conductance of the blood in the LV. The volume calibration of

the conductance measurements was performed in vitro as described by Yang et al. [18] and parallel conductance was determined by the hypertonic saline method [16,19,20].

Hemodynamic measurements

Steady state

After instrumentation, LV pressure-volume signals were acquired in steady state to quantify general hemodynamic conditions: Heart rate (HR), stroke volume (SV), cardiac output (CO), end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), end-diastolic pressure (EDP) and end-systolic pressure (ESP) were determined. Stroke work (SW) was determined as the area of the pressure-volume loop, and the maximal and minimal rate of LV pressure change, dP/dt_{MAX} and dP/dt_{MIN}, and the isovolumic relaxation time constant Tau were calculated. Mean aortic pressure (MAP) was determined from the pressure signal recorded just prior to the catheter insertion into the LV. Systemic vascular resistance (SVR) was calculated as MAP/CO, without correction for central venous pressure. In addition, we determined effective arterial elastance (E_A) as ESP/SV.

Pressure-volume relationships

To obtain indices of systolic and diastolic LV function we determined pressure-volume relations by recording pressure-volume loops during a gradual preload reduction by gently compressing the inferior caval vein. By this procedure we reduced systolic pressure typically by 20-30mmHg within 2s (~15 beats). To quantify systolic function we used the end-systolic pressure-volume relation (ESPVR), the relation between dP/dt_{MAX} and EDV, and the preload recruitable stroke work relation (PRSWR: SW vs. EDV). The slopes of these linear relations, E_{ES} (end-systolic elastance), S-dP, and S-PR, respectively, are sensitive measures of intrinsic systolic LV function [21-23]. In addition, the position of the ESPVR was quantified by its intercept at ESP=75mmHg (ESV_{75}), whereas the positions of the dP/dt_{MAX}-EDV and SW-EDV relations were quantified by calculating their intercepts at EDV=70µL, respectively $dP/dt_{MAX,70}$ and SW_{70} [24]. The values (75mmHg and 70µL) were selected retrospectively as the typical mean values of ESP and EDV for these rats. For diastolic function the chamber stiffness constant k was determined by fitting the end-diastolic pressure-volume points with an exponential relation.

Wall stress

Time-varying wall stress, WS(t), was calculated from the LV pressure and volume signals (P(t), V(t)) as described by Arts et al. [25]: $WS(t)=P(t)\cdot(1+3\cdot V(t)/V_{WALL})$. Ventricular wall volume (V_{WALL}) was determined post-mortem as described below. Peak WS is the maximum of the WS(t) curve.

Parallel conductance

To determine parallel conductance we performed intravenous hypertonic saline injections (10% saline, 20µL) [18,20]. Parallel conductance was calculated as the mean of three consecutive assessments.

Echocardiographic measurements

To support the conductance catheter-derived findings we performed echocardiographic measurements in a small group of additional animals (3 Dex- and 3 Sal-treated rats). The animals were sedated with 1.5% isoflurane, and placed in a supine position. Imaging was performed in the parasternal long- and short-axis plane through the shaved anterior chest using a Vingmed echocardiography system FIVE with a 10 MHz phased-array transducer. After two-dimensional imaging, a single M-mode line was directed across the ventricle at the level of the papillary muscles perpendicular to the anterior and posterior walls to determine LV end-systolic and end-diastolic diameters. The measurements were performed by an experienced echocardiographer (RFMB) who was blinded to the treatment.

Histopathological analysis

After the hemodynamic measurements the heart was arrested by slowly infusing 1ml 0.1M $CdCl_2$ via the jugular vein. The chest was opened and the heart excised and immersion fixed in phosphate-buffered formalin 4%. After removal of extracardiac structures and atria the hearts were weighed and then embedded in paraffin. Subsequently the hearts were sectioned parallel to the equator in 3-mm slices and stained with hematoxylin and eosin for conventional histopathological analysis. A slice at the level of the papillary muscles was selected for measurement of LV free wall thickness.

Statistics

All data are expressed as the mean±SD. Comparisons between group's means were performed by unpaired t-tests and statistical significance was defined as $p<0.05$.

RESULTS

Rats

Experiments were performed in Dex-treated (Dex, n=12) and saline (Sal)-treated male rats (Sal, n=10). Figure 1 shows body weight curves for both groups. No significant differences were found on day 0 and day 1, but from day 2 onward the Dex animals had a significantly lower body weight. At 4 weeks the mean body weight for Dex was 75±6 versus 85±10g for Sal ($p=0.013$).

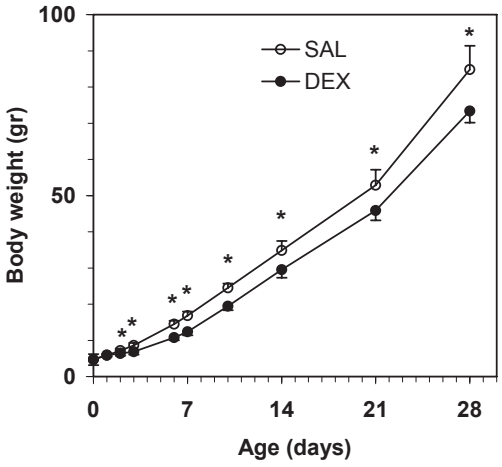

Figure 1: Body weight in saline-treated (Sal, n=10) and dexamethasone-treated (Dex, n=12) rats during 4 weeks. Differences were significant ($p<0.05$) from day 2 onward.

Table 1: Hemodynamic indices: saline-treated (Sal) vs dexamathasone-treated (Dex) rats

	Sal	Dex	P-values
HR (bpm)	509±36	488±46	NS
MAP (mmHg)	63±11	60±11	NS
ESP (mmHg)	76±10	74±12	NS
EDP (mmHg)	3.8±2.7	4.1±2.1	NS
dP/dt_{MAX} (mmHg/s)	9599±1433	9702±2793	NS
dP/dt_{MIN} (mmHg/s)	7067±1662	6959±1903	NS
Tau (ms)	9.1±2.0	9.4±1.6	NS
ESV (µL)	34±11	54±18	0.005
EDV (µL)	59±19	84±23	0.012
CO (mL/min)	12.9±4.6	15.0±5.1	NS
EF (%)	43±7	37±8	0.078 (NS)
SW (µl.mmHg)	2128±982	2481±1106	NS
PWS (mmHg)	124±21	154±32	0.015
SVR (mmHg/mL/min)	5.1±1.5	4.3±1.4	NS
E_A (mmHg/µl)	3.3±1.1	2.6±0.7	0.091 (NS)

Values are mean±SD. HR indicates heart rate; MAP, mean arterial pressure; ESP, end-systolic pressure; EDP, end-diastolic pressure; dP/dt_{MAX} and dP/dt_{MIN}, maximal and minimal rate of LV pressure change; Tau, time constant of isovolume relaxation; ESV, end-systolic volume; EDV, end-diastolic volume; CO, cardiac output; EF, ejection fraction; SW, stroke work; PWS, peak wall stress; SVR, systemic vascular resistance; E_A, effective arterial elastance.

Hemodynamics

Typical LV pressure and volume signals acquired during steady state and followed by a preload reduction induced by caval occlusion are shown in Figure 2. Mean hemodynamic indices derived from the steady state signals are given in Table 1. Typical pressure-volume

Table 2: Pressure-volume relations: saline-treated (Sal) vs dexamethasone-treated (Dex) rats

		SAL	DEX	P-values
ESPVR	E_{ES} (mmHg/µL)	2.50±1.39	1.24±0.43	0.028
	ESV_{75} (µl)	34±13	53±20	0.016
dP/dt_{MAX}-EDV	S-dP (mmHg/s/µL)	176±90	102±30	0.032
	$dP/dt_{MAX,70}$ (mmHg)	12444±4186	8520±3226	0.027
PRSWR	S-PR (mmHg)	45±13	41±10	NS
	SW_{70} (mmHg.µL)	2649±560	1915±933	0.050
EDPVR	k (1/µL)	0.017±0.006	0.029±0.026	0.154 (NS)

ESPVR, end-systolic pressure-volume relation; dP/dt_{MAX}-EDV, relation between dP/dt_{MAX} and EDV; PRSWR, preload recruitable stroke work relation; EDPVR, end-diastolic pressure-volume relation. E_{ES}, end-systolic elastance; ESV_{75}, volume intercept of ESPVR at 75mmHg; S-dP, slope of dP/dt_{MAX}-EDV relation; $dP/dt_{MAX,70}$ intercept dP/dt_{MAX}-EDV relation at 70µL; S-PR, slope of PRSWR; SW_{70}, intercept of PRSWR at 70µL; k = diastolic chamber stiffness constant.

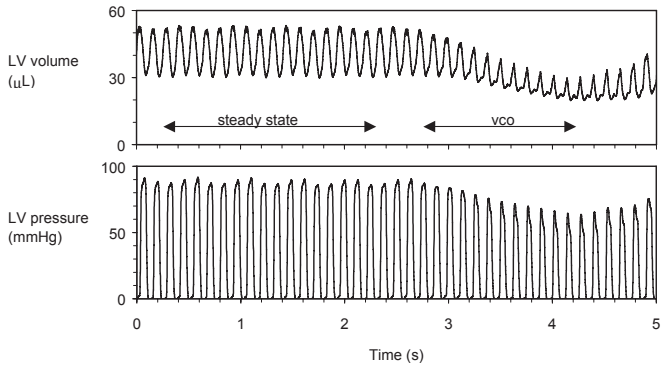

Figure 2: Typical LV pressure and volume signals recorded during steady state, followed by vena caval occlusion (vco). Steady state signals were used to determine the various hemodynamic indices as summarized in Table 1. Data acquired during the preload reduction are used to derive the pressure-volume relations as shown in Figure 3 and summarized in Table 2.

loops obtained during preload reductions for Sal and Dex are depicted in Figure 3, which also shows the derived pressure-volume relationships ESPVR, EDPVR, dP/dt_{MAX}-EDV and PRSWR. The summarized results for these relations are given in Table 2. The most apparent differences between the groups were found for the ventricular volumes. Compared to the Sal animals, both ESV and EDV were significantly higher (58% and 43%, respectively) in the Dex animals indicating substantial dilatation. Pressure-derived indices (ESP, EDP, Tau, dP/dt_{MAX} and dP/dt_{MIN}) were largely unchanged. Heart rate, cardiac output and ejection fraction were also not significantly altered, although the latter tended to be lower in the Dex animals. SVR and E_A did not change significantly. Calculated peak wall stress was significantly higher. The contractility indices E_{ES} and S-dP (the slopes of the ESPVR and dP/dt_{MAX}-EDV relation respectively) clearly show a reduced intrinsic systolic function in the Dex animals (-50%, respectively -42%, vs. Sal). The decrease in S-PR (the slope of the PRSWR) did not reach statistical significance. Reduced contractile state is further supported by the significant rightward and downward shift of the pressure-volume relations as quantified by their intercepts: x-intercept ESV_{75} increased by as much as 56%, whereas y-intercepts $dP/dt_{MAX,70}$ and SW_{70} decreased by 32%

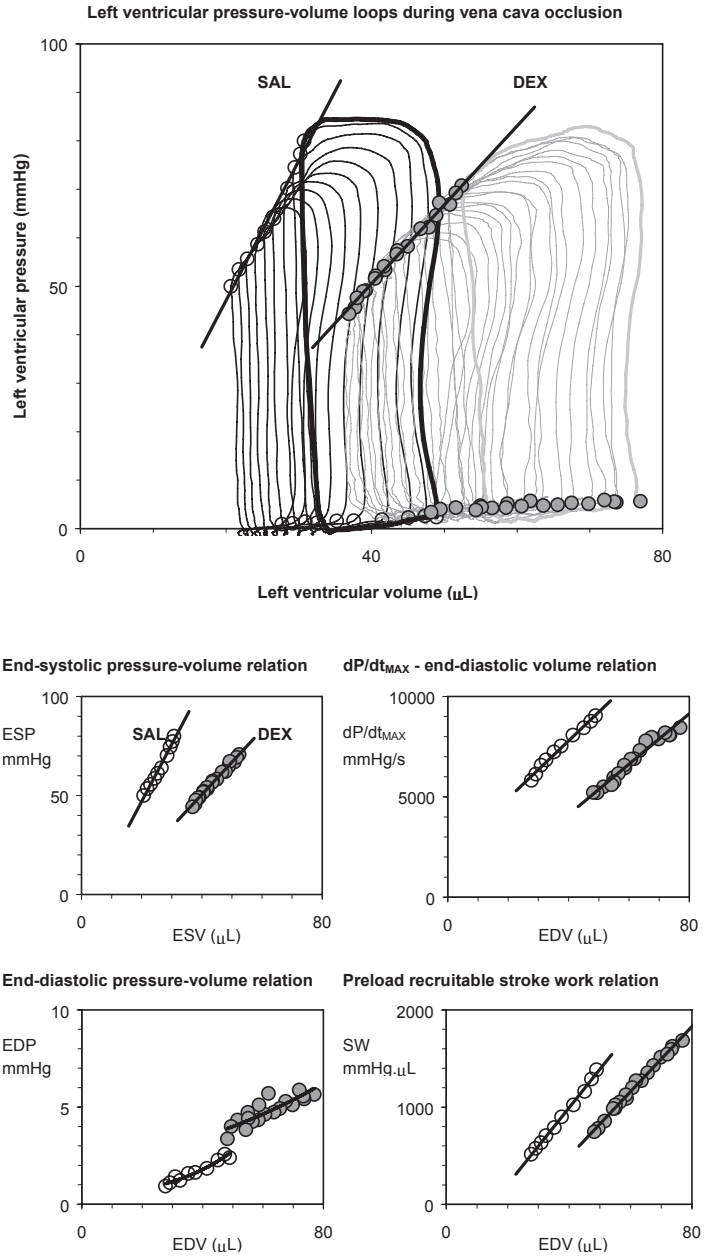

Figure 3: Representative data from a saline-treated rat (Sal, black) and a dexamethasone-treated rat (Dex, grey). The top panel shows pressure-volume loops during preload reduction. The end-systolic and end-diastolic points are marked and the ESPVR is indicated. The first loop (in bold) represents the preceding steady state condition. The rightward position of the Dex loops on the volume axis indicate substantially enlarged volumes. The reduced slope and rightward shift of the ESPVR indicate a decreased systolic function in Dex compared to Sal. The lower panels show the various pressure-volume relations. The ESPVR, the dP/dt_{MAX}-EDV relation and the PRSWR all show a rightward shift and a decreased slope consistent with reduced contractile state. The EDPVR is shifted upwards, however the overall difference in diastolic stiffness constant between the two groups did not reach statistical significance.

and 28%, respectively. The increased chamber stiffness constant k may suggest a slightly reduced diastolic compliance, but the increase did not reach statistical significance. The significant rightward shift of EDPVR (Figure 3) indicates substantial remodeling in the Dex-treated animals.

Echocardiography

Compared to the Sal rats, the Dex rats showed increased end-systolic (3.0±0.7 vs 2.6±0.5mm) and end-diastolic diameters (4.6±0.5 vs 3.9±0.4mm). The 17% increase in end-diastolic diameters in the Dex rats appears to be fully consistent with the 42% increase in end-diastolic volume found in the invasive studies. The two dimensional ultrasound images did not allow for reliable and reproducible measurements of long axis ventricular lengths in all of these rats, but assuming a fixed LV end-diastolic long axis of 7mm would predict (spheroidal model) an end-diastolic volume of 77µl in the Dex and 56µl in the Sal animals.

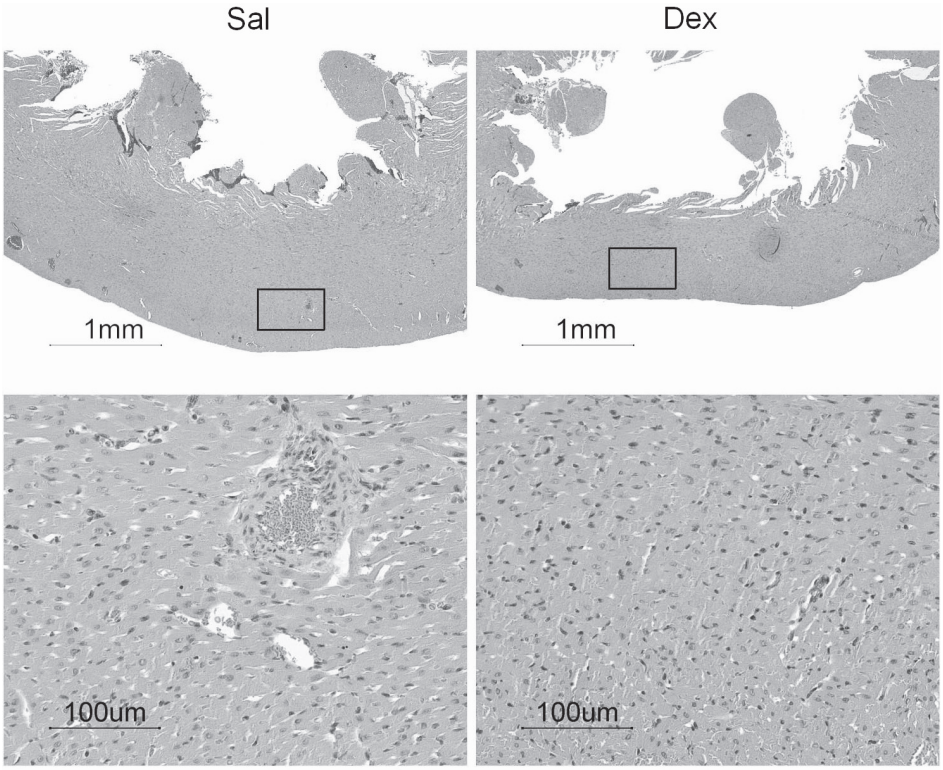

Figure 4: Hematoxylin-eosin-stained histological slides. The top panels show crosssections of the LV free wall at the level of the papillary muscles. Note the substantially thinner wall in Dex compared to Sal. The lower panel show the myocardium tissue at a higher magnification. No clear signs of cellular necrosis or fibrosis are visible.

Histopathology

Heart weight (HW) was significantly lower in the Dex animals as compared to the Sal animals (270±40 vs. 371±23mg, p<0.001). After normalization for body weight (BW), which was also reduced in the Dex animals, HW/BW was still significantly lower in the Dex animals: (3.62±0.48 vs. 4.40±0.49mg/g, p<0.001). Histopathological inspection of the hematoxylin-eosin-stained slides showed reduced wall thickness (1.07±0.10 vs. 1.40±0.12mm p<0.001) but no clear signs of cellular necrosis or fibrosis (see Figure 4).

DISCUSSION

A recent study showed gross histological abnormalities in myocardial cells of adult rats that had been treated with Dex in the neonatal period [14]. These findings are consistent with a preliminary report [15] indicating reduced survival of Dex-treated rats. However, further data on the intermediate and long-term cardiovascular effects of neonatal Dex treatment are lacking. Currently, no human data on intermediate and long-term cardiovascular effects are available. However, substantial cohorts of patients are currently reaching puberty and adulthood. We therefore studied cardiac function in 4-week-old rats to investigate whether the late effects as described by de Vries et al. [14] are preceded by detectable alterations in cardiac function at a younger (prepubertal) age.

We found that rats treated with Dex in the neonatal period have a significantly reduced ventricular wall volume and reduced contractility at 4 weeks of age. Cardiac output was maintained in the Dex-treated animals as compared to the controls, but end-diastolic volume was increased by 43%, clearly indicating a state of compensatory dilatation. The reduced wall volume and increased cavity volume result in a substantially increased wall stress. Heart rate, diastolic function and systemic vascular resistance were largely unchanged. We did not normalize our volumetric and contractility parameters for body weight of ventricular weight. However, body weight and ventricular weight were significantly lower in the Dex-treated rats and therefore the differences between the two groups would be even more pronounced when using normalized parameters.

Myocardial growth in the fetus is obtained by hyperplasia, which changes to hypertrophy after birth. This is shown by an immunohistochemical study in human hearts by Huttenbach et al. [26] which reports a comparatively high rate of proliferation from 12 to 28 weeks of gestation, with a significant decrease in proliferation after 28 weeks of gestation. This finding is in line with stereological studies in fetal and early postnatal human hearts by Mayhew et al. [27] which suggest that proliferation ceases approximately 2-3 weeks after birth. Thus, Dex treatment in preterm infants occurs during a developmental period in which substantial myocyte hyperplasia would be expected. In rat pups a rapid switch from myocyte hyperplasia to hypertrophy occurs between postnatal day 3 and 4, as described by Li et al. [28]. They

found that the number of myocytes increased by 68% during the first 3 days and remained constant thereafter, whereas myocyte volume remained relatively constant during the first 3 days and increased 2.5-fold from day 3 to day 12. These studies support that Dex treatment in term rat pups on day 1,2 and 3 (as performed in our study) corresponds with the clinical Dex treatment of preterm human babies.

With regard to the effects of Dex on ventricular function several mechanisms may be important. Rudolph et al. [29] investigated the effects of cortisol on fetal and neonatal myocardial growth and found that cortisol inhibits myocyte replication. We found a reduced myocardial wall volume which fits with the hypothesis that neonatal Dex treatment leads to an earlier than normal transition from hyperplasia to hypertrophy resulting in fewer myocytes. In addition, Dex treatment alters the number of steroid receptors in the hypothalamic-pituitary-adrenal axis which may affect, e.g., the renin-angiotensin system which in time would lead to various cardiovascular disorders including hypertension [30]. Dex is also associated with inhibition of angiogenesis and capillary growth [31,32], which may result in relatively poor vascularization. Oxygen supply to the myocardium may then become a limiting factor especially when the myocardial cells are hypertrophied. This situation may be further aggravated when oxygen demand is enhanced by increased wall stress as found in our study.

Our results in 4-week-old rats show a substantial reduction in intrinsic systolic function in the Dex-treated animals, however cardiac output and pressures are normal. The latter presumably is a result of compensatory dilatation as evident from the significantly increased ventricular volumes, indicating that the animals invoke their Starling mechanism. Combined with the reduced wall volume these alterations markedly increased wall stress. The rightward shifts of both the ESPVR, evidenced by its increased volume intercept ESV_{75}, and the EDPVR indicate a process of remodeling. The finding that end-diastolic pressures and stiffness were relatively normal despite an increased end-diastolic volume suggests structural changes such as slippage of cardiomyocytes [33]. We speculate that excessive hypertrophy initially fully compensates, but in a later stage this mechanism may fail and lead to a state of compensatory dilation (as found in our prepubertal rats) and eventually may progress to cardiac failure as suggested by the study of de Vries et al. [14].

The pressure-volume loops in our study were obtained with a miniature conductance catheter. This methodology has been extensively validated in various species including mice which are substantially smaller than 4-week-old rats [17-19]. However, the experience with the conductance catheter applied in young rats is limited. Therefore, to support our main findings, we also performed echocardiographic measurements in a small group of additional animals. The echocardiographic findings were fully consistent with the hemodynamic data obtained by the conductance catheter method.

Whether our findings are applicable to humans is unknown. At this point no cardiac abnormalities have been reported in children in the age group (pre-puberty, 10-12 year-old) corresponding to the 4-week-old rats in our study. However, from the lack of such reports we

would not draw the conclusion that findings similar to those found in our rats can be excluded in humans. First, widespread routine Dex treatment started in the early 90's, therefore the number of children that have reached puberty is still fairly limited. Second, the effects in this age group may be subclinical (as suggested by our findings in rats) and therefore such studies may not have seemed to be warranted. This may explain that, to our knowledge, no studies are available regarding the long-term effects of Dex on cardiac function (or cardiac dimensions) in humans. In contrast to possible late side effects, the early cardiac adverse effects of neonatal Dex treatment have been well studied in human babies. Serial echocardiographical measurements have documented a transient phase of LV hypertrophy which starts during treatment and is generally resolved 2-3 weeks after Dex weaning [12,13,34,35]. The same phenomenon of transient early hypertrophy has also been shown to be present in the rat model [11]. This finding supports the validity of the rat model and the treatment dose. The rat model has also been used extensively in neurological studies and these studies have demonstrated important late side effects of Dex treatment [6]. Importantly, recent clinical studies indicate very similar findings in humans [5,8,9,36], again showing the correspondence with the rat model.

The Dex treatment in our study was based on extrapolation of the human treatment protocol. The 3-day duration of the Dex exposure represents about 15% of the lactation period in the rat (21 days), which corresponds with a typical 21-days treatment period in humans. Similar to the typical clinical treatment, a tapering dose of 0.5, 0.3 and 0.1µg/g was used. The same approach was used in most previous cardiovascular and neurological studies in the rat.

Obviously, extrapolation of our data to humans must be done with extreme caution, but we feel that further hemodynamic studies in humans should be considered. Screening of children previously treated with Dex may be relevant in order to initiate early medical intervention if necessary. It is important to realize that in our study alterations in systolic function were derived from pressure-volume relations, which are difficult to obtain in clinical practice. Our data regarding ejection fraction and cardiac output, systolic and diastolic pressures, which are more commonly used in patient studies, did not reveal abnormal function. Therefore determination of absolute cardiac volumes, that were found to be enlarged, presumably as a compensatory mechanism, might be a realistic and useful diagnostic possibility. In addition, recently developed echocardiographic methods like tissue-Doppler myocardial velocity imaging or strain rate imaging may prove to be of value.

Limitations

We used term male rat pups as a model for premature human babies. This model has been proven useful for neurologic studies because with regard to brain development, a 7-day old rat is roughly equivalent to a full-term human infant and consequently term rat pups may correspond well to premature infants [37]. Furthermore, at day 1-3 after birth the rat myocardium

still shows substantial hyperplasia [28], similar to the preterm human myocardium [26,27]. Thus, with respect to the transition from myocyte hyperplasia to hypertrophy the term rat pup appears to be a good model for the preterm human infant. However, potential differences in the degree of maturation of neurohormonal systems, receptor mechanisms and innervation [38] may also be of influence in the response to Dex treatment. The development of these systems has been studied in the rat [39], but very few data are available for the (preterm) human situation. Thus, species differences may be present, but other studies [11,14,40,41] have employed the same model and shown that several of the effects of Dex treatment found in humans, including early transient hypertrophy, were also found in the rat model.

We used only male pups to limit the within-group variability of the various parameters, but potentially gender-specific responses could be present. Neurological studies appear to indicate that males are more susceptible to adverse effects of neonatal GC treatment, however we are not aware of studies showing gender-specific effects on the cardiovascular system.

The relatively low ejection fraction and mean arterial pressure in the control (Sal) animals indicate that cardiac function was somewhat depressed presumably as a result of general anesthesia. We cannot rule out that the Dex animals are more sensitive to our anesthesia resulting in relatively more depressed cardiac function. However by using fentanyl-fluanisone-midazolam anesthesia we aimed at minimizing this effect [42]. Furthermore, our additional echocardiographic measurements were obtained during isoflurane inhalation and indicated very similar differences between Dex- and Sal- treated rats as the invasive data.

In conclusion

Neonatal Dex treatment in rats leads to unfavorable changes in systolic left ventricular function in the prepubertal period. Whether this represents an early stage of heart failure remains to be demonstrated. Further studies are required to investigate the long-term cardiac effects of Dex treatment and the underlying mechanisms. If applicable to humans, a rather large patient population is involved and early cardiac screening of children treated with Dex may be indicated.

