**Joepe Kaandorp**

# Allopurinol for fetal neuro- and cardiovascular protection

Voor myn moeder

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# Allopurinol for fetal neuro- and cardiovascular protection

*Allopurinol voor foetale neuro- en cardiovasculaire protectie*

(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. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 30 januari 2014 des middags te 4 uur

**door**

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# CONTENTS

# Chapter 1
General introduction

Despite advances in obstetric practice, acute intra-partum fetal hypoxia-ischemia remains one of the most common forms of fetal stress and is associated with the development of cerebral palsy and cognitive disabilities in later life [1-3]. Hypoxia-ischemia (HI) or birth asphyxia has an incidence of 4-to-9 per 1000 neonates and is defined as an impaired maternal-fetal respiratory gas exchange resulting in hypoxemia, accompanied by the development of a combined respiratory and metabolic acidosis [1-5]. Asphyxia will only lead to functional and structural organ damage when the period of circulatory failure is prolonged enough to result in a significant production of toxic free radicals.

## Pathophysiology of acute fetal asphyxia

Free radicals are atoms or molecules that are highly reactive with other cellular structures because they contain unpaired electrons. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can wreak havoc on a broad range of macromolecules. There are many types of radicals, but those of most concern in biological systems are derived from oxygen, and known collectively as *reactive oxygen species* (ROS). Oxygen has two unpaired electrons in separate orbits in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation. Free radicals can cause damage to parts of cells such as proteins, DNA, and cell membranes by stealing their electrons through a process called oxidation. This is why free radical damage is called 'oxidative stress'. When free radicals oxidize important components of the cell, those components lose their ability to function normally, and the accumulation of such damage may cause the cell to die.
Although asphyxia is a global insult, it is the brain, the heart and the kidneys that are particularly at risk of damage from ischemia-reperfusion injury associated with asphyxia [5].
There is increasing evidence from experimental and clinical studies that a substantial part of birth asphyxia-related brain damage occurs upon and immediately after birth, when brain perfusion and oxygenation are recovering. The excessive formation of reactive oxygen species (ROS) and other toxic compounds peaks 30-to-60 minutes after birth and plays an important causative role in this reperfusion-reoxygenation damage to the developing brain [6-12]. These pathways are described in more detail in **Chapter 2** of this thesis.

## A brief history of perinatal neuroprotection

Earlier and recent experimental studies in newborn animals suffering HI have suggested that postnatal pharmacologic treatment or moderate hypothermia to prevent or reduce the excessive production of free radicals can reduce post-HI damage to the newborn brain if treatment was started within 6 hours after the start of reperfusion and reoxygenation, the so-called "window of therapeutic opportunity" [13,14]. Up to now, in the clinical setting, only moderate hypothermia proved to be beneficial to reduce post-HI damage to the brain in mildly and moderately asphyxiated human neonates [15,16]. Most pharmacologic treatment modalities, even started within the window of opportunity of 6 hours after birth, did not result in an improved long-term neurodevelopmental outcome [14,17]. Postnatally administered neuroprotective agents may be given too late to be fully effective in terms of reduction of free radical formation, like superoxide and peroxynitrite, which have their maximal production immediately (within 30 minutes) after birth [9].

Earlier (pharmacologic) treatment, i.e. antenatally via the mother in case of suspected fetal hypoxia, might be a more rational approach since the concentration of neuroprotective drugs is already near or even within the therapeutical range at birth and during the reperfusion-reoxygenation period shortly after birth. It thus provides the opportunity to limit or pre-

vent the consequences of potentially injuring mechanisms. This approach may reduce birth asphyxia-related damage to the newborn heart and brain and improve neurodevelopmental outcome of the threatened fetus and newborn in an earlier stage of the process.

To successfully neuroprotect the hypoxic fetus in utero and during reperfusion and reoxygenation around the process of birth, a 'candidate' drug should qualify to the following criteria:
1. it should readily cross the placenta;
2. it should not be toxic to the mother;
3. it should have a therapeutic effect on the fetus, and
4. it should not be toxic to the fetus.

To date there is not much reported in experimental or clinical studies about antenatal pharmacologic therapies to reduce or prevent fetal hypoxia-induced neonatal brain damage.

## Allopurinol

The xanthine oxidase (XO) inhibitor allopurinol has been the cornerstone of the clinical management of gout and conditions associated with hyperuricemia since it was approved by the Food and Drug Administration for that specific indication in 1966 [18]. In the succeeding decades an increasing number of researchers suggested potential additional beneficial effects of allopurinol for various forms of ischemic tissue and vascular injuries, and congestive heart failure [18].
In the early 1980's Granger *et al.* demonstrated that ischemic bowel injury occurred especially on reperfusion and was attenuated by superoxide dismutase (SOD) [19]. On the basis of this observation, the hypothesis was put forward that XO-derived reactive oxygen species (ROS) contribute to the ischemic injury via ATP catabolism during hypoxia and increasing electron acceptor availability on reperfusion. Since the introduction of the concept of ischemia-reperfusion injury, several lines of evidence support the role of XO-derived ROS generation, and beneficial effects of XO inhibitors against ischemic damage of the heart, brain, intestine, liver, kidney, lung, and other tissues were shown [18,20].

### Mechanism of action

Formation of the superoxide free radical due to conversion of hypoxanthine into xanthine by xanthine oxidase has an important role in the occurrence of hypoxia-ischemia induced injury of the brain [21]. Administration of the xanthine oxidase inhibitor allopurinol reduces the production of superoxide free radical formation, thereby potentially limiting the amount of hypoxia-reperfusion damage [22,23]. Furthermore, allopurinol also has a non-protein bound iron (pro-radical) chelating and direct free radical (hydroxyl) scavenging effect, especially when high dosages are administered [18,24].

### Earlier studies

The first human studies investigating potential beneficial effects of allopurinol on ischemia-reperfusion damage focused on the pathogenic role of XO in myocardial ischemia and showed that inhibiting XO activation during cardiac surgery by allopurinol treatment improved cardiac performance, scored by cardiac index, and reduced the need for inotropic or mechanical support [25]. A more recently performed study by Clancy *et al.* showed that allopurinol provided significant neurocardiac protection in higher-risk hypoplastic heart infants who underwent cardiac surgery using deep hypothermic circulatory arrest [26].

Allopurinol made its way to neonatal medicine already in the early 1990's [22,27,28] when Palmer *et al.* showed that high-dose allopurinol administered at 15 min after recovery from cerebral hypoxia-ischemia markedly reduced both acute brain edema and long-term cerebral injury in immature rats. A prospective randomized study in human neonates, examining the effects of allopurinol in term asphyxiated neonates, showed an improvement of electrocortical brain activ-

ity, and a reduction in free radical formation after early neonatal (<4 hours of life) allopurinol administration [29]. A more recent paper by Gunes *et al.* [30] reports an improved neurological outcome after postnatal allopurinol administration compared to placebo in term moderately asphyxiated neonates. A subsequent randomized clinical trial by Benders *et al.* [17] however could not confirm these results and suggested that when birth asphyxia had been too severe, the inflicted brain damage could no longer be reversed. A recent follow-up study of the two clinical trials performed by van Bel and Benders *et al.,* showed a potential beneficial effect of postnatally administered allopurinol on mortality and severe neurological disabilities at 4-8 years of age, but only in a subgroup of moderately asphyxiated infants (**Chapter 3** of this thesis). These beneficial effects are in line with previous findings on neonatal head cooling after acute fetal hypoxia at term [15]. Most likely no advantage of treatment occurs anymore when the interval to the initiation of treatment is too long or when brain damage is already too severe. It is conceivable that earlier allopurinol treatment, e.g. maternally administered allopurinol during labor in case of suspected fetal hypoxia, may earlier limit the amount of free radical production and subsequent hypoxia-reperfusion injury.

Pharmacologic data have been published by Boda *et al.*, which showed that therapeutical plasma levels were reached within 20 minutes in the human fetus after oral allopurinol administration (600 mg) to the mother [31]. Some animal studies have been performed using piglets (neonatal administration), sheep and sows (maternal administration) [32-35]. These studies showed an adequate placental passage of allopurinol and an improved outcome after allopurinol treatment on cerebral energy status and recovery of umbilical blood flow respectively. Furthermore, we showed reduced fetal cardiac oxidative stress in chronically instrumented fetal sheep after antenatal allopurinol administration to the pregnant ewe during repeated periods of ischemia [35]. In addition our research group performed a prospective randomized placebo controlled double blind pilot study, in which we administered intravenously allopurinol (500 mg) to pregnant women when fetal asphyxia was suspected. Data from this pilot study showed an inverse correlation between levels of allopurinol and the amount of S100ß, a biomarker for brain tissue damage, in cord blood [36].

**Potential side effects**

Established side effects of allopurinol are rare and most commonly include symptoms of hypersensitivity like skin rashes [37]. Stevens-Johnson syndrome has been reported, but only after prolonged treatment (> 10 days) in case of gout [38]. During previously performed studies in pregnant women and neonates, including those performed by our group, no adverse reactions were seen [17,26,29,31,32,36].

## In conclusion

It becomes increasingly clear that postnatal (pharmacologic) treatment with antioxidative drugs or with NMDA-receptor antagonists of birth asphyxia-related damage to the newborn brain has a limited neuroprotective effect, even when therapy has been started within the "window of opportunity", being within 6 hours after birth. This might be due to the maximal formation of reactive (oxygen) species and other toxic agents like peroxynitrite upon and within the first 30-to-60 minutes after reperfusion and reoxygenation. Moreover, also during fetal hypoxia, an important determinant of birth asphyxia, free radicals and other toxic agents are produced albeit in much lower concentrations as compared to the early reperfusion phase. The fetus of pregnant women on the brink of delivery, with signs of (imminent) hypoxia may therefore benefit from additional maternal treatment with drugs reducing excessive neurotransmitter formation and formation of free radicals. If necessary, early postnatal hypothermia with or

without pharmacologic neuroprotective agents can further reduce or even prevent fetal hypoxic and birth asphyxia-related brain damage of the newborn.

## AIM OF THIS THESIS

The aim of this thesis is to investigate the applicability of maternally administered allopurinol for fetal neuroprotection and to define strategies for future practice.

## OUTLINE OF THIS THESIS

**Chapter 2** summarizes the molecular mechanisms underlying early reperfusion-reoxygenation damage and focuses on the most investigated pharmacological agents (phenobarbital, vitamin C and E, allopurinol, melatonin and xenon) to be given antenatally to the mother to neuroprotect the hypoxic fetus.
**Chapter 3** investigates the long-term effects of neonatal allopurinol-treatment in infants suffering from moderate to severe birth asphyxia.

**Part I: Animal studies**
**Chapter 4** provides a model that can be used to investigate the role of xanthine oxidase derived reactive oxygen species in the pathogenesis of fetal hypoxic brain injury and the long-term neurological effects in rats.
**Chapter 5** investigated the contribution of reactive oxygen species, derived from xanthine oxidase, to the fetal peripheral vasoconstrictor response to hypoxia via interaction with Nitric Oxidase-dependent mechanisms in the ovine fetus.
In **Chapter 6** we tested the hypothesis that maternal treatment with allopurinol during repeated episodes of fetal asphyxia would limit ischemia-reperfusion damage to the fetal brain in chronically instrumented fetal sheep in late gestation.

**Part II: Clinical trial**
**Chapter 7** describes the protocol of the multicenter randomized double blind placebo controlled clinical trial, which we performed to assess whether maternal allopurinol treatment during fetal hypoxia would reduce the release of biomarkers associated with neonatal brain damage.
**Chapter 8** focuses on the placental transfer of allopurinol.
**Chapter 9** shows the main outcome measures of the multicenter randomized double blind placebo controlled clinical trial.

Finally, in **Chapter 10** the results and conclusions of the conducted studies are discussed and suggestions for future practice and research with respect to the use of allopurinol in perinatal medicine are made.

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# Chapter 2

# Fetal hypoxia is an important determinant of birth asphyxia and subsequent adverse outcome: antenatal neuroprotection at term

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## ABSTRACT

Hypoxic ischemic encephalopathy is directly associated with the development of cerebral palsy and cognitive disabilities later in life, therefore remaining an important problem in perinatal medicine. Postnatal neuroprotective strategies have been investigated elaborately, but up to now, only moderate hypothermia proved to be beneficial in reducing post hypoxic-ischemic encephalopathy in a selected group of asphyxiated neonates. Since the vast amount of toxic free radicals is produced in the reperfusion and reoxygenation period upon and immediately (30-to-60 minutes) after birth, we postulate that antenatal (i.e. maternal) pharmacological neuroprotection of the fetus, combined with postnatal hypothermia, might be a more optimal approach to prevent this free radical induced brain damage. This review summarizes the molecular mechanisms underlying early reperfusion-reoxygenation damage and focuses on the most promising pharmacological agents (phenobarbital, vitamin C and E, allopurinol, melatonin and xenon) to be given antenatally to the mother to neuroprotect the hypoxic fetus.

With an incidence of 4-to-9 per 1000 neonates, perinatal hypoxia-ischemia (HI) or birth asphyxia is one of the main causes of neonatal HI encephalopathy, which is associated with the development of cerebral palsy and cognitive disabilities later in life [1-4]. It therefore is one of the most fundamental problems in perinatal medicine.
Asphyxia is defined as an impaired maternal-fetal respiratory gas exchange resulting in hypoxemia, accompanied by the development of a combined respiratory and metabolic acidosis [5]. Asphyxia will only lead to functional and structural organ damage when the period of circulatory failure is prolonged enough to result in a significant production of toxic free radicals [4]. Although asphyxia is a global insult, it is the brain, the heart and the kidneys that are particularly at risk of damage from ischemia-reperfusion injury associated with asphyxia [5].

Possible causes of fetal hypoxia during labor include umbilical cord compression, poor placental function, placental abruption, inadequate relaxation of the uterus (leading to poor placental oxygenation), inadequate maternal oxygenation, and low maternal blood pressure [6]. There is increasing evidence from experimental and clinical studies that a substantial part of birth asphyxia-related brain damage occurs upon and immediately after birth when brain perfusion and oxygenation are recovering. The excessive formation of reactive oxygen species, such as free radicals, and other toxic compounds peaks 30-to-60 minutes after birth and plays an important causative role in this reperfusion-reoxygenation damage to the immature brain [7-10].

Earlier and recent experimental studies in newborn animals suffering HI have suggested that postnatal pharmacologic treatment or moderate hypothermia to prevent or reduce the excessive production of free radicals can reduce post-HI damage to the newborn brain if treatment was started within 6 hours after the start of reperfusion and reoxygenation, the so-called "window of therapeutic opportunity" [11,12]. Up to now, in clinical trials, only moderate hypothermia proved to be beneficial to reduce post-HI damage to the brain in mildly and moderately asphyxiated human neonates [13,14]. Most pharmacologic treatment modalities, even started within the therapeutic window of 6 hours after birth, did not result in an improved long-term neurodevelopmental outcome in human infants [11,15]. We postulate that postnatally administered neuroprotective agents are likely to be given too late to be really effective in terms of reduction of free radical formation or other toxic compounds like superoxide and peroxynitrite (see also below), which have their maximal production immediately (within 30 minutes) after birth.

We therefore hypothesize that antenatal (pharmacologic) treatment of the hypoxic fetus, via the mother, is a more rational approach since the concentration of neuroprotective drugs are already near or even within the therapeutical range at birth and during the reperfusion-reoxygenation period shortly after birth.

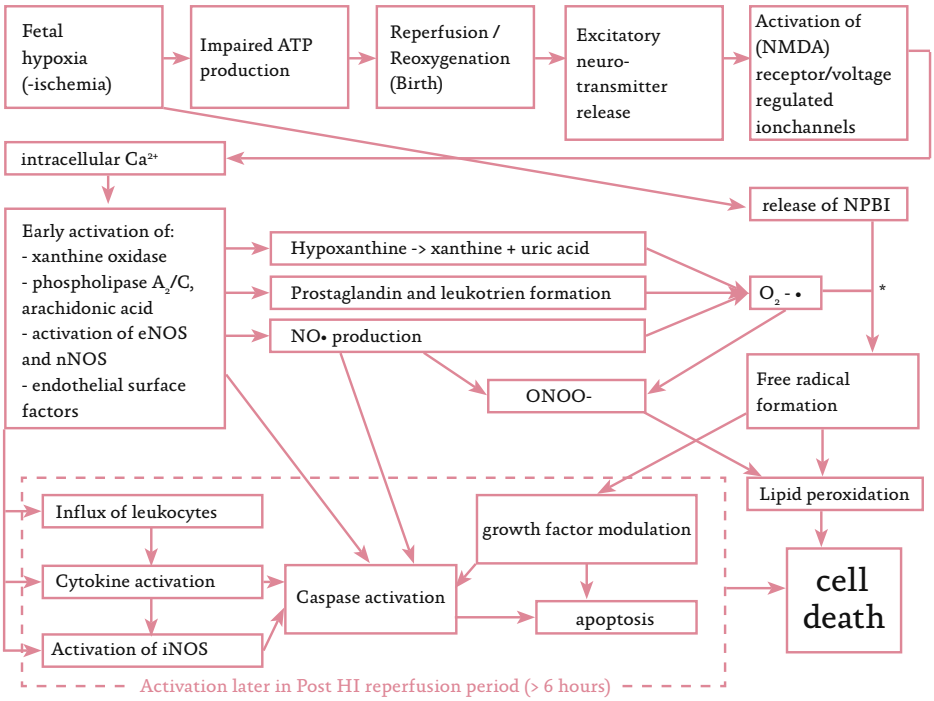
This review briefly summarizes the molecular mechanisms underlying early reperfusion-reoxygenation damage to the newborn brain and the current knowledge with respect to early recognition of fetal hypoxia during labor. The paper also reviews the most promising pharmacologic agents to be administered antenatally to the mother to reduce the consequences of fetal hypoxia, i.e. to reduce the production of reactive (oxygen) species like free radicals during fetal hypoxia and especially upon reperfusion and reoxygenation shortly after birth.

## Molecular mechanisms of early post hypoxic-ischemic reperfusion injury to the newborn brain

Birth asphyxia-induced brain injury is caused by a chain of events. During sustained hypoxia (with or without ischemia) formation of ade-

nosine triphosphate (ATP) is impaired, leading to the failure of ATP-dependent sodium-potassium pumps that normally maintain the polarity of neuronal membranes. Depolarization of the neuronal membrane induces a release of excitatory neurotransmitters causing an excessive influx of calcium into the neuronal and microglial cells through voltage regulated and receptor regulated (N-methyl-D-aspartate [NMDA]) calcium channels. This influx of calcium leads to a massive production of neurotransmitters such as glutamate, which induces an activation of many enzymatic reactions including the activation of lipases, proteases and endonucleases. These events further enhance the production of free radicals, which leads to lipid peroxidation and eventually to damage of neuronal cell membranes and nucleotides [16-18].

**Figure 2-1. Possible injuring mechanisms induced upon and after reperfusion- reoxygenation after fetal and perinatal hypoxia-ischemia**




During fetal hypoxia, but especially upon reperfusion-reoxygenation opening of ion channels leads to influx of calcium into neurons with subsequent excessive neurotransmitters production and activation of enzymatic reactions. This activation gives rise to subsequent metabolisation of hypoxanthine, fatty acids originating from neuronal cell membranes, of nitric oxide production and the Fenton reaction (*) This leads to excessive free radicals formation, lipid peroxidation and ultimate neuronal cell death.

NMDA= N-methyl-D-aspartate; NO= nitric oxide; NOS= nitric oxide synthase; eNOS= endothelial NOS; nNOS= neuronal NOS; iNOS=inducible NOS, NPBI=non-protein bound iron.

Upon reoxygenation an important early source of free radical production occurs due to metabolisation of hypoxanthine (a degradation product of ATP), accumulated during the actual HI-period, into xanthine and uric acid by the activated enzyme xanthine oxidase, a process that needs oxygen. During this process large amounts of the superoxide free radical ($O_2^{-}\cdot$) are formed. The simultaneous occurring metabolisation of arachidonic acid, freed from damaged cell membranes, into prostaglandins leads to a further production of the superoxide free radical. This chain of reactions, causing an excess production of superoxide, culminates commonly within the first 30-60 minutes after the start of reperfusion and reoxygenation and plays a central role in further formation of free radicals [10,19]

1. Superoxide-derived hydrogen peroxide ($H_2O_2$) reacts with the pro-radical non protein-bound iron (NPBI), accumulated in neuronal and microglial cells during the actual HI insult due to a low intracellular pH, to form the very toxic hydroxyl (OH) free radical. This reaction is also known by the Fenton Reaction [20,21]. Several experimental and clinical studies indeed show that NPBI is related to brain damage in the immediate post-HI period [22,23].
2. Finally superoxide will also react with the free radical Nitric Oxide (NO) to form the toxic compound peroxynitrite (ONOO-). Peroxynitrite causes lipid peroxidation of neuronal cell membranes eventually leading to neuronal cell death [24]. Nitric Oxide is produced due to the activation of nitric oxide synthase (NOS), available in neurons, astrocytes (neuronal NOS) and endothelial cells (endothelial NOS) [25]. Further production of NO, somewhat later in the post-HI period, occurs because of activation of inducible NOS available in microglial cells and invaded white blood cells [24,26]. Although post-HI reperfusion-reoxygenation injury to the brain continues up to days and even weeks after the completion of HI by a pro- and anti-inflammatory response and subsequent inappropriate neuronal cell apoptosis [27], these processes will not be discussed here in detail because the are beyond the scope of this review.

Figure 2-1 summarizes schematically the injuring mechanisms induced upon and early after reperfusion and reoxygenation.

## Diagnosing fetal hypoxia

To timely start antenatal (maternal) neuroprotective treatment, it is important to appropriately identify fetal hypoxia in an early stage. Besides marked loss of fetal movements, a number of intrapartum surveillance techniques to diagnose fetal hypoxia are used nowadays.

Cardiotocography (CTG), introduced in the early 70's, allows continuous recording of the fetal heart rate, based on the R-R interval of the fetal electrocardiogram (ECG). Fetuses with progressive heart rate abnormalities caused by hypoxia such as decreased short-term variability or fetal tachycardia, followed by fetal bradycardia (due to a redistribution of organ blood perfusion), are often born with low Apgar scores and/or substantial metabolic acidosis. However, since the interpretation of the fetal heart rate trace is subject to poor inter- and intra-observer agreement on classification of tracings and a high percentage of false-positive registrations, CTG monitoring alone does not always provide us with adequate information to discriminate between normal or abnormal fetal condition during labor [28-30]. Therefore additional techniques to assess fetal well-being are necessary to obtain more detailed information.

Fetal blood sampling (FBS) is one of the oldest techniques to obtain complementary information about fetal oxygenation in case of a non-reassuring fetal heart rate tracing by measuring the pH in fetal scalp blood [31,32]. In case of a pH lower than 7.20, fetal asphyxia is imminent [32-34]

and termination of pregnancy is advised [35].
A recent Cochrane meta-analysis [36] reported data from 12 randomized controlled trials on the effectiveness of the CTG. They found that continuous monitoring by means of CTG without FBS leads to a significant increase the number of caesarean sections, without a marked positive effect in neonatal outcome. If, however, FBS was used alongside the CTG, a less prominent increase in caesarean section rate was seen with a reduction in neonatal seizures of 50%.

Another candidate tool for a more reliable intrapartum assessment of fetal condition is fetal pulse oximetry (FPO), in which the actual level of fetal hypoxemia is recorded. Two large trials with respect to the usefulness of FPO did not show any effectiveness in reducing neonatal morbidity or overall incidence of caesarean sections (CS) [37,38].
Since the 1990's intrapartum fetal monitoring by recording the fetal electrocardiogram has been possible. In case of overt fetal hypoxia, changes in the ST segment and PR interval of the fetal ECG are present. Several investigators have assessed the value of analyzing the parameters as an adjunct to conventional cardiotocography. A large randomized trial by Amer-Wahlin *et al.* [39] showed that analysis of the ST segment, in addition to conventional CTG monitoring, significantly reduced caesarean delivery rates as well as metabolic acidemia in umbilical arterial blood. A recent Dutch randomized clinical trial of nearly 6000 patients also showed a decrease in metabolic acidosis, although there was no improvement in short term neonatal outcome [40]. In addition, there was a 50 % reduction in fetal scalp sampling in patients where ST-analysis was used as intrapartum fetal monitoring compared to patients who were only monitored by CTG. For a more reliable conclusion regarding the value of this technique to appropriately diagnose fetal hypoxia in an early stage long-term follow-up data of the included children seems mandatory.
Given the above mentioned considerations fetal monitoring by means of CTG or fetal ECG, combined with fetal blood sampling to obtain information about fetal acidosis seems the most optimal approach to timely diagnose fetal distress due to hypoxia at this moment.

## Non-specific measures to reduce or prevent fetal hypoxia

If fetal hypoxia is suspected during labor, several general measures can be performed to optimize fetal condition, depending on the underlying problem.