REFERENCE LIST

1. American Academy of Pediatrics Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics 2002;109:330-338
2. Mammel MC, Green TP, Johnson DE, Thompson TR Controlled trial of dexamethasone therapy in infants with bronchopulmonary dysplasia. Lancet 1983;1:1356-1358
3. Liley HG, White RT, Benson BJ, Ballard PL Glucocorticoids both stimulate and inhibit production of pulmonary surfactant protein A in fetal human lung. Proc Natl Acad Sci U S A 1988;85:9096-9100
4. Cummings JJ, D'Eugenio DB, Gross SJ A controlled trial of dexamethasone in preterm infants at high risk for bronchopulmonary dysplasia. N Engl J Med 1989;320:1505-1510
5. Bakker JM, Van Bel F, Heijnen CJ Neonatal glucocorticoids and the developing brain: short-term treatment with life-long consequences? Trends Neurosci 2001;24:649-653
6. Kamphuis PJ, Gardoni F, Kamal A, Croiset G, Bakker JM, Cattabeni F, Gispen WH, Van Bel F, Di Luca M, Wiegant VM Long-lasting effects of neonatal dexamethasone treatment on spatial learning and hippocampal synaptic plasticity: involvement of the NMDA receptor complex. FASEB J 2003;17: 911-913
7. Halliday HL The effect of postnatal steroids on growth and development. J Perinat Med 2001;29:281-285
8. Barrington KJ The adverse neuro-developmental effects of postnatal steroids in the preterm infant: a systematic review of RCTs. BMC Pediatr 2001;1:1
9. Yeh TF, Lin YJ, Huang CC, Chen YJ, Lin CH, Lin HC, Hsieh WS, Lien YJ Early dexamethasone therapy in preterm infants: a follow-up study. Pediatrics 1998;101:E7
10. Doyle L, Davis P Postnatal corticosteroids in preterm infants: systematic review of effects on mortality and motor function. J Paediatr Child Health 2000;36:101-107
11. La Mear NS, MacGilvray SS, Myers TF Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol Neonate 1997;72:175-180
12. Werner JC, Sicard RE, Hansen TW, Solomon E, Cowett RM, Oh W Hypertrophic cardiomyopathy associated with dexamethasone therapy for bronchopulmonary dysplasia. J Pediatr 1992;120: 286-291
13. Israel BA, Sherman FS, Guthrie RD Hypertrophic cardiomyopathy associated with dexamethasone therapy for chronic lung disease in preterm infants. Am J Perinatol 1993;10:307-310
14. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr Res 2002;52:900-906
15. Kamphuis PJ, Croiset G, Bakker JM, Wiegant VM, Van Bel F Neonatal dexamethasone treatment results in reduction of life-span in rats. Pediatr-Res 2001;49:299A (abstr)
16. Baan J, Van Der Velde ET, De Bruin H, Smeenk G, Koops J, Van Dijk AD, Temmerman D, Senden J, Buis B Continuous measurement of left ventricular volume in animals and humans by conductance catheter. Circulation 1984;70:812-823
17. Georgakopoulos D, Mitzner WA, Chen CH, Byrne BJ, Millar HD, Hare JM, Kass DA In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. American Journal of Physiology 1998;274:1416-22
18. Yang B, Larson DF, Beischel J, Kelly R, Shi J, Watson RR Validation of conductance catheter system for quantification of murine pressure-volume loops. J Invest Surg 2001;14:341-355
19. Feldman MD, Erikson JM, Mao Y, Korcarz CE, Lang RM, Freeman GL Validation of a mouse conductance system to determine LV volume: comparison to echocardiography and crystals. Am J Physiol Heart Circ Physiol 2000;279:H1698-H1707
20. Steendijk P, Baan J Comparison of intravenous and pulmonary artery injections of hypertonic saline for the assessment of conductance catheter parallel conductance. Cardiovascular Research 2000;46:82-89

21. Kass DA, Maughan WL, Guo ZM, Kono A, Sunagawa K, Sagawa K Comparative influence of load versus inotropic states on indexes of ventricular contractility: experimental and theoretical analysis based on pressure-volume relationships. Circulation 1987;76:1422-1436
22. Little WC The left ventricular dP/dtmax-end-diastolic volume relationship in closed-chest dogs. Circ Res 1985;56:808-815
23. Glower DD, Spratt JA, Snow ND, Kabas JS, Davis JW, Olsen CO, Tyson GS, Sabiston DC, Rankin SJ Linearity of the Frank-Starling relationship in the intact heart: the concept of preload recruitable stroke work. Circulation 1985;71:994-1009
24. Steendijk P, Baan Jj, Van Der Velde ET, Baan J Effects of critical coronary stenosis on global systolic left ventricular function quantified by pressure-volume relations during dobutamine stress in the canine heart. J Am Coll Cardiol 1998;32:816-828
25. Arts T, Bovendeerd PH, Prinzen FW, Reneman RS Relation between left ventricular cavity pressure and volume and systolic fiber stress and strain in the wall. Biophys J 1991;59:93-102
26. Huttenbach Y, Ostrowski ML, Thaller D, Kim HS Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. Cardiovasc Pathol 2001;10:119-123
27. Mayhew TM, Pharaoh A, Austin A, Fagan DG Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors. J Anat 1997;191 (Pt 1):107-115
28. Li F, Wang X, Capasso JM, Gerdes AM Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28:1737-1746
29. Rudolph AM, Roman C, Gournay V Perinatal myocardial DNA and protein changes in the lamb: effect of cortisol in the fetus. Pediatr Res 1999;46:141-146
30. Dodic M, Peers A, Coghlan JP, Wintour M Can excess glucocorticoid predispose to cardiovascular and metabolic disease in middle age? Trends Endocrinol Metab 1999;10:86-91
31. Hasan Q, Tan ST, Xu B, Davis PF Effects of five commonly used glucocorticoids on haemangioma in vitro. Clin Exp Pharmacol Physiol 2003;30:140-144
32. Lansink M, Koolwijk P, van H, V, Kooistra T Effect of steroid hormones and retinoids on the formation of capillary-like tubular structures of human microvascular endothelial cells in fibrin matrices is related to urokinase expression. Blood 1998;92:927-938
33. Olivetti G, Quaini F, Lagrasta C, Cigola E, Ricci R, Maestri R, Anversa P Cellular basis of ventricular remodeling after myocardial infarction in rats. Cardioscience 1995;6:101-106
34. Zecca E, Papacci P, Maggio L, Gallini F, Elia S, De Rosa G, Romagnoli C Cardiac adverse effects of early dexamethasone treatment in preterm infants: a randomized clinical trial. J Clin Pharmacol 2001;41:1075-1081
35. Skelton R, Gill AB, Parsons JM Cardiac effects of short course dexamethasone in preterm infants. Arch Dis Child Fetal Neonatal Ed 1998;78:F133-F137
36. Heide-Jalving M, Kamphuis PJ, van der Laan MJ, Bakker JM, Wiegant VM, Heijnen CJ, Veen S, Van Bel F Short- and long-term effects of neonatal glucocorticoid therapy: is hydrocortisone an alternative to dexamethasone? Acta Paediatr 2003;92:827-835
37. Flagel SB, Vazquez DM, Watson SJ, Jr., Neal CR, Jr. Effects of tapering neonatal dexamethasone on rat growth, neurodevelopment, and stress response. Am J Physiol Regul Integr Comp Physiol 2002;282:R55-R63
38. Segar JL, Lumbers ER, Nuyt AM, Smith OJ, Robillard JE Effect of antenatal glucocorticoids on sympathetic nerve activity at birth in preterm sheep. Am J Physiol 1998;274:R160-R167
39. Robinson RB Autonomic receptor--effector coupling during post-natal development. Cardiovasc Res 1996;31:E68-E76
40. Muangmingsuk S, Ingram P, Gupta MP, Arcilla RA, Gupta M Dexamethasone induced cardiac hypertrophy in newborn rats is accompanied by changes in myosin heavy chain phenotype and gene transcription. Mol Cell Biochem 2000;209:165-173

41. Sicard RE, Werner JC Dexamethasone induces a transient relative cardiomegaly in neonatal rats. Pediatr Res 1992;31:359-363
42. Jong WM, Zuurbier CJ, De Winter RJ, Van Den Heuvel DA, Reitsma PH, Ten Cate H, Ince C Fentanyl-fluanisone-midazolam combination results in more stable hemodynamics than does urethane alpha-chloralose and 2,2,2-tribromoethanol in mice. Contemp Top Lab Anim Sci 2002;41:28-32

CHAPTER 4

LEFT VENTRICULAR PRESSURE-VOLUME RELATIONSHIPS DURING NORMAL GROWTH AND DEVELOPMENT IN THE ADULT RAT: STUDIES IN 8- AND 50-WEEK-OLD MALE WISTAR RATS

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Acta Physiol Scand 2005; 185: 181-91

ABSTRACT

Left ventricular (LV) pressure-volume relations provide relatively load-independent indexes of systolic and diastolic LV function, but few data are available on pressure-volume relations during growth and development in the normal adult heart. Furthermore, to quantify intrinsic ventricular function the indexes should be normalized for heart weight. However, in many studies the indexes are reported in absolute terms, or body weight-correction is used as a surrogate for heart weight-correction.

We determined pressure-volume relations in young (8 weeks old, n=13) and middle-aged (50 weeks old, n=19) male Wistar rats in relation to their heart and body weights. The animals were anesthetized and a 2F pressure-conductance catheter was introduced into the LV to measure pressure-volume relations.

Heart and body weights were significantly higher in the 50-week-old rats, whereas the heart-to-body weight ratio was significantly lower (2.74±0.32 vs. 4.41±0.37 mg/g, $p<0.001$). Intrinsic systolic function, quantified by the slopes of the end-systolic pressure-volume relation (E_{ES}), the dP/dt_{MAX} vs. end-diastolic volume relation (S-dP), and the preload recruitable stroke work relation (PRSW), normalized for heart weight, was slightly decreased in the 50-week-old rats (S-dP: -26%, $p<0.004$; PRSW: -13%, $p<0.06$). Heart weight-corrected diastolic indexes were not significant different. The absolute indexes qualitatively showed the same results, but body-weight corrected pressure-volume indexes showed improved systolic function and significantly depressed diastolic function.

Intrinsic systolic function slightly decreases from the juvenile to the middle-aged period in normal male Wistar rats. Furthermore, correction of pressure-volume indexes for body weight is not an adequate surrogate for heart weight-correction in these animals.

INTRODUCTION

Systolic and diastolic left ventricular pressure-volume relations reflect inotropic and lusitropic properties of the left ventricle [37]. These relations are relatively independent of loading conditions and therefore they are considered the best way to quantify intrinsic ventricular function [22]. Indexes derived from pressure-volume relations have shown to be highly sensitive to changes in myocardial function induced by various diseases and pharmacological interventions, and consequently these indexes are widely used in physiological and clinical studies. However, few data are available on the changes in pressure-volume relations during growth and development in the normal adult heart. Most studies focus either on the maturation in very early stages, or on the pathology in the senescent heart [12,30]. In the intermediate age-range, representing the young to middle-aged adults, it is often implicitly assumed that cardiac function is relatively constant.

Furthermore, theoretical and experimental studies indicate that indexes derived from pressure-volume relations should be normalized for heart (or ventricular) weight [6,17,38,45]. However, in many studies the indexes are either reported in absolute terms or are indexed for body weight (or body surface area) as a surrogate for heart weight. Justification for these corrections is generally not given and, to our knowledge, no previous study directly compared the effect of the various corrections on the derived indexes.

Therefore, in the present paper we studied systolic and diastolic pressure-volume relations in normal 8- and 50-week-old rats, representing the young adult and middle-aged stages. Data were obtained invasively in closed-chest, anesthetized animals, by miniaturized pressure-conductance catheters [1,2,15]. We determined the age, heart-weight and body-weight dependence of the derived indexes and investigated the influence of normalization.

MATERIALS AND METHODS

The study protocol was approved by the Animal Research Committee of the University of Leiden. The investigation conforms to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985).

Animals and instrumentation

Male Wistar rats were obtained from our in-house breeding and were studied at 8 weeks (n=13) and 50 weeks (n=19) of age. Prior to the studies the animals were kept under constant temperature and humidity conditions and had free access to food and water. The rats were sedated by inhalation of a mixture of halothane (4%) and oxygen. Subsequently, general anesthesia was initiated by intraperitoneal (IP) injection of a fentanyl-fluanison-midazolam

mixture (FFM). The FFM mixture consisted of 2 parts Hypnorm® (0.315 mg/mL fentanyl + 10 mg/mL fluanison), 1 part Dormicum® (5 mg/mL midazolam) and 1 part saline. This mixture was administered in a dose of 0.4 mL/100 g body weight. Supplemental IP injections (one-third of the initial dose) were provided if necessary so that the animals remained unresponsive to tail pinch by forceps. The animals were placed on a controlled warming pad to keep body temperature constant. A tracheostomy was performed, a 25G cannula was inserted and connected to a pressure-controlled respirator, and ventilation was started with an air/oxygen mixture (FiO_2 = 0.5). The animals were placed under a microscope (Zeiss, Germany) and the left jugular vein and the right carotid artery were exposed via a midline cervical incision. The jugular vein was cannulated for infusion of hypertonic saline to determine parallel conductance (see below). Via the carotid artery a miniaturized combined pressure-conductance catheter (SPR-878, Millar Instruments, Houston, TX, USA) was introduced and positioned into the left ventricle (LV) guided by on-line pressure and volume signals. The abdomen was opened via a small incision just below the diaphragm to enable temporary preload reductions by directly compressing the inferior vena cava using a cotton-tipped stick. The pressure-conductance catheter was connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, The Netherlands) for on-line display and registration of LV pressure and volume signals. All data were acquired using Conduct-NT software (CD Leycom) at a sample rate of 2000 Hz and analyzed off-line by custom-made software (CircLab).

Hemodynamic measurements

Steady state

After instrumentation, LV pressure-volume signals were acquired in steady state to quantify general hemodynamic conditions: Heart rate, stroke volume, cardiac output, end-diastolic volume, end-systolic volume, ejection fraction, end-diastolic pressure, and end-systolic pressure were assessed. Mean arterial pressure was determined from the aortic pressure recording obtained just prior to insertion of the catheter into the LV. Stroke work was determined as the area of the pressure-volume loop, and the maximal and minimal rate of LV pressure change, dP/dt_{MAX} and dP/dt_{MIN}. The relaxation time constant τ was calculated as the time-constant of mono-exponential pressure decay during isovolumic relaxation: $P(t) = A+B\cdot\exp(-t/\tau)$. The isovolumic period was defined as the time-period between the moment of dP/dt_{MIN} and the time-point at which $dP(t)/dt$ reached 10% of dP/dt_{MIN} [23].

Pressure-volume relationships

To obtain load-independent indices of systolic and diastolic LV function we determined pressure-volume relations by recording pressure-volume loops during a gradual preload reduction obtained by gently compressing the inferior caval vein. By this procedure we reduced systolic pressure typically by 20-30 mmHg within 2 s (~15 beats). To quantify systolic function

we used the end-systolic pressure-volume relation (ESPVR), the relation between dP/dt_{MAX} and end-diastolic volume, and the preload recruitable stroke work relation (stroke work vs. end-diastolic volume). The slopes of these linear relations, E_{ES} (end-systolic elastance), S-dP, and PRSW, respectively, are sensitive and relatively load-independent measures of systolic LV function [16,24,39]. For diastolic function the chamber stiffness constant k_{ED} was determined by fitting the end-diastolic pressure-volume points with an exponential relation [27]. Since some previous studies have used a linear approach, we also determined end-diastolic stiffness E_{ED} as the slope of a linear relation fitted to the end-diastolic pressure-volume points.

Calibration of the conductance catheter

To derive absolute volumes from the conductance catheter, the signals must be calibrated for parallel conductance (V_p) and slope factor (α). Parallel conductance was determined by the hypertonic saline method [2]. We performed intravenous injections via the jugular vein cannula (10% saline, 20μL) and parallel conductance was calculated as the mean of three consecutive assessments [43,48]. To determine slope factor α we placed a transit-time ultrasonic flow probe (Transonic Systems, Maastricht, The Netherlands) on the ascending aorta, after opening the thorax at the end of the experiment, via a midsternal incision: α was calculated as cardiac output determined by conductance divided by cardiac output determined by aortic flow.

Heart preparation

After all hemodynamic measurements, the abdominal aorta was cannulated and we performed a perfusion fixation of the heart with 10% formalin diluted 1:1 with NaCl. Subsequently, the heart was excised, extracardiac structures were carefully removed and weight was obtained.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Variability of a specific index was quantified by the coefficient of variation calculated as 100%·SD/mean. Comparisons between group means were performed by unpaired t-tests and statistical significance was defined as $p<0.05$.

RESULTS

Heart and body weights

As expected heart weight and body weight were significantly higher in the 50-week-old rats, whereas heart-to-body weight ratio was significantly reduced (Table 1, Figure 1). Plotting heart weight versus body weight showed a positive correlation in the 8-week-old rats, however the correlation was lost in the 50-week-old animals (Figure 2).

Table 1: Body weight, heart weight, and heart-to-body weight ratio in 8- and 50-week-old rats

	8 weeks	50 weeks	p
BW (g)	261 ± 32	541 ± 40	< 0.001
HW (mg)	1,146 ± 140	1,476 ± 103	< 0.001
HW/BW (mg/g)	4.41 ± 0.37	2.74 ± 0.32	< 0.001

BW = body weight; HW = heart weight

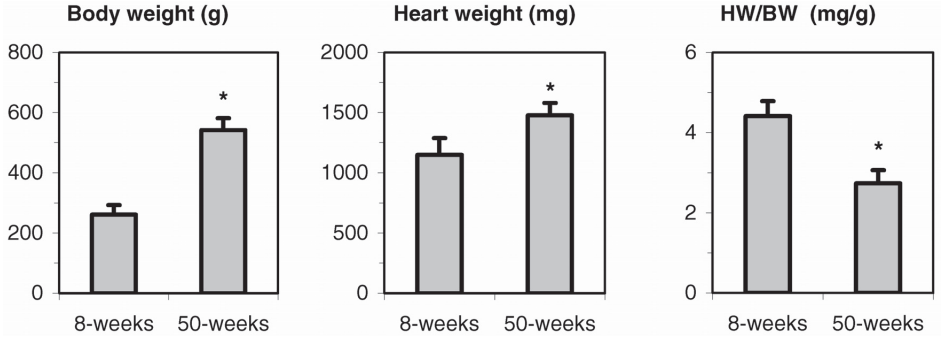

Figure 1: Body weight (BW), heart weight (HW) and HW/BW ratio in 8- and 50-week-old rats

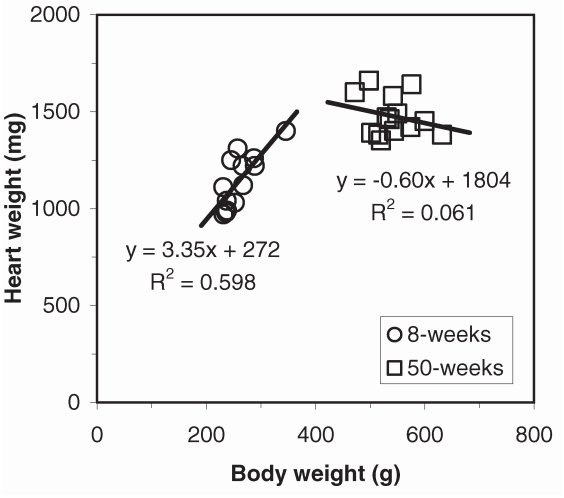

Figure 2: Relation between heart weight and body weight in 8- and 50-week-old rats

Pressure-derived indexes

Heart rate and pressure-derived indexes are shown in Table 2. Heart rate, mean arterial pressure, total peripheral resistance, end-systolic pressure, dP/dt_{MAX} and dP/dt_{MIN} were all significantly lower in the 50-week-old rats. We did not find a significant correlation between heart weight or the heart-to-body weight ratio and mean arterial pressure in the 8-week-old or the 50-week-old rats. End-diastolic pressure and τ tended to be increased but differences were not statistically significant.

Table 2: Heart rate and pressure-derived indexes in 8- and 50-week-old rats

	8 weeks	50 weeks	p
HR (beats/min)	444 ± 21	415 ± 24	< 0.001
MAP (mmHg)	72 ± 8	52 ± 13	< 0.001
TPR (mmHg/ml/min)	0.96 ± 0.18	0.59 ± 0.25	<0.001
LVESP (mmHg)	82 ± 14	57 ± 8	< 0.001
LVEDP (mmHg)	4.4 ± 1.7	5.0 ± 1.5	0.300
dP/dt_{MAX} (mmHg/s)	11,933 ± 1,612	9,973 ± 2,651	0.016
dP/dt_{MIN} (mmHg/s)	6,810 ± 1,776	4,588 ± 930	0.001
τ (ms)	10.4 ± 1.9	10.9 ± 3.2	0.637

HR = heart rate; MAP = mean arterial pressure; TPR = total peripheral resistance; LVESP = left ventricular end-systolic pressure; LVEDP = left ventricular end-diastolic pressure; τ = relaxation time constant

Volumetric indexes

Volumetric indexes (cardiac output, stroke volume, end-diastolic volume and end-systolic volume) are shown in Table 3. They are presented in absolute terms, after correction for body weight, and after correction for heart weight, respectively (note that ejection fraction, which is the ratio of stroke volume and end-diastolic volume, is invariant to weight correction). The results show that all absolute volumes were significantly increased in the 50-week-old rats. However, the indexed (i.e. divided by body weight) cardiac output and stroke volumes were significantly decreased, whereas indexed end-diastolic volume was unchanged, and indexed end-systolic volume was increased in the 50-week-old rats. After normalization for heart weight, cardiac output and stroke volume were the same in both groups, whereas end-systolic and end-diastolic volumes were significantly higher in the 50-week-old rats.

Pressure-volume relations

Typical examples of pressure-volume loops and pressure-volume relations in 8- and 50-week-old rats are shown in Figure 3. Summarized results of the slopes of the LV pressure-volume relations are presented in Table 4. Like the volumetric indexes they are given in absolute terms and after correcting for body weight and heart weight, respectively. To avoid confusion, note that the slope of the ESPVR, E_{ES}, represents the change in end-systolic pressure divided by the change in end-systolic volume. Since the correction for body (respectively heart) weight

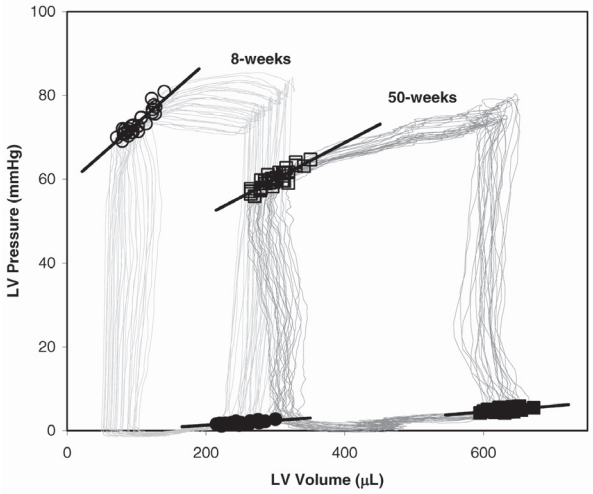

Figure 3: Typical examples of pressure-volume loops and end-systolic (ESPVR) and end-diastolic (EDPVR) pressure-volume relations. Open circles: ESPVR in an 8-week-old rat; closed circles: EDPVR in an 8-week-old rat; open squares: ESPVR in a 50-week-old rat; closed squared: EDPVR in a 50-week-old rat.

is done by dividing end-systolic volume by body (heart) weight, the slope E_{ES} is corrected by multiplying its absolute value by body (heart) weight. The same reasoning also holds for S-dP, k_{ED} and E_{ED}. PRSW, however, is unaffected by weight corrections because the determinants of this relation (stroke work and end-diastolic volume) are both volumetric indexes.

In absolute terms there was a significant decrease in S-dP and PRSW in the 50-week-old rats, whereas the other indices remained constant. Indexing the pressure-volume relations for body weight results in a significant increase in all indexes in the 50-week-old rats. After normalization for heart weight S-dP showed a significant decrease in the 50-week-old rats, whereas the other indexes tended to be increased, but this effect did not reach statistical significance.

Variability

We calculated the coefficient of variation of the various volumetric and pressure-volume indexes to investigate whether correction for body weight or heart weight reduced the within-group variability. Results are shown in Figure 4. For the volumetric indexes correction for body weight resulted in a small but systematic reduction in variability: On the average variability was reduced from 33.1 to 30.2% in the 8-week-old animals, and from 35.3 to 33.6% in the 50-week-old animals. Correction for heart weight also tended to reduce variability, but this effect was even smaller and not consistent for all indexes. For the slopes of the pressure-volume relations, E_{ES} and S-dP, correction for body weight and for heart weight both consistently reduced variability, with a larger reduction by the correction for heart weight. In the 8-week-old animals, variability reduced from 45.0 to 40.4% with body weight correction, and to 38.5% with heart weight correction. In the 50-week-old animals, the variability reduced from 53.3 to 52.6% and to 49.3%, respectively. As mentioned earlier ejection fraction and PRSW are invariant to weight corrections, but these indexes are shown in Figure 4 for comparison.

Table 3: Volumetric indexes in 8- and 50-week-old rats

	Absolute volumes			Indexed volumes (per g body weight)			Normalized volumes (per g heart weight)		
	8 weeks	50 weeks	p	8 weeks	50 weeks	p	8 weeks	50 weeks	p
CO (mL/min)	77 ± 14	99 ± 33	0.016	0.30 ± 0.05	0.18 ± 0.06	<0.001	68 ± 12	64 ± 19	0.554
SV (µL)	175 ± 34	238 ± 75	0.003	0.67 ± 0.12	0.44 ± 0.13	<0.001	153 ± 27	154 ± 44	0.962
LVEDV (µL)	292 ± 97	599 ± 209	<0.001	1.12 ± 0.33	1.11 ± 0.37	0.938	255 ± 80	383 ± 136	0.007
LVESV (µL)	123 ± 76	368 ± 151	<0.001	0.47 ± 0.27	0.68 ± 0.28	0.036	107 ± 63	234 ± 102	0.001
LVEF (%)	61 ± 11	40 ± 9	<0.001						

CO = cardiac output; SV = stroke volume; LVEDV = left ventricular end-diastolic volume; LVESV = left ventricular end-systolic volume; LVEF = left ventricular ejection fraction

Table 4: Pressure-volume indexes in 8- and 50-week-old rats

	Absolute			Indexed for body weight (g)			Normalized for heart weight (g)		
	8 weeks	50 weeks	p	8 weeks	50 weeks	p	8 weeks	50 weeks	p
E_{ES} (mmHg/µL)	0.188±0.068	0.168±0.090	0.483	48.0±16.1	89.6±47.0	0.002	0.213±0.076	0.270±0.131	0.174
S-dP (mmHg/s/µL)	20.4±11.0	10.9±5.8	<0.001	5123±2424	5842±3080	0.006	22.4±9.9	16.6±8.3	0.004
PRSW (mmHg)	47.0±8.6	41.0±8.3	0.060						
k_{ED} (1/µL)	0.0056±0.0031	0.0058±0.0061	0.890	1.42±0.68	3.10±3.24	0.041	0.0063±0.0030	0.0094±0.0097	0.264
E_{ED} (mmHg/µL)	0.020±0.010	0.022±0.018	0.671	5.11±2.81	11.60±9.14	0.008	0.022±0.012	0.033±0.028	0.198

E_{ES} = end-systolic elastance; S-dP = slope of the dP/dt_{MAX} vs. end-diastolic volume relation; PRSW = preload recruitable stroke work; k_{ED} = diastolic stiffness constant; E_{ED} = diastolic stiffness

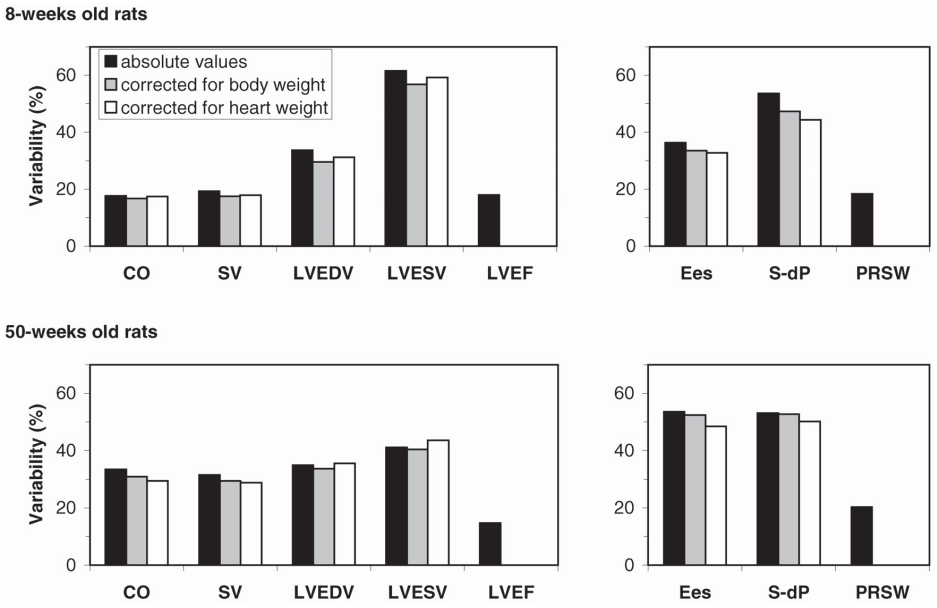

Figure 4: Coefficient of variation of various volumetric and pressure-volume indexes in 8- and 50-week-old rats

DISCUSSION

This study was conducted to gain insight into the possible changes in cardiac function during normal growth and development in adult rats. In addition, we investigated the influence of correction of hemodynamic indexes by body weight and heart weight. Left ventricular function was quantified by indexes derived from pressure-volume relations in 8- and 50-week-old rats. We used these two age groups because they represent the young adult and middle-aged stages. Our findings regarding heart weight and body weight show, as expected, that heart and body weight were significantly higher in the 50-week-old rats, whereas the heart-to-body weight ratio was significantly lower in the 50-week-old rats. In the 8-week-old rats a close correlation was found between heart weight and body weight, but this relation was absent in the 50-week-old animals. The relation between heart weight and body weight in rats was studied previously by other groups [14,35] showing a decline in heart-to-body weight ratio with increasing age consistent with our findings. In contrast, Capasso et al. [9] found a remarkably constant heart-to-body weight ratio in female Wistar rats from 2 to 10 months of age. A linear increase in heart weight with body weight in young rats was previously demonstrated [34]. A similar effect was also observed in human studies, showing that in infancy virtually the entire variability of left ventricular mass is explained by body size whereas with advancing age the ability of body size to predict left ventricular mass decreases [13].