Management of non-reassuring fetal heart rate patterns consists of correcting any fetal hypoxic-ischemic insult, if possible. Moving the mother to lateral position to decompress the vena cava is one of the first measures to optimize uteroplacental perfusion [6]. Correcting iatrogenic maternal hypotension after regional anesthesia, administrating oxygen to the mother, discontinuing oxytocin administration and a single intravenous injection of tocolysis to relax the uterus can further improve fetal oxygenation [6,41]. In case of umbilical cord compression a change of maternal position or amnioinfusion can give relief. A recent Cochrane review shows that amnioinfusion appears to be useful to reduce the occurrence of variable heart rate decelerations and lower the use of caesarean section. The trials reviewed, however, were too small to address the possibility of rare but serious maternal adverse effects of amnioinfusion [42]. Transvaginal amnion infusion can also serve to dilute or wash out thick meconium to possibly prevent meconium aspiration syndrome. Several studies however conclude that, although prophylactic amnioinfusion does dilute meconium, it does not improve perinatal outcome if standard peripartum surveillance is used [43-45].

## Specific pharmacologic therapy to reduce or prevent fetal hypoxia-induced brain damage

As already hypothesized, antenatal pharma-

cologic treatment of the hypoxic fetus, via the mother, is a more rational approach since the concentration of neuroprotective drugs are already near or within the therapeutical range during the process of birth and during reperfusion and reoxygenation shortly after birth. This is the point of time where a maximal formation of reactive (oxygen) species such as free radicals and other toxic compounds can be expected, eventually leading to substantial damage to the newborn brain.

To successfully neuroprotect the hypoxic fetus in utero and during reperfusion and reoxygenation around the process of birth, a 'candidate' drug should qualify to the following criteria: 1) it should readily cross the placenta; 2) it should not be toxic to the mother; and 3) it should not be toxic to the fetus. To date there is not much reported in experimental or clinical studies about antenatal pharmacologic therapies to reduce or prevent fetal hypoxia-induced neonatal brain damage. Below we will summarize the scarce reports concerning this topic and discuss the most relevant drugs that may classify for this purpose.

*Phenobarbital* is a potential neuroprotective drug that easily passes the placenta and reaches concentrations that are equal to maternal concentrations [46]. It is reported to be an NMDA-receptor antagonist and also has gamma-aminobutyric-acid (GABA)-receptor activating properties [47-49]. It might furthermore reduce fluctuations in blood pressure [50] and cerebral perfusion, prevent intracranial hemorrhage [51], and ischaemic brain injury due to a possible free radical scavenging effect [52]. Especially its effectiveness in preterm infants by reducing brain damage such as periventricular/intraventricular hemorrhages (PIVHs) is elaborately investigated. Postnatal treatment with phenobarbital has been the subject of a Cochrane review by Whitelaw and Odd [53], who stated that postnatal administration of phenobarbital cannot be recommended as prophylaxis to prevent PIVH in preterm infants since there is no significant improvement in neurodevelopmental outcome and appeared to be associated with an increased need for mechanical ventilation due to respiratory depression. Early studies on maternal phenobarbital showed promising results in preventing PIVH in preterm infants [54-59]. However, adverse effects of antenatal phenobarbital were seen in a trial by Reinisch *et al* [60], who showed a possible detrimental influence on intelligence in adult males after in utero exposure to phenobarbital. Ajayi *et al.* [61] stated that early phenobarbital administration in term newborns with perinatal asphyxia increased the incidence of seizures with a trend towards increased mortality. However, up to now, no antenatal trials are known directed to reduce neonatal brain damage during and after fetal hypoxia. From above mentioned data we must conclude that it seems unrealistic to expect a reducing effect on the newborn brain of maternal phenobarbital treatment in case of fetal hypoxia and subsequent development of neonatal hypoxic-ischemic encephalopathy.

*Vitamin C and E* are important antioxidative agents that cross the placenta easily because these water-soluble vitamins are actively transported across the placenta from mother to fetus [62]. Therapeutic or prophylactic suppletion of vitamin C and E to pregnant women was suggested to have a protective effect in case of pre-eclampsia, since it should reduce oxidative-induced placental endothelial dysfunction [63]. However, a large study by Poston *et al.* showed no reduction in the incidence of preeclampsia, but did increase the rate of babies born with a low birth weight [64]. Also a large WHO study did not find any reduction in preeclampsia after administration of vitamin C and E to women with poor nutritional status [65].

*Allopurinol*. Formation of the superoxide free radical due to conversion of hypoxanthine into xanthine by xanthine oxidase plays a very important role in the occurrence of hypoxia- isch-

emia induced injury of the brain [66]. Administration of the xanthine oxidase inhibitor allopurinol (ALLO) reduces the production of superoxide free radical formation, thereby limiting the amount of hypoxia-reperfusion damage [67,68]. Furthermore, ALLO has also a non-protein bound iron (pro-radical) chelating and direct free radical (hydroxyl) scavenging effect, especially when high dosages are administered [19,69].

A prospective randomized study in human neonates, examining the effects of ALLO in term asphyxiated neonates, showed an improvement of electrocortical brain activity and a reduction in free radical formation after early neonatal (<4 hours of life) ALLO administration [70]. A recent paper by Gunes *et al.* [71] reports an improved neurological outcome after postnatal ALLO administration compared to a placebo in term moderately asphyxiated neonates. Moreover a randomized clinical trial by Benders *et al.* [15] had comparable results and showed a beneficial effect of ALLO on neurological development when the most severely asphyxiated neonates were excluded. Apparently, when asphyxia has been too severe, the inflicted brain damage can no longer be reversed. It is conceivable, that earlier ALLO treatment, i.e. the use of ALLO during labor in the case of suspected hypoxia, gives the opportunity to start even earlier with the treatment, thereby limiting the amount of hypoxia-reperfusion injury and further improving neurological outcome.

Pharmacologic data have been published by Boda *et al.*, which showed that therapeutical plasma levels were reached within 20 minutes in the human fetus after ALLO administration (600 mg) to the mother [72]. Some animal studies have been performed using piglets (neonatal administration), sheep and sows (maternal administration) [73-76]. These studies showed an adequate placental passage of ALLO and an improved outcome after ALLO treatment on cerebral energy status and recovery of umbilical blood flow respectively.

Our research group recently performed a prospective randomized placebo controlled double blind pilot study, in which we administered intravenously ALLO (500 mg) to pregnant women when fetal asphyxia was imminent. Data from this pilot study showed an inverse correlation between levels of ALLO and the amount of S100ß, a biomarker for brain tissue damage, in cord blood [77]. In addition, we performed a study in chronically instrumented fetal sheep, in which we showed evidence of cardio- and neuroprotection after antenatal ALLO administration to the pregnant ewe during repeated periods of ischemia [74].

Allopurinol has been used in internal medicine for many years for the treatment of gout. Side effects of ALLO are rare and most commonly include symptoms of hypersensitivity like skin rashes[78]. Stevens-Johnson syndrome has been reported, but only after prolonged treatment (> 10 days) in case of gout [79]. During previously performed studies in pregnant women and neonates, including those performed by our group, no adverse reactions were seen [72,77,80-82]. Together with the fact that the costs of ALLO-treatment are low, antenatal allopurinol seems to be promising as a neuroprotective agent.

Currently a national double blind placebo controlled multicenter trial has started in The Netherlands in which pregnant women on the brink of delivery are treated with 500 mg allopurinol or a placebo in case of suspected fetal distress.

*Melatonin* (N-acetyl-5-methoxytryptamine) is a small lipophilic indoleamine generated primarily in the pineal gland and secreted in a circadian manner [83]. Melatonin plays an important role in a variety of physiologic functions, including regulation of circadian rhythms as well as visual, reproductive, cerebrovascular, neuro-endocrine and neuro-immunological actions. Furthermore melatonin acts as a powerful scavenger of oxygen and nitrogen free radicals [84].

In adult animals melatonin is neuroprotective in models of focal cerebral ischemia [85] and the pre-treatment of melatonin (10 mg/kg intraperitoneal injection) reduces microglia activation in the hippocampus after kainic acid-induced inflammation in rats [86].

Previous experimental and clinical reports show that melatonin is rapidly transferred from maternal to fetal circulation in pregnant near term women up to 80% of the maternal concentration [87,88]. Prophylactic maternally administered melatonin (10 mg/kg intraperitoneally) prevents oxidative lipid peroxidation, DNA and mitochondrial damage in the brain of mature and premature fetal rats [89]. Besides proven benefits of prophylactic treatment, Hamada *et al.* [90] showed that maternal administration of melatonin within one hour after an HI-episode can prevent reperfusion-reoxygenation-induced oxidative cerebral damage in neonatal rats. Miller *et al.* [91] showed that intravenously administered melatonin to the mother (1 mg bolus, then 1 mg/h for 2 h) reduces hydroxyl free radical generation and lipid peroxidation in fetal sheep in response to asphyxia following umbilical cord occlusion. Another experimental study [92] demonstrated that post-asphyxial antenatal melatonin treatment attenuates the increase in activated microglia and 8-isoprostane (a marker of lipid peroxidation) production in mid-gestation fetal sheep. These protective actions of melatonin are likely due to its direct scavenging activity [93] and its indirect effects on antioxidant enzyme activities. Okatani *et al* showed that melatonin given to the pregnant rat increased antioxidant enzyme activity in the fetal brain which, thereby, provided indirect protection against free radical induced injury [94].

Many experimental studies have shown that maternal administration of melatonin before hypoxia significantly decreases oxidative damage in the fetal brain [88-94]. The choice of maternal melatonin treatment used as a single drug or in combination with other antioxidants such as ALLO and conventional treatments may lead to the development of a new approach for neuronal injury induced by fetal hypoxia and subsequent birth asphyxia.

*Xenon*. The noble anesthetic gas xenon (Xe) is an upcoming neuroprotectant in both in vitro and in vivo experimental studies [95-98].

Several incompletely understood characteristics of xenon might explain its neuroprotective effects [99]. Xenon is directly neuroprotective by being an NMDA-receptor antagonist [100], reducing overall neurotransmitter release [101], thereby limiting the excitotoxic apoptotic activity in the cascade of neuronal damage. Some authors postulate that xenon can also have a preconditioning effect, if administered before a hypoxic-ischemic insult, by inducing a pro-survival response by upregulation of a 'defensive' gene [102].

For these reasons xenon is an attractive candidate for neuroprotection because of the lack of adverse reactions and the absence of fetotoxicity [103]. Most studies are experimental and studied xenon in combination with hypothermia in neonatal rats [95-98]. Thoresen *et al.* [99] showed an optimal neuroprotective long-term effect on functional and pathological outcome in neonatal rats after 3 hours of hypothermia combined with 3 hours of xenon administration, with the neuroprotective effects of the two agents being additive. They even found a significant beneficial effect after 3 hours of hypothermia combined with one hour of xenon administration, thereby allowing transport to a specialized center and lowering costs. The latter being very important, since xenon administration is very expensive. When xenon concentrations are 70% or more xenon has a sedative effect. To our knowledge no human studies with xenon as a neuroprotective agent have been performed until now. At this moment one trial using postnatal xenon in combination with hypothermia in human neonates is running in the UK (trial register; Azzopardi, UK).

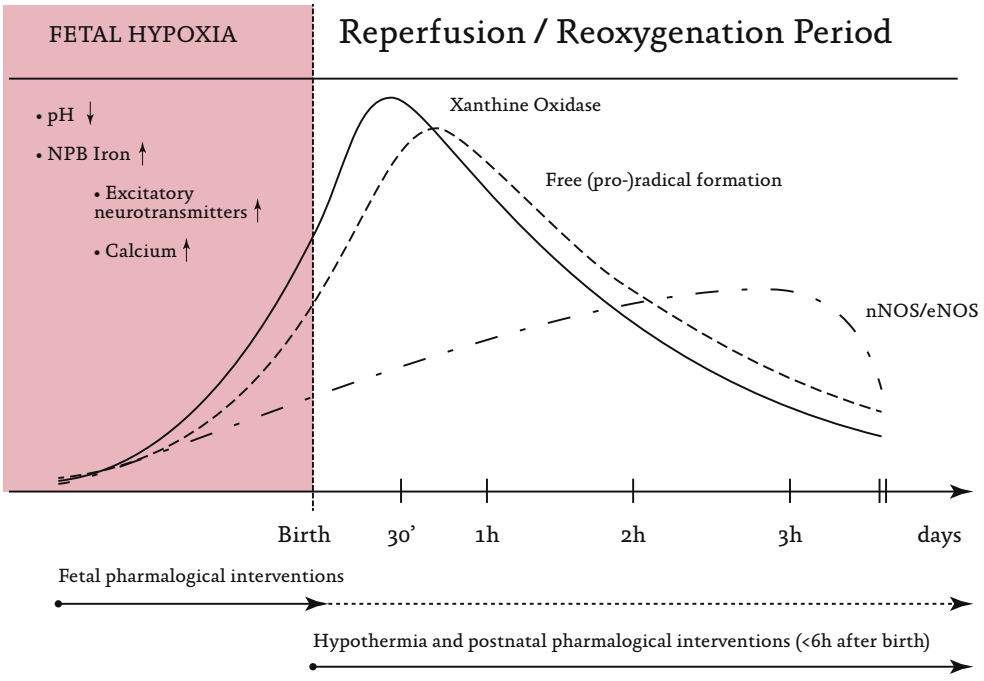
Besides the promising neuroprotective effects of postnatal treatment there might be a possible role for xenon as an antenatal neuroprotective agent. Xenon has received a marketing license as an anesthetic drug in several countries in Europe [103] and can for example be used as an anesthetic during caesarean section in case of fetal hypoxia, thereby allowing neuroprotection in an even earlier phase.

We are fully aware of the fact that our choice of drugs and agents discussed in this section may appear arbitrary and that it may be worthwhile to consider other drugs like N-acetylcysteine and other compounds with antioxidative properties for maternal treatment of the consequences of fetal hypoxia. On the other hand, the above-mentioned agents are drugs with which most experience has been obtained in maternal administration. Moreover in most instances knowledge of placental passage, a pre-requisite to treat the fetus via the mother, was best investigated in the drugs and agents discussed above.

## Conclusions and recommendations

It becomes increasingly clear that postnatal (pharmacologic) treatment with antioxidative or with NMDA-receptor antagonists of birth asphyxia-related damage to the newborn brain

**Figure 2-2. Proposed treatment scheme in case of fetal hypoxia with or without a subsequent occurrence of hypoxic-ischemic encephalopathy due to severe reperfusion-reoxygenation damage to the newborn brain**




NPB Iron= Non Protein Bound Iron; NOS= nitric oxide synthase; nNOS= neuronal NOS; eNOS= endothelial NOS.

has a limited neuroprotective effect, even when therapy has been started within the "window of opportunity", being within 6 hours after birth. This is probably because the maximal formation of reactive (oxygen) species and other toxic agents like peroxynitrite occurs upon and within the first 30-to-60 minutes after reperfusion and reoxygenation. Moreover, also during fetal hypoxia, an important determinant of birth asphyxia, free radicals and other toxic agents are produced albeit in much lower concentrations as compared to the early reperfusion phase. The fetus of pregnant women on the brink of delivery, with signs of (imminent) hypoxia may therefore benefit from additional maternal treatment with drugs reducing excessive neurotransmitter formation and formation of free radicals. If necessary, early postnatal hypothermia with or without pharmacologic neuroprotective agents can further reduce or even prevent fetal hypoxic and birth asphyxia-related brain damage of the newborn. Figure 2-2 summarizes schematically our proposed treatment protocol.

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# Chapter 3

# Long-term neuroprotective effects of allopurinol after moderate perinatal asphyxia. Follow-up of two randomized controlled trials

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## ABSTRACT

**Objective**
Free-radical-induced reperfusion injury has been recognized as an important cause of brain tissue damage after birth asphyxia. Allopurinol reduces the formation of free radicals, thereby potentially limiting the amount of hypoxia–reperfusion damage. In this study the long-term outcome of neonatal allopurinol treatment after birth asphyxia was examined.

**Design**
Follow-up of 4 to 8 years of two earlier performed randomized controlled trials.

**Setting**
Leiden University Medical Center, University Medical Center Groningen and University Medical Center Utrecht, The Netherlands.

**Patients**
Fifty-four term infants were included when suffering from moderate-to-severe birth asphyxia in two previously performed trials.

**Intervention**
Infants either received 40 mg/kg allopurinol (with an interval of 12h) starting within 4h after birth or served as controls.

**Main outcome measures**
Children, who survived, were assessed with the Wechsler Preschool and Primary Scales of Intelligence test or Wechsler Intelligence Scale for Children and underwent a neurological examination. The effect of allopurinol on severe adverse outcome (defined as mortality or severe disability at the age of 4–8 years) was examined in the total group of asphyxiated infants and in a predefined subgroup of moderately asphyxiated infants (based on the amplitude integrated electroencephalogram).

**Results**
The mean age during follow-up (n=23) was 5 years and 5 months (SD 1 year and 2 months). There were no differences in long-term outcome between the allopurinol-treated infants and controls. However, subgroup analysis of the moderately asphyxiated group
showed significantly less severe adverse outcome in the allopurinol-treated infants compared with controls (25% vs 65%; RR 0.40, 95%CI 0.17 to 0.94).

**Conclusions**
The reported data may suggest a (neuro)protective effect of neonatal allopurinol treatment in moderately asphyxiated infants.

Hypoxia-ischemia (HI) during birth asphyxia damages the susceptible developing brain. Reperfusion of previously ischemic brain tissue is now recognized as an important mechanism for substantial additional brain injury due to the formation of toxic free radicals [1,2]. This reperfusion injury may be ameliorated by neuroprotective strategies such as hypothermia and/or early post-asphyxial pharmacological intervention like allopurinol [3]. Experimental studies investigating specific pharmacological therapies showed promising results [4,5], but appeared to be less successful in studies in the human neonate [6]. Up to now only therapeutic hypothermia of the brain or the whole body proved to reduce post-HI damage to the brain in moderately asphyxiated human neonates [7-10]. In severely asphyxiated neonates (defined by a severely abnormal amplitude integrated electroencephalogram (aEEG) plus seizures) hypothermia, however, did not seem to significantly improve neurodevelopmental outcome [7].

We previously performed two randomized controlled trials on the effect of high dose allopurinol (40mg/kg/day) for reducing post-HI brain damage in respectively 22 and 32 asphyxiated human neonates [11,12]. The xanthine oxidase inhibitor allopurinol potentially protects against reperfusion-induced brain injury by reducing free radical formation and in high dosages also acts as a free radical scavenger and free iron chelator [4,13,14]. A recent Cochrane meta-analysis, involving our two studies and a comparable Turkish trial [15], concluded that the currently available data are insufficient to determine whether allopurinol is beneficial as a neuroprotective treatment for birth asphyxia-induced HI encephalopathy [6]. However, the neuroprotective effect of allopurinol in the subgroup of moderately asphyxiated children was not yet analyzed.

In this study we investigated neurodevelopmental and cognitive outcome between 4 and 8 years of age in the surviving patients of our two previously performed randomized controlled trials. Because recent studies showed that in particular infants suffering from moderate birth asphyxia benefit from neuroprotection with hypothermia in contrast to those suffering from severe birth asphyxia, we analyzed differences in mortality and long-term developmental outcome between allopurinol-treated and non-treated children not only in the entire group of asphyxiated infants, but also in a subgroup of moderately asphyxiated infants [7].

## Methods

### Participants

The infants included in this follow-up study were the participants in our two randomized controlled trials concerning the effect of early neonatal allopurinol-treatment in reducing post-HI brain damage [11,12]. The inclusion criteria of these studies were similar according to the following criteria; (near) term infants, without known chromosomal anomalies, who suffered from birth asphyxia defined as fetal distress (late decelerations on fetal monitoring or meconium staining; the need for resuscitation for > 2 minutes; cord or lowest pH < 7.00 and multi-organ failure).

After admission, electrical brain activity was monitored by aEEG (CFM, Lectromed; Oxford Instruments, Oxford, UK). Amplitude intergraded EEG has been proven to be of value for evaluating background and seizure activity. The type of background pattern predicts long-term outcome [16,17]. The following patterns, of increasing abnormality, can be distinguished; continuous normal voltage, discontinuous normal voltage, burst suppression, continuous extremely low voltage and flat trace. The last three patterns, which are abnormal, are induced by hypoxic-ischemic encephalopathy. Van Bel *et al.* included all children with the above mentioned inclusion criteria, irrespective of the aEEG. In the study by Benders *et al.* only those children, with the above-mentioned inclusion criteria,

which had a burst suppression pattern or worse on the amplitude-integrated electroencephalogram (aEEG) were included.

**Intervention**

If all inclusion criteria were met, the infants received either two dosages of 20 mg/kg allopurinol (Apurin; Multipharma, Copenhagen, Denmark) intravenously starting within 4 hours after birth (with an interval of 12 hours) or served as controls. Because high dosages were used for the first time, the study by van Bel *et al.* was performed randomized, but not blinded to monitor possible side effect. Benders *et al.* performed the study randomized, double blind and placebo controlled.

**Outcome assessment**

Children, who survived and could be tested, were assessed with the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III, an individually administered instrument for assessing the intelligence of children aged 2:6-7:3) [18] or the Wechsler Intelligence Scale for Children (WISC-III-NL for children aged 6-17) by psychologists [19]. With these tests Verbal, Performance and Full Scale Intelligence Quotients (IQs) were measured. Total IQs were classified according to the guidelines of the Dutch Professional Association of Psychologists; IQ was defined normal if ≥ 90, moderately delayed if 70 ≤ IQ < 90 and severely delayed if < 70 [20].

Every child underwent a standardized neurological examination by a neonatologist trained in neurodevelopmental assessments. Cerebral Palsy (CP) was classified using the Gross Motor Function Classification System (GMFCS). The GMFCS describes the major functional characteristics of children with CP. It is a five-level pattern-recognition system. Children that are classified as GMFCS levels I and II have the potential to walk independently both indoors and outdoors, and in the community as well. In contrast, children classified in GMFCS levels III to V are limited in their self mobility. They walk with a mobility device and are potential wheelchair users [21,22]. The psychologists and neonatologists who assessed neurodevelopmental outcome were independent and not informed about patient allocation.

Neurodevelopmental outcome was classified as normal outcome, mild disability or severe disability. A Full Scale IQ of ≥ 90 without any physical abnormalities was defined as normal. Cerebral Palsy classified as GFMCS levels I or II, epilepsy with good response to treatment, hearing impairment and/or 70 ≤ IQ < 90 were classified as mild disabilities. Cerebral Palsy classified as GFMCS levels III to V, epilepsy not responding to treatment, blindness, deafness and/or a Full Scale IQ < 70 were regarded as severe disabilities.

The combined frequency of mortality or severe disability in survivors at 4-8 years of age was considered as severe adverse outcome.

**Table 3-1. Survival rates of two RCTs concerning neonatal allopurinol after birth asphyxia**

| | Van Bel et al. | | Benders et al. | |
|---|---|---|---|---|
| | ALLO (n=11), n | CONT (n=11), n | ALLO (n=17), n | CONT (n=15), n |
| Died | 2 | 6 | 13 | 10 |
| Survivors | 9 | 5 | 4 | 5 |

ALLO, allopurinol; CONT, controls.

**Table 3-2. Baseline Characteristics**

| | ALLO (n=28) | CONT (n=25)* | p |
|---|---|---|---|
| Gestational age (wks.days) | 40.0 (37.0-41.4) | 39.6 (36.0-42.0) | 0.253 |
| Birth weight (g) | 3500 (2425-5270) | 3240 (2300-4720) | 0.747 |
| Cord pH | 6.90 (6.49-7.05) | 6.94 (6.60-7.15) | 0.112 |
| Apgar score at 5 minutes | 4 (2-7) | 4 (1-8) | 0.471 |
| aEEG | | | |
| CNV, n (%) | 8 (29%) | 5 (20%) | 0.405 |
| BS, n (%) | 8 (29%) | 12 (48%) | 0.371 |
| CLV, n (%) | 2 (7%) | 4 (16%) | 0.414 |
| FT, n (%) | 10 (36%) | 4 (16%) | 0.109 |

Data are reported as median (range), unless otherwise stated. *One child was excluded because of a suspected syndrome.

ALLO, allopurinol; BS, burst suppression; CLV, continuous low voltage; CNV, continuous normal voltage; CONT, controls; FT, flat trace.

Survival rates and long-term neurodevelopmental outcome were compared in both groups. All infants were then divided in two predefined groups for subgroup analysis based on the aEEG-signal; moderately asphyxiated neonates were defined by having a burst suppression pattern or better on the aEEG and severely asphyxiated neonates were defined by having continuous low voltage or flat trace patterns on the aEEG. The analysis of the tracings was done by two neonatologists (FvB, MB) with expertise in the interpretation of the aEEG. The aEEGs were classified using well-established criteria as published by Toet *et al* [16]. This is a clinically used classification system to distinguish moderately asphyxiated infants from severely asphyxiated infants [17]. Disagreements were solved by discussion. This subgroup analysis was performed to test the hypothesis that no advantage of neonatal allopurinol treatment is seen when brain damage is too severe.

**Statistical analysis**

Statistical analyses were performed with the SPSS 15.0 statistical package. Categorical variables were compared by Fisher's exact test. Differences regarding continuous data were assessed by Student's t test or Mann-Whitney U test depending on normality. The analyses concerning severe adverse outcome were stratified by trial. We report separate risk ratios (RR) per trial and a pooled RR with 95% confidence interval (95%CI), generated by using a Mantel Haenszel approach (Review Manager (RevMan) [Computer program]. Version 5.1. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). Statistical significance was based on two-sided tests with a cut-off level of 0.05.

**Ethical Approval**

Both studies were approved by the local ethical committees of the three participating hospi-

tals (the Leiden University Medical Center, the University Medical Center Groningen and the University Medical Center Utrecht [the Netherlands]). Informed consent was given by the parents of all participating children.