Our findings regarding heart and body weights already predict that comparison of hemodynamic indexes between the groups will strongly depend on whether correction for body weight or for heart weight is performed. This may complicate the answer to the question whether cardiac function was different between the age groups. With regard to the contractile indexes derived from pressure-volume relations, theoretically correction for heart weight is optimal to derive the 'intrinsic' cardiac properties and correction for body weight should be regarded as a surrogate method [6,17,38,45]. In addition to the attempt to obtain an index that reflects the 'intrinsic' properties of the muscle, an (often implicit) aim of normalization in many studies is to reduce the within-group variability. Typically two groups are compared (e.g. treated vs. untreated) and the variation in heart (of body) weight in each group is assumed to be random and not systematically different between the groups. By correcting contractile indexes for heart (body) weight one hopes to reduce the within-group variability and increase the sensitivity to detect differences between groups. Our analysis of within-group variability shown in Figure 4 reveals that the reduction in variability by weight corrections was only very limited. It shows that for the volumetric indexes the variability is reduced mostly by body weight correction, whereas in the pressure-volume derived indexes heart weight correction produced a better reduction in variability. Apparently, biological variability and variability induced by other sources, such as experimental conditions (anesthesia, temperature, etcetera), play a more important role.

In this study we used multiple pressure-volume relations to compare LV function in the two age groups. For LV systolic function, we determined the end-systolic pressure-volume relation, the preload recruitable stroke work relation, and the relation between dP/dt_{MAX} and end-diastolic volume, and for LV diastolic function the end-diastolic pressure-volume relation. In the literature, the systolic relations are frequently used interchangeably and they typically show the same directional changes after positive or negative inotropic stimulation [25,44,46,47]. However, inotropic sensitivity and relative load independency were reported to vary between these relations. Takeuchi et al. [46] reported a comparable inotropic sensitivity of the end-systolic pressure-volume relation and the preload recruitable stroke work relation, but found the latter to be less load-dependent. Wallace et al. [47] found an equal load-independency for all three relations. Kass et al. [22] described the end-systolic pressure-volume relations as the least sensitive with regard to inotropic changes, but the most load-independent relation. In contrast, Little et al. [25] found the preload recruitable stroke work relation to be the least sensitive to intropic stimulation, and the end-systolic pressure-volume relation the most dependent on load changes. Partial inconsistencies between these studies may be related to species and methodological differences. Moreover, although all of these relations are used to quantify systolic function, they are not fully comparable because the end-systolic pressure-volume relation is defined at end-ejection, dP/dt_{MAX} vs. end-diastolic volume at pre-ejection, and the preload recruitable stroke work relation describes more globally the entire cardiac cycle.

Our data show that intrinsic systolic function, as quantified by the slopes of the systolic relations (E_{ES}, S-dP, and PRSW) normalized for heart weight, appeared to be slightly decreased in the 50-week-old rats: although E_{ES} was not significantly changed, S-dP was reduced by 26% ($p<0.004$), and PRSW by 13% ($p<0.06$). The heart weight-normalized diastolic pressure-volume indexes k_{ED} and E_{ED} both increased by approximately 50% suggesting increased diastolic stiffness (i.e. reduced diastolic function), but these changes were not significant. In a qualitative sense, the absolute indexes (i.e. without correction for weight) showed the same results. However, after correction for body weight the systolic indexes E_{ES} and S-dP both improved significantly, whereas the diastolic indexes showed a significantly increased diastolic stiffness. This indicates that correction for body weight is not an adequate surrogate for heart weight-correction when comparing 8-week-old and 50-week-old animals. This is due to the fact that the heart-to-body weight ratio was substantially lower in the 50-week-old rats and thus the body weight-corrected pressure-volume indexes were relatively overestimated in the older animals.

The decrease in systolic function obtained from the heart weight-normalized pressure-volume relations is consistent with the reduction in ejection fraction and dP/dt_{MAX}. The finding that the normalized volumes were significantly increased, whereas normalized CO was unchanged, suggests the use of the Frank-Starling mechanism to compensate for reduced systolic function.

To our knowledge no previous studies have investigated intrinsic LV function by pressure-volume relations in a similar adult age range in intact animals. The effects of more advanced aging on myocardial function were studied with pressure-volume loops by Pacher et al. [30]. They found decreased systolic function accompanied by delayed relaxation and increased diastolic stiffness in 2-years-old male Fischer 344 rats, compared to 6-months-old animals. Cardiac function in these aging rats reflected a condition of heart failure consistent with the fact that ~50% of the animals in this group died during the observation period with dilated hearts and marked fluid accumulation. In a recent study [32] it was shown that these effects may be importantly counteracted by inhibition of poly(ADP-ribose) polymerase (PARP), indicating a prominent role of oxidant-induced cell injury in cardiac dysfunction with aging. Capasso et al. [8] documented hemodynamic function at 4, 12, 20 and 29 months of age in male Fischer rats. Their data confirm the findings of Pacher et al. in the senescent animals and show that the decline in cardiac function occurs largely after 20 months. Data from 12- and 20-months-old animals only show a tendency towards reduced diastolic function, but no change in systolic function. However, no load-independent pressure-volume indexes were obtained. The changes in the 12-months old group are in line with our findings in the 50-week old animals for cardiac output, cardiac index, dP/dt_{MIN}, diastolic pressure, end-systolic and end-diastolic volume. The drop in dP/dt_{MAX} was similar in both studies but it did not reach statistical significance in Capasso's study. Moreover, systolic pressure was unchanged in Capasso's study but dropped significantly in ours. These differences may be related to the

different anesthetic approaches (see Limitations section), but they may also be dependent on the rat strain (Fischer vs. Wistar).

Additional insight into the age-associated changes in contractility may be obtained from basal *in vitro* studies. Heller et al. [18] studied cardiac cell preparations from 53 and 153-day-old rats and found a 20% decrease in actomyosin ATPase activity. Since myofilament ATPase activity is related to contractile function, including the speed and duration of contraction [40] this suggests an age-related decrease in contractility comparable to our findings. Studies by Chesky et al. [10,36] suggest that the declining actomyosin ATPase activity represents an age-related diminished ability of calcium to reverse the inhibitory action of the regulatory proteins. Altered ATPase activity may also be related to myosin iso-enzyme make-up shift from fast cycling V1 to a slower cycling V3, which occurs with increasing age [19]. Qi et al. [33] proposed that this shift in myosin iso-enzymes underlies the changes in contractile function found in papillary muscles isolated from 7 weeks, 4 months and 9 months old rats. However, the differences between preparations from younger and older rats also depended on extracellular calcium concentrations, suggesting that the age-related changes are at least partly the result of differences in calcium availability and handling. In line with this, recent studies show that alterations in intracellular calcium handling in older animals are compensated by prolongation of the action potential [20] leading to maintained contractile function. In contrast, a study by Nair et al. [29] shows an age-associated increase in contractility in isolated myocytes from 2, 6 and 12-months old rats. Their data suggest that this age-dependent variation may be mediated by variation in the distribution and function of sarcolemmal calcium channels. Thus, the reported molecular and cellular changes are largely in line with our findings, but mechanical, electrical and structural changes may interact and serve as compensatory mechanisms. Thus, it remains difficult to predict how these findings from *in vitro* studies translate to changes in cardiac function in the intact animal.

Limitations

The blood pressures in our study were relatively low in comparison to other studies in anesthetized rats [4,8,11,14,26,30-32,35]. This could be related to the type of anesthesia. We used a fentanyl-fluanison-midazolam (FFM) mixture whereas most other comparable studies in rats used barbiturates (pentobarbital) or ketamine/xylazine. We preferred FFM because it is reported to have only relatively mild cardiac depressant effects, and is associated with stable, near-physiological hemodynamic conditions [7,21]. In contrast, barbiturates may interfere with calcium transports and tend to decrease contractility, whereas ketamine is reported to have a strong peripheral vasopressor effect that is considered to be centrally mediated. In line with our findings, in comparison to pentobarbital, FFM is reported to produce lower blood pressure mainly related to a better preserved tissue perfusion [42]. The dP/dt_{MAX} and heart rate values in our study are in the same range or even higher than most other studies in anesthetized rats, suggesting that indeed myocardial depression is limited and that the

low blood pressures are mainly the result of peripheral vasodilatory effects. This is confirmed by the calculated total peripheral resistance in our study which was substantially lower than values reported in rats under barbiturate anesthesia [5,30]. Since our outcome variables derived from pressure-volume relations are relatively load-independent, the lower blood pressures do not importantly affect with our main conclusions. We cannot exclude possible age-dependent cardiovascular effects of the anesthesia used in our study, however we have no indications from the literature that this would be the case. Another potential bias could be related to the body weight differences between the two groups: the anesthesia dosage (in ml/g body weight) was the same in both groups, but differences in body composition may have resulted in differences in pharmacokinetics.

Stroke volumes (and cardiac output) in the present study appear to be somewhat higher than recently reported values in anesthetized rats. The 8-week-old rats in our study had an average stroke volume of 175±34 µL. In young rats of similar body weight (~300g) Nahrendorf et al. [28] found 127±8 µL, whereas Pacher et al. [30] reported 124±9 µL, and from a study by Batkai et al. [4] we derived a stroke volume of approximately 160 µL. These differences may be related to anesthesia since these mentioned studies all used barbiturates, whereas we applied FFM. In addition, differences may be related to rat strain and/or gender, since the previous studies were performed in female Wistar rats, male Fischer 344 rats, and male Spray-Dawley rats, respectively, whereas we used male Wistar rats.

Body weight, absolute and relative heart weights, and age-dependent weight gains vary substantially between different rat strains [41]. Our findings were obtained in male Wistar rats and therefore further studies are required to investigate whether our conclusions can be extrapolated to other strains.

The 4-electrode pressure-conductance catheter used in the present study registers only a single volume segment, in contrast to multiple segments that may be obtained with 10-12 electrode catheters used in larger hearts. The implicit assumption is that this segment is representative for the heart as a whole. This assumption seems very reasonable, especially in view of the fact that in many (small animal) studies volume estimates are based on a single diameter or cross-sectional area. Underestimation was corrected by comparing the stroke volume obtained with the conductance catheter with stroke volume (SV) obtained from a flow probe placed on the ascending aorta. The gain factor ($SV_{CONDUCTANCE}/SV_{FLOW\ PROBE}$) was used to calibrate the volume readings from the catheter. This approach of using an independent stroke volume or cardiac output measurement to calibrate the conductance catheter is used in most previous conductance catheter studies. However, in small animals, particularly in the mice, reliable independent cardiac output measurements may be difficult to obtain. Therefore, several studies have used *in-vitro* calibration methods based on conductance measurements in cylindrical holes with known diameters [30,48]. In a previous study in 4-week-old rats we have used a similar *in-vitro* calibration method [3], however in the present study the larger

size of the animals allowed us to place a flow probe on the ascending aorta, and we preferred this more direct *in-vivo* calibration method.

In conclusion, our study indicates that intrinsic systolic left ventricular function slightly decreases in the adult male Wistar rat during normal growth and development from the juvenile to the middle-aged period, whereas diastolic function does not change significantly. Pressure-volume indexes for intrinsic LV function require normalization for heart weight. Correction for body weight is not a good surrogate because the decreased heart-to-body weight ratio in the older male Wistar rats causes a relative overestimation of the body-weight corrected pressure-volume indexes.

REFERENCE LIST

1. Baan J, Jong TT, Kerkhof PL, Moene RJ, van Dijk AD, van der Velde ET, Koops J Continuous stroke volume and cardiac output from intra-ventricular dimensions obtained with impedance catheter. Cardiovasc Res 1981;15:328-334
2. Baan J, van der Velde ET, de Bruin HG, Smeenk GJ, Koops J, van Dijk AD, Temmerman D, Senden J, Buis B Continuous measurement of left ventricular volume in animals and humans by conductance catheter. Circulation 1984;70:812-823
3. Bal MP, de Vries WB, van der Leij FR, van Oosterhout MF, Berger RM, Baan J, van der Wall EE, van Bel F, Steendijk P Neonatal glucocorticosteroid treatment causes systolic dysfunction and compensatory dilation in early life: studies in 4-week-old prepubertal rats. Pediatr Res 2005;58:46-52
4. Batkai S, Pacher P, Jarai Z, Wagner JA, Kunos G Cannabinoid antagonist SR-141716 inhibits endotoxic hypotension by a cardiac mechanism not involving CB1 or CB2 receptors. Am J Physiol Heart Circ Physiol 2004;287:H595-H600
5. Batkai S, Pacher P, Osei-Hyiaman D, Radaeva S, Liu J, Harvey-White J, Offertaler L, Mackie K, Rudd MA, Bukoski RD, Kunos G Endocannabinoids acting at cannabinoid-1 receptors regulate cardiovascular function in hypertension. Circulation 2004;110:1996-2002
6. Beyar R, Sideman S Relating left ventricular dimension to maximum elastance by fiber mechanics. Am J Physiol 1986;251:R627-R635
7. Brammer A, West CD, Allen SL A comparison of propofol with other injectable anaesthetics in a rat model for measuring cardiovascular parameters. Lab Anim 1993;27:250-257
8. Capasso JM, Palackal T, Olivetti G, Anversa P Severe myocardial dysfunction induced by ventricular remodeling in aging rat hearts. Am J Physiol 1990;259:H1086-H1096
9. Capasso JM, Remily RM, Sonnenblick EH Alterations in mechanical properties of rat papillary muscle during maturation. Am J Physiol 1982;242:H359-H364
10. Chesky JA, Rockstein M Reduced myocardial actomyosin adenosine triphosphatase activity in the ageing male Fischer rat. Cardiovasc Res 1977;11:242-246
11. Cingolani OH, Yang XP, Liu YH, Villanueva M, Rhaleb NE, Carretero OA Reduction of cardiac fibrosis decreases systolic performance without affecting diastolic function in hypertensive rats. Hypertension 2004;43:1067-1073
12. Colan SD, Parness IA, Spevak PJ, Sanders SP Developmental modulation of myocardial mechanics: age- and growth-related alterations in afterload and contractility. J Am Coll Cardiol 1992;19:619-629
13. de Simone G, Devereux RB, Kimball TR, Mureddu GF, Roman MJ, Contaldo F, Daniels SR Interaction between body size and cardiac workload: influence on left ventricular mass during body growth and adulthood. Hypertension 1998;31:1077-1082
14. Forman DE, Cittadini A, Azhar G, Douglas PS, Wei JY Cardiac morphology and function in senescent rats: gender-related differences. J Am Coll Cardiol 1997;30:1872-1877
15. Georgakopoulos D, Mitzner WA, Chen CH, Byrne BJ, Millar HD, Hare JM, Kass DA In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. Am J Physiol 1998;274:H1416-H1422
16. Glower DD, Spratt JA, Snow ND, Kabas JS, Davis JW, Olsen CO, Tyson GS, Sabiston DC, Jr., Rankin JS Linearity of the Frank-Starling relationship in the intact heart: the concept of preload recruitable stroke work. Circulation 1985;71:994-1009
17. Goto Y, Slinker BK, LeWinter MM Similar normalized Emax and O2 consumption-pressure-volume area relation in rabbit and dog. Am J Physiol 1988;255:H366-H374
18. Heller LJ, Whitehorn WV Age-associated alterations in myocardial contractile properties. Am J Physiol 1972;222:1613-1619
19. Hoh JF, McGrath PA, Hale PT Electrophoretic analysis of multiple forms of rat cardiac myosin: effects of hypophysectomy and thyroxine replacement. J Mol Cell Cardiol 1978;10:1053-1076

20. Janczewski AM, Spurgeon HA, Lakatta EG Action potential prolongation in cardiac myocytes of old rats is an adaptation to sustain youthful intracellular Ca2+ regulation. J Mol Cell Cardiol 2002;34:641-648
21. Jong WM, Zuurbier CJ, De Winter RJ, Van Den Heuvel DA, Reitsma PH, Ten Cate H, Ince C Fentanyl-fluanisone-midazolam combination results in more stable hemodynamics than does urethane alpha-chloralose and 2,2,2-tribromoethanol in mice. Contemp Top Lab Anim Sci 2002;41:28-32
22. Kass DA, Maughan WL, Guo ZM, Kono A, Sunagawa K, Sagawa K Comparative influence of load versus inotropic states on indexes of ventricular contractility: experimental and theoretical analysis based on pressure-volume relationships. Circulation 1987;76:1422-1436
23. Leeuwenburgh BP, Steendijk P, Helbing WA, Baan J Indexes of diastolic RV function: load dependence and changes after chronic RV pressure overload in lambs. Am J Physiol Heart Circ Physiol 2002;282:H1350-H1358
24. Little WC The left ventricular dP/dtmax-end-diastolic volume relation in closed-chest dogs. Circ Res 1985;56:808-815
25. Little WC, Cheng CP, Mumma M, Igarashi Y, Vinten-Johansen J, Johnston WE Comparison of measures of left ventricular contractile performance derived from pressure-volume loops in conscious dogs. Circulation 1989;80:1378-1387
26. Liu P, Xu B, Cavalieri TA, Hock CE Age-related difference in myocardial function and inflammation in a rat model of myocardial ischemia-reperfusion. Cardiovasc Res 2002;56:443-453
27. Mandinov L, Eberli FR, Seiler C, Hess OM Diastolic heart failure. Cardiovasc Res 2000;45:813-825
28. Nahrendorf M, Hiller KH, Greiser A, Kohler S, Neuberger T, Hu K, Waller C, Albrecht M, Neubauer S, Haase A, Ertl G, Bauer WR Chronic coronary artery stenosis induces impaired function of remote myocardium: MRI and spectroscopy study in rat. Am J Physiol Heart Circ Physiol 2003;285:H2712-H2721
29. Nair RR, Nair P Age-dependent variation in contractility of adult cardiac myocytes. Int J Biochem Cell Biol 2001;33:119-125
30. Pacher P, Mabley JG, Liaudet L, Evgenov OV, Marton A, Hasko G, Kollai M, Szabo C Left ventricular pressure-volume relationship in a rat model of advanced aging-associated heart failure. Am J Physiol Heart Circ Physiol 2004;287:H2132-H2137
31. Pacher P, Mabley JG, Soriano FG, Liaudet L, Komjati K, Szabo C Endothelial dysfunction in aging animals: the role of poly(ADP-ribose) polymerase activation. Br J Pharmacol 2002;135:1347-1350
32. Pacher P, Vaslin A, Benko R, Mabley JG, Liaudet L, Hasko G, Marton A, Batkai S, Kollai M, Szabo C A new, potent poly(ADP-ribose) polymerase inhibitor improves cardiac and vascular dysfunction associated with advanced aging. J Pharmacol Exp Ther 2004;311:485-491
33. Qi XL, Rouleau JL Age-dependent changes in the effects of endocardial endothelium on the contractile characteristics of its adjacent myocardium in rats. J Mol Cell Cardiol 1997;29:823-829
34. Rabkin DG, Jia CX, Cabreriza SE, Hart JP, Starr JP, Spotnitz HM A novel arresting solution for study of postmortem pressure--volume curves of the rat left ventricle. J Surg Res 1998;80:221-228
35. Raya TE, Gaballa M, Anderson P, Goldman S Left ventricular function and remodeling after myocardial infarction in aging rats. Am J Physiol 1997;273:H2652-H2658
36. Rockstein M, Chesky JA, Lopez T Calcium sensitivity of myocardial actomyosin ATPase in young and mature male Fischer rats. A brief note. Mech Ageing Dev 1978;8:413-416
37. Sagawa K, Maughan L, Suga H, and Sunagawa K 1988 Cardiac Contraction and the Pressure-Volume Relationship. Oxford Univ Press, New York,
38. Sagawa K The ventricular pressure-volume diagram revisited. Circ Res 1978;43:677-687
39. Sagawa K The end-systolic pressure-volume relation of the ventricle: definition, modifications and clinical use. Circulation 1981;63:1223-1227
40. Scheuer J, Bhan AK Cardiac contractile proteins. Adenosine triphosphatase activity and physiological function. Circ Res 1979;45:1-12

41. Schoeffner DJ, Warren DA, Muralidara S, Bruckner JV, Simmons JE Organ weights and fat volume in rats as a function of strain and age. J Toxicol Environ Health A 1999;56:449-462
42. Skolleborg KC, Gronbech JE, Grong K, Abyholm FE, Lekven J Distribution of cardiac output during pentobarbital versus midazolam/fentanyl/fluanisone anaesthesia in the rat. Lab Anim 1990;24: 221-227
43. Steendijk P, Baan J Comparison of intravenous and pulmonary artery injections of hypertonic saline for the assessment of conductance catheter parallel conductance. Cardiovasc Res 2000;46:82-89
44. Steendijk P, Baan J, Jr., van der Velde ET, Baan J Effects of critical coronary stenosis on global systolic left ventricular function quantified by pressure-volume relations during dobutamine stress in the canine heart. J Am Coll Cardiol 1998;32:816-826
45. Suga H, Hisano R, Goto Y, Yamada O Normalization of end-systolic pressure-volume relation and Emax of different sized hearts. Jpn Circ J 1984;48:136-143
46. Takeuchi M, Odake M, Takaoka H, Hayashi Y, Yokoyama M Comparison between preload recruitable stroke work and the end-systolic pressure-volume relationship in man. Eur Heart J 1992;13 Suppl E:80-84
47. Wallace A, Lam HW, Mangano DT Linearity, load dependence, hysteresis, and clinical associations of systolic and diastolic indices of left ventricular function in man. Multicenter Study of Perioperative Ischemia (McSPI) Research Group. J Card Surg 1995;10:460-467
48. Yang B, Larson DF, Beischel J, Kelly R, Shi J, Watson RR Validation of conductance catheter system for quantification of murine pressure-volume loops. J Invest Surg 2001;14:341-355

CHAPTER 5

LONG-TERM HEMODYNAMIC FOLLOW-UP BY LEFT VENTRICULAR PRESSURE-VOLUME LOOPS AFTER NEONATAL DEXAMETHASONE TREATMENT IN RATS

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ABSTRACT

Dexamethasone treatment is clinically applied to wean preterm infants from mechanical ventilation. However, concern has emerged about adverse side-effects. The cardiovascular short-term side-effects of neonatal dexamethasone treatment are well documented, but the long-term consequences are unknown. Previous studies showed suppressed mitosis during dexamethasone treatment leading to reduced ventricular weight, depressed systolic function and compensatory dilatation in prepubertal rats. In addition, recent experimental data indicated a reduced life expectancy. Therefore, we investigated the long-term effects of neonatal dexamethasone treatment on cardiovascular function.

Neonatal rat pups were treated with dexamethasone or received saline. Cardiac function was determined in 8-, 50-, and 80-week-old animals, representing young adult, middle-aged and elderly stages. A pressure-conductance catheter was introduced into the left ventricle to measure pressure-volume loops. Subsequently, the hearts were collected for histological examination. Our results showed reduced ventricular and body weights in dexamethasone-treated rats at 8 and 80 weeks, but not at 50 weeks. Cardiac output and diastolic function were unchanged in all age groups, but systolic function was depressed at 50 and 80 weeks evidenced by reduced ejection fractions and rightward shifts of the end-systolic pressure-volume relationships.

Early adverse effects of neonatal dexamethasone treatment are transient but reduced ventricular weight and systolic dysfunction become manifest again in elderly rats. Presumably, cellular hypertrophy initially compensates for the dexamethasone treatment-induced lower number of cardiomyocytes but fails at a later stage leading to systolic dysfunction. If applicable to humans, cardiac screening of a relatively large patient group to enable secondary prevention may be indicated.

INTRODUCTION

Glucocorticoids, in particular dexamethasone, are widely used to treat or prevent chronic lung disease in preterm infants. However, due to adverse long-term effects on cognition and motoneuron development, neonatal glucocorticoid treatment remains controversial [3]. The American Academy of Pediatrics and the Canadian Pediatric Society recently published guidelines for the use of postnatal steroid treatment in preterm infants [1]. With regard to the long-term effects on the brain, studies have documented adverse outcome including cerebral palsy and delayed and abnormal neurological development [5,6,12,18,41]. The short-term cardiovascular effects after neonatal dexamethasone treatment include hypertension and hypertrophic cardiomyopathy. These effects were described both in animal and human studies and are generally thought to be transient [7,8,17,28,29,35,39]. However, recent data showed a significantly reduced life expectancy of rats treated with dexamethasone in the neonatal period suggesting permanent damage and detrimental long-term effects [20]. The cause of premature death in these rats remained speculative, but was considered to be due to cardiac or kidney failure. In a recent study in rats we have shown that neonatal dexamethasone treatment inhibits cardiomyocyte mitosis and results in a reduced number of cardiomyocytes at adult age [10]. Cardiovascular effects of neonatal dexamethasone treatment were previously studied in 4-week-old rats, representing the pre-pubertal period [4]. These young animals showed reduced heart weights and reduced left ventricular wall thickness. Hemodynamic measurements revealed impaired systolic function and increased left ventricular volumes with maintained cardiac output, indicating a state of compensatory dilatation [4]. However, the long-term cardiovascular effects are unknown. Therefore, in the present study we investigated cardiac function in 8-, 50-, and 80-week-old rats that were treated with dexamethasone in the neonatal period and compared those with age-matched control animals.

METHODS

Animals

The study protocol was approved by the Animal Research Committee of the University of Leiden. Pregnant Wistar rats (270–300 g) were housed individually and kept under conventional housing conditions. Pups were born on day 21–22 of gestation. On the day of birth, male pups were selected and randomly divided into treatment and control groups. Treatment and control animals were kept separately and placed with foster mothers in groups of four to six pups. Rat pups in the treatment group were injected intraperitoneally with dexamethasone using a 3-day tapering dose following a protocol as used before [19]. Consequently, the treated animals received 0.5, 0.3, and 0.1 µg/g body weight dexamethasone on day 1, 2, and

3 of life, respectively. The animals in the control group received equal volumes (10 µL/g body weight) sterile pyrogen-free saline. Temperature and humidity were kept constant and the rats had free access to food and water. An artificial 12 h-light/12 h-dark cycle was used. The rats were weaned on day 21 and studied at 8 weeks, 50 weeks or 80 weeks of age.

Animal Preparation

The rats were sedated by inhalation of a mixture of halothane (4%) and oxygen. Subsequently, general anesthesia was initiated by intraperitoneal (IP) injection of a fentanyl-fluanison-midazolam mixture (FFM). The FFM mixture consisted of 2 parts Hypnorm® (0.315 mg/mL fentanyl + 10 mg/mL fluanison), 1 part Dormicum® (5 mg/mL midazolam) and 1 part saline. This mixture was administered in a dose of 0.4 mL/100 g body weight. Supplemental IP injections (one-third of the initial dose) were provided if necessary so that the animals remained unresponsive to tail pinch by forceps. The animals were placed on a controlled warming pad to keep body temperature constant. A tracheostomy was performed, a cannula was inserted and connected to a pressure-controlled respirator, and ventilation was started with an air/oxygen mixture (FiO_2=0.5). The animals were placed under a microscope (Zeiss, Germany) and the left jugular vein and the right carotid artery were exposed via a midline cervical incision. The jugular vein was cannulated for infusion of hypertonic saline to determine parallel conductance (see below). Via the carotid artery a miniaturized combined pressure-conductance catheter (SPR-878, Millar Instruments, Houston, TX, USA) was introduced and positioned into the left ventricle (LV) guided by on-line pressure and volume signals [14]. The abdomen was opened via a small incision just below the diaphragm to enable temporary preload reductions by directly compressing the inferior vena cava using a cotton-tipped stick. The pressure-conductance catheter was connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, The Netherlands) for on-line display and registration of LV pressure and volume signals. All data were acquired using Conduct-NT software (CD Leycom) at a sample rate of 2000 Hz and analyzed off-line by custom-made software.