## Results

Fifty-four full term infants were included in the two previously performed studies; 22 children in the study by van Bel *et al.* and 32 infants in the study by Benders *et al.* Twenty-eight asphyxiated infants received allopurinol (ALLO) and 26 asphyxiated infants served as controls (CONT) (table 3-1).
There were no significant differences between the groups regarding gestational age, birth weight, cord pH, Apgar scores, distribution of aEEG patterns (table 3-2) or the age at follow-up.

In total, 31 infants died in the neonatal period, leaving 23 survivors (13 in the ALLO-group and 10 in the CONT-group) available for long-term follow-up. During the years it became clear

**Table 3-3. Mortality and developmental outcome in survivors of two RCTs concerning neonatal allopurinol after birth asphyxia**

| | Total group of asphyxiated infants | | |
|---|---|---|---|
| | ALLO (n=28) | CONT (n=26) | *p* |
| Mortality, n (%) | | | |
| Died | 15 (54%) | 16 (62%) | 0.376 |
| Survivors | 13 (46%) | 10 (39%) | |
| Excluded | 0 | 1[b] | |
| IQ [c] | (n=11) | (n=8) | |
| Verbal | 92.4 (13.4) | 100.1 (21.3) | 0.361 |
| Performance | 94.3 (13.6) | 94.0 (16.4) | 0.957 |
| Full Scale | 92.8 (13.8) | 96.6 (15.7) | 0.590 |
| Long-term neurodevelopmental outcome, n (%) | (n=13) | (n=9) | |
| Severe disabled [d] | 1 (8%) | 1 (11%) | 1.000 |
| Mild disabled [e] | 5 (39%) | 4 (44%) | 1.000 |
| Normal [f] | 7 (54%) | 4 (44%) | 0.680 |
| Overall long-term outcome, n (%) | | | |
| Severe adverse outcome [g] | 16 (57%) | 17 (68%) | 0.571 |

[a] Subgroup of moderately asphyxiated children; children with a burst suppression pattern or better on the aEEG.
[b] Excluded because of suspected syndrome.
[c] Values are reported as mean (SD).
[d] The two children with a severe disability both had Cerebral Palsy (GMFCS ≥ 3).

that one child in the CONT-group, with consanguine parents, was suspected to have a syndrome. The infant had mental retardation and epilepsy (not responding to anti-epileptic treatment), which could not be solely explained by perinatal asphyxia since the neonatal MRI was normal. He was therefore excluded from further analyses.

The age of the children at follow-up was 5 years 5 months (SD 1y 2mo) in the ALLO-treated and 5 years 6 months (SD 1y 1mo) in the non-treated children. There were no infants with Full Scale IQ-scores lower than 70 as tested with the WISC or WPPSI. Full Scale IQs of ≥ 70 and < 90 were found in seven infants; three in the CONT-group and four in the ALLO-group. Hearing impairment was apparent in one child with normal IQ-scores in the ALLO-group. In the CONT-group one child, with normal IQ-scores, suffered from epilepsy well responding to treatment. In the ALLO-group one child had developed a spastic quadriplegic Cerebral Palsy classified as GMFCS level V with cortical blind-

| **Subgroup of moderately asphyxiated infants** [a] | | | |
|---|---|---|---|
| ALLO (n=16) | CONT (n=17) | *p* | RR (95%CI) |
| | | | |
| 4 (25%) | 10 (59%) | 0.053 | |
| 12 (75%) | 7 (41%) | | |
| | | | |
| (n=11) | (n=6) | | |
| 92.4 (13.4) | 98.8 (17.1) | 0.414 | |
| 94.3 (13.6) | 99.8 (13.9) | 0.470 | |
| 92.8 (13.8) | 98.7 (14.3) | 0.429 | |
| | | | |
| (n=12) | (n=7) | | |
| 0 (0%) | 1 (14%) | 0.368 | |
| 5 (42%) | 2 (29%) | 0.656 | |
| 7 (58%) | 4 (57%) | 1.000 | |
| | | | |
| 4 (25%) | 11 (65%) | 0.047 | 0.40 (0.17-0.94)[h] |

[e] The group of mild disabled children included children with epilepsy well responding to treatment, hearing impairment or 70 ≤ IQ < 90.
[f] Normal outcome: IQ ≥ 90, no physical impairment. [g] Severe adverse outcome was defined as mortality or severe disability. [h] Pooled RR, generated by using a Mantel Haenszel approach to stratify by trial.
ALLO, allopurinol; CONT, controls.

ness and severe mental retardation. One child in the CONT-group developed a spastic quadriplegic Cerebral Palsy classified as GMFCS level IV. Both children were not tested because severe neurodevelopmental delay was already established and therefore the parents refused to cooperate.

One child in the ALLO-group did not speak Dutch as his first language so the verbal IQ could not be tested reliably. In this specific case the performal IQ, instead of the total IQ, was therefore used to classify outcome.

One child, in the ALLO-group in the study by van Bel *et al.* did not undergo the psychological test. The parents refused to cooperate, because the child did not have neurological problems at 5 years of age and received normal education. At the age of 12 all children in the Netherlands undergo a CITO-test [23], which is a standardized Dutch test to assess the intelligence of children in different subareas (e.g. mathematics, language). The outcome is a transformed scale score. The raw score (i.e. the number of questions answered correctly) is converted to a percentile. Individual scores are compared with scores of children of the same age in the Netherlands. This child scored p70, which means 70% of all other children of that age who performed the same test scored lower. Her outcome was therefore classified as "normal".

No significant differences in long-term outcome were seen in children in the ALLO-group compared to the CONT-group (table 3). However, after excluding the most severely asphyxiated children based on the aEEG, significantly less severe adverse outcome was seen in the allopurinol treated infants compared to controls (25% vs 65%; p = 0.047). Separate risk ratios (RR) for severe adverse outcome per trial are shown

**Figure 3-1. Severe adverse outcome in two randomized controlled trials concerning neonatal allopurinol after birth asphyxia**

| Study or Subgroup | Experimental | | Control | | Weight |
|---|---|---|---|---|---|
| | Events | Total | Events | Total | |
| **Moderate asphyxia** | | | | | |
| van Bel 1998 | 1 | 9 | 4 | 9 | 21.9% |
| Benders 2006 | 3 | 7 | 7 | 8 | 35.8% |
| Subtotal (95% CI) | | 16 | | 17 | 57.7% |
| Total events | 4 | | 11 | | |
| Heterogeneity: $Chi^2$=0.42, df=1 (P=0.52); $I^2$=0% | | | | | |
| Test for overall effect: Z=2.10 (P=0.04) | | | | | |
| **Total asphyxia** | | **28** | | **26** | **100%** |
| Total events | 16 | | 17 | | |
| Heterogeneity: $Chi^2$=7.80, df=3 (P=0.05); $I^2$=62% | | | | | |
| Test for overall effect: Z=0.72 (P=0.47) | | | | | |
| Test for subgroup differences: $Chi^2$=6.70, df=1 (P=0.010), $I^2$=85.1% | | | | | |

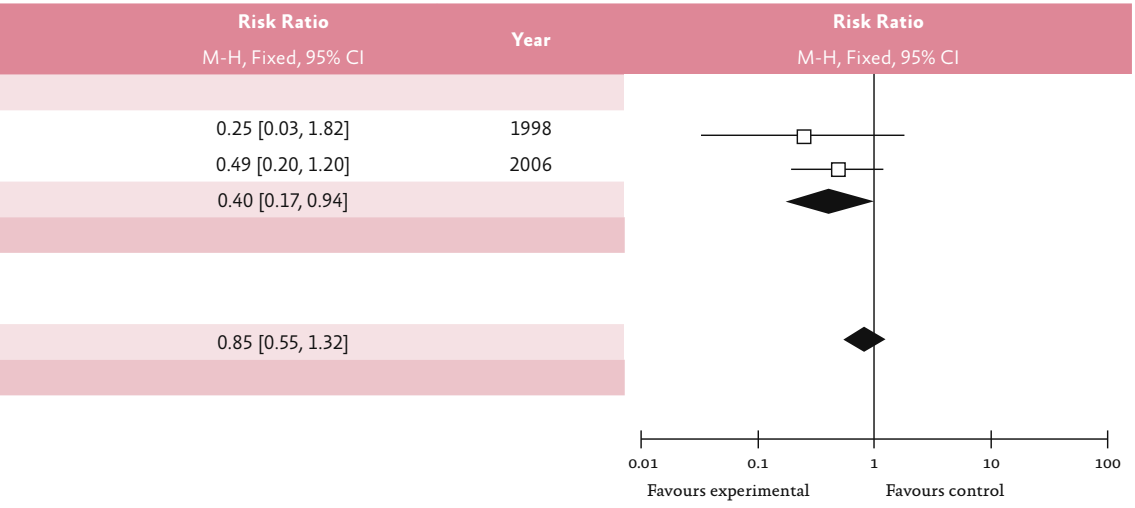
Occurrence of severe adverse outcome (indicated as 'events') in a subgroup of moderately asphyxiated infants, stratified by trial. A pooled RR for severe adverse outcome in the experimental (allopu-

in figure 3-1. The pooled RR for severe adverse outcome in the ALLO-group was 0.40 (0.17-0.94) (table 3-3, figure 3-1).

## Discussion

Although no significant effect of neonatal allopurinol treatment is seen in reducing mortality or developmental disability in the overall group of asphyxiated infants, the present data suggest that neonatal treatment with allopurinol does lower the risk of severe adverse outcome (i.e. mortality or severe disability) in moderately asphyxiated infants. These findings are in line with the hypothesis that no advantage of neonatal treatment is seen when birth asphyxia is too severe, as stated earlier by Gluckman *et al.* when treating them with moderate hypothermia [7]. However, the number of children involved in the analyses is small, so the conclusions drawn have to be taken with caution. There were also some small differences in inclusion criteria between both studies. The study by Benders *et al.* only included children with a burst suppression pattern or worse on the amplitude-integrated electroencephalogram (aEEG). This might explain the differences in overall mortality rate between the two studies, since it probably resulted in the inclusion of more severely asphyxiated infants in the study by Benders *et al.* Furthermore, there was a substantial variation between subjects in follow-up age, but since developmental scores calculated by WPPSI and WISC are adjusted for age, this should not be regarded as a big drawback.

In a true intention-to-treat analysis, the infant who was excluded because of a suspected syndrome should have been included. We nevertheless decided to exclude this child from further analyses. The child was diagnosed with epilepsy not responding to treatment, so would therefore be classified in the "severe adverse out-




rinol) group was generated using a Mantel Haenszel approach.

come"-group. With a small sample size, like in the present study, including this child could have had enormous influence on the final results and conclusions. Since the child made part of the control group, this would have made the results more significantly in favor of allopurinol, which could not be justified.
It must be stated that there are limitations of aEEG interpretation depending on experience, especially in interpreting baseline drift, seizure recognition (with or without seizure detection) and the fact that it is only possible to give a general and not localized assessment of background pattern of brain activity. However, in our opinion, this method to assess the severity of brain damage is reliable in both clinical care and research settings if used by well-trained neonatologists [17,24].

To our knowledge, this is the first report concerning follow-up data after neonatal allopurinol treatment and, despite the above-mentioned limitations, we are of the opinion that this follow-up study provides us with some interesting data.
As already stated we could not find a neuroprotective effect of neonatal treatment with allopurinol in the overall group of asphyxiated infants such as is the case with hypothermia. Our study, however, had insufficient power to exclude a modest but important effect size.
Another possible explanation might be the relatively late administration of allopurinol with a median of 3 hours postnatally. Since the vast amount of toxic free radicals, an important reason for post-HI damage to the developing brain is produced during the hypoxic ischemic event itself and in particular upon reperfusion and reoxygenation in the first 30 to 60 minutes after birth, we suggest that earlier treatment might be more effective. By intravenous administration of allopurinol to the mother when fetal distress, an important determinant of perinatal asphyxia, is suspected, therapeutic levels of allopurinol and its active metabolite oxypurinol can be reached even before birth [25,26]. In this way, therapeutic levels of allopurinol are already available upon reperfusion, thereby reducing the formation of toxic free radicals in an earlier and crucial stage, namely during maximal free radical formation.
A recent pilot study in this respect, performed by our research group, showed a significant inversed correlation between levels of fetal allopurinol and S100ß (a clinically used marker for brain damage) in cord blood [26]. A large multicenter randomized clinical trial on maternal treatment with allopurinol in case of signs of fetal hypoxia is now running in the Netherlands (NCT00189007) to investigate whether or not maternal allopurinol-treatment can further improve long-term outcome of neonates exposed to (severe) fetal hypoxia [27].

In conclusion, the effects of neonatal treatment with allopurinol seem promising in moderately asphyxiated infants. Although the window of opportunities is probably quite small it is not clear whether the more severely asphyxiated children can also benefit from allopurinol treatment if treatment is started upon or even before birth in case of (severe) fetal hypoxia. Given the fact that superoxide production occurs upon and during the early reperfusion/reoxygenation phase, it seems to be appropriate to already treat the mother in case of suspected fetal distress during labor to reach therapeutic levels in the newborn before reoxygenation, thereby reducing free radical formation in the earliest possible state. Therapeutic hypothermia did not play any role in this follow-up study, since both initial trials were performed before the introduction of therapeutic mild hypothermia in clinical practice. For future research, however, it is very important to take the impact of mild therapeutic hypothermia on allopurinol metabolism and pharmacokinetics into account.

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# Chapter 4
# Maternal to fetal allopurinol transfer and xanthine oxidase suppression in the late gestation pregnant rat

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## ABSTRACT

Fetal brain hypoxic injury remains a concern in high risk delivery. There is significant clinical interest in agents that may diminish neuronal damage during birth asphyxia, such as in allopurinol, an inhibitor of the pro-oxidant enzyme xanthine oxidase. Here, we established in a rodent model the capacity of allopurinol to be taken up by the mother, cross the placenta, rise to therapeutic levels and suppress xanthine oxidase activity in the fetus. On day 20 of pregnancy, Wistar dams were given 30 or 100 $mg.kg^{-1}$ allopurinol orally. Maternal and fetal plasma allopurinol and oxypurinol concentrations were measured, and xanthine oxidase activity in the placenta and maternal and fetal tissues determined. There were significant strong positive correlations between maternal and fetal plasma allopurinol ($r=0.97$, $P<0.05$) and oxypurinol ($r=0.88$, $P<0.05$) levels. Under baseline conditions, maternal heart ($2.18\pm0.62$ mU $mg^{-1}$), maternal liver ($0.29\pm0.08$ mU $mg^{-1}$), placenta ($1.36\pm0.42$ mU $mg^{-1}$), fetal heart ($1.64\pm0.59$ mU $mg^{-1}$) and fetal liver ($0.14\pm0.08$ mU $mg^{-1}$) samples all showed significant xanthine oxidase activity. This activity was suppressed in all tissues 2h after allopurinol administration and remained suppressed 24h later ($P<0.05$), despite allopurinol and oxypurinol levels returning towards baseline. The data establish a mammalian model of xanthine oxidase inhibition in the mother, placenta and fetus, allowing investigation of the role of xanthine oxidase-derived reactive oxygen species in the maternal, placental and fetal physiology during healthy and complicated pregnancy.

Despite advances in obstetric practice, acute intra-partum fetal hypoxia remains one of the most common forms of fetal stress, with substantial morbidity and mortality [1]. One possible strategy to combat the detrimental effects of fetal hypoxia would be to decrease the associated excessive generation of reactive oxygen species (ROS) from stimulated pro-oxidant mechanisms within the cell, such as the xanthine oxidase pathway [2]. Indeed, maternal treatment with the xanthine oxidase inhibitor allopurinol is being considered in human pregnancy complicated by intra-partum hypoxia in order to protect the infant from excessive generation of ROS [3 - 9]. This clinical interest in antenatal maternal administration of allopurinol follows previous studies which reported that allopurinol treatment in the asphyxiated neonate improved neonatal outcome [10], but if the time-interval between hypoxia and treatment had been too long, or when fetal hypoxia had been too severe, no reduction in serious morbidity or mortality was observed [4]. Therefore, there has been growing clinical and scientific interest in establishing whether perinatal outcome may be improved in complicated labor if the window of treatment with allopurinol could be initiated before birth, for instance via maternal treatment to cover the actual period of fetal hypoxia and reperfusion [7,11].

Whilst there have been studies in large animals including the pregnant ewe [11,12] and sow [13], no small animal model of allopurinol administration to the mother during pregnancy has been established. Such a model is indispensable in species with a comparatively shorter lifespan to allow follow up of the effects of xanthine oxidase inhibition during pregnancy on the physiology of the offspring in later life. This is essential when many of the conditions associated with a suboptimal fetal environment such as cardiovascular disease and type 2 diabetes only emerge in later life.

Therefore, the aims of this study were to investigate in rodent pregnancy: 1) whether xanthine oxidase is active in the placenta, maternal and fetal tissues in late gestation; 2) if maternal oral administration of allopurinol could increase maternal plasma levels of allopurinol and its active metabolite oxypurinol; 3) if maternal allopurinol and oxypurinol could cross the placenta, yielding therapeutic levels in the fetal circulation, and 4) whether elevations in maternal and fetal plasma levels of allopurinol and oxypurinol resulted in suppression of xanthine oxidase activity in the placenta, maternal and fetal tissues. These aims were determined using a clinically-relevant dose of allopurinol (30 mg $kg^{-1}$), comparable to the dose given in previous human studies [4,10], and a larger dose (100 mg $kg^{-1}$) to investigate any pharmacological benefit in terms of either greater fetal levels of allopurinol and/or longer inhibition of xanthine oxidase activity.

## Materials and methods

All procedures involving animals were carried out under the Animals (Scientific Procedures) Act 1986 and approved by the Local Ethics Review Committee of the University of Cambridge, UK. Thirty-five time-mated pregnant Wistar rats (Charles River Limited, UK) were delivered to the University of Cambridge between 10-14 days of gestation and were individually housed under standard conditions (21±1°C, 55% humidity, 12h/12h light/dark cycle) with free access to food and water.

On day 20 of pregnancy all animals were randomized to either control or allopurinol treatment. Allopurinol (30mg.$kg^{-1}$ or 100mg.$kg^{-1}$, Sigma, UK, suspended in 3ml Hartley's Strawberry Jelly) was placed in a clear glass bowl inside the cage at 8am. Animals had been given an untreated jelly dose the day before to condition them to eat it. Following allopurinol treatment, animals underwent euthanasia ($CO_2$ followed by cervical dislocation) at either 2h, 6h or 24h following administration (n=5 all time points and doses).

A further five animals underwent euthanasia at 8am on day 20 of pregnancy to act as controls. Following euthanasia, a maternal blood sample was taken by cardiac puncture for the measurement of maternal circulating allopurinol and oxypurinol levels. Fetal rats were then exposed by laparotomy. The fetuses were subjected to cervical transection and blood was collected and pooled from all fetuses. Maternal and fetal blood samples were anti-coagulated with ethylenediaminetetraacetic acid (EDTA), centrifuged (3000g for 5 minutes), and the plasma was frozen in liquid nitrogen and subsequently stored at -80°C until analysis for allopurinol and oxypurinol. Samples of placenta, maternal heart, maternal liver, and fetal liver of *ca*. 100mg were taken, weighed and homogenized in buffer (1ml of 100mM TRIS-HCl, pH 7.5 with 10ml protease inhibitor cocktail, SIGMA, P8340). Fetal heart samples were *ca*. 20-25mg. All further analysis was adjusted for the weight of tissue taken. Fetal tissue samples and associated placentas were taken only from male pups to reduce any differences attributable to sex. The sex of each fetal pup was determined by their ano-genital distance as previously described [14]. Following centrifugation of the tissue homogenate (30 min, 10 000g at 4°C), the supernatant was decanted and frozen in aliquots at -80°C until further analysis.

**Plasma allopurinol and oxypurinol measurements**

Allopurinol and oxypurinol plasma concentrations were determined by using reversed-phase, high-performance liquid chromatography with UV-detection at 254 nm for the quantification of allopurinol and oxypurinol in plasma [15]. The method was linear between 0.5 and 25 $mg.ml^{-1}$ with a lower limit of detection of 0.2 $mg.ml^{-1}$ for both compounds.

**Xanthine oxidase assay**

Fetal plasma xanthine oxidase activity levels were measured using a commercially available assay kit (A22182, Invitrogen, UK). The assay works on the principle that xanthine oxidase produces superoxide anions. In vitro, superoxide spontaneously degrades to hydrogen peroxide ($H_2O_2$). $H_2O_2$, in the presence of horseradish peroxidase (HRP), reacts stoichiometrically with Amplex Red reagent to generate the red-fluorescent oxidation product, resorufin. In brief, 50 ml of tissue homogenate or xanthine oxidase standard solution was added to 50 ml of 100 µM Amplex Red reagent solution which also contained 0.4 $U.mL^{-1}$ HRP and 200 µM hypoxanthine. The resulting solutions were mixed and then incubated for 30 min at 37°C. The presence of resorufin was detected by fluorescence using excitation in the range of 530–560 nm and emission detection at 590 nm. Activity was then expressed per mg of wet tissue.

**Data and statistical analysis**

All values are expressed as mean ± S.E.M. The correlation between fetal and maternal allopurinol and oxypurinol levels was assessed using the Pearson Product Moment correlation (Sigma-Stat 3.5; Chicago, IL, USA). Allopurinol, oxypurinol and xanthine oxidase activity levels were compared using one-way ANOVA comparing with reference to time 0h. Where significant differences were found, the Student-Newman-Keuls *post-hoc* test was applied (Sigma-Stat 3.5; Chicago, IL, USA). Statistical significance was accepted when $P<0.05$.

## Results

**Allopurinol and oxypurinol levels in maternal and fetal plasma**

Administration of 30 $mg.kg^{-1}$ and 100 $mg.kg^{-1}$ allopurinol to the pregnant rat led to increases in both maternal and fetal allopurinol and oxypurinol by 2h (Figure 4-1). Following the administration of allopurinol at both doses of 30 $mg.kg^{-1}$ and 100 $mg.kg^{-1}$, allopurinol levels returned to baseline at 6h following dosing, however oxypurinol levels remained significantly elevated in both mother and fetus. Although maternal and fetal plasma oxypurinol levels were detect-

able 24h following dosing, they were not significantly different from baseline.
When all paired maternal and fetal plasma concentrations of allopurinol and oxypurinol were related to determine maternal to fetal transfer, a significant positive correlation was obtained for both compounds ($P<0.05$; Figure 4-2). The correlation coefficient (r) for the relationship between maternal and fetal allopurinol was 0.97, and between maternal and fetal oxypurinol was 0.88, indicating a high degree of correlation (Figure 4-2).

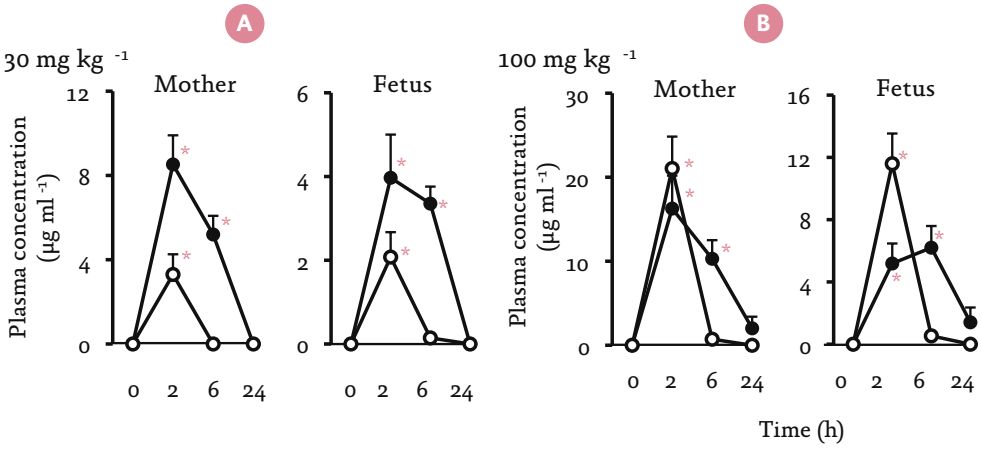
**Plasma xanthine oxidase levels**

Xanthine oxidase activity was detectable in maternal heart, maternal liver, placenta, fetal heart and fetal liver (Figure 3). Maternal and fetal cardiac tissue displayed the greatest activity (2.17±0.6 mU.mg$^{-1}$ and 1.65±0.59 mU.mg$^{-1}$, respectively), followed by placenta (1.36±0.41 mU.mg$^{-1}$) and then maternal liver and fetal liver (0.29±0.09 mU.mg$^{-1}$ and 0.14±0.08 mU.mg$^{-1}$). Treatment with allopurinol at 30 mg.kg$^{-1}$ and 100 mg.kg$^{-1}$ significantly depressed activity in all tissues by 2 h and this suppression was sustained 6 and 24 h following treatment.