Hemodynamic measurements

Steady state

After instrumentation, LV pressure-volume signals were acquired in steady state to quantify hemodynamic conditions: Heart rate, cardiac output, end-diastolic volume, end-systolic volume, ejection fraction, end-diastolic pressure, and end-systolic pressure were assessed. Stroke work was determined as the area of the pressure-volume loop, and the maximal and minimal rates of LV pressure change, dP/dt_{MAX} and dP/dt_{MIN}, and the isovolumic relaxation time constant τ were calculated.

Pressure-volume relationships

To obtain load-independent indices of systolic and diastolic LV function we determined pressure-volume relations by recording pressure-volume loops during a gradual preload reduction obtained by gently compressing the inferior caval vein [9]. By this procedure we reduced systolic pressure typically by 20-30 mmHg within 2-3 s (~15 beats). To quantify systolic function we used the end-systolic pressure-volume relation (ESPVR), and the preload recruitable stroke work relation (PRSW, stroke work vs. end-diastolic volume). The slopes of these linear relations, E_{ES} (end-systolic elastance) and S_{PRSW}, respectively, and their positions yield sensitive and relatively load-independent measures of systolic LV function [15,24,32]. The position of the ESPVR was quantified by its volume intercept (ESV_{INT}) at a fixed end-systolic pressure, whereas the position of the PRSW was determined as its stroke work intercept (SW_{INT}) at a fixed end-diastolic volume. The fixed values for end-systolic pressure and end-diastolic volume were determined retrospectively as the corresponding overall mean values for all animals. Diastolic LV function was quantified by the linear slope of the end-diastolic pressure-volume relationship (EDPVR) and its position defined as its end-diastolic pressure intercept (EDP_{INT}) at the fixed end-diastolic volume [25].

Calibration of the conductance catheter

To derive absolute volumes from the conductance catheter, the signals were calibrated for parallel conductance (V_p) and slope factor (α). Parallel conductance was determined by the hypertonic saline method [2]. We performed intravenous injections via the jugular vein cannula (10% saline, 5-10 µL) and parallel conductance was calculated as the mean of three consecutive assessments [36,40]. To determine slope factor α we placed a transit-time ultrasonic flow probe (Transonic Systems, Maastricht, The Netherlands) on the ascending aorta, after opening the thorax at the end of the experiment, via a midsternal incision: Slope factor α was calculated as cardiac output determined by conductance divided by cardiac output determined by aortic flow.

Heart preparation

After the hemodynamic measurements a cannula was inserted retrogradely into the abdominal aorta to allow external perfusion of the heart. The hearts were arrested in diastole by slowly infusing 1 mL 0.1 M Cadmium chloride via a needle introduced in the apex of the left ventricle. Subsequently, the right atrium was cut to allow drainage and external perfusion via the aortic cannula was started using a reservoir at approximately 70 cm height. A mixture of saline and nitroprusside (0.1 mg/ml) was infused for 3 minutes to achieve coronary vasodilatation followed by 3 min perfusion with formalin solution (2%). The hearts were then excised and immersion fixed in phosphate-buffered formalin 4%. After at least 48h of fixation any remaining extra-cardiac structures and the atria were carefully removed from the hearts and ventricular weight was determined.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Comparisons between group means were performed by unpaired t-tests and statistical significance was defined as $p<0.05$.

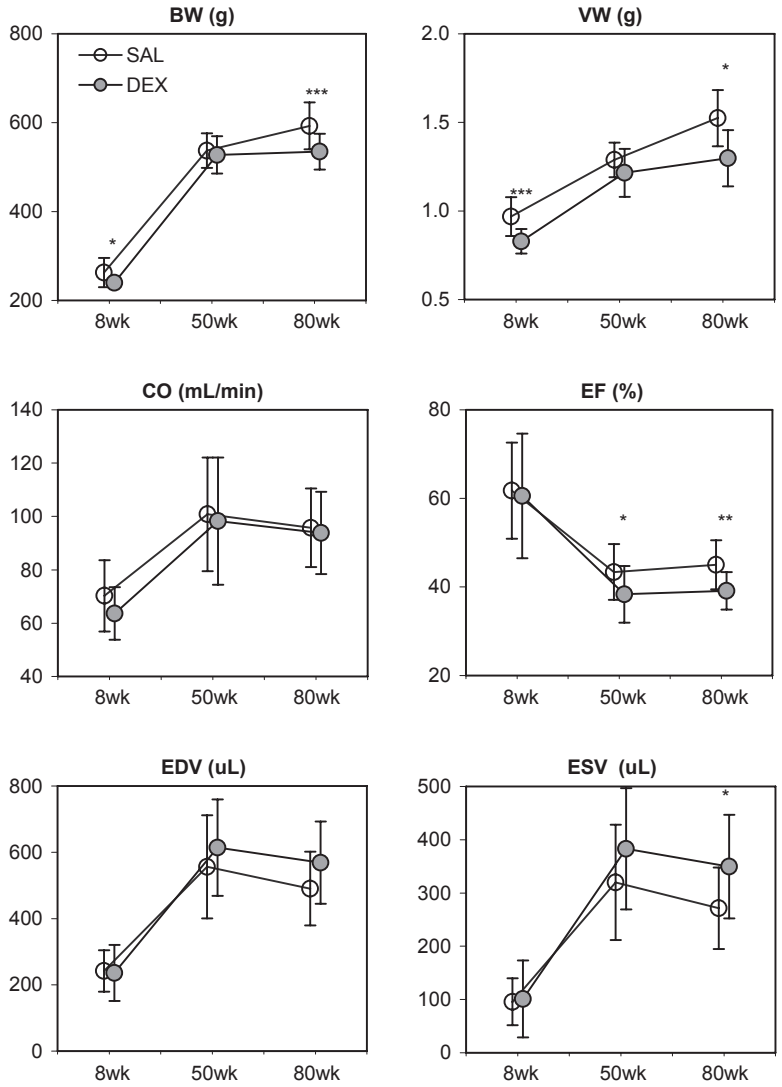

Figure 1: Weight and hemodynamic characteristics in 8-, 50-, and 80-week-old saline- and dexamethasone-treated rats. BW indicates body weight; VW, ventricular weight; CO, cardiac output; EF, ejection fraction; EDV, end-diastolic volume; ESV, end-systolic volume; *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

RESULTS

Experiments were performed in dexamethasone- and saline-treated male rats in three age groups: the 8-week-old group (8wk; saline: n=12, dexamethasone: n=10); the 50-week-old group (50wk; saline: n=18, dexamethasone: n=18), and the 80-week-old group (80wk; saline: n=14, dexamethasone: n=14).

Heart and body weights

Anatomical data for dexamethasone- and saline-treated rats in the three groups are summarized in Table 1 and are depicted in Figure 1. In the 8wk group, body weight, ventricular weight, and the ventricular-to-body weight ratio were all significantly lower in dexamethasone-treated rats ($p<0.05$). No significant differences between dexamethasone- and saline-treated rats were found in the 50wk group. In the 80wk group however, the dexamethasone-treated animals again showed a significantly reduced body weight ($p<0.01$) and ventricular weight ($p<0.05$), but no difference in the ventricular-to-body weight ratio.

Hemodynamics

Hemodynamic indices for the three age groups are listed in Table 2. In the 8wk group no significant differences were found between saline- en dexamethasone-treated rats. In the 50wk group, ejection fraction was decreased in the dexamethasone-treated rats and the ESPVR and PRSW relations were shifted significantly towards larger volumes as indicated by the increased ESV_{INT} and decreased SW_{INT}, respectively. These changes reflect a decrease in systolic function. In the 80wk group these differences were more pronounced (as shown in

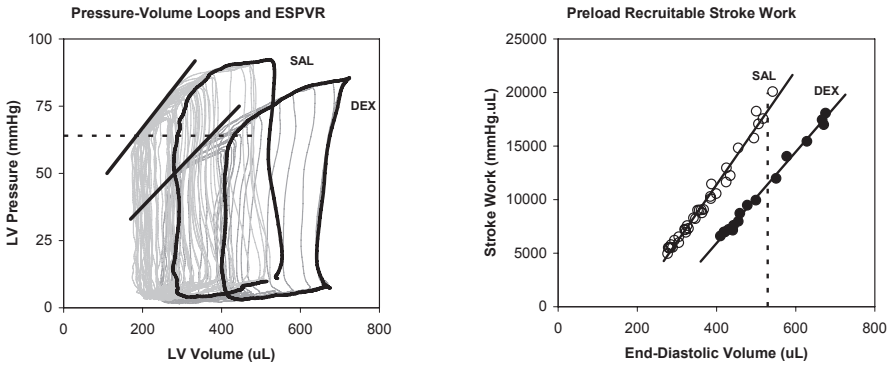

Figure 2: Typical examples of pressure-volume loops during gradual caval vein occlusion to derive pressure-volume relations in saline-treated and dexamethasone-treated 80-week-old rats. ESPVRs (left panel) and PRSWs (right panel) are indicated. The slopes and positions of these relations are load-independent indicators of ventricular function. Positions were quantified as the intercept of the relations at the level of the overall mean end-systolic pressure and end-diastolic volume, respectively, in the group (indicated by the dotted lines).

Table 1: Body weight (BW), ventricular weight (VW), and ventricular-to-body weight ratio in 8-, 50 and 80-week-old saline-treated and dexamethasone-treated rats.

	8wk			**50wk**			**80wk**		
	saline	dexamethasone		saline	dexamethasone		saline	dexamethasone	
	(n=12)	(n=10)	p	(n=18)	(n=18)	p	(n=14)	(n=14)	p
BW (g)	263±33	240±11	<0.05	537±39	528±42	NS	593±53	535±40	<0.005
VW (mg)	0.97±0.11	0.83±0.07	<0.005	1.29±0.10	1.22±0.14	NS	1.52±0.16	1.30±0.16	<0.05
VW/BW (mg/g)	3.69±0.21	3.45±0.25	<0.05	2.39±0.30	2.32±0.22	NS	2.55±0.33	2.40±0.17	NS

Table 2: Hemodynamic indices in 8-, 50 and 80-week-old saline-treated and dexamethasone-treated rats.

	8wk			50wk			80wk		
	saline	dexamethasone		saline	dexamethasone		saline	dexamethasone	
	(n=12)	(n=10)	p	(n=18)	(n=18)	p	(n=14)	(n=14)	p
Pump function									
HR, beats/min	443±22	424±32	NS	415±25	419±30	NS	411±27	401±27	NS
CO, ml/min	70±13	64±10	NS	101±21	98±24	NS	96±15	94±15	NS
SW, µl.mmHg	13839±3966	11762±1604	NS	15875±4280	14677±4459	NS	17442±3832	16595±3214	NS
Systolic function									
ESV, µL	96±44	101±72	NS	320±108	383±114	NS	271±77	350±97	<0.05
ESP, mmHg	82±14	79±17	NS	60±13	57±15	NS	64±11	63±7	NS
EF, %	62±11	61±14	NS	43±6	38±6	<0.05	45±6	39±4	<0.01
dP/dt_{MAX}, mmHg/s	12639±2016	10186±3016	NS	10174±2751	9358±3354	NS	8254±1917	8309±2395	NS
E_{ES}, mmHg/µL	0.212±0.081	0.256±0.076	NS	0.153±0.052	0.158±0.057	NS	0.185±0.051	0.183±0.066	NS
ESV_{INT}, µL	82±92	92±127	NS	301±143	405±140	<0.05	253±81	335±97	<0.05
S_{PRSW}, mmHg	49±12	47±6	NS	38±21	41±11	NS	52±9	48±11	NS
SW_{INT}, mmHg. µL	13714±3328	12131±4479	NS	19194±7182	12986±5381	<0.01	19021±3502	14859±4131	<0.01
Diastolic function									
EDV, µL	242±62	236±85	NS	556±156	614±145	NS	490±111	568±124	NS
EDP, mmHg	4.6±1.6	4.2±1.8	NS	4.5±1.4	4.2±1.6	NS	7.5±2.0	8.1±3.0	NS
$-dP/dt_{MIN}$, mmHg/s	6609±1694	5899±2124	NS	4550±841	3953±1327	NS	3676±707	3912±889	NS
τ, ms	10.6±1.9	12.1±2.6	NS	11±3.2	12.0±2.9	NS	11.7±2.8	11.0±1.2	NS
E_{ED}, mmHg/µL	0.027±0.017	0.029±0.012	NS	0.019±0.011	0.021±0.012	NS	0.014±0.019	0.019±0.007	NS
EDP_{INT}, mmHg	4.0±2.1	3.1±3.3	NS	6.2±3.3	4.5±4.0	NS	8.5±3.3	8.4±3.6	NS

HR indicates heart rate; CO, cardiac output; SW, stroke work; ESV, end-systolic volume; ESP, end-systolic pressure; EF, ejection fraction; E_{ES}, end-systolic elastance; ESV_{INT}, volume intercept of end-systolic pressure-volume relation (ESPVR) (NB: calculated at mean ESP: 81 mmHg for 8wk, 58 mmHg for 50wk, 64 mmHg for 80wk); PRSW, preload recruitable stroke work relation; SW_{INT} intercept of PRSWR (calculated at mean EDV: 239 µL for 8wk, 585 µL for 50wk, 529 µL for 80wk); EDV, end-diastolic volume; EDP, end-diastolic pressure; τ, time constant of isovolumic relaxation; E_{ED} end-diastolic elastance; EDP_{INT}, pressure intercept of end-diastolic pressure-volume relation (EDPVR) (calculated at mean EDV).

the typical examples in Figure 2), whereas the increase in end-systolic volume also reached statistical significance, and end-diastolic volume showed a clear tendency to increase (p=0.091). Despite the reductions in systolic function in the dexamethasone-treated rats, cardiac pump function was maintained in all age groups as shown by the unchanged cardiac output and stroke work. Diastolic function was also not significantly affected.

DISCUSSION

Short-term adverse cardiovascular side effects of neonatal dexamethasone treatment, including hypertension and hypertrophic cardiomyopathy, are well documented both in experimental studies and in humans [7,8,17,28,29,35,39]. However, possible detrimental long-term effects have not been studied in detail. Clinical data, in this respect, are not available since neonatal dexamethasone treatment started in the early 90's. However, a substantial patient cohort is current reaching adulthood. Our previous studies in 4-week-old prepubertal rats revealed reduced heart weight and depressed systolic function with compensatory dilatation and maintained cardiac output [4]. Histopathological studies indicated that these cardiovascular effects were presumably due to suppression of cardiomyocyte mitosis during dexamethasone treatment resulting in a significantly reduced number of cardiomyocytes [10]. In addition, recent data indicated a reduced life span in rats treated neonatally with dexamethasone [20]. The present longitudinal study investigated cardiovascular function during life span in 8- 50- and 80-week-old rats. Our findings indicated that at 8 weeks the rats still had reduced heart and body weights, but much less pronounced than at 4 weeks. Our previous studies documented 27% reduction in ventricular weight and 12% reduction in body weight at 4 weeks, whereas in the present study we found, respectively, 14% and 9% reduction at 8 weeks. At 50 weeks the ventricular and body weights were fully normalized, but at 80 weeks the dexamethasone-treated rats again showed significantly reduced ventricular and body weights by 14% and 10%, respectively. In line with these findings, the cardiac function studies revealed that the systolic dysfunction previously found at 4 weeks was normalized at 8 weeks, but reappeared at 50 weeks and, more pronouncedly, at 80 weeks. In all age groups global pump function quantified by cardiac output and stroke work was maintained. However, the animals with depressed systolic function showed evidence of compensatory cardiac dilatation although the increase in end-diastolic volume did not reach statistical significance (P=0.091), indicating that these animals used their Frank-Starling mechanism to maintain normal cardiac output.

Thus, our study supports a previous hypothesis that neonatal dexamethasone treatment may lead to cardiovascular dysfunction later in life. However, the present data did not show a continuous decline of cardiac function, but rather indicated that the systolic dysfunction at 4 weeks was followed by normalization in the adult phase and gradual development of

systolic dysfunction only in the elderly animals. Importantly, these effects were not only evident from a reduced ejection fraction, but also from significant rightward shifts of the ESPVR and PRSW relationships. The positions of these relations are relatively load-independent [9,21,38] markers of systolic function and the shifts therefore confirm a true decline in intrinsic ventricular function and exclude that the reduced ejection fraction merely results from, e.g., increased afterload in the dexamethasone-treated animals. The slopes of the ESPVR and PRSW were not affected, however previous studies indicate that the positions of these relationships particularly when determined at a physiological level yield more sensitive and consistent indexes for detection of changes in contractile state [9,37].

Presumably, the early effects of dexamethasone on ventricular function are gradually masked by compensatory cellular hypertrophy in the adult animals, but become manifest again in the elderly animals. We would speculate that the depressed systolic function was linked with a substantially lower number of cardiomyocytes in the dexamethasone-treated rats resulting from suppression of proliferation during dexamethasone treatment [10]. The ventricular weight measurements indicated that the lower number of cardiomyocytes was partly compensated by cellular hypertrophy in the 8-week-old group, but fully compensated only in the 50-week-old animals. Apparently, the development of cellular hypertrophy in this model is relatively slow and not fully completed at 8 weeks. The reduced ventricular weight at 80 weeks may reflect that possibilities for cellular hypertrophy become exhausted in the elderly dexamethasone-treated rats or that these animals show increased cell loss, as previously suggested, reflecting early aging.

Interestingly, in this study we did not observe increased mortality in the dexamethasone-treated rats as was seen in a previous study [20]. Whereas in the earlier study 25% of dexamethasone-treated rats died prematurely before 80 weeks (18 months), none of the rats in the present study died prior to the hemodynamic studies. A possible explanation could be that the rats in the present study were housed in groups of 4-6 rats, whereas in the earlier study each rat was housed individually. Based on behavioural studies [31], solitary animals are exposed to more stress which could have amplified the differences between dexamethasone-treated and control animals and lead to reduced life span.

Extrapolation of the results found in this study in rats to the clinical practice of dexamethasone treatment in humans remains speculative. We used term rat pups as a model for premature human babies. With regard to brain development, a 7-day-old rat is roughly equivalent to a full-term human infant and thus in this respect term rat pups correspond well to premature infants [13]. Furthermore, at day 1-3 after birth, the period during which dexamethasone treatment was installed, the rat myocardium still shows substantial hyperplasia [23], similar to the preterm human myocardium [16,26]. Thus, with respect to the transition from myocyte hyperplasia to hypertrophy the term rat pup is also a good model for the preterm human infant. However, potential differences in the degree of maturation of neurohormonal systems, receptor mechanisms and innervation [33] may also be of influence

in the response to dexamethasone treatment. The development of these systems has been studied in the rat [30], but very few data are available for the (preterm) human situation. Thus, although species differences may be of influence, this model is frequently used and several effects of dexamethasone treatment found in humans, including early transient hypertrophy, were also found in the rat model [11,22,27,34].

We conclude that the reduced ventricular weight and systolic dysfunction previously found in 4-week-old rats after neonatal dexamethasone treatment is transient, presumably due to compensatory cellular hypertrophy, but becomes manifest again in elderly rats. The elderly animals with depressed systolic function showed evidence of compensatory cardiac dilatation, indicating that these animals have to invoke the Frank-Starling mechanism to maintain normal cardiac output. If our results are applicable to humans, a relatively large patient population is involved and early screening and a cardiovascular follow-up program may be warranted to enable secondary prevention.

REFERENCE LIST

1. Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics 2002;109:330-338
2. Baan J, van der Velde ET, de Bruin HG, Smeenk GJ, Koops J, van Dijk AD, Temmerman D, Senden J, Buis B Continuous measurement of left ventricular volume in animals and humans by conductance catheter. Circulation 1984;70:812-823
3. Bakker JM, van Bel F, Heijnen CJ Neonatal glucocorticoids and the developing brain: short-term treatment with life-long consequences? Trends Neurosci 2001;24:649-653
4. Bal MP, de Vries WB, van der Leij FR, van Oosterhout MF, Berger RM, Baan J, van der Wall EE, van Bel F, Steendijk P Neonatal glucocorticosteroid treatment causes systolic dysfunction and compensatory dilation in early life: studies in 4-week-old prepubertal rats. Pediatr Res 2005;58:46-52
5. Barrington KJ Postnatal steroids and neurodevelopmental outcomes: a problem in the making. Pediatrics 2001;107:1425-1426
6. Barrington KJ The adverse neuro-developmental effects of postnatal steroids in the preterm infant: a systematic review of RCTs. BMC Pediatr 2001;1:1
7. Bensky AS, Kothadia JM, Covitz W Cardiac effects of dexamethasone in very low birth weight infants. Pediatrics 1996;97:818-821
8. Brand PL, van Lingen RA, Brus F, Talsma MD, Elzenga NJ Hypertrophic obstructive cardiomyopathy as a side effect of dexamethasone treatment for bronchopulmonary dysplasia. Acta Paediatr 1993;82:614-617
9. Burkhoff D, Mirsky I, Suga H Assessment of systolic and diastolic ventricular properties via pressure-volume analysis: a guide for clinical, translational, and basic researchers. Am J Physiol Heart Circ Physiol 2005;289:H501-H512
10. de Vries WB, Bal MP, Homoet-van der Kraak P, Kamphuis PJ, van der Leij FR, Baan J, Steendijk P, de Weger RA, van Bel F, van Oosterhout MF Suppression of physiological cardiomyocyte proliferation in the rat pup after neonatal glucocorticosteroid treatment. Basic Res Cardiol 2006;101:36-42
11. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr Res 2002;52:900-906
12. Doyle LW, Halliday HL, Ehrenkranz RA, Davis PG, Sinclair JC Impact of postnatal systemic corticosteroids on mortality and cerebral palsy in preterm infants: effect modification by risk for chronic lung disease. Pediatrics 2005;115:655-661
13. Flagel SB, Vazquez DM, Watson SJ, Jr., Neal CR, Jr. Effects of tapering neonatal dexamethasone on rat growth, neurodevelopment, and stress response. Am J Physiol Regul Integr Comp Physiol 2002;282:R55-R63
14. Georgakopoulos D, Mitzner WA, Chen CH, Byrne BJ, Millar HD, Hare JM, Kass DA In vivo murine left ventricular pressure-volume relations by miniaturized conductance micromanometry. Am J Physiol 1998;274:H1416-H1422
15. Glower DD, Spratt JA, Snow ND, Kabas JS, Davis JW, Olsen CO, Tyson GS, Sabiston DC, Jr., Rankin JS Linearity of the Frank-Starling relationship in the intact heart: the concept of preload recruitable stroke work. Circulation 1985;71:994-1009
16. Huttenbach Y, Ostrowski ML, Thaller D, Kim HS Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. Cardiovasc Pathol 2001;10:119-123
17. Israel BA, Sherman FS, Guthrie RD Hypertrophic cardiomyopathy associated with dexamethasone therapy for chronic lung disease in preterm infants. Am J Perinatol 1993;10:307-310
18. Jones RA Randomized, controlled trial of dexamethasone in neonatal chronic lung disease: 13- to 17-year follow-up study: I. Neurologic, psychological, and educational outcomes. Pediatrics 2005;116:370-378

19. Kamphuis PJ, Bakker JM, Broekhoven MH, Kunne C, Croiset G, Lentjes EG, Tilders FJ, van Bel F, Wiegant VM Enhanced glucocorticoid feedback inhibition of hypothalamo-pituitary-adrenal responses to stress in adult rats neonatally treated with dexamethasone. Neuroendocrinology 2002;76:158-169
20. Kamphuis PJ, de Vries WB, Bakker JM, Kavelaars A, van Dijk JE, Schipper ME, van Oosterhout MF, Croiset G, Heijnen CJ, Van Bel F, Wiegant VM Reduced life expectancy in rats after neonatal dexamethasone treatment. Pediatr Res (in press)
21. Kass DA, Maughan WL, Guo ZM, Kono A, Sunagawa K, Sagawa K Comparative influence of load versus inotropic states on indexes of ventricular contractility: experimental and theoretical analysis based on pressure-volume relationships. Circulation 1987;76:1422-1436
22. La Mear NS, MacGilvray SS, Myers TF Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol Neonate 1997;72:175-180
23. Li F, Wang X, Capasso JM, Gerdes AM Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28:1737-1746
24. Little WC The left ventricular dP/dtmax-end-diastolic volume relation in closed-chest dogs. Circ Res 1985;56:808-815
25. Mandinov L, Eberli FR, Seiler C, Hess OM Diastolic heart failure. Cardiovasc Res 2000;45:813-825
26. Mayhew TM, Pharaoh A, Austin A, Fagan DG Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors. J Anat 1997;191 (Pt 1):107-115
27. Muangmingsuk S, Ingram P, Gupta MP, Arcilla RA, Gupta M Dexamethasone induced cardiac hypertrophy in newborn rats is accompanied by changes in myosin heavy chain phenotype and gene transcription. Mol Cell Biochem 2000;209:165-173
28. Ohning BL, Fyfe DA, Riedel PA Reversible obstructive hypertrophic cardiomyopathy after dexamethasone therapy for bronchopulmonary dysplasia. Am Heart J 1993;125:253-256
29. Papile LA, Tyson JE, Stoll BJ, Wright LL, Donovan EF, Bauer CR, Krause-Steinrauf H, Verter J, Korones SB, Lemons JA, Fanaroff AA, Stevenson DK A multicenter trial of two dexamethasone regimens in ventilator-dependent premature infants. N Engl J Med 1998;338:1112-1118
30. Robinson RB Autonomic receptor--effector coupling during post-natal development. Cardiovasc Res 1996;31 Spec No:E68-E76
31. Ruis MA, te Brake JH, Buwalda B, De Boer SF, Meerlo P, Korte SM, Blokhuis HJ, Koolhaas JM Housing familiar male wildtype rats together reduces the long-term adverse behavioural and physiological effects of social defeat. Psychoneuroendocrinology 1999;24:285-300
32. Sagawa K The end-systolic pressure-volume relation of the ventricle: definition, modifications and clinical use. Circulation 1981;63:1223-1227
33. Segar JL, Lumbers ER, Nuyt AM, Smith OJ, Robillard JE Effect of antenatal glucocorticoids on sympathetic nerve activity at birth in preterm sheep. American Journal of Physiology-Regulatory Integrative and Comparative Physiology 1998;43:R160-R167
34. Sicard RE, Werner JC Dexamethasone induces a transient relative cardiomegaly in neonatal rats. Pediatr Res 1992;31:359-363
35. Skelton R, Gill AB, Parsons JM Cardiac effects of short course dexamethasone in preterm infants. Arch Dis Child Fetal Neonatal Ed 1998;78:F133-F137
36. Steendijk P, Baan J Comparison of intravenous and pulmonary artery injections of hypertonic saline for the assessment of conductance catheter parallel conductance. Cardiovasc Res 2000;46:82-89
37. Steendijk P, Baan J, Jr., van der Velde ET, Baan J Effects of critical coronary stenosis on global systolic left ventricular function quantified by pressure-volume relations during dobutamine stress in the canine heart. J Am Coll Cardiol 1998;32:816-826
38. van der Velde ET, Burkhoff D, Steendijk P, Karsdon J, Sagawa K, Baan J Nonlinearity and load sensitivity of end-systolic pressure-volume relation of canine left ventricle in vivo. Circulation 1991;83:315-327

39. Werner JC, Sicard RE, Hansen TW, Solomon E, Cowett RM, Oh W Hypertrophic cardiomyopathy associated with dexamethasone therapy for bronchopulmonary dysplasia. J Pediatr 1992;120: 286-291
40. Yang B, Larson DF, Beischel J, Kelly R, Shi J, Watson RR Validation of conductance catheter system for quantification of murine pressure-volume loops. J Invest Surg 2001;14:341-355
41. Yeh TF, Lin YJ, Lin HC, Huang CC, Hsieh WS, Lin CH, Tsai CH Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity. N Engl J Med 2004;350:1304-1313

CHAPTER 6

HISTOPATHOLOGICAL CHANGES OF THE HEART AFTER NEONATAL DEXAMETHASONE TREATMENT: STUDIES IN 4-, 8- AND 50-WEEK-OLD RATS

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Submitted

ABSTRACT

Dexamethasone is widely used to treat or prevent chronic lung disease in preterm infants, but recent studies have raised concerns regarding potential negative long-term effects. Multiple short-term cardiovascular side effects have been described both in rat pups and humans, but studies regarding long-term effects on the heart are lacking. The aim of this study was to investigate the histopathological myocardial changes after neonatal dexamethasone treatment in the young and adult rat heart.

Rat pups were treated with dexamethasone on day 1, 2 and 3 (0.5, 0.3 and 0.1 µg/g) of life. Control pups received saline. At 4, 8 and 50 weeks of life the rats were sacrificed and anatomic data collected. Heart tissue was stained with HE, Cadherin-PAS and Sirius Red for cardiomyocyte morphometry and collagen determination. Cardiomyocyte length of the dexamethasone-treated rats was significantly increased ($p<0.05$) compared to controls in all three age groups, whereas ventricular weight was reduced. This was accompanied by a significant increase in cardiomyocyte width ($p<0.01$) in the 50-week-old rats. These changes resulted in significantly increased cardiomyocyte volume at 50 weeks ($p<0.01$) indicating cellular hypertrophy. Collagen content gradually increased with age and was 62% higher ($p<0.01$) in the dexamethasone-treated rats at 50 weeks of age.

Neonatal dexamethasone treatment affects normal growth of the heart resulting in cellular hypertrophy and increased collagen deposition in the adult rat heart. Since previous studies in rats have shown premature death and suggested cardiac failure, cardiovascular follow-up programs for preterm infants treated with glucocorticoids should be considered.

INTRODUCTION

In preterm infants suffering from severe respiratory distress syndrome (RDS), chronic lung disease is a serious complication [16,20]. In the pathogenesis of chronic lung disease an underlying excessive pro-inflammatory process seems to be involved [37]. Glucocorticoids (GCs), in particular dexamethasone (Dex), are widely used to treat or prevent chronic lung disease in preterm infants because of their anti-inflammatory action. Moreover, GCs stimulate lung maturation and enhance surfactant production [10].

However concerns have been raised about the wide range of side effects of GCs [4]. These concerns included findings of abnormal brain development in newborn animals treated with systemic steroids [19]. Recent reports on follow-up in ex-preterm children neonatally treated with Dex confirm its adverse effects on brain growth [28] and neuromotor developmental outcome [6]. With regard to the cardiovascular system, short-term side effects such as myocardial hypertrophy, manifested by increased ventricular septal and left ventricular wall thickness, and hypertension have been reported in animal and human studies [15,21,38]. Recent studies from our group suggested that neonatal Dex treatment may have detrimental long-term effects on the heart, possibly initiated by temporary suppression of the proliferative capacity of cardiomyocytes during and early after treatment [11].

We performed histopathological and immunohistochemical studies on hearts of rats sacrificed 4, 8 and 50 weeks after neonatal Dex treatment to delineate the cardiac effects in the pre- and postpubertal and middle-aged period. In this study we show increased cellular hypertrophy and collagen deposition in the Dex-treated rats of all three age groups, being most pronounced in the 50-week-old rats.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Animal Research Committee of the University of Leiden. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No.85–23, revised 1996). Pregnant Wistar rats (270 –300 g) were housed individually and kept under conventional housing conditions. Pups were born on day 21–22 of gestation. On the day of birth, male pups were selected and randomly divided into treatment and control groups. Treatment and control animals were kept separately and placed with foster mothers in groups of four to six pups. Rat pups in the treatment group were injected intraperitoneally with Dex using a 3-day tapering dose following a protocol as used before [18]. Consequently, the treated animals received 0.5, 0.3, and 0.1 µg/g body weight Dex on day 1, 2, and 3 of life respectively. The animals in the control group received equal volumes (10 µl/g body weight) sterile pyrogen-free saline (Sal).

Temperature and humidity were kept constant and the rats had free access to food and water. An artificial 12h-light/12h-dark cycle was used. The rats were weaned on day 21 and studied at 4 weeks, 8 weeks or 50 weeks of age. Prior to sacrifice for the current histopathological study, hemodynamic measurements were performed that were reported elsewhere [5]. All groups at the various ages (4-, 8-, and 50- weeks-old) consisted of eight rats.

The animals were sedated by inhalation of a mixture of halothane (4%) and oxygen, subsequently general anesthesia was initiated by intraperitoneal injection (IP) of a fentanyl-fluanison-midazolam mixture. This mixture consisted of 2 parts Hypnorm® (0.315 mg/ml fentanyl + 10 mg/ml fluanison), 1 part Dormicum® (5 mg/ml midazolam) and 1 part water and was administered in a dose of 0.4 ml/100 g body weight. Supplemental injections (one-third of initial dose) were provided if necessary. Before instrumentation, body weight (BW) was measured.

Instrumentation and histopathological preparation

Under general anesthesia a midsternal thoracotomy was performed and the abdomen of the rats was opened with a medial incision. A 20G cannula was inserted retrogradely into the abdominal aorta to allow external perfusion of the heart. Subsequently, the hearts were arrested in diastole by slowly infusing 1 ml 0.1 M Cadmium chloride via a needle (25G) introduced in the apex of the left ventricle. Subsequently, the right atrium was cut to allow drainage and external perfusion via the aortic cannula was started using a reservoir at approximately 70 cm height. A mixture of NaCl and nitroprusside (0.1 mg/ml) was infused for 3 minutes to achieve coronary vasodilatation followed by 3 min perfusion with formalin solution (2%). The hearts were then excised and immersion fixed in phosphate-buffered formalin 4%. After at least 48h of fixation any remaining extra-cardiac structures and the atria were carefully removed from the hearts and ventricular weight (Vw) was determined. The ventricles were cut in 2 or 3 coronal sections of approximately 2 mm thickness and embedded in paraffin. Subsequently, the hearts were sectioned parallel to the equator in 3 µm slices.

Staining procedure

Hematoxilin and Eosin (HE) staining was performed using routine techniques for general histopathological assessment. Subsequently a HE-stained slice at the level of the papillary muscles was selected for measurement of LV free wall thickness.

Sirius Red staining (Polysciences, Warrington, PA) was performed in 3 µm sections after pre-treatment with picrinic acid to determine collagen content. The collagen-positive area was quantified using Image-Pro analysis software (Media Cybernetics, Inc) in a total of 40 fields of 4 predefined regions (left ventricular free wall, anterior wall, posterior wall, and interventricular septum) using a final magnification of x200. Collagen content was expressed as fraction of the total (measured) myocardial area.

Cadherin-Periodic Acid Schiff (PAS) staining, modified from Brüel et al. [8] was used for the morphometric measurements of the cardiomyocytes. The Cadherin-PAS provides staining of the intercalated discs and the lateral sarcolemma. First, 3-µm sections of the ventricles were deparaffinized. EDTA boiling was used for antigen retrieval. The slides were then incubated with Cadherin (pan, C1821, Sigma-Aldrich, Denmark) for 1h at room temperature. After applying this primary antibody, the slides were incubated with RAMPO (P0161, dakocytomation) for 30 minutes. Subsequently, a Horse Radish-labeled antibody (powervision poly HRP-anti-Rabbit IgG, immunologic) was used. After developing the slides with di-aminobenzidene acid (DAB) and washing, the PAS staining was performed. The slides were incubated with 1% periodic Acid for 10 minutes, followed by incubation with Schiff's reagent (Merck) for 30 minutes. Finally the slides were counter-stained with hematoxylin.

Cardiomyocyte Morphometry

Morphometry was performed as described by van Oosterhout et al. [35] using the Cadherin-PAS staining. In brief, myocytes from predefined myocardial areas (see below) were visualized with a microscope (Zeiss Axiomat, final magnification 400x) equipped with a digital camera (Nikon Eclipse E800) and coupled to a personal computer equipped with dedicated software (QProdit, Leica micro-systems). In the middle part of the left ventricular free wall and the interventricular septum, longitudinally oriented myocytes (40 from each site, rendering 80 cell measurements for each animal) were selected and the cell boundaries were traced. Diameter and longitudinal sectional area of these cells were automatically determined by dedicated software (QProdit, Leica micro-systems). Only those myocytes in which the nucleus was centrally located within the cell and with intercalated discs visible at both ends of the cell were used to ensure that the long axis of the myocyte was perpendicular to the microscope objective [36]. Effective cardiomyocyte length was defined in this study as longitudinal sectional area divided by the diameter of the longitudinally oriented cells. Cardiomyocyte volume was calculated as area (in the longitudinal section) multiplied by width times $\pi/4$ on the assumption of a cylindrical configuration. For each heart the median of volume, length and width was calculated and used for statistical analysis.

Statistics

Data are presented as mean ± SD. Anatomical data of the age groups were compared using unpaired t-tests. Morphometric data and collagen content for each age group were analyzed using Mann-Whitney U tests because of a non-linear distribution of the data. P-values of <0.05 were considered statistically significant.

RESULTS

Anatomical parameters

Anatomical data for Dex-treated and Sal-treated rats are summarized in Table 1. In the 4-week-old rats, Bw, Vw and the ratio of Vw/Bw were lower in the Dex-treated rats by, respectively, 16% ($p<0.001$), 22% ($p<0.001$) and 8% ($p<0.05$). In the 8-week-old rats, Vw was 11% lower in Dex ($p<0.05$), but no difference was found for Bw or Vw/Bw. No differences between Dex and Sal were found for Bw, Vw or ratio Vw/Bw in the 50-week-old rats.

Ventricular wall thickness was lower by 20% ($p<0.01$) in the Dex-treated rats at 4 weeks, but no differences were found in the 8- and 50-week-old rats.

Cardiomyocyte morphometry

No significant differences for width and length of longitudinally oriented cardiomyocytes (80 for each animal) between the free wall and interventricular septum were detected (data not shown) thus pooled data are presented.

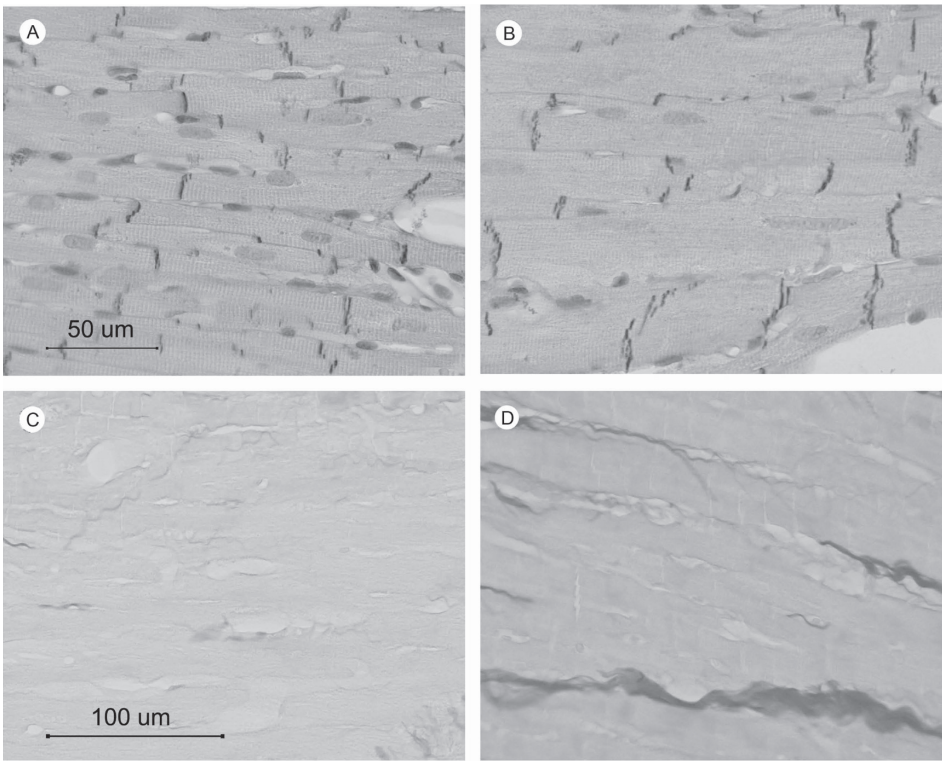

Figure 1: Histology of the hearts of 50-week-old rats neonatally treated with dexamethasone (Dex) or saline (Sal). Upper panel: Representative examples of the Cadherin-Pas staining for 50-week-old Sal- and Dex-treated rats (A and B). Longitudinally sectioned myocytes showed an increased distance between intercalated discs in the Dex-treated rats (B) compared to Sal (A), indicating increased length of the cardiomyocytes. Lower panel: Increased collagen content in the 50-week-old Dex-treated rat (D) compared to the Sal-treated rat (C).

Table 1: Anatomical parameters of the heart (mean ± SD) in 4-, 8- and 50-week-old rats neonatally treated with dexamethasone or saline

	4-week-old rats			8-week-old rats			50-week-old rats		
	Sal	Dex	p	Sal	Dex	p	Sal	Dex	p
BW (g)	86±9	72±3	0.001	259±23	243±8	NS	518±36	515±32	NS
VW (g)	0.36±0.02	0.28±0.03	0.001	0.95±0.09	0.84±0.07	0.05	1.32±0.13	1.23±0.10	NS
VW/BW (g/kg)	4.2±0.3	3.8±0.34	0.05	3.7±0.2	3.5±0.36	NS	2.6±0.32	2.4±0.16	NS
WT (mm)	1.47±0.19	1.18±0.18	0.01	2.45±0.14	2.34±0.10	NS	2.74±0.25	2.63±0.23	NS
Cardiomyocyte dimensions									
Length (µm)	60.9±5.5	69.3±4.1	0.05	79.7±6.7	90.9±6.1	0.01	93.9±11.5	111.9±9.9	0.05
Width (µm)	12.0±0.7	12.1±0.6	NS	15.7±1.3	15.9±1.6	NS	17.7±1.8	20.7±1.6	0.01
Volume (*10^3µm^3)	7.1±0.9	8.1±1.2	NS	15.7±4.0	18.1±3.1	NS	23.8±6.0	38.6±9.0	0.01

Dex: dexamethasone; Sal: saline; BW: body weight; VW: ventricular weight; WT: wall thickness; NS: not significant. p-value Dex vs Sal

In Figure 1, representative examples of the Cadherin-Pas staining for 50-week-old Sal- and Dex-treated rats are shown. Longitudinally sectioned myocytes showed an increased distance between intercalated discs in the Dex-treated rats compared to Sal, indicating increased length of the cardiomyocytes.

Figure 2 shows the relation between cardiomyocyte volume and ventricular weight. In the Sal-treated rats a linear relation between cell volume and ventricular weight was found. In the Dex-treated rats however, this relation was non-linear with a disproportional increase in cell volume between 8 and 50 weeks of age, indicating relatively smaller hearts and larger cardiomyocytes. Following Dex treatment, cell volume was higher, though not significantly, at 4 and 8 weeks by 15%. At 50 weeks, cell volume was significantly increased by 62% ($p<0.01$).

At all ages this increased cell volume in the Dex-groups was accompanied by a significant increase of the cardiomyocyte long axis (4 weeks: +14% ($p<0.05$), 8 weeks: +14% ($p<0.01$), 50 weeks: +19% ($p<0.05$). The width of the cardiomyocytes did not increase in the 4- and 8-week-old rats, but eventually increased by 17% ($p<0.01$) in the 50-week-old Dex-treated rats. The relation between the myocyte width and length is depicted in Figure 3. It shows that cellular enlargement in the Dex-groups was predominantly due to lengthening in the 4- and 8-week-old rats, whereas at 50 weeks both length and width were increased compared to Sal. These findings suggest eccentric hypertrophy at the younger ages and concentric hypertrophy in the older animals.

Collagen content measured as a fraction of the total myocardial area tended to be higher already at 4 weeks (Dex: 0.74 ± 0.46% vs Sal: 0.50 ± 0.33%, $p=0.248$) and 8 weeks (Dex: 1.16 ± 0.70% vs Sal: 0.74 ± 0.51%, $p=0.141$). In the 50-week-old Dex-treated rats the collagen content was 62% higher than in control rats (Dex: 2.20 ± 0.60% vs Sal: 1.36 ± 0.21% ($p<0.01$)). Figure 2 (bottom) shows typical examples to illustrate the increased collagen content in the 50-week-old Dex-treated rats.

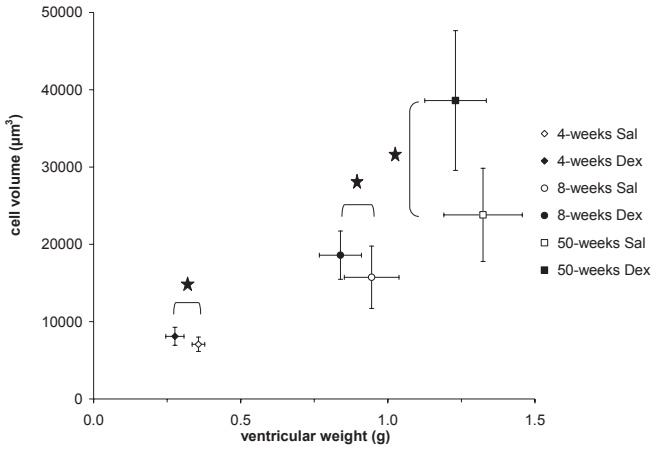

Figure 2: Cardiomyocyte volume versus ventricular weight. At all ages the dexamethasone (Dex) treated rats had smaller hearts with larger cardiomyocytes as compared to saline (Sal) treated rats. At 50 weeks of age this difference was most pronounced. Open symbols = Sal; closed symbols = Dex. * $p<0.05$ Dex vs Sal

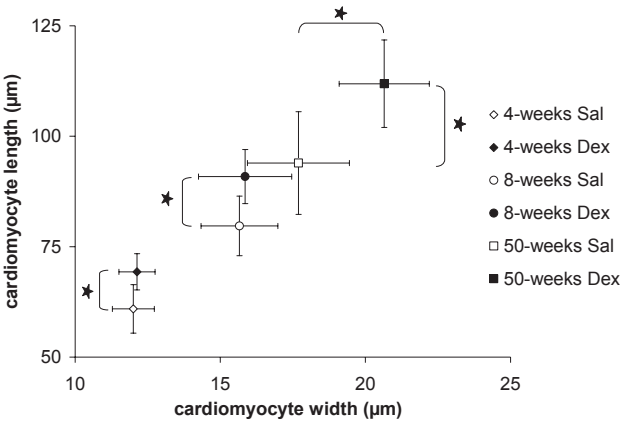

Figure 3: Cardiomyocyte length versus width. At all ages cardiomyocyte length was significantly increased in the dexamethasone (Dex) treated rats compared to saline (Sal) treated rats. At 50 weeks both cell length and cell width were significantly increased in Dex-treated rats. Open symbols = Sal; closed symbols = Dex. * $p<0.05$ Dex vs Sal

DISCUSSION

Our study shows that neonatal Dex treatment leads to a significant lower ventricular weight at 4 weeks, which is still present at 8 weeks, but no longer at 50 weeks. These differences were only partly explained by a lower body weight in the Dex-treated animals, because the ventricular weight:body weight ratio remained significantly reduced in the 4-week-old animals. At the cellular level, however, cardiomyocyte cell volume was increased in the Dex-treated animals, but most pronouncedly and significantly in the 50-week-old rats. This increased cell volume, compared to the age-matched controls, was caused primarily by an increase in cell length, whereas in the 50-week-old animals cardiomyocyte width was increased as well. Combining the increased cell volume with the lower ventricular weight clearly suggests a lower number of cardiomyocytes in the Dex-treated rats, which presumably is explained by a suppression of cardiomyocyte proliferation during Dex treatment as recently reported by our group [11]. Furthermore, our data show a proportional increase in cell volume and ventricular weight in the control animals (as would be expected over this age range), but the increase in cell volume is relatively accelerated in the Dex-treated animals suggesting additional cardiomyocyte loss in the 50-week-old rats. This finding is consistent with previously reported early aging after neonatal Dex treatment [12]. In order to interpret the long-term effects of neonatal Dex treatment, normal myocardial development and growth should be considered. In fetal and neonatal rats, the increase in myocardial mass occurs mainly by hyperplasia [9,24,25]. In the transition period after birth, proliferation is replaced by hypertrophy as evidenced by an increase in the percentage of binucleated myocytes, although hyperplasia continues in the first week of life [9,22,23,40].

In humans, the myocardial growth involves continuous proliferation of myocyte nuclei from 16 weeks of gestation to term. At or soon after full term birth proliferation ceases and thereafter growth occurs by hypertrophy of individual myocytes. In preterm infants however,

cardiomyocyte proliferative activity remains present and constant during the early preterm period and only decreases in the late preterm and early postnatal period [14,25]. The transition between hyperplasia and hypertrophy during the early postnatal period is influenced by nutritional, hemodynamic and humoral factors [31]. The increasing mechanical load [3], the perinatal increase in plasma catecholamine [34], triidothyronine concentrations [7] and GCs [27,29] accelerate the conversion to hypertrophic growth. Although several studies suggest that a significant fraction of myocytes retain the ability to divide [17,30] a decrease in the total number of cardiomyocytes occurs in the aging heart [2]. In view of the above mentioned factors neonatal Dex treatment is expected to affect myocardial development through the negative effect on mitosis. Indeed perinatal administration of cortisol in fetal lambs was shown to inhibit myocyte hyperplasia and to stimulate the hypertrophic myocardial growth pattern which normally starts postnatally [33]. As a consequence, Dex may interfere not only with the number of cardiomyocytes retaining the ability to divide but also with the total number of cardiomyocytes. Together with a premature transition to hypertrophy demonstrated in this study, postnatal Dex treatment in rats, results in a reduced number of cardiomyocytes later in life [11].

The results of the present study confirm the presence of a lower number of cardiomyocytes in Dex-treated adult rats, whereas cell length and cell volume were increased, indicating cellular hypertrophy to compensate for the lower number of cells. In the young Dex-treated rats ventricular weight was reduced despite this cellular hypertrophy, but in the 50-week-old animals in which also myocyte width was increased substantially, these hypertrophic processes are thought to be compensatory. Consequently, we suggest that the early cellular hypertrophy becomes more pronounced at a later stage (50-week-old rats) due to additional physiological cell loss during aging [2]. On top of this, the increased collagen content seen in the 50-week-old Dex-treated rats in this study may indicate premature aging and may be associated with impaired diastolic function (unpublished data).

Although these findings may explain why the Dex-treated rats might be prone to cardiac failure at a later stage in life, the underlying mechanism of the persisting hypertrophy found in this study is not entirely clear. It has been suggested that Dex may influence the intracellular calcium concentration and the transcription of growth factors [32,39]. Normally the width and length of rat myocytes increase isomorphically during cellular hypertrophy with a constant length-to-width ratio [1]. However, in this study the cardiomyocytes of the 4- and 8-week-old Dex-treated rats mainly show an increased cardiomyocyte length, whereas the 50-week-old cardiomyocytes show a more isomorphical growth with a substantial increase in cardiomyocyte volume. Further studies are necessary to reveal the actual mechanism of this growth pattern.

To date no follow-up studies have been reported in human subjects regarding the cardiovascular status after neonatal Dex treatment. Dodic et al. [13] found hypertension, left ventricular hypertrophy and reduced cardiac functional reserve in adult sheep after brief

prenatal exposure to Dex. These animals also showed an increased collagen content. This is in accordance with our study showing an increase of collagen in the 50-week-old Dex-treated rats.

The results obtained from this as well as earlier experimental studies by our group, put to question the safety of early neonatal GC-treatment in the human setting. One cannot simply extrapolate the reported findings in the newborn and adult rat model to the preterm infant and adult human since the myocardial tissue of term rat pups may have a different affinity to Dex than the myocardial tissue of the preterm infant. However, the proliferative pattern of cardiomyocytes in the perinatal period in human tissue generally mirrors that seen in rat hearts, in which hyperplasia in the early postnatal period changes to hypertrophy. Moreover, Dex administration to newborn pups is producing cardiac hypertrophy during the treatment similar to that seen in premature infants treated with GCs [21,26,38].

In conclusion, neonatal Dex treatment leads to permanent histopathological changes during growth and development of the rat heart. At all ages investigated, Dex-treated rats have smaller hearts with larger cardiomyocytes compared to control rats. Since a substantial number of the preterm infants have been treated with Dex in the late nineties up to recently, a mandatory cardiovascular follow-up program in preterm infants treated with Dex in neonatal period should therefore be seriously considered.