## Discussion

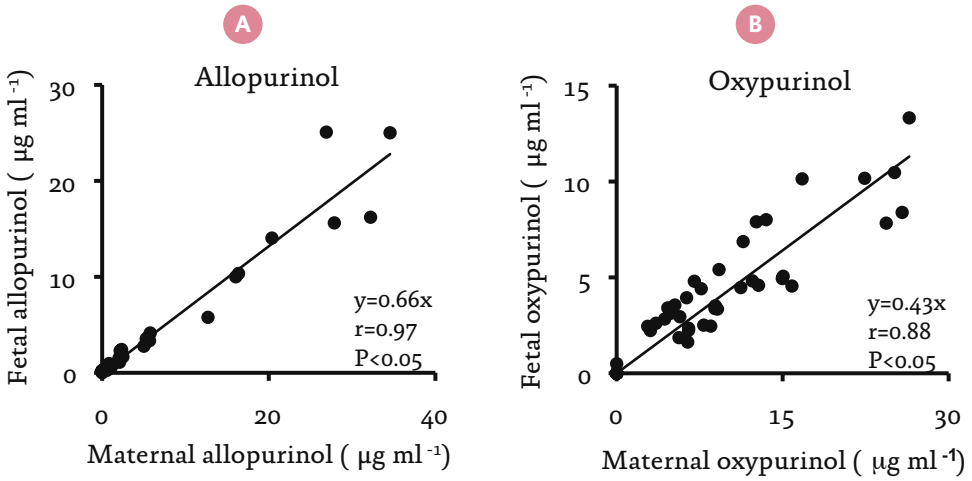
Despite intense basic science research and clinical trials investigating the potential neuroprotective effects of the xanthine oxidase inhibitor allopurinol on the fetal brain during and following acute fetal hypoxia [4,7,8,10,13,16,17], no animal model exists that offers the possibility of maternal administration of allopurinol and long term follow up of the treated offspring. Here, we show that allopurinol administered orally to the pregnant rat during late pregnancy leads to therapeutic rises in circulating allopurinol and oxypurinol in the mother and fetus. The increased levels achieved were functionally relevant as demonstrated by significantly reduced, xanthine oxidase activity levels in the placenta, and maternal and fetal tissues following 24h of maternal allopurinol administration. The level

**Figure 4-1. Maternal and fetal plasma allopurinol and oxypurinol measurements**




Values are mean ± SEM for allopurinol (○) and oxypurinol (●) concentrations in maternal and fetal plasma (n=5 per time point and group) at 0h, 2h, 6h and 24h post administration of (A) 30 mg kg$^{-1}$, and (B) 100 mg kg$^{-1}$ of allopurinol to the mother at time 0h. One way ANOVA with post-hoc Student-Newman-Keuls where appropriate. Significant differences ($P<0.05$): *, *vs.* 0 h control.

**Figure 4-2. Maternal to fetal allopurinol and oxypurinol correlations**




Values are paired maternal and fetal (A) allopurinol and (B) oxypurinol concentrations at all time points and both 30 mg $kg^{-1}$ and 100 mg $kg^{-1}$ allopurinol dosing protocols. N=35 paired samples per analysis. Both relationships show significant positive correlation (P<0.05, Pearson correlation).

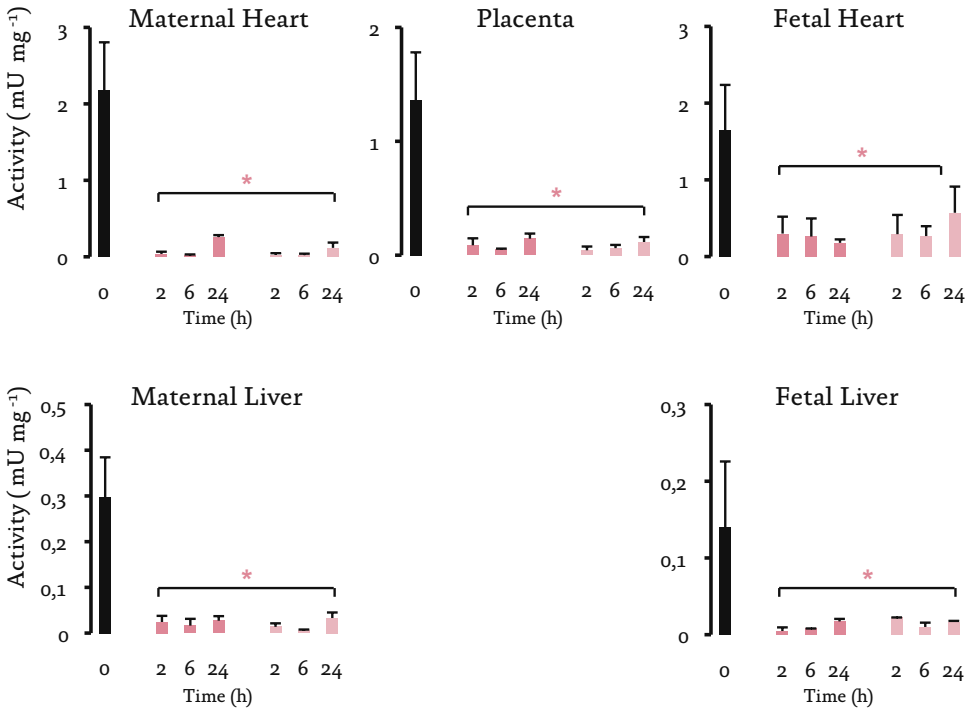
of suppression of xanthine oxidase activity was similar with the low and high doses of maternal allopurinol administration.

Currently, human clinical studies based in the Netherlands are assessing whether antenatal allopurinol administration during intra-partum fetal hypoxia can reduce overall morbidity and mortality in the newborn [7, 9, 18]. Recent follow-up data from those trials suggests that there may be a reduction in the relative risk of severe disability between 4 and 8 years of age in children who were moderately asphyxiated intra-partum but received postnatal allopurinol treatment starting within 4 h after birth [9]. Importantly, postnatal allopurinol was not associated with any negative side effects in the children who were followed up [9]. The rationale behind moving clinical therapy from postnatal to antenatal allopurinol administration is that by shortening the period between the beginning of ROS production and the antioxidant cover, the overall tissue injury, and thereby clinical disability, may be reduced. In support of this hypothesis, we have recently reported that maternal antenatal allopurinol administration can reduce hippocampal brain damage in an ovine model of repeated birth asphyxia [18].

Previous reports have confirmed that maternal treatment with allopurinol crosses the ovine [11,12], porcine [13] and human [19, 20] placenta to increase fetal plasma concentrations of allopurinol and oxypurinol. In sheep, a dose of 20 mg kg-1 i.v. to the maternal ewe led to peak maternal allopurinol and oxypurinol concentrations of 47 and 17 mg $ml^{-1}$ respectively and allopurinol concentrations of approximately 5 mg $ml^{-1}$ in the fetus [11]. In a pregnant sow and piglet model, 15 mg $kg^{-1}$ allopurinol given intravenously to the mother led to fetal peak concentrations of 5 mg $mg^{-1}$ [13]. In the present study, the plasma levels of allopurinol and oxypurinol reached 3.3 mg $mg^{-1}$ and 8.5 mg $mg^{-1}$ respectively in maternal plasma and 2.1 mg mg-1 and 4.0 mg $mg^{-1}$ respectively in fetal plasma for the 30 mg $kg^{-1}$ oral dose, values that are slightly lower than those measured in the sheep and pig for doses of sim-

ilar magnitude. This may reflect species differences in the pharmacokinetics of allopurinol [21]. For example, there may be reduced bioavailability of allopurinol and oxypurinol following oral administration relative to other routes of administration and/or differences in drug transfer across the placenta. It is also possible that basal xanthine oxidase activity in the rodent dam and its fetus may be different, altering the rate of conversion of allopurinol to oxypurinol. Finally, differences in the clearance of allopurinol and oxypurinol from the circulation may also exist. Following treatment with 100 mg $kg^{-1}$ of allopurinol to the mother, fetal values of allopurinol and oxypurinol were 11.6 mg $mg^{-1}$ and 5.2 mg $mg^{-1}$ at 2h, respectively. It might have been expected that these values would be approximately three times greater than those recorded following 30 mg $kg^{-1}$. However, given that xanthine oxidase catalyses the conversion of allopurinol to oxypurinol, and that this reaction is prevented by oxypurinol as the active metabolite, the production of oxypurinol may prevent conversion of allopurinol to oxypurinol, lead-

**Figure 4-3. Maternal, fetal and placental xanthine oxidase activity**




Values are mean ± SEM for tissue xanthine oxidase activity in samples of maternal heart and liver, placenta, fetal heart and liver maternal and fetal organ activities of xanthine oxidase assayed *in vitro* at 0, 2, 6 and 24 h following oral administration of allopurinol to the pregnant dam on day 20 of pregnancy (■, 0 h control; ■, 30 mg $kg^{-1}$; ■, 100 mg $kg^{-1}$; n=5 per time point). One way ANOVA with post-hoc Student-Newman-Keuls where appropriate. Significant differences ($P<0.05$): *, *vs.* 0 h control.

ing to a relatively high allopurinol concentration and therefore decreasing the rate of further generation of oxypurinol [2,22]. There was also a high correlation between paired maternal and fetal concentrations of allopurinol and oxypurinol, independent of time point of dose, indicating that placental transfer is reliable and consistent. However, fetal levels of allopurinol and oxypurinol were lower than maternal, reflected by the coefficient of the straight line relationship being < 1.

Effective inhibition of xanthine oxidase has been previously reported in the adult rat over the range of allopurinol doses from 2-50 mg $kg^{-1}$ [23]. In the sheep fetus, concentrations of allopurinol of 2.25 mg $ml^{-1}$ achieved a lowering of ROS production [12], and 5 mg $ml^{-1}$ allopurinol increased umbilical blood flow following umbilical cord occlusion [11]. In ovine pregnancy, larger doses of allopurinol led to suppression of a1 mediated vasoconstriction in the fetal femoral vascular bed via decreasing ROS mediated NO depletion in the circulation [24]. In this study, allopurinol concentrations as low as 2.1 mg $ml^{-1}$ were sufficient to inhibit xanthine oxidase activity in the fetal rat, with higher doses not offering greater inhibition. Despite the decrease in allopurinol and oxypurinol levels over the 24h period examined in this experiment, xanthine oxidase activity was still suppressed in all tissues investigated. Therefore, doses of allopurinol as low as 30 mg $kg^{-1}$ administered to the mother are effective to promote the long-term suppression of xanthine oxidase activity in placenta and the fetal organs.

In summary, the current study in rodent pregnancy has established a human clinically relevant model of maternal allopurinol administration that leads to therapeutic concentrations of allopurinol and oxypurinol in the fetal circulation. A single dose of 30 mg $kg^{-1}$ allopurinol is sufficient to suppress xanthine oxidase activity in the mother, placenta and fetus for at least 24h. The model is indispensable to investigate the long-term consequences of maternal treatment with allopurinol on the physiology of the offspring, and to establish the lasting safety of xanthine oxidase inhibition in human high-risk pregnancy in current clinical obstetric practice.

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# Chapter 5
# Xanthine oxidase and the fetal cardiovascular defense to hypoxia in late gestation ovine pregnancy

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## ABSTRACT

**Background**

Hypoxia is a common challenge to the fetus, promoting a physiological defense to redistribute blood flow towards the brain and away from peripheral circulation. During acute hypoxia, reactive oxygen species (ROS) interact with nitric oxide (NO) to provide an oxidant tone, which contributes to the mechanisms redistributing the fetal cardiac output, however the source of ROS is unknown. This study investigated whether ROS derived from xanthine oxidase contribute to the fetal peripheral vasoconstrictor response to hypoxia via interaction with NO-dependent mechanisms.

**Methods and Results**

Fifteen pregnant ewes and their fetuses were surgically prepared for long term recording at 118 days of gestation (term approximately 145 days). After 5 days recovery, mothers were infused i.v. for 30 min with either vehicle, low (30 $mg.kg^{-1}$) or high (150$mg.kg^{-1}$) allopurinol with or without fetal NO blockade. Allopurinol inhibited the increase in fetal plasma uric acid and suppressed the fetal femoral vasoconstrictor, glycemic and lactate acidemic responses during hypoxia (all $P<0.05$), effects that were restored to control levels with fetal NO blockade.

**Conclusions**

The data provide evidence for the activation of fetal XO in vivo during hypoxia and for XO-derived ROS in contributing to the fetal peripheral vasoconstriction, part of the fetal defense to hypoxia. The data are of significance to the understanding of the physiological control of the fetal cardiovascular system during hypoxic stress. The findings are also of clinical relevance in the context of obstetric trials in which allopurinol is being administered to pregnant women when the fetus shows signs of hypoxic distress.

Fetal hypoxia can result in marked fetal cardiovascular compromise with subsequent hypoxic-ischemic encephalopathy [1], which is associated with cerebral palsy and cognitive disability in later life [2] Therefore the prevention and management of fetal hypoxia remain major concerns in obstetric practice today.