REFERENCE LIST

1. Anversa P, Olivetti G, Loud AV Morphometric study of early postnatal development in the left and right ventricular myocardium of the rat. I. Hypertrophy, hyperplasia, and binucleation of myocytes. Circ Res 1980;46:495-502
2. Anversa P, Palackal T, Sonnenblick EH, Olivetti G, Meggs LG, Capasso JM Myocyte cell loss and myocyte cellular hyperplasia in the hypertrophied aging rat heart. Circ Res 1990;67:871-885
3. Baba HA, Takeda A, Schmid C, Nagano M Early proliferative changes in hearts of hypertensive Goldblatt rats: an immunohistochemical and flow-cytometrical study. Basic Res Cardiol 1996;91: 275-282
4. Bakker JM, van Bel F, Heijnen CJ Neonatal glucocorticoids and the developing brain: short-term treatment with life-long consequences? Trends Neurosci 2001;24:649-653
5. Bal MP, de Vries WB, van der Leij FR, van Oosterhout MF, Berger RM, Baan J, van der Wall EE, van Bel F, Steendijk P Neonatal glucocorticosteroid treatment causes systolic dysfunction and compensatory dilation in early life: studies in 4-week-old prepubertal rats. Pediatr Res 2005;58:46-52
6. Barrington KJ The adverse neuro-developmental effects of postnatal steroids in the preterm infant: a systematic review of RCTs. BMC Pediatr 2001;1:1
7. Breall JA, Rudolph AM, Heymann MA Role of thyroid hormone in postnatal circulatory and metabolic adjustments. J Clin Invest 1984;73:1418-1424
8. Bruel A, Nyengaard JR Design-based stereological estimation of the total number of cardiac myocytes in histological sections. Basic Res Cardiol 2005;100:311-319
9. Clubb FJ, Jr., Bishop SP Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. Lab Invest 1984;50:571-577
10. Committee on Fetus and Newborn Postnatal Corticosteroids to Treat or Prevent Chronic Lung Disease in Preterm Infants. Pediatrics 2002;109:330-338
11. de Vries WB, Bal MP, Homoet-van der Kraak P, Kamphuis PJ, van der Leij FR, Baan J, Steendijk P, de Weger RA, van Bel F, van Oosterhout MF Suppression of physiological cardiomyocyte proliferation in the rat pup after neonatal glucocorticosteroid treatment. Basic Res Cardiol 2006;101:36-42
12. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr Res 2002;52:900-906
13. Dodic M, Samuel C, Moritz K, Wintour EM, Morgan J, Grigg L, Wong J Impaired cardiac functional reserve and left ventricular hypertrophy in adult sheep after prenatal dexamethasone exposure. Circ Res 2001;89:623-629
14. Huttenbach Y, Ostrowski ML, Thaller D, Kim HS Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. Cardiovasc Pathol 2001;10:119-123
15. Israel BA, Sherman FS, Guthrie RD Hypertrophic cardiomyopathy associated with dexamethasone therapy for chronic lung disease in preterm infants. Am J Perinatol 1993;10:307-310
16. Jobe AH, Bancalari E Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001;163:1723-1729
17. Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P Myocyte proliferation in end-stage cardiac failure in humans. Proc Natl Acad Sci U S A 1998;95:8801-8805
18. Kamphuis PJ, Bakker JM, Broekhoven MH, Kunne C, Croiset G, Lentjes EG, Tilders FJ, van Bel F, Wiegant VM Enhanced glucocorticoid feedback inhibition of hypothalamo-pituitary-adrenal responses to stress in adult rats neonatally treated with dexamethasone. Neuroendocrinology 2002;76:158-169
19. Kamphuis PJ, Gardoni F, Kamal A, Croiset G, Bakker JM, Cattabeni F, Gispen WH, van Bel F, Di Luca M, Wiegant VM Long-lasting effects of neonatal dexamethasone treatment on spatial learning and hippocampal synaptic plasticity: involvement of the NMDA receptor complex. FASEB J 2003;17: 911-913

20. Kinsella JP, Greenough A, Abman SH Bronchopulmonary dysplasia. Lancet 2006;367:1421-1431
21. La Mear NS, MacGilvray SS, Myers TF Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol Neonate 1997;72:175-180
22. Li F, Wang X, Capasso JM, Gerdes AM Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28:1737-1746
23. Li F, Wang X, Gerdes AM Formation of binucleated cardiac myocytes in rat heart: II. Cytoskeletal organisation. J Mol Cell Cardiol 1997;29:1553-1565
24. Loud AV, Anversa P, Giacomelli F, Wiener J Absolute morphometric study of myocardial hypertrophy in experimental hypertension. I. Determination of myocyte size. Lab Invest 1978;38:586-596
25. Mayhew TM, Pharaoh A, Austin A, Fagan DG Stereological estimates of nuclear number in human ventricular cardiomyocytes before and after birth obtained using physical disectors. J Anat 1997;191:107-115
26. Muangmingsuk S, Ingram P, Gupta MP, Arcilla RA, Gupta M Dexamethasone induced cardiac hypertrophy in newborn rats is accompanied by changes in myosin heavy chain phenotype and gene transcription. Mol Cell Biochem 2000;209:165-173
27. Murphy BE Ontogeny of cortisol-cortisone interconversion in human tissues: a role for cortisone in human fetal development. J Steroid Biochem 1981;14:811-817
28. Murphy BP, Inder TE, Huppi PS, Warfield S, Zientara GP, Kikinis R, Jolesz FA, Volpe JJ Impaired cerebral cortical gray matter growth after treatment with dexamethasone for neonatal chronic lung disease. Pediatrics 2001;107:217-221
29. Murphy VE, Smith R, Giles WB, Clifton VL Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocr Rev 2006;27:141-169
30. Nadal-Ginard B, Kajstura J, Leri A, Anversa P Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ Res 2003;92:139-150
31. Penney DG Postnatal modification of cardiac development: a review. J Appl Cardiol 1990;5:324-327
32. Re RN Cellular biology of the renin-angiotensin systems. Arch Intern Med 1984;144:2037-2041
33. Rudolph AM, Roman C, Gournay V Perinatal myocardial DNA and protein changes in the lamb: effect of cortisol in the fetus. Pediatr Res 1999;46:141-146
34. van Bel F, Dorrepaal CA, Benders MJ, Zeeuwe PE, van de BM, Berger HM Changes in cerebral hemodynamics and oxygenation in the first 24 hours after birth asphyxia5. Pediatrics 1993;92:365-372
35. van Oosterhout MFM, Prinzen FW, Arts T, Schreuder JJ, Vanagt WYR, Cleutjens JPM, Reneman RS Asynchronous Electrical Activation Induces Asymmetrical Hypertrophy of the Left Ventricular Wall. Circulation 1998;98:588-595
36. Vliegen HW, van der LA, Huysman JA, Wijnvoord EC, Mentar M, Cornelisse CJ, Eulderink F Morphometric quantification of myocyte dimensions validated in normal growing rat hearts and applied to hypertrophic human hearts. Cardiovasc Res 1987;21:352-357
37. Watterberg KL, Demers LM, Scott SM, Murphy S Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics 1996;97:210-215
38. Werner JC, Sicard RE, Hansen TW, Solomon E, Cowett RM, Oh W Hypertrophic cardiomyopathy associated with dexamethasone therapy for bronchopulmonary dysplasia. J Pediatr 1992;120:286-291
39. Whitehurst RM, Jr., Zhang M, Bhattacharjee A, Li M Dexamethasone-induced hypertrophy in rat neonatal cardiac myocytes involves an elevated L-type Ca(2+) current. J Mol Cell Cardiol 1999;31:1551-1558
40. Zak R Development and proliferative capacity of cardiac muscle cells. Circ Res 1974;35:suppl-26

CHAPTER 7

NEONATAL GLUCOCORTICOID TREATMENT OF RATS INDUCES LIFE-LONG CHANGES IN EXPRESSION OF CARDIAC CONTRACTILE PROTEINS

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To be submitted

ABSTRACT

Glucocorticoids, in particular dexamethasone (Dex), are often used to improve chronic lung disease in premature infants. Hypertrophic cardiomyopathy is an acute negative side effect thought to be transient. However, studies in rats suggest that neonatal treatment with Dex has long-term effects on cardiac structure and function. Dex-treated rats show a reduction in cardiomyocyte number and an increase in cardiomyocyte volume persisting throughout life.

To investigate whether the increase in cardiomyocyte cell volume induced by neonatal Dex treatment is associated with long-lasting changes in the ventricular expression of contractile and structural proteins. Rat pups were injected with Dex or saline (Sal) on day 1, 2 and 3 of life. At 4, 8 and 50 weeks rats were sacrificed and ventricular expression of α-actin, total myosin heavy chain (MHC), β-MHC, desmin, α- and β tubulin proteins were determined by Western blotting.

Dex treatment increased the expression of the contractile protein α-actin in all age groups; 4-week (33%, $p<0.01$), 8-week (45%, $p<0.05$) and 50-week (228%, $p<0.001$). This increase in α-actin was accompanied by an increase in total MHC (40%, $p<0.05$; 143%, $p<0.01$; 105%, $p<0.05$) and in β-MHC (94%, $p<0.01$; 60%, $p<0.05$; 61%, $p<0.05$) at all respective ages. The ratio of α-MHC/β-MHC mRNA was significantly decreased by neonatal Dex treatment in 8-week-old rats. Increase in contractile proteins was not associated with a Dex-induced change in the expression of structural proteins such as desmin and tubulin.

Neonatal Dex treatment induced an increase in the contractile proteins MHC and α-actin that persisted into adulthood. The change in MHC protein expression resembles the classical isoform switch that has been described in pressure or volume overload of the heart as well as in aging.

INTRODUCTION

Glucocorticoids (GCs), in particular dexamethasone (Dex), have been widely used to treat or prevent chronic lung disease in preterm infants suffering from severe infant respiratory distress syndrome [41].

Despite the short-term beneficial effects of neonatal Dex, there is increasing concern with respect to long-term deleterious side effects such as an impaired neurodevelopmental outcome of children [29,47]. In addition, short-term cardiovascular side effects have been described both in animals and humans although it has been speculated that the myocardial hypertrophy detected in preterm infants during neonatal Dex treatment is transient, long-term follow-up data are lacking [17,22,39,46]. The mechanism underlying the acute myocardial hypertrophy during treatment is as yet unknown, but is thought to be either due to a transient hypertension associated with steroid treatment or to cell specific effects of corticosteroids irrespective of hypertension [21].

It has been suggested that the newborn rat is a suitable animal model to study the possible long-term (adverse) effects of neonatal GC therapy in humans [19]. Using this model we have previously shown that neonatal Dex treatment causes systolic dysfunction and compensatory dilatation in juvenile rats [1]. Moreover, we observed a significantly reduced life-expectancy of rats treated neonatally with Dex as compared to Saline (Sal)-treated control rats [20]. A temporary suppression of normal physiological cardiomyocyte proliferation during and early after treatment with Dex on day 1, 2 and 3 of life was detected in the same model, contributing to a reduced number of cardiomyocytes in adulthood [9]. This finding was confirmed in another study, in which we showed that neonatal Dex treatment in rats induced a life long hypertrophy of the cardiomyocytes presumably because of the lower amount of cardiomyocytes (chapter 6).

We hypothesized that the observed functional and histopathological changes of the heart after neonatal Dex treatment are associated with long lasting changes in contractile- or structural proteins or both. The contractile protein myosin is responsible for energy transduction and force development in cardiac and skeletal muscles [12,14]. It is composed of two myosin heavy chains (MHC): α and β. In rats during fetal life β-MHC is the predominant isoform, whereas in adult life a switch occurs towards decreased β- and increased α-MHC [4]. Pressure-and volume-overload induced hypertrophy and senescence are known to change the developmental pattern towards a return of fetal gene expression, hence increased β-MHC and decreased α-MHC [8,35,36]. The contractile activity of α-MHC is considered to be fast while β-MHC is more economical though considerably slower than the α-MHC [25,31,42]. Another contractile protein, α-actin, can be present as two isoforms in the cardiac muscle cell, the skeletal isoform and the cardiac isoform [37]. In the rat heart, both isoforms are expressed in utero but at adult age the mRNA expression of the cardiac isoform is predominant [3]. An increase in α-actin mRNA of the skeletal isoform has been demonstrated in a variety of

experimental models of hypertension accompanied by myocardial hypertrophy [30,35,36,38]. Because the skeletal isoform of α-actin is normally only active in fetal life and not during adult life, the α-actin expression switch by hemodynamic overload represents another example of a fetal program reactivation [38,40].

The intermediate filament protein desmin and the microtubule proteins α- and β-tubulin are examples of structural proteins, also called true cytoskeletal proteins. The desmin filaments play an important role in maintaining the structural integrity of myocytes [45] and tubulin, which is composed of α and β-tubulin dimers, is responsible for the transmission of mechanical and chemical stimuli within and between cells [34,43]. In numerous experimental studies, the role of cytoskeletal alterations especially of microtubules and desmin, in cardiac hypertrophy and congestive heart failure has been described [7,34,44].

The aim of the present study was to characterize the cardiomyocyte hypertrophy induced by neonatal Dex treatment at the protein level. Given that pathologic stimuli can cause shifts in the expression of the contractile proteins MHC as well as changes in expression of α-actin, we analyzed changes in expression of these contractile proteins throughout life in rats treated neonatally with Dex in comparison to rats treated with Saline (Sal). We compared these data with the expression of the structural proteins desmin and tubulin in the same animals.

MATERIALS AND METHODS

Animals

The study protocol was approved by the Animal Research Committee of the University of Leiden. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No.85–23, revised 1996).

Pregnant Wistar rats (270 –300 g) were housed individually and kept under conventional housing conditions. Pups were born on day 21–22 of gestation. On the day of birth (d0), male pups were selected and randomly divided between treatment and control groups. Treatment and control animals were kept separately and placed with foster mothers in groups of six pups. Rat pups in the treatment group were injected intraperitoneally with Dex using a 3-day tapering dose following the protocol we used before [19]. Consequently, the treated animals received 0.5, 0.3, and 0.1 μg/g body weight Dex on day 1, 2, and 3 of life, respectively. The animals in the control group received equal volumes (10 μL/g body weight) sterile pyrogen-free Sal. Temperature and humidity were kept constant and the rats had free access to food and water. An artificial 12 h-light/12 h-dark cycle was used. Body weight was measured daily during the first week and regularly from day 7 onward. The rats were weaned on day 21 and the groups (n=7-8/group) were sacrificed by decapitation at 4 weeks (juvenile), 8 weeks (adult) or 50 weeks (middle aged).

Antibodies

The following primary antibodies were used: mouse-anti-α-Actin (Sigma-Aldrich, Saint Louis, USA), mouse-anti-α-Tubulin (Sigma-Aldrich), mouse-anti-β-Tubulin (Sigma-aldrich), mouse-anti-myosin-heavy chain-α/β (α-β-MHC) (Chemicon International Huissen, Netherlands), mouse-anti-myosin-heavy chain-β (β-MHC) (Chemicon International), mouse-anti-Desmin (Sigma-Aldrich).

The secondary antibody used was goat-anti-mouse-IgG conjugated with horseradish peroxidase (Sigma-Aldrich).

Western Blotting

After decapitation the hearts were rapidly excised and the atria were removed. Subsequently, ventricles were freeze-clamped in liquid nitrogen. Samples were preserved on dry ice during the procedure and stored at -80 °C afterwards. Frozen ventricular tissue was crushed under liquid nitrogen using a mortar and pestle. Crushed tissue was homogenized in a buffer (20 mmol/L Tris pH 7.4, 5 mmol/L EDTA, and 100 mmol/L NaCl, 0.1% SDS, 1% sodium-deoxycholate, 1% Triton-x-100) supplemented with protease inhibitors (leupeptin 10µg/mL, pepstatin 5 µg/mL, benzamidine 200µg/mL and 1 mmol/L PMSF). Homogenates were centrifuged at 13,000*g* for 15 mins to remove nuclei and debris. Supernatants were collected and aliquots were stored at -80°C. Protein concentration was determined using Bio-Rad protein assay using bovine serum albumin (BSA) as standard. Western blot analyses were performed using 5-20 µg aliquots of protein, resolved on 10% and 7.5% SDS-PAGE gels. Proteins were then transferred to nitrocellulose membranes (Hybond-C, Amersham, UK) by electroblotting. Membranes were incubated either 2 hrs at room temperature or overnight at 4°C with 5% dry milk-TBST or 2% BSA-TBST with the primary antibodies followed by incubation for 1 hour with the secondary antibody. Bands were visualized using the enhanced chemiluminescence detection system (ECL, Amersham, UK) and exposure to X-ray film. Chemiluminographs were scanned using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

Reverse Transcription and Real-Time PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carsbad, USA). Using random primers cDNA was synthesized from 0.5ug total RNA with the Superscript RNase H-reverse transcriptase kit (invitrogen). Real-time polymerase chain reaction (PCR) was performed with the use of DNA Master SYBR-green kits and a light cycler apparatus (Roche applied science). The following primer pairs were used: CAG ATT TTC CCG GTG GAG AG for α-MHC and ACA GTC ACC GTC TTG CCA TTC for β-MHC. The ratio of alpha to beta-MHC was determined.

Statistics

Data for Western blot analyses were normalized to the average densitiy of bands in the Sal group of each age group and are presented as means ± SD. Data for Sal- and Dex-treated

animals in each age group were analyzed using unpaired t-tests. P values of <0.05 were considered statistically significant.

RESULTS

Differences in body weight (BW), ventricular weight (VW) and the ratio VW/BW between Dex- and Sal-treated rats have been described in an earlier study (Chapter 6). Briefly, Dex-treated rats had significantly lower BW (16%), VW (22%) and VW/BW (8%) at 4 weeks. In the 8-week-old rats, only VW was significantly lower (11%) in Dex-treated rats, whereas at 50 weeks no differences were detected in BW, VW or ratio VW/BW. Cardiomyocyte volume was increased at 4 weeks of age (18%) and further increased in 8- and 50-week-old Dex-treated rats to respectively 29% and 67% larger than the cardiomyocyte volume of saline-treated rats.

Expression of contractile proteins

In the Dex-treated rats, α-actin was increased at 4 weeks (33% $p<0.01$) and continued to increase at 8 weeks (45% $p<0.05$) and at 50 weeks of age (228% $p<0.001$) (Figure 1a). The expression of MHC was analyzed using an antibody that recognizes both the α as well as the β isoform of MHC. The expression of MHC was significantly increased at 4 weeks of age in the Dex-treated animals by 40% ($p<0.05$). The increase in MHC was even more pronounced in the 8- and 50-week-old Dex-treated rats with 143% ($p<0.01$) and 105% ($p<0.05$) respectively (Figure 1b). We also performed Western Blot analyses with an antibody specific for β-MHC. Similarly the expression of β-MHC increased significantly at 4 weeks (94% $p<0.01$), 8 weeks (60% $p<0.05$) and 50 weeks (61% $p<0.05$) (Figure 1c). We were not able to test α-MHC separately since no commercial antibody against this particular isoform is available. Therefore, to address the question whether Dex treatment induces a change in the ratio of α- to β-MHC we performed real-time PCR analyses on reverse transcribed RNA from hearts from 4-week- and 8-week-old rats. Neonatal Dex treatment did not alter the ratio of α-MHC to β-MHC mRNA as determined at 4 weeks. However, at the age of 8 weeks, the ratio α-MHC to β-MHC was significantly decreased ($p<0.01$) in the Dex-treated rats, suggesting a shift towards an increase in β-MHC expression, consistent to what is seen during hypertrophy (Figure 2).

Expression of cytoskeletal proteins

In contrast to the contractile proteins, the mean expression of the cytoskeletal proteins desmin and β-tubulin did not differ between the Dex-treated rats and Sal-treated rats at all ages. In addition, the expression of α-tubulin in the 4- and 50 weeks-old Dex-treated rats was not different from Sal-treated rats. Only at 8 weeks we observed significantly higher values for α-tubulin in the Dex-treated rats (75%; $p<0.05$) (Figure 3 a-c).

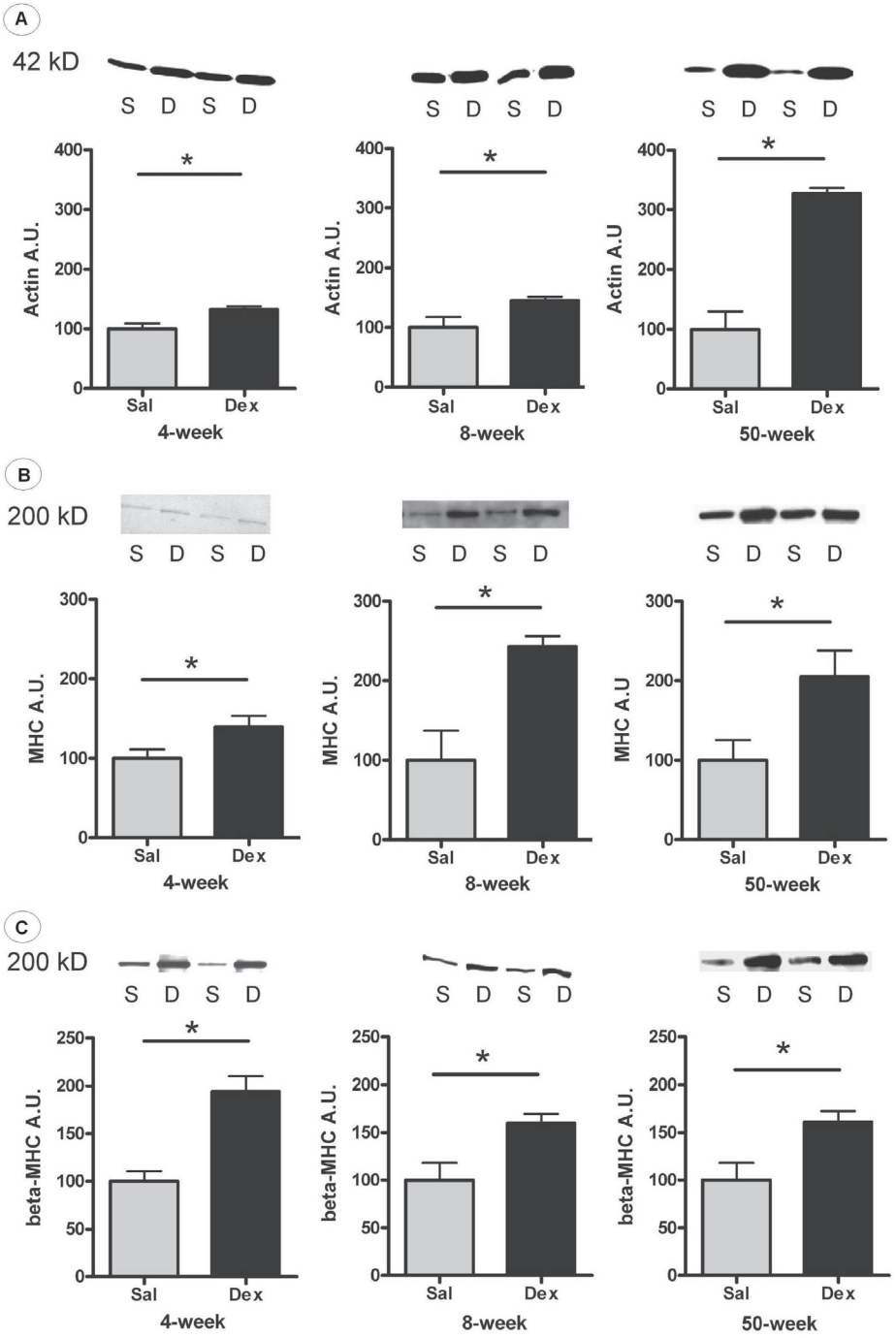

Figure 1a-c: Increased α-actin, total-MHC and β-MHC expression was observed in 4-, 8- and 50-week-old Dex-treated rats. Data are presented as mean ± sd and are depicted as percentage of the control group. The Western blots represent the individual animals either treated with Sal or Dex and are shown as such in an alternating way.

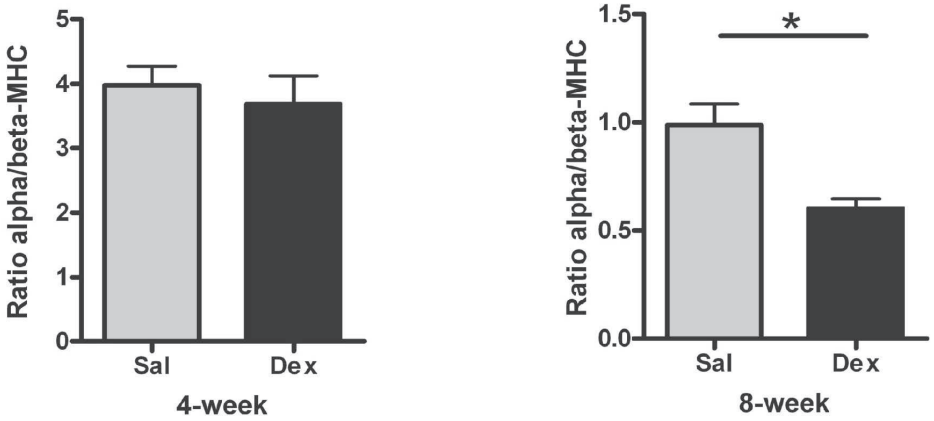

Figure 2: The ratio of α-MHC to β-MHC mRNA in the 4-week-old Dex-treated rats was not different as compared to Sal-treated rats. At 8 weeks the ratio α-MHC to β-MHC was significantly decreased ($p<0.01$) in the Dex-treated rats, suggesting a shift towards an increase in β-MHC expression.

DISCUSSION

The data presented here demonstrated that neonatal Dex treatment in rats induced a life-long increase in the cardiac contractile proteins MHC and α-actin, whereas the expression of the structural proteins desmin and tubulin remained largely unchanged. Dex treatment induced an increase in the expression of proteins recognized by an antibody directed against both α and β-MHC at all ages. Using a specific antibody against β-MHC, we showed that at all ages this protein was significantly increased in the Dex-treated rats. In addition, the ratio of the α- and β-MHC isoforms at the mRNA-level was not changed at 4 weeks, but increased at 8 weeks in the Dex-treated rats, indicating that a shift towards increased β-MHC isoform levels occurred. A significant increase in the contractile protein α-actin was also observed in all age groups. In contrast, the relative expression of the two structural proteins tubulin and desmin did not change in the Dex-treated rats compared to the Sal-treated rats, although α-tubulin was significantly increased in the 8-week-old Dex-treated rats. The latter may reflect the active involvement of tubulin in the reparation of cardiomyocytes [11], which would not be surprising in view of the partly restored cardiac function at this age (Bal et al. submitted).

The long-lasting Dex-induced increase in contractile proteins and not structural proteins, is suggesting that the effect of Dex is specific for certain proteins and does not reflect a general change in the expression of all cytoskeleton associated proteins.

A former study of our group showed that neonatal Dex treatment in rats resulted in inhibition of cardiomyocyte mitosis and a reduction in the total number of cardiomyocytes present at the age of 3 weeks [9]. In addition, cellular hypertrophy was observed in 4-, 8- and 50-week-old Dex-treated rats (Chapter 6). It has been described that during hypertrophy the expression of contractile cardiac proteins changes and that re-expression of a fetal

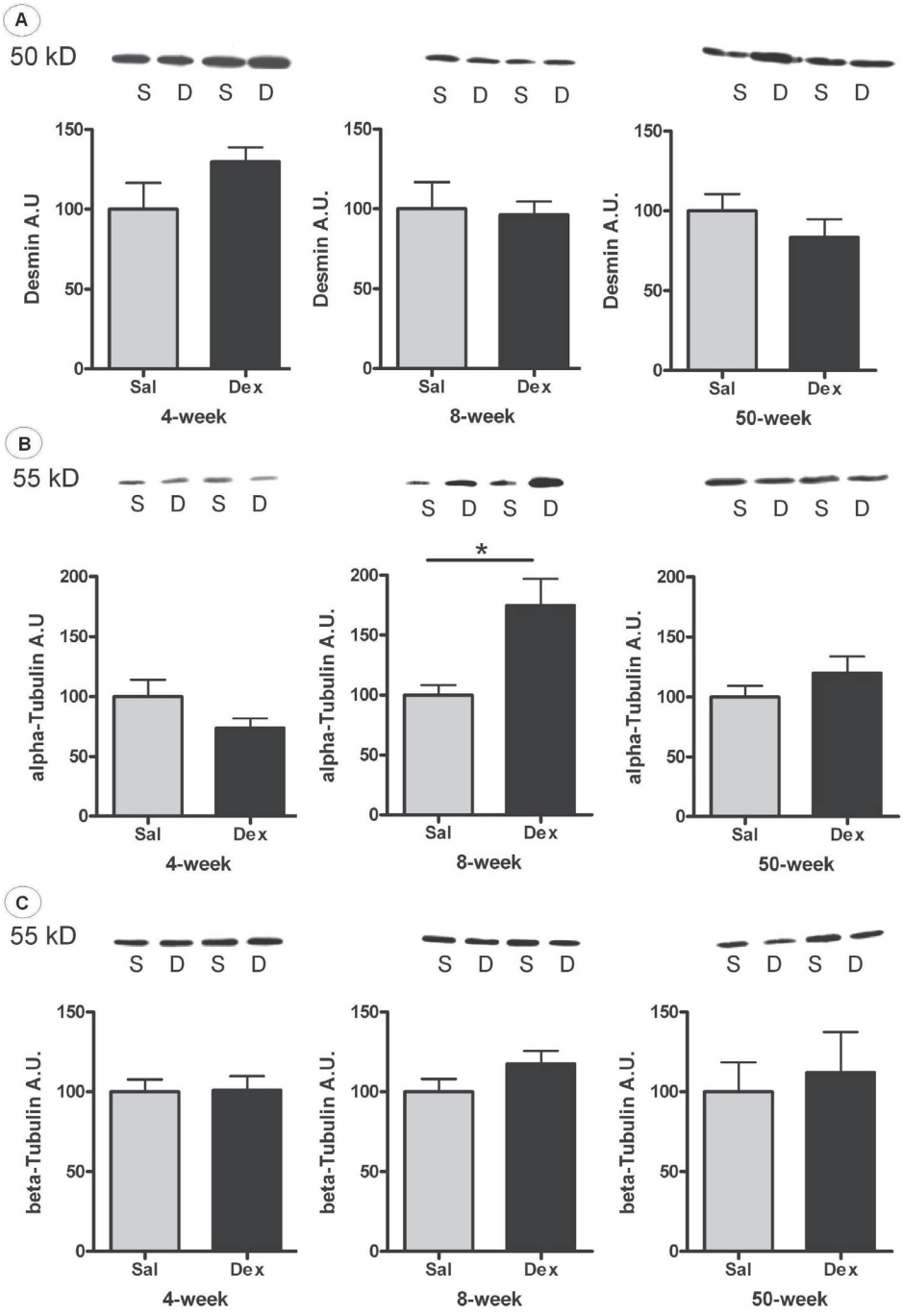

Figure 3a-c: No changes in expression of the cytoskeletal proteins desmin and β-tubulin between the Dex- and Sal-treated rats in all groups. In the 4-, and 50-week-old rats also α-tubulin expression did not change, whereas in the 8-week-old Dex-treated rats a significant higher expression was observed ($p<0.05$)

phenotype occurs during this process. The mechanism and factors involved in these changes are still largely unknown [5,26]. The neonatal Dex-induced increase in β-MHC protein at 4, 8 and 50 weeks and the decrease in the ratio of alpha/beta MHC mRNA observed at 8 weeks resembles the classical isoform switch from α to β-MHC that has been described in pressure- and volume-overload induced hypertrophy and in senescence [25,27,42]. Such changes in proportion of MHC proteins are thought to be directly related to the level of mechanical performance of the heart [27]. A shift towards more β-MHC could be advantageous since less energy is required for force maintenance. It reduces the speed of contraction and relaxation in the hypertrophied fiber [13,24] resulting in improved economy of force development that is usually considered adaptive [18,36].

In a recent study performed in 4-week-old rats treated neonatally with Dex we observed systolic dysfunction and compensatory dilatation with increased volumes [1]. Interestingly, at this time point, we did already observe increased β-MHC and α-actin expression. It may well be possible that this change in contractile proteins results from the volume overload and reflects an adaptive response of the cardiomyocytes. At 4 weeks the ratio of α/β-MHC mRNA was similar in Sal- and Dex-treated animals. At 8 weeks, however, the α/β MHC mRNA ratio showed a shift towards increased β-MHC concomitant with normal hemodynamic function (submitted). Interestingly, at 50 weeks the increased expression in β-MHC was still observed in the Dex-treated group, but at that age systolic function was depressed (Bal et al. submitted), suggesting that compensatory mechanisms start to fail. Unfortunately, we do not have data yet on the ratio of alpha-and beta MHC at this time point.

Not only MHC expression, but also α-actin expression was increased throughout life after neonatal Dex treatment. It has been suggested that an upregulation of α-skeletal actin isoform in hypertrophic myocardium may serve to achieve a high degree of myocardial contractility [2,15]. Since we used an antibody that recognizes both alpha-skeletal and alpha cardiac muscle actins, we do not know which subtype of actin was altered. We hypothesize, however, that the increase in MHC protein, the decrease in α/β MHC ratio and the increase in actin reflect adaptation of the cardiomyocytes to enhance contractile performance.

During aging continuous loss of myocytes has been suggested to generate a greater workload on the remaining myocyte, thereby increasing the level of β-MHC [33,37,40]. It has been shown that with an increase in body size and age the expression of the slow β-MHC isoform becomes more pronounced [42]. Moreover, a shift towards increased β-MHC isoform has been reported in accelerated senescence [8]. Interestingly, we have shown that the life span of rats treated neonatally with Dex was markedly reduced and that cardiomyocytes showed early degeneration during adulthood [10,20]. It may well be possible that neonatal Dex treatment leads to earlier aging, and that the difference between Dex-treated and Sal-treated rats with respect to cardiac contractile proteins reflects a process of premature aging. Interestingly, Muangmingsuk et al. [28] described an acute Dex treatment induced increase in α-MHC mRNA expression and a decreased β-MHC mRNA expression after neonatal Dex

treatment in rats, which were sacrificed 1, 3, 5, 7 and 9 days after treatment. Apparently, the acute effects of neonatal Dex treatment (increased ratio α-MHC to β-MHC) are different from the long term effect of treatment we observe here (decreased ratio α-MHC to β-MHC). Clark et al. [6] have shown that Dex treatment in *young adult* rats also induces an increase in contractile proteins, and preferentially MHC. They speculate that the increase in contractile proteins and preferentially MHC is exerted through a direct effect of GCs on protein metabolism and that GCs produce heterogeneous regulation of specific cardiac proteins [32]. In this study we have shown a long-lasting increase in contractile protein level after *neonatal* Dex treatment. It is therefore not likely that direct effects of Dex on protein metabolism are responsible. The possibility remains however, that acute Dex treatment induces epigenetic modifications in the α and /or β-MHC gene that have consequences into adulthood.

Based on the present and other studies performed by our group we question the safety of early neonatal GC-treatment in humans. We used a rat model to investigate the long term cardiac effects after neonatal Dex treatment. Extrapolation of the results from this and other experimental studies must be exerted with caution, since the myocardial tissue of term rat pups may have a different affinity to Dex than the myocardial tissue of the preterm infant. However, several studies have shown that the proliferative pattern of cardiomyocytes in the perinatal period in human tissue generally mirrors the pattern seen in rat hearts, in which hyperplasia in the early postnatal period changes to hypertrophy after birth [16,23]. It has also been demonstrated that during Dex treatment newborn pups develop cardiac hypertrophy during the treatment similar to that seen in premature infants treated with GCs [22,28,46].

In conclusion, neonatal Dex treatment induces an increase in contractile proteins that persists into adulthood. It is conceivable that the increase in contractile proteins contributes to maintain cardiac function as an adaptive response to the reduced cardiac cell number but eventually will fail in adulthood.

REFERENCE LIST

1. Bal MP, de Vries WB, van der Leij FR, van Oosterhout MF, Berger RM, Baan J, van der Wall EE, van Bel F, Steendijk P Neonatal glucocorticosteroid treatment causes systolic dysfunction and compensatory dilation in early life: studies in 4-week-old prepubertal rats. Pediatr Res 2005;58:46-52
2. Black FM, Packer SE, Parker TG, Michael LH, Roberts R, Schwartz RJ, Schneider MD The vascular smooth muscle alpha-actin gene is reactivated during cardiac hypertrophy provoked by load. J Clin Invest 1991;88:1581-1588
3. Carrier L, Boheler KR, Chassagne C, de la BD, Wisnewsky C, Lakatta EG, Schwartz K Expression of the sarcomeric actin isogenes in the rat heart with development and senescence. Circ Res 1992;70:999-1005
4. Chien KR 1999 Molecular Basis of Cardiovascular Disease: A Companion to Braunwald's Heart Disease. In: Breslow JL, Leiden JM, Rosenberg RD, Seidman CE (eds) W.B. Saunders Company, pp 191-209
5. Chien KR, Knowlton KU, Zhu H, Chien S Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. FASEB J 1991;5:3037-3046
6. Clark AF, DeMartino GN, Wildenthal K Effects of glucocorticoid treatment on cardiac protein synthesis and degradation. Am J Physiol 1986;250:C821-C827
7. Collins JF, Pawloski-Dahm C, Davis MG, Ball N, Dorn GW, Walsh RA The role of the cytoskeleton in left ventricular pressure overload hypertrophy and failure. J Mol Cell Cardiol 1996;28:1435-1443
8. Compagno V, Di L, I, Cestelli A, Donatelli M Effect of aging and hypertension on beta-myosin heavy chain in heart of spontaneously hypertensive rats. Int J Mol Med 2001;7:507-508
9. de Vries WB, Bal MP, Homoet-van der Kraak P, Kamphuis PJ, van der Leij FR, Baan J, Steendijk P, de Weger RA, van Bel F, van Oosterhout MF Suppression of physiological cardiomyocyte proliferation in the rat pup after neonatal glucocorticosteroid treatment. Basic Res Cardiol 2006;101:36-42
10. de Vries WB, van der Leij FR, Bakker JM, Kamphuis PJ, van Oosterhout MF, Schipper ME, Smid GB, Bartelds B, van Bel F Alterations in adult rat heart after neonatal dexamethasone therapy. Pediatr Res 2002;52:900-906
11. Dudnakova TV, Lakomkin VL, Tsyplenkova VG, Shekhonin BV, Shirinsky VP, Kapelko VI Alterations in myocardial cytoskeletal and regulatory protein expression following a single Doxorubicin injection. J Cardiovasc Pharmacol 2003;41:788-794
12. Eisenberg E, Greene LE The relation of muscle biochemistry to muscle physiology. Annu Rev Physiol 1980;42:293-309
13. Fitzsimons DP, Patel JR, Moss RL Cross-bridge interaction kinetics in rat myocardium are accelerated by strong binding of myosin to the thin filament. J Physiol 2001;530:263-272
14. Harrington WF, Rodgers ME Myosin. Annu Rev Biochem 1984;53:35-73
15. Hewett TE, Grupp IL, Grupp G, Robbins J Alpha-skeletal actin is associated with increased contractility in the mouse heart. Circ Res 1994;74:740-746
16. Huttenbach Y, Ostrowski ML, Thaller D, Kim HS Cell proliferation in the growing human heart: MIB-1 immunostaining in preterm and term infants at autopsy. Cardiovasc Pathol 2001;10:119-123
17. Israel BA, Sherman FS, Guthrie RD Hypertrophic cardiomyopathy associated with dexamethasone therapy for chronic lung disease in preterm infants. Am J Perinatol 1993;10:307-310
18. Izumo S, Lompre AM, Matsuoka R, Koren G, Schwartz K, Nadal-Ginard B, Mahdavi V Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. Interaction between hemodynamic and thyroid hormone-induced signals. J Clin Invest 1987;79:970-977
19. Kamphuis PJ, Bakker JM, Broekhoven MH, Kunne C, Croiset G, Lentjes EG, Tilders FJ, van Bel F, Wiegant VM Enhanced glucocorticoid feedback inhibition of hypothalamo-pituitary-adrenal responses to stress in adult rats neonatally treated with dexamethasone. Neuroendocrinology 2002;76:158-169

20. Kamphuis PJ, de Vries WB, Bakker JM, Kavelaars A, van Dijk JE, Schipper ME, van Oosterhout MF, Croiset G, Heijnen CJ, Van Bel F, Wiegant VM Reduced life expectancy in rats after neonatal dexamethasone treatment. Pediatr Res (in press)
21. Komamura K, Shirotani-Ikejima H, Tatsumi R, Tsujita-Kuroda Y, Kitakaze M, Miyatake K, Sunagawa K, Miyata T Differential gene expression in the rat skeletal and heart muscle in glucocorticoid-induced myopathy: analysis by microarray. Cardiovasc Drugs Ther 2003;17:303-310
22. La Mear NS, MacGilvray SS, Myers TF Dexamethasone-induced myocardial hypertrophy in neonatal rats. Biol Neonate 1997;72:175-180
23. Li F, Wang X, Capasso JM, Gerdes AM Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. J Mol Cell Cardiol 1996;28:1737-1746
24. McDonald KS, Herron TJ It takes "heart" to win: what makes the heart powerful? News Physiol Sci 2002;17:185-190
25. Mercadier JJ, Lompre AM, Wisnewsky C, Samuel JL, Bercovici J, Swynghedauw B, Schwartz K Myosin isoenzyme changes in several models of rat cardiac hypertrophy. Circ Res 1981;49:525-532
26. Morgan HE, Baker KM Cardiac hypertrophy. Mechanical, neural, and endocrine dependence. Circulation 1991;83:13-25
27. Morkin E Control of cardiac myosin heavy chain gene expression. Microsc Res Tech 2000;50:522-531
28. Muangmingsuk S, Ingram P, Gupta MP, Arcilla RA, Gupta M Dexamethasone induced cardiac hypertrophy in newborn rats is accompanied by changes in myosin heavy chain phenotype and gene transcription. Mol Cell Biochem 2000;209:165-173
29. Murphy BP, Inder TE, Huppi PS, Warfield S, Zientara GP, Kikinis R, Jolesz FA, Volpe JJ Impaired cerebral cortical gray matter growth after treatment with dexamethasone for neonatal chronic lung disease. Pediatrics 2001;107:217-221
30. Nadal-Ginard B, Mahdavi V Molecular basis of cardiac performance. Plasticity of the myocardium generated through protein isoform switches. J Clin Invest 1989;84:1693-1700
31. Narolska NA, van Loon RB, Boontje NM, Zaremba R, Penas SE, Russell J, Spiegelenberg SR, Huybregts MA, Visser FC, de Jong JW, van d, V, Stienen GJ Myocardial contraction is 5-fold more economical in ventricular than in atrial human tissue. Cardiovasc Res 2005;65:221-229
32. Nichols NR, Tracy KE, Funder JW Glucocorticoid effects on newly synthesized proteins in muscle and non-muscle cells cultured from neonatal rat hearts. J Steroid Biochem 1984;21:487-496
33. O'Neill L, Holbrook NJ, Fargnoli J, Lakatta EG Progressive changes from young adult age to senescence in mRNA for rat cardiac myosin heavy chain genes. Cardioscience 1991;2:1-5
34. Schaper J, Froede R, Hein S, Buck A, Hashizume H, Speiser B, Friedl A, Bleese N Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. Circulation 1991;83:504-514
35. Schiaffino S, Samuel JL, Sassoon D, Lompre AM, Garner I, Marotte F, Buckingham M, Rappaport L, Schwartz K Nonsynchronous accumulation of alpha-skeletal actin and beta-myosin heavy chain mRNAs during early stages of pressure-overload--induced cardiac hypertrophy demonstrated by in situ hybridization. Circ Res 1989;64:937-948
36. Schwartz K, Boheler KR, de la BD, Lompre AM, Mercadier JJ Switches in cardiac muscle gene expression as a result of pressure and volume overload. Am J Physiol 1992;262:R364-R369
37. Schwartz K, Chassagne C, Boheler KR The molecular biology of heart failure. J Am Coll Cardiol 1993;22:30A-33A
38. Schwartz K, de la BD, Bouveret P, Oliviero P, Alonso S, Buckingham M Alpha-skeletal muscle actin mRNA's accumulate in hypertrophied adult rat hearts. Circ Res 1986;59:551-555
39. Skelton R, Gill AB, Parsons JM Cardiac effects of short course dexamethasone in preterm infants. Arch Dis Child Fetal Neonatal Ed 1998;78:F133-F137

40. Takahashi T, Schunkert H, Isoyama S, Wei JY, Nadal-Ginard B, Grossman W, Izumo S Age-related differences in the expression of proto-oncogene and contractile protein genes in response to pressure overload in the rat myocardium. J Clin Invest 1992;89:939-946
41. Truffert P, Empana JP, Breart G, Saugstad OD, Goelz R, Halliday HL, Anceschi M Treatment strategies for bronchopulmonary dysplasia with postnatal corticosteroids in Europe: the EURAIL survey. Acta Paediatr 2003;92:948-951
42. van d, V, Moorman AF, Stienen GJ Age-dependent changes in myosin composition correlate with enhanced economy of contraction in guinea-pig hearts. J Physiol 1998;507 (Pt 2):497-510
43. Wang N, Ingber DE Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension. Biophys J 1994;66:2181-2189
44. Wang X, Li F, Campbell SE, Gerdes AM Chronic pressure overload cardiac hypertrophy and failure in guinea pigs: II. Cytoskeletal remodeling. J Mol Cell Cardiol 1999;31:319-331
45. Wang X, Osinska H, Gerdes AM, Robbins J Desmin filaments and cardiac disease: establishing causality. J Card Fail 2002;8:S287-S292
46. Werner JC, Sicard RE, Hansen TW, Solomon E, Cowett RM, Oh W Hypertrophic cardiomyopathy associated with dexamethasone therapy for bronchopulmonary dysplasia. J Pediatr 1992;120: 286-291
47. Yeh TF, Lin YJ, Lin HC, Huang CC, Hsieh WS, Lin CH, Tsai CH Outcomes at school age after postnatal dexamethasone therapy for lung disease of prematurity. N Engl J Med 2004;350:1304-1313

CHAPTER 8

SUMMARY, FUTURE PERSPECTIVES AND CONCLUDING REMARKS

SUMMARY

The present thesis describes the issue of "neonatal glucocorticoid treatment and predisposition to cardiovascular disease in rats". Glucocorticoid treatment, in particular dexamethasone (Dex), is widely used to treat chronic lung disease in premature infants. However, short and long-term side effects have been reported both in animal and human studies. Especially, the long-term neurological side effects such as cerebral palsy and delayed and abnormal neurological development have been described. With regard to the cardiovascular system, the short-term side effects are well documented, but the long-term effects are still largely unknown. However, recent studies in adult rats indicated negative long-term effects of neonatal Dex treatment on the heart and a severely reduced life expectancy. In this thesis we have therefore investigated the functional, histopathological and biochemical consequences of neonatal Dex treatment on the heart during life span in rats.

An earlier study of our group showed that neonatal Dex treatment in rat pups caused a decrease in heart weight, probably due to inhibition of myocyte mitotic activity during treatment. Moreover this treatment resulted in permanent histopathological changes, in particular cellular hypertrophy, in the adult heart.

In **chapter 2** we investigated the early effects of neonatal Dex treatment in rats on cell proliferation after birth. We tested the hypothesis that a lower number of cardiomyocytes later in life was caused by a Dex-induced, diminished cardiomyocyte proliferation and/or early cell death. This is a plausible assumption in view of the fact that GCs are administered during a critical period of cardiac development. In an experimental but established model rat model, histopathological and immunohistochemical studies were performed to look at signs of apoptosis and/or differences in proliferation capacity of cardiomyocytes before, during and after Dex treatment.

Male rat pups were injected intraperitoneally with Dex on day 1, 2, and 3 (0.5, 0.3 and 0.1 µg/g) of life. Dosages and duration of treatment were proportional to those used in human preterm infants. Control pups received Sal in equal volumes. The rats were sacrificed at day 0, 2, 4, 7 and 21, and hearts were harvested and immersion fixed in formalin.

The cardiomyocytes of the Dex-treated pups showed a reduced proliferation as indicated by a lower mitotic index and reduced number of Ki-67-positive cardiomyocytes on day 2 and 4 compared to day 0 and day 7 and also as compared to the age-matched Sal-pups. On day 7 and day 21 the mitotic index was not different between groups. From day 2 onward up to day 21 Dex-treated pups showed a lower number of cardiomyocytes. No signs of cardiomyocyte apoptosis were found in any group.

From this study we concluded that Dex treatment causes temporary suppression of cardiomyocyte hyperplasia which will lead to a reduced number of cardiomyocytes during life.

In **chapter 3** we determined the effects of neonatal Dex treatment on cardiac function in 4-week-old rats (pre-pubertal), measured by pressure-volume loops. This study was designed to investigate if neonatal Dex treatment causes detectable alterations in cardiac function at a young age. Using the same animal model as described before; the animals were anesthetized, intubated and ventilated, and a miniature pressure-conductance catheter was introduced into the left ventricle to measure pressure-volume loops. Cardiac function was measured and systolic and diastolic pressure-volume relations (ESPVR/EDPVR) were determined to quantify intrinsic systolic and diastolic function, virtually independent of loading conditions. Subsequently, hearts were excised for histological examination. Compared to Sal-treated animals, Dex-treated rats had a reduced ventricular weight (Dex vs Sal: 270±40 vs. 371±23mg, $p<0.001$) and reduced systolic function (end-systolic elastance: 1.24±0.43 vs. 2.50±1.39 mmHg/µL, $p=0.028$; end-systolic volume intercept at 75 mmHg: 34±13 vs. 53±20 µl, $p=0.016$). Cardiac output was maintained and end-diastolic volume was increased (84±23 vs. 59±19 µL, $p=0.012$) indicating a state of compensatory dilatation. Heart rate, diastolic function and systemic vascular resistance were unchanged. From these findings we concluded that cardiac dysfunction was present already in the prepubertal period.

In **chapter 4** we investigated the pressure-volume relations during growth and development in the normal rat. Whereas it is known that left ventricular pressure-volume relations provide relatively load-independent indexes of systolic and diastolic LV function, little data are available on pressure-volume relations during growth and development in the normal adult heart. Furthermore, in order to quantify intrinsic ventricular function in animals of different size and weight, the indexes derived from pressure-volume relations ideally should be normalized for heart weight (i.e. volume is replaced by volume/heart weight). In many studies, however, the indexes are reported in absolute terms, or body-weight correction is used as a surrogate for heart weight-correction. Therefore, the effects of normalization on pressure-volume relations were also examined. We determined pressure-volume relations in young adult (8-week-old) and middle-aged (50-week-old) normal Wistar rats.

Heart and body weights were significantly higher in the 50-week-old rats, whereas the heart-to-body weight ratio was significantly lower (2.74±0.32 vs. 4.41±0.37 mg/g, $p<0.001$). Intrinsic systolic function, quantified by the slopes of the end-systolic pressure-volume relation (E_{ES}), the dP/dt_{MAX} vs. end-diastolic volume relation (S-dP), and the preload recruitable stroke work relation (PRSW), normalized for heart weight, was slightly decreased in the 50-week-old rats (S-dP: -26%, $p<0.004$; PRSW: -13%, $p<0.06$). Heart weight-corrected diastolic indexes were not significantly different. The absolute indexes qualitatively showed the same results, but body-weight corrected pressure-volume indexes showed improved systolic function and significantly depressed diastolic function.

This experimental study showed that intrinsic systolic function slightly decreases from the juvenile to the middle-aged period in normal male Wistar rats. Furthermore we found

that correction of pressure-volume indexes for body weight is not an adequate surrogate for heart weight-correction in these animals.

In the previous chapters we reported suppressed mitosis during Dex treatment leading to significantly reduced ventricular weight, depressed systolic function and compensatory dilatation in prepubertal (4-week-old) rats. In addition, recent experimental data indicated a significantly reduced life expectancy of rats treated with Dex in the neonatal period [2]. Therefore the aim of the study described in **Chapter 5** was to investigate the long-term effects of neonatal Dex treatment on cardiovascular function. Neonatal rat pups were treated with Dex or received Sal. Cardiac function was determined in 8-, 50-, and 80-week-old animals, representing young adult, middle-aged, and elderly stages. We determined cardiac function as described before.

Our results showed reduced ventricular and body weights in Dex-treated rats at 8 and 80 weeks, but not at 50 weeks. Compared to control animals, cardiac output and diastolic function were unchanged in all age groups, but systolic function was depressed at 50 and 80 weeks evidenced by reduced ejection fractions and rightward shifts of the end-systolic pressure-volume relationships.

From this study we concluded that the early adverse effects of neonatal Dex treatment, as found in the 4-week-old rats, are transient but reduced ventricular weight and systolic dysfunction become manifest again in elderly rats. We speculate that cellular hypertrophy and possible other factors (see below chapter 7) initially compensate for the Dex treatment-induced lower number of cardiomyocytes but fail at a later stage leading to reappearance of systolic dysfunction.

In the previous chapters we investigated hemodynamic function in normal and Dex-treated rats in different age groups. Using the same animals as before, **Chapter 6** describes histopathological myocardial characteristics after neonatal Dex treatment during life span (4-, 8- and 50-week-old rats) in comparison with Sal-treated rats.

Heart tissue was stained with HE, Cadherin-PAS and Sirius Red for cardiomyocyte morphometry and collagen determination. Cardiomyocyte length of the Dex-treated rats was significantly increased ($p<0.05$) compared to controls in all three age groups, whereas ventricular weight was reduced. This was accompanied by a significant increase in cardiomyocyte width ($p<0.01$) in the 50-week-old rats. These changes resulted in significantly increased cardiomyocyte volume at 50 weeks ($p<0.01$) indicating cellular hypertrophy. Collagen content gradually increased with age and was 62% higher ($p<0.01$) in the Dex-treated rats at 50 weeks of age.

We concluded that neonatal Dex treatment affects normal growth of the heart resulting in reduced heart weight, cellular hypertrophy, and increases in collagen deposition in the adult rat heart, confirming earlier hypotheses.

The study described in **Chapter 7** was performed to investigate whether the increase in cardiomyocyte cell volume induced by neonatal Dex treatment as reported earlier (chapter 6) is associated with long-lasting changes in the ventricular expression of contractile and structural proteins.

Ventricular expression of the contractile proteins α-actin, total myosin heavy chain (MHC) and β-MHC were determined. In addition, we determined the expression of the structural proteins desmin and α- and β tubulin by Western Blotting.

Dex treatment in rat pups increased the expression of the contractile protein α-actin in all age groups; 4-week (33%, $p<0.01$), 8-week (45%, $p<0.05$) and 50-week (228%, $p<0.001$). This increase in α-actin was accompanied by an increase in total MHC (40%, $p<0.05$; 143%, $p<0.01$; and 105%, $p<0.05$, respectively) and in β-MHC (94%, $p<0.01$; 60%, $p<0.05$; and 61%, $p<0.05$, respectively) in all age groups. The ratio of α-MHC/β-MHC mRNA was significantly decreased by neonatal Dex treatment in the 8-week-old rats. The increase in contractile proteins was not associated with Dex-induced changes in the expression of structural proteins such as desmin and tubulin.

This study demonstrated that neonatal Dex treatment induced an increase in the contractile proteins MHC and α-actin that persisted into adulthood. The change in MHC protein expression resembles the classical isoform switch that has been described in pressure or volume overload of the heart as well as in aging.

It is conceivable that the increases in contractile proteins contribute to maintain cardiac function as an adaptive or compensatory response to the reduced cardiac cell number previously found.

Concluding remarks and future directions

The studies described in this thesis support our hypothesis that neonatal exposure to Dex in rats has serious life-long adverse consequences on the heart. This hypothesis was tested at the functional, histopathological and biochemical level.

Neonatal Dex treatment caused a suppression of mitosis during a phase in which normally substantial hyperplasia occurs resulting in a lower number of cardiomyocytes compared to control. Presumably compensatory mechanisms, i.e cellular hypertrophy and upregulation of the expression of contractile proteins were found at various time points during life span. At the functional level depressed systolic function at prepubertal age was found in Dex-treated rats. In the young adults normal cardiac function was found but systolic dysfunction reappeared in middle-aged and elderly rats with maintained cardiac output and compensatory dilatation.

GC treatment started in the early nineties. With the expanding technical and medical knowledge an increasing number of extremely premature babies are being treated with GCs. However, because of the frequently reported long-term neurological adverse side effects, the American Academy of Pediatrics recommended more restrictive use of GCs [1].

The results obtained from the experimental studies in this thesis also question the use of early neonatal GC treatment in the human setting in view of the cardiovascular adverse side effects. Extrapolation of the findings in our rat studies to the human situation must, however, be done with caution.

If our findings are confirmed in humans, this may have consequences for a potentially large patient population that has been treated with Dex during the neonatal period in the early nineties up to now. On the basis of our data, we strongly recommend a large controlled clinical study focusing on cardiac screening before and after puberty of prematurely born Dex-treated children to enable secondary prevention.

Since, GC treatment can not always be avoided, alternatives to Dex such as hydrocortisone should be considered. A very recent retrospective matched cohort study performed by our group indicated that hydrocortisone-treated children had better behavioral and motor developmental outcome at school age than Dex-treated children [3].

Long-term effects of hydrocortisone on the cardiovascular system are unknown. We would therefore recommend to include cardiovascular investigations in future multicenter randomized controlled clinical trials regarding the use of hydrocortisone to prevent CLD.

REFERENCE LIST

1. Committee on Fetus and Newborn Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics 2002;109:330-338
2. Kamphuis PJ, de Vries WB, Bakker JM, Kavelaars A, van Dijk JE, Schipper ME, van Oosterhout MF, Croiset G, Heijnen CJ, Van Bel F, Wiegant VM Reduced life expectancy in rats after neonatal dexamethasone treatment. Pediatr Res;(in press)
3. Karemaker R, Heijnen CJ, Veen S, Baerts W, Samsom JF, Visser GH, Kavelaars A, van Doornen LJ, Van Bel F Differences in behavioral outcome and motor development at school age after neonatal treatment for chronic lung disease with dexamethasone versus hydrocortisone. Pediatr Res;(in press)

CHAPTER 9

NEDERLANDSE SAMENVATTING, CONCLUSIE EN TOEKOMSTPERSPECTIEVEN

SAMENVATTING

Dit proefschrift beschrijft de effecten van neonatale glucocorticosteroïd behandeling en de gevolgen voor het cardiovasculaire systeem bij ratten. Glucocorticosteroïd behandeling en vooral dexamethason (Dex) wordt wereldwijd gebruikt ter behandeling van chronische longziekte bij premature kinderen. Echter, het gebruik van glucocorticosteroïden heeft zowel korte als lange termijn bijwerkingen, welke worden beschreven in zowel dier als in humane studies. In het bijzonder zijn de neurologische lange termijn bijwerkingen zoals cerebrale parese en vertraagde en abnormale neurologische ontwikkeling beschreven. De korte termijn bijwerkingen op het cardiovasculaire systeem zijn goed gedocumenteerd, de lange termijn bijwerkingen echter zijn nog steeds niet goed bekend. Recente studies in volwassen ratten laten negatieve lange termijn bijwerkingen zien na neonatale behandeling met Dex. Bovenal werd een verminderde levensverwachting gezien in de met Dex behandelde dieren. Naar aanleiding hiervan onderzochten wij in dit proefschrift gedurende de levenscyclus van de rat de functionele, histopathologische en biochemische effecten van neonatale Dex behandeling op het hart.

Een eerder verschenen studie van onze groep liet zien, dat neonatale Dex behandeling in de rat een verminderd hartgewicht veroorzaakt, waarschijnlijk tengevolge van vroegtijdige remming van myocyt proliferatie gedurende de behandeling. Bovenal leidde deze behandeling tot permanente histopathologische veranderingen, zich vooral uitend in hypertrofie van de myocyt, in het volwassen rattenhart.

In **hoofdstuk 2** onderzochten wij in ratten de vroegtijdige effecten van neonatale Dex behandeling op cel proliferatie na de geboorte. De hypothese dat een verminderd aantal cardiomyocyten op volwassen leeftijd werd veroorzaakt door een door Dex-geïnduceerde verminderde cardiomyocyt proliferatie en/of vroegtijdige celdood werd getoetst. Dit is een logische aanname gezien het feit, dat glucocorticosteroïden toegediend worden in een kritische fase van de ontwikkeling van het hart. Histopathologische en immunohistochemische studies werden uitgevoerd in een bestaand rattenmodel om te kijken naar tekenen van apoptose en/of verschillen in proliferatie capaciteit van cardiomyocyten voor, gedurende en na Dex behandeling.

Mannelijke ratten pups werden intraperitoneaal geïnjecteerd met Dex op de eerste, tweede en derde levensdag (0.5, 0.3 and 0.1 µg/g). De dosis en de duur van de behandeling waren in verhouding met hetgeen gegeven wordt aan te vroeggeboren kinderen. Controle dieren kregen gelijkwaardige hoeveelheden van een zoutoplossing ingespoten (Saline). De ratten werden opgeofferd op dag 0, 2, 7 en dag 21, waarna de harten werden verzameld en gefixeerd in formaline.

De cardiomyocyten van de Dex behandelde pups toonden op dag 2 en 4 van hun leven een verminderde proliferatie, aangeduid door een lagere mitotische index en de aanwezigheid van een verminderd aantal van Ki-67-positieve cardiomyocyten vergeleken met de controle dieren. Op dag 7 en dag 21 was de mitotische index niet verschillend meer tussen de behandelde en onbehandelde groepen. Vanaf dag 2 tot dag 21 lieten de Dex behandelde dieren een verminderd aantal cardiomyocyten zien. Tekenen van cardiomyocyt-apoptose werd in geen enkele groep gevonden.

Deze studie toonde aan, dat behandeling met Dex in de neonatale periode tijdelijk de cardiomyocyt hyperplasie onderdrukt, wat uiteindelijk zal leiden tot een verminderd aantal cardiomyocyten tijdens het verdere leven.

In **hoofdstuk 3** werden de effecten bestudeerd van neonatale Dex behandeling op de cardiale functie in 4 weken oude ratten (pre-puberteit) d.m.v. druk-volume curven. Deze studie werd uitgevoerd om te kijken of neonatale Dex behandeling al op jonge leeftijd veranderingen in de cardiale functie te weeg brengt. Gebruik makende van hetzelfde diermodel zoals boven beschreven, werden de dieren onder narcose gebracht, geïntubeerd en beademd. Een miniatuur druk–conductantie catheter werd in de linker ventrikel gebracht om druk-volume curven te meten.

Cardiale functie werd gemeten en systolische en diastolische druk-volume relaties (ESPVR/EDPVR) werden bepaald om de intrinsieke systolische en diastolische functie te kwantificeren onafhankelijk van de belasting van het hart. Vervolgens werden na opoffering de harten uitgesneden en gebruikt voor histologisch onderzoek.

De met Dex behandelde ratten hadden in vergelijking met de controle dieren een lager ventrikel gewicht (Dex vs Sal: 270±40 vs. 371±23mg, $p<0.001$) en een verminderde systolische functie (eind-systolische weerstand: 1.24±0.43 vs. 2.50±1.39 mmHg/µL, $p=0.028$; eind-systolisch volume intercept at 75 mmHg: 34±13 vs. 53±20 µl, $p=0.016$).

Het hartminuut volume bleef gehandhaafd en het eind-diastolisch volume was toegenomen (84±23 vs. 59±19 µL, $p=0.012$) duidend op compensatoire dilatatie. Hartslag, diastolische functie en systemische vasculaire weerstand waren onveranderd.

Uit deze bevindingen concludeerden wij, dat cardiale dysfunctie al aanwezig is in de prepuberale periode.

In **hoofdstuk 4** onderzochten wij druk-volume relaties van het hart gedurende groei en ontwikkeling in de normale rat. We weten, dat linker ventrikel druk-volume relaties relatief belasting onafhankelijke indexen weergeven van de systolische en diastolische linker ventrikel (LV) functie. Er is echter weinig data voorhanden betreffende druk-volume relaties gedurende groei en ontwikkeling in het normale volwassen rattenhart.

Om de intrinsieke kamer functie in dieren van verschillende grootte en gewicht te kwantificeren, moeten de indexen afgeleid van druk-volume relaties idealiter worden genormaliseerd

voor hart gewicht (bv. volume wordt vervangen door volume/hart gewicht). Echter in vele studies worden de indexen gegeven in absolute waarden of lichaamsgewicht wordt gebruikt als surrogaat voor hartgewicht correctie.

In deze studie werden daarom ook de effecten van normalisatie van de druk-volume relaties bestudeerd.

Druk-volume relaties werden bepaald op de jong volwassen (8-weken-oud) en op middelbare leeftijd (50-weken-oud) in normale Wistar ratten.

Hart- en lichaamsgewicht waren significant hoger in de 50-weken oude ratten, terwijl de hartgewicht/lichaamsgewicht ratio significant was afgenomen in de 50-weken oude ratten (2.74±0.32 vs. 4.41±0.37 mg/g, $p<0.001$).

De intrinsieke systolische functie, bepaald door de hellingen van eind-systolische druk-volume relatie (Ees), de dP/dt_{MAX} versus eind-diastolische volume relatie (S-dP), en de "preload recruitable stroke work" relatie (PRSW) genormaliseerd voor hart gewicht, was lichtelijk afgenomen in de 50-weken oude ratten (S-dP: -26%, $p<0.004$; PRSW: -13%, $p<0.06$). De diastolische indexen gecorrigeerd voor het hartgewicht waren niet significant verschillend. De absolute indexen lieten over het geheel dezelfde resultaten zien, terwijl lichaamsgewicht gecorrigeerde druk-volume indexen een verbeterde systolische functie en een significant verslechterde diastolische functie lieten zien in de 50-weken oude ratten.

Deze experimentele studie toonde aan, dat in normale mannelijke Wistar ratten de intrinsieke systolische functie geleidelijk afneemt vanaf de jong volwassen leeftijd tot de middelbare leeftijd. Verder constateerden wij, dat lichaamsgewicht correctie van druk-volume relaties niet een goed alternatief is voor hartgewicht correctie in deze dieren.

In de voorafgaande hoofdstukken rapporteerden wij, dat een onderdrukte mitose gedurende neonatale Dex behandeling leidt tot een significant verminderd ventrikel gewicht, verminderde systolische functie en compensatoire dilatatie in prepuberale (4-weken- oud) ratten. Daarnaast toont een recente studie van onze groep aan, dat de levensverwachting van ratten, die neonataal behandeld zijn met Dex, significant is verminderd [2]. Daarom was het doel van de studie in **hoofdstuk 5** om de lange termijn effecten van neonatale Dex behandeling op de cardiovasculaire functie te onderzoeken.

Neonatale ratten pups werden behandeld met Dex of kregen een zoutoplossing (Sal) ingespoten. De cardiale functie werd bepaald (zoals boven beschreven) in 8-, 50-, en 80-weken oude ratten. Zij vertegenwoordigen jong volwassen, middelbare en bejaarde ratten.

Onze resultaten toonden in de 8- en 80 weken oude Dex behandelde dieren een lager ventrikel en lichaamsgewicht, maar niet in de 50 weken oude ratten. Vergeleken met de controle dieren, waren hart minuut volume en de diastolische functie ongewijzigd in alle groepen. Echter de systolische functie was onderdrukt in de 50- en 80-weken oude ratten wat te zien was aan een verminderde ejectie fractie en een rechtsverschuiving van eind-sytolische druk-volume relaties.

Uit deze studie concludeerden wij, dat de vroege negatieve bijwerkingen van neonatale Dex behandeling zoals gevonden in de 4-weken oude ratten voorbijgaand zijn, maar zich opnieuw manifesteren in bejaarde ratten. We speculeren, dat cellulaire hypertrofie en mogelijk andere factoren (zie ook hoofdstuk 7) in eerste instantie compenseren voor het verminderde aantal cardiomyoyten ten gevolge van de neonatale Dex behandeling. Uiteindelijk faalt ook dit mechanisme en dit resulteert in heroptreden van systolische dysfunctie op latere leeftijd.

In de voorafgaande hoofdstukken onderzochten we de hemodynamische functie in normale en neonatale Dex behandelde ratten in verschillende leeftijdsgroepen. **Hoofdstuk 6** beschrijft histopathologische myocard karakteristieken na neonatale Dex behandeling gedurende de levensloop van Wistar ratten (4-, 8- en 50-weken oud). De resultaten werden vergeleken met de uitkomsten van de controle dieren.

Hartweefsel werd gekleurd met HE, Cadherin-PAS en Sirius Red voor bepaling van cardiomyocyt morfometrie en collageen gehalte. Op alle leeftijden was de lengte van de cardiomyocyt in de Dex behandelde dieren significant toegenomen ($p<0.05$) vergeleken met de controle dieren, terwijl het ventrikel-gewicht verminderd was. De toename in lengte in de Dex behandelde ratten werd vergezeld met een significante toename ($p<0.01$) in breedte van de cardiomyocyt in de 50-weken oude ratten. Deze veranderingen leidden in de 50-weken oude met Dex behandelde ratten tot een significante toename ($p<0.01$) in volume van de cardiomyocyt, duidende op hypertrofie. Het collageen gehalte nam geleidelijk toe met de leeftijd en was vergeleken met de controle dieren met 62% toegenomen in deze 50-weken oude Dex behandelde ratten.

We concludeerden, dat neonatale Dex behandeling de normale groei van het hart beïnvloed resulterend in een verminderd hart gewicht, cellulaire hypertrofie en toegenomen collageen afzetting in het volwassen rattenhart. Deze bevindingen bevestigden onze eerdere hypothesen.

In de studie beschreven in **hoofdstuk 7** onderzochten wij of de eerder gerapporteerde toename in cel volume van de cardiomyocyt (hoofdstuk 6), ontstaan door neonatale Dex behandeling, geassocieerd is met langdurige veranderingen in de expressie van contractiele en structurele eiwitten in de ventrikel.

De ventriculaire expressie van de contractiele eiwitten α-actine, totaal myosin heavy chain (MHC) and β-MHC werd bepaald. Daarnaast onderzochten wij de expressie van de structurele eiwitten zoals desmine, α- and β tubuline d.m.v. Western Blotting.

De expressie van het contractiele eiwit α-actine nam toe in alle leeftijdsgroepen in Dex behandelde ratten; 4-weken (33%, $p<0.01$), 8-weken (45%, $p<0.05$) and 50-weken (228%, $p<0.001$). Deze toename in α-actine werd vergezeld door een toename in de expressie van totaal MHC in alle groepen met respectievelijk 40%, $p<0.05$; 143%, $p<0.01$; en 105%, $p<0.05$.

De expressie van β-MHC nam ook toe in alle groepen met respectievelijk 94%, $p<0.01$; 60%, $p<0.05$; en 61%, $p<0.05$.

In de 8-weken oude ratten was de ratio α-MHC/β-MHC mRNA significant afgenomen door neonatale Dex behandeling.

De toename in contractiele eiwitten was niet geassocieerd met Dex-geïnduceerde veranderingen in de expressie van structurele eiwitten zoals desmine en tubuline.

Deze studie toonde aan, dat neonatale Dex behandeling een blijvende toename veroorzaakte van de contractiele eiwitten MHC en α-actine. De verandering in expressie van MHC komt overeen met de klassieke isoform verandering, die beschreven wordt bij druk of volume overbelasting van het hart of bij veroudering.

Mogelijk dat de toename in contractiele eiwitten dient als een adaptieve of compensatoire respons op het verminderde aantal cardiale cellen om de hartfunctie te waarborgen.

Conclusies en toekomstperspectieven

De studies beschreven in dit proefschrift ondersteunen onze hypothese dat neonatale blootstelling aan het glucocorticosteroïd Dex ernstige, levenslange bijwerkingen heeft op het hart. Deze hypothese werd in dit proefschrift getest op functioneel, histopathologisch en biochemisch niveau.

Neonatale Dex behandeling veroorzaakte een onderdrukking van de mitose in een fase, waarin normaal gesproken substantiële hyperplasie optreedt. Dit leidde tot een uiteindelijk verminderd aantal cardiomyocyten op volwassenen leeftijd vergeleken met controle dieren.

Compensatoire mechanismen zoals cellulaire hypertrofie en verhoging van de expressie van contractiele eiwitten werden gevonden in verschillende fasen van het leven. Op functioneel niveau werd een verminderde systolische functie gevonden in Dex behandelde ratten in de prepuberale periode. Op jong-volwassenen leeftijd werd een normale hart functie gevonden, maar op middelbare leeftijd en in bejaarde ratten trad opnieuw systolische dysfunctie op met een normaal hartminuut volume maar met compensatoire dilatatie.

Glucocorticosteroïd behandeling en, zoals gezegd, vooral Dex behandeling werd gestart begin jaren negentig. Door de toegenomen technische en medische kennis wordt een toegenomen aantal ernstig te vroeggeboren kinderen behandeld met deze glucocorticosteroïden. Echter vanwege de frequent gerapporteerde neurologische bijwerkingen op lange termijn, heeft de American Academy of Pediatrics aanbevelingen gedaan in zake het gebruik van GSs [1].

De hier genoemde cardiovasculaire bijwerkingen van Dex stellen het gebruik van neonatale GC behandeling in de humane setting ter discussie.

Uiteraard moet extrapolatie van de bevindingen in onze ratten studies naar de humane situatie met beleid gebeuren. Als onze bevindingen in mensen wordt bevestigd, heeft dit consequenties voor een potentieel grote patiënten populatie die behandeld zijn met Dex vanaf de jaren negentig tot nu toe in de neonatale periode.

Op basis van onze data, adviseren wij een grote gecontroleerde klinische studie voor cardiale screening van ex-premature kinderen behandeld met Dex in de neonatale periode. Deze screening is van belang om eventuele secundaire preventie mogelijk te maken.

Alternatieven voor Dex, zoals hydrocortison, een minder potent corticosteroïd, moet worden overwogen, aangezien GC behandeling niet altijd kan worden voorkomen. Een door onze groep recent uitgevoerde retrospectieve matched controlled cohort studie in ex-premature kinderen, wel of niet behandeld met glucocorticosteroïden, laat zien dat kinderen op schoolleeftijd, die behandeld zijn met hydrocortison een betere cognitieve en motorische ontwikkeling laten zien dan kinderen behandeld met Dex [3].

De lange termijn effecten van hydrocortison op het cardiovasculaire systeem zijn niet bekend. Wij adviseren daarom om cardiovasculaire onderzoeken aangaande het gebruik van hydrocortison voor preventie van chronische longziekte in te vroeggeborenen in de toekomst te includeren in multicenter gerandomiseerde klinische trials.

REFERENTIE LIJST

1. Committee on Fetus and Newborn Postnatal corticosteroids to treat or prevent chronic lung disease in preterm infants. Pediatrics 2002;109:330-338
2. Kamphuis PJ, de Vries WB, Bakker JM, Kavelaars A, van Dijk JE, Schipper ME, van Oosterhout MF, Croiset G, Heijnen CJ, Van Bel F, Wiegant VM Reduced life expectancy in rats after neonatal dexamethasone treatment. Pediatr Res; (in press):
3. Karemaker R, Heijnen CJ, Veen S, Baerts W, Samsom JF, Visser GH, Kavelaars A, van Doornen LJ, Van Bel F Differences in behavioral outcome and motor development at school age after neonatal treatment for chronic lung disease with dexamethasone versus hydrocortisone. Pediatr Res; (in press):

DANKWOORD

Promoveren doe je nooit alleen en gezien de lijst met vele namen kan ik het met dit statement slechts hartgrondig eens zijn. Ondankbaar is de plek die aan het dankwoord, doorgaans achter in het proefschrift, wordt toebedacht. Dankbaar daarentegen is de "wetenschap" dat het dankwoord wel het meest gelezen deel van het proefschrift is. En met dit laatste aspect ben ik oprecht blij omdat zo extra wordt benadrukt wie de mensen zijn die voor mij onmisbaar waren en mij liefdevol hebben gesteund met raad en daad bij de totstandkoming van dit proefschrift.

Met wie heb ik de eer gehad te mogen samenwerken, in de hoop dat ik niemand vergeet, gedurende mijn zwerftocht aan de Universiteiten van Utrecht, Leiden en Groningen.

Prof. dr. F. van Bel, beste Frank, jij was mijn promotor in Utrecht en dat heb je geweten ook. Zo maakte je altijd grapjes als ik weer eens aan de telefoon hing om iets te vragen. Jouw vooruitziende blik dat dit zelfs nog tijdens mijn huwelijksreis zou gebeuren kwam door omstandigheden ook uit. To the point tijdens vergaderingen en de reputatie hebben van "shortest discussion writer ever" maakte je waar door menigmaal behoorlijke stukken tekst in de discussie te verwijderen. Niet lang nadenken welke hotels het best in aanmerking kwamen tijdens congressen deed de kreet "brakke Braxton" geboren worden. Frank, bedankt voor je steun, ook om in opleiding te komen, omdat jij altijd hebt geloofd dat ik mij als AIO daar geen zorgen over hoefde te maken. Ik heb het als een eer ervaren dat jij mij persoonlijk voor dit promotieonderzoek hebt gevraagd!

Prof. dr. J. Baan, beste Jan, jij was mijn tweede promotor in Leiden en ik had de eer je laatste promovendus te zijn, omdat je tijdens mijn promotieonderzoek met emeritaat ging. Jouw kennis van de cardiophysica was voor mij van onschatbare waarde. Bedankt dat je in vergaderingen altijd rake zaken wist te benoemen waardoor de lijn voor mij weer helder werd. Beste Jan, bedankt voor al je steun, ik ben trots dat ik je laatste promovendus mocht zijn.

Dr. P. Steendijk, beste Paul, jij was mijn co-promotor in Leiden en ik heb ontzettend veel aan jou te danken. Jouw rustige uitstraling, je immer aanwezige bereidheid mij te helpen en je altijd goede humeur zijn een paar eigenschappen, waar ik steun uit kon putten. Altijd stond je mij te woord en hielp je mij uit de brand. Samen hebben we met veel plezier vele proeven gedaan die altijd vlekkeloos verliepen. Jouw stimulans daarbij wist je bij mij iedere keer weer goed over te brengen, een prestatie temeer omdat ik de tel ben kwijtgeraakt hoeveel ratten er door onze handen zijn gegaan. Paul, bedankt voor het doornemen van de stukken, de tijd die je me bood om mijn hart te luchten en je optimistische instelling waardoor ik weer de positieve kant van het verhaal kon zien. Beste Paul, ik had geen betere co-promotor kunnen hebben dan jij! Ik hoop dat wij ook in de toekomst contact zullen blijven houden.

Dr. F. R. van der Leij, beste Feike, na anderhalf jaar Leiden vond ik het een hele stap om naar Groningen te gaan. Leek die ik ben in de moleculaire celbiologie en als pure clinicus heb jij getracht mij het één en ander te leren, omdat PCR en Western Blotting voor mij allemaal totaal nieuwe begrippen waren.

Helaas zaten sommige zaken niet echt mee, want wat moet je nu met ratten die zich in Groningen anders gedragen dan in Leiden. Gronings aanspreken? We zijn er nooit achter gekomen. Maar tegenslagen maken een mens sterker en uiteindelijk kunnen we terugkijken op een mooi resultaat. Beste Feike, bedankt voor je steun als co-promotor!

Prof. C. J. Heijnen, beste Cobi, wat een warm onthaal heb ik van jou en je medewerkers gekregen in jouw laboratorium in Utrecht! Zomaar geïnstalleerd door Frank van Bel hadden jullie ineens weer zo'n "domme clinicus" in het laboratorium. Ik kan niet anders zeggen dan dat ik het enorm getroffen heb om in jullie laboratorium terecht te zijn gekomen. Al ben ik dan geen echte onderzoeker pur sang, jullie hebben mij oprecht weten te motiveren tot het echte onderzoekswerk. Dank daarvoor!

Dr. A. Kavelaars, beste Annemieke, ongelofelijk hoeveel tijd jij hebt gestoken in het project. Hartverwarmend om te zien dat iemand die "geen verstand heeft van harten zaken" toch zo ontzettend veel weet en enthousiast wordt bij elk leuk resultaat. Ik moet zeggen, gaandeweg stak jij mij aan met jouw enthousiasme wat heeft geleid tot een heel mooi artikel. Bedankt!

Dr. B. Bartelds, beste Beatrijs, jou wil ik hartelijk bedanken voor je enorme kennis die je hebt ingebracht, waarbij elke invalshoek werd bekeken. Op je vrije dag naar een vergadering komen in Utrecht vanuit Groningen, dan moet je wel echt hart hebben voor onderzoek.

Prof. dr. R. M. F. Berger, beste Rolf, de echo's die door jou zijn uitgevoerd in 4 weken oude ratten vormden een mooie aanvulling in het verschenen artikel. Dank voor je tijd en energie die je erin hebt gestoken.

Drs. W. B. de Vries, beste Willem, als mede promovendus op hetzelfde onderwerp hebben wij aan vele vergaderingen samen deelgenomen. Het heeft geleid tot prachtige resultaten. Als ik het even niet meer zag zitten, was jij er om mij ervan te overtuigen dat alles goed kwam, waarbij jij mij toesprak als mijn "grote broer". Ik hoop dat we er met z'n allen een mooi symposium van maken en in de toekomst zullen wij als collega's zeker verder gaan.

Dr. F. M. van Oosterhout, beste Matthijs, jou wil ik uit de grond van mijn hart bedanken als ik weer eens met mijn potten vol organen kwam aanzetten die door jou en medewerkers van het pathologielaboratorium behandeld moesten worden. Jij bleef er altijd heel rustig onder en bewaakte de kwaliteit van de preparaten. Wie had gedacht dat ik ooit nog zelf de

preparaten zou gaan beoordelen? Menigmaal heb ik bij jou aangeklopt als ik er met een pathologisch preparaat niet uitkwam en even zovele keren was jij mij daarbij behulpzaam. Alice in wonderland maar ik was het meisje in de microscopiekamer waar ik menig uurtje in het donker heb doorgebracht.

Prof. dr. E. E. van der Wall, beste Ernst door jou leuke grappen kon ik de tegenvallers die bij een promotie onderzoek horen weer relativeren.

Collega's van de tuin cardiologie, bedankt voor de gezelligheid. We hebben een leuke tijd gehad en veel gelachen. Pascalle, je had altijd een luisterend oor en fijn dat we een vriendschap hieraan over hebben gehouden.

Iedereen van het laboratorium psychoneuroimmunologie, al was mijn tijd bij jullie maar kort, toch hebben jullie mij het gevoel gegeven dat ik er thuis hoorde. Bedankt daarvoor. Cora en Wendy, jullie wil ik speciaal bedanken voor jullie eindeloze geduld wat betreft mijn vragen over de Western Blotting. En, voor jullie is straks ook het einde van de promotie in zicht. Zet 'm op!

Mede-onderzoekers van de AIO kamer en alle medewerkers van het laboratorium metabole ziekten in Groningen, als was mijn tijd in Groningen niet altijd even happy, met jullie heb ik mij altijd reuze vermaakt. Wij "chicks en uberchick" hebben een leuke tijd gehad! Anniek, grote bewondering heb ik voor jouw doorzettingsvermogen en je altijd grote interesse in anderen. Maaike, "never a dull moment" met jou in de buurt!

Yan, thank you for the nice time and all the work we did together. I hope that your thesis is going well and I'm sure you will succeed. Good luck in your home country China. May all your wishes come true.

Inge-Lot, jouw interesse in mijn onderzoek was hartverwarmend. Bedankt, dat ik af en toe mijn hart kon luchten als zaken tegen zaten. Over een paar jaar ben ik getuige van jouw promotie.

Het dierenlaboratorium in Leiden wil ik bedanken voor de uitstekende samenwerking. Peter de Jong, jij hebt mij geleerd hoe ik mannetjes en vrouwtjes ratten kan onderscheiden. Helaas heb ik nooit mijn angst voor de Wistar rat overwonnen, ondanks de hoeveelheid ratten die in de studie zijn opgenomen. Gelukkig hielp jij mij iedere keer weer trouw met het wegen van de ratten. Ik geloof dat dat beter was, zowel voor mij als voor de ratten. Ben en Fred, bedankt dat jullie de verzorging van de ratten ter hand hebben genomen toen ik in Groningen was en ik vanwege de afstand niet iedere keer kon terug komen om te wegen.

De leden van de beoordelingscommissie: Prof. dr. W. A. Helbing, Prof. dr. J. M. Wit, Prof. dr. G. H. A. Visser, Prof. dr. A. F. Bos, Prof. dr. C. J. Heijnen dank ik voor het kritisch beoordelen van mijn proefschrift.

De secretaressen Danielle Nijssen, Ineke Krijgsman en Lot Hassert bedank ik voor jullie meeleven tijdens de afgelopen vier enerverende jaren.

Dr. W. Maertzdorf, beste Wiel, wie had kunnen denken dat een stage neonatologie in het tweede jaar van mijn studie zou leiden tot groot enthousiasme voor de kindergeneeskunde? Direct vanaf het eerste moment toen wij elkaar zagen, hadden wij een klik en die is er nog steeds. Mijn eerste congres in Belfast als student-assistent zal ik nooit vergeten. Toen is de basis gelegd voor mijn verdere carrière. Zie hier het resultaat!

Mijn twee lieve oudste broers, Martijn en Peter-Christiaan, ik vind het een hele eer dat jullie mijn paranimfen zijn. Ik had geen betere "steunpilaren" kunnen uitzoeken. Bedankt voor jullie hulp en fijn dat we in alle omstandigheden op elkaar kunnen rekenen!

Mijn twee lieve jongste broers, David en Constantin, jullie zijn natuurlijk erg blij dat jullie zus gaat promoveren, want dan hebben jullie weer een vrije dag op school. David, ik zou het heel "cool" vinden je als toekomstige collega te consulteren. Constantin, ongelofelijk hoeveel interesse jij altijd hebt getoond in mijn studie. Geniet van deze dag!

Lieve mama en papa, als twee mensen een plaats verdienen in dit dankwoord, dan zijn jullie dat wel. Dit boekje begint met jullie en is met recht ook jullie eer! Mama, wat een tijd en energie heb jij gestoken in schoolwerk, regelen van bijlessen, tijdschema's e.d. Het is ook niet verwonderlijk dat je er uiteindelijk je beroep van hebt gemaakt. Remedial teacher ben je met recht in hart en nieren! Jullie hebben altijd in mij geloofd, ook als ik het zelf niet meer zag zitten. Jullie betrokkenheid is ongelofelijk. Niet alleen wat betreft deze promotie, maar ook met andere zaken kan ik altijd bij jullie terecht. Ik ben er trots op, dat ik uit zo'n hecht en warm gezin kom!

Tot slot, lieve Tim. Wat hebben wij een turbulent jaar achter de rug! Trouwen, verhuizen en een auto-ongeluk tijdens onze huwelijksreis doen je nog dichter bij elkaar brengen. Bedankt voor je zorgzaamheid, geduld en kalmte als ik weer eens opstandig was. Jij ging meestal uit van het standpunt "de soep wordt niet zo heet gegeten als ze wordt opgediend". Jij kon mij altijd weer relativeren door te zeggen: slaap er maar eens een nachtje over, dan ziet alles er weer anders uit. Ik heb alle vertrouwen in een mooie toekomst voor ons samen, waarin we elkaar goed aanvullen.

CURRICULUM VITAE

Miriam Petronella Bal werd op 25 maart 1974 geboren te Den Haag. Na het behalen van het VWO-diploma in 1993 startte zij met de studie geneeskunde aan de Rijksuniversiteit Limburg te Maastricht, waar zij in 1998 haar doctoraal met genoegen heeft behaald. Tijdens deze periode deed zij een wetenschapsstage op de afdeling Klinische Genetica. Tussen 1998 en 2000 deed zij haar co-schappen in diverse ziekenhuizen, alsmede een onderzoeksstage aan het instituut "The Centre of Fetal Origins of Adult Disease (FOAD)" in Southampton. Na het behalen van haar diploma tot basisarts werd zij AGNIO kindergeneeskunde in het Rijnstate ziekenhuis te Arnhem. Vanaf 2002 werkte zij als onderzoeker op de afdeling Neonatologie aan het Universitair Medisch Centrum Utrecht (afdelingshoofd: Prof. dr. F. van Bel). Voor dit onderzoekstraject was zij anderhalf jaar werkzaam op de afdeling Cardiologie van het Leids Universitair Medisch Centrum (afdelingshoofd: Prof. dr. E.E. van der Wall). Vervolgens werkte zij anderhalf jaar op de afdeling Metabole ziekten (kindergeneeskunde) van het Universitair Medisch Centrum Groningen (afdelingshoofd: Prof. dr. P.J. Sauer). De afronding van het onderzoek vond plaats in Utrecht op de afdeling Psychoneuroimmunologie (afdelingshoofd: Prof. dr. C. J. Heijen) en Leiden (wederom afdeling Cardiologie).

In januari 2007 zal zij starten met de opleiding kindergeneeskunde aan het Leids Universitair Medisch Centrum.