The beneficial effects of the xanthine oxidase inhibitor allopurinol in reducing hypoxic damage in adult cardiology and in pediatric and adult cardiothoracic surgery have long been established [3-6]. In contrast, the effects of allopurinol in protecting the physiology of the fetus against hypoxia during the developmental period have been less well described. One study reported that treatment with allopurinol of the hypoxic neonate following complicated labor improved neonatal outcome [7]. However, if the time-interval between the hypoxic challenge and treatment was too long, or the hypoxia too severe, no reductions in serious morbidity or mortality were reported [8]. Consequently, there has been growing clinical interest in establishing whether perinatal outcome in complicated pregnancy may be improved if the window of treatment with allopurinol is advanced, for instance via maternal treatment to cover the actual period of fetal hypoxia in complicated labor. Maternal treatment with allopurinol crosses the placenta yielding therapeutic levels in the neonatal circulation [9] justifying this route of administration for preventative therapy in obstetric practice. However, virtually nothing is known about the effects of maternal treatment with allopurinol on the maternal or fetal physiology, or on maternal and fetal cardiovascular function.

We have discovered that the interaction between the superoxide anion (•O2-) and nitric oxide (NO) provides an oxidant tone in the fetal vasculature that controls blood flow in several circulations during basal and hypoxic conditions [10-13]. Maternal treatment with allopurinol under basal conditions increased umbilical blood flow and the gain of the fetal cardiac baroreflex, but it impaired fetal α adrenergic mediated pressor and femoral vasopressor responses via increasing NO bioavailability [10]. However, the effect of maternal treatment with allopurinol on the fetal cardiovascular defense to acute hypoxia remains unknown. Therefore, in this study we tested the hypothesis that xanthine oxidase has a role in the regulation of fetal cardiovascular function during acute hypoxia. The hypothesis was tested by investigating the in vivo effects of maternal treatment with high and low doses of allopurinol on the fetal cardiovascular responses to hypoxia in the chronically catheterized ewe and fetus during late gestation. To determine whether enhanced NO bioavailability was an involved mechanism mediating the effects of allopurinol on fetal cardiovascular function, maternal treatment with allopurinol was repeated in the presence of fetal in vivo NO blockade with a NO clamp [14-16].

## Methods

### Surgical preparation

Experiments were conducted on pregnant Welsh Mountain ewes using procedures approved by the Local Ethics Review Committee of the University of Cambridge and licensed by the Home Office under the UK Animals (Scientific Procedures) Act, 1986.

Fifteen Welsh Mountain sheep fetuses and their mothers were surgically instrumented under general anesthesia for long-term recording between 118 and 120 days of gestation (term ca. 145 days) using strict aseptic conditions, as described in detail [10,17]. Midline abdominal and uterine incisions were made, the fetal hind limbs were exteriorized and the right femoral artery and vein were isolated and catheterized (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products, NSW, Australia). A further catheter was anchored onto the fetal hind limb for measurement of amniotic pressure and for ad-

ministration of antibiotics into the amniotic cavity (600 mg in 2 ml, benzylpenicillin; Crystapen, Schering-Plough, Animal Health Division, Welwyn Garden City, UK). A transonic flow probe was also implanted around the contra-lateral femoral artery (Type 2RS, Transonic Systems Inc.). The dead space of the catheters was filled with heparinized saline (80 i.u. heparin. $ml^{-1}$ in 0.9% NaCl) and the catheter ends were plugged. A Teflon catheter (i.d. 1.0 mm, o.d. 1.6 mm, Altec, UK) was inserted in the maternal femoral artery and placed in the descending aorta, and a maternal venous catheter placed in the inferior vena cava (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products, NSW, Australia). The catheters and flow probe cable were then exteriorized via a keyhole incision in the maternal flank, and placed in a bag sutured to the skin of the ewe. Antibiotics were administered daily to the ewe (0.20-0.25 $mg.kg^{-1}$ I.M. Depocillin; Mycofarm, Cambridge, UK), to the fetus I.V. and into the amniotic cavity (300 mg Penbritin; SmithKline Beecham Animal Health, Welwyn Garden City, Hertfordshire, UK). Mean fetal arterial blood pressure (corrected for amniotic pressure), femoral blood flow, fetal heart rate (triggered from the arterial blood pressure or femoral blood flow pulse), maternal blood pressure and maternal heart rate (triggered from blood pressure) were continuously recorded using a custom made computerized Data Acquisition System (DAS; Department of Physiology, Development and Neuroscience, Cambridge University, UK).

**Experimental protocol**

Following 5 days of post-operative recovery, ewes and fetuses were subjected to the acute hypoxia protocol which consisted of 2 h normoxia, 0.5 h hypoxia and 1 h recovery (Figure 5-1). Acute hypoxia in the fetus was induced by maternal inhalational hypoxia [17], changing the concentrations of gases breathed by the ewe to 6% $O_2$ in $N_2$ with small amounts of $CO_2$ (15 $l.min^{-1}$ air, 35 $l.min^{-1}$ $N_2$, 1.5-2.5 $l.min^{-1}$ $CO_2$). This mixture was designed to reduce the fetal $PaO_2$ to ca. 10 mmHg whilst maintaining fetal $PaCO_2$. Following the 0.5 h period of hypoxia, the ewe was returned to breathing air for the 1 h recovery period.

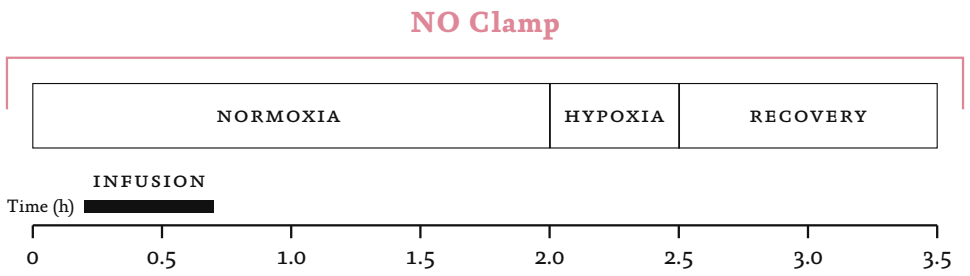
Acute hypoxia was induced following maternal I.V. infusion with vehicle (n=11), the low (30 $mg.kg^{-1}$, n=5) or the high dose of allopurinol (150 $mg.kg^{-1}$, n=9; Sigma, UK). Allopurinol was dissolved in the minimum volume of 4M sodium hydroxide (NaOH) and made up with saline [10,18] Vehicle was saline treated with 4M NaOH to achieve the same pH of the allopurinol solution [10,18] The 30 min infusion period started 20 min following the onset of recording during normoxia. The low dose of allopurinol was adapted from recent studies of allopurinol compatible with human treatment [7,8]. The high dose of allopurinol was 5 times the low dose. The timing of the acute hypoxic challenge coincided with peak concentrations of oxypurinol (the active metabolite of allopurinol) measured in fetal plasma following maternal treatment with a comparable dose of allopurinol in the same breed of sheep [19,20].

The experiment with the high dose of allopurinol was repeated following fetal treatment with the NO clamp (n=6). The NO clamp is an established technique that combines fetal treatment with the NO synthase inhibitor NG-nitro-L-arginine methyl ester (L-NAME; 100 $mg.kg^{-1}$ bolus dissolved in 2 ml saline, fetal i.a; Sigma) with the NO donor sodium nitroprusside (5.1+2.0 $\mu g.kg^{-1}.min^{-1}$, mean+SD dissolved in saline, fetal I.V.; Sigma). The technique blocks de novo synthesis of NO while compensating for the tonic production of the gas and thereby maintaining basal cardiovascular function [14-16].

**Blood sampling regimen and assays**

During any acute hypoxia protocol, descending aortic blood samples (1.0 ml) were taken from the mother and fetus at set time intervals: 0, 50 and 120 min of normoxia, 5, 15 and 30 min of hypoxia, and 15 and 60 min of recovery. Arte-

**Figure 5-1. Diagrammatic representation of the acute hypoxia protocol**




The experimental protocol consisted of 2 hours of normoxia, 0.5 hours of hypoxia (6% O2) and 1 hour of recovery, with maternal infusion from 20-50 min of: saline vehicle (n=11), the low dose of allopurinol (30mg.kg$^{-1}$; n=5), the high dose allopurinol (150mg.kg$^{-1}$; n=9) or high dose of allopurinol during fetal blockade of NO synthase with the NO clamp (n=6).

rial blood gas and acid base status (ABL5 Blood Gas Analyzer, Radiometer; Copenhagen, Denmark; measurements corrected to 39.5 °C for fetal blood and 38 °C for maternal blood), percentage saturation of hemoglobin with oxygen (Sat Hb) and the blood hemoglobin concentration [Hb] (Hemoximeter OSM3, Radiometer; Copenhagen, Denmark), and blood glucose and lactate concentrations (Yellow Springs 2300 Stat Plus Glucose/Lactate Analyzer; YSI Ltd, Farnborough, UK) were determined for each sample. Given xanthine oxidase catalyzes the conversion of hypoxanthine to uric acid, plasma concentrations of urate were also measured using HPLC with electrochemical detection [21]. In brief, aliquots of maternal and fetal plasma (acidified 1:1 with ice-cold 10% metaphophoric acid, centrifuged and the supernatant stored at -80°C) were diluted 1:4 with ice-cold 5% metaphophoric acid; final dilution of plasma 1 in 10. To this 100 µl HPLC-grade heptane was added and following vortex mixing for 40 s, the samples were centrifuged (13,000rpm; 5 min) and the lower (aqueous) layer removed and treated with heptane again until the supernatant was clear. This clear supernatant was transferred to a 0.8 ml HPLC vial. An electrochemical detector (EG&G Instruments; Wokingham, Berkshire) with a working electrode (set at 400 mV and sensitivity of 0.5 µA) was used for detection. Final concentrations for urate were calculated with external standards, which were run simultaneously. The limit of sensitivity for the assay was 0.1 µmol.l$^{-1}$ for urate, and the inter-assay coefficient of variation was less than 5%. An additional 1 ml of arterial blood was withdrawn at set intervals for fetal plasma catecholamine analyses during acute hypoxia following maternal I.V. infusion with vehicle (n=6) or the high dose of allopurinol (n=6) only to minimize fetal blood loss. These samples were collected under sterile conditions into chilled heparin tubes (2 ml Li+-heparin tubes; LIP Ltd., Shipley, UK) containing reduced glutathione (4 nmol per tube; G-4251; Sigma, UK) and EGTA (5 nmol per tube; E-4378; Sigma, UK). Samples were then centrifuged at 4000 rpm for 4 min at 4°C and stored at -80°C until analysis. Fetal plasma catecholamine concentrations were measured by a commercially available catecholamine radioimmunoassay previously validated for use with sheep plasma (2-CAT RIA, Diasourse) and as previously described in detail [11].

**Data and statistical analyses**

Values for blood gas, acid-base and metabolic

status are the mean+S.E.M. for normoxia before (Pre i), during (During i) or after (Post i,) infusion, hypoxia (H, the mean value of 5, 15 and 30 min of hypoxia) and recovery (R, the mean value of 15 and 60 min of recovery). Values for plasma uric acid are the mean+S.E.M. at 0, 50 and 120 min of normoxia, 30 min of hypoxia, and 60 min of recovery. Fetal and maternal cardiovascular variables were recorded continually, and were compiled into line graphs of the mean+S.E.M. for every minute, for all animals. The serial cardiovascular variables were analyzed using summary measures to focus the number of comparisons [22]. To determine the effects of allopurinol on basal maternal and fetal cardiovascular function, the mean of minute means were determined before (Pre i, 0-20 min), during (During i, 21-50min) and after (Post i, 51-120 min) infusion. To determine the effects of allopurinol on maternal and fetal cardiovascular function during acute hypoxia, the means ± S.E.M. for the absolute change in the area under the curve (AUC) from normoxic baseline in cardiovascular variables were calculated. These were determined as 30 minute epochs during normoxia (N, 90-120 min), hypoxia (H, 121-150 min) and recovery (R, 151-180 min). For all data, comparisons within (effect of time) and between (effect of treatment) groups were assessed statistically using two-way ANOVA with repeated measures. Where a significant effect of time or treatment was indicated, the post hoc Tukey test was used to isolate the statistical differences. For all comparisons, statistical significance was accepted when $P<0.05$.

## Results

### Maternal arterial blood gas, acid base and metabolic status

Pre-infusion values for maternal arterial blood gas, acid base and metabolic status were similar in all ewes and were within the normal range for pregnant Welsh Mountain sheep at this stage of gestation (Table 5-1). Infusion with saline vehicle or the low dose of allopurinol had no effect on arterial blood gas, acid base or metabolic status. In contrast, infusion with the high dose of allopurinol, with or without fetal treatment with the NO clamp, significantly increased maternal pHa for the duration of the protocol. Further, high allopurinol infusion significantly increased maternal acid-base excess (ABE) and decreased maternal $PaO_2$ without affecting maternal hemoglobin saturation with oxygen (Sat Hb; Table 5-1).

In all ewes, acute hypoxia induced significant falls of similar magnitude in maternal $PaO_2$ and Sat Hb without any alteration to $PaCO_2$ (Table 5-1). During recovery, infusion with the high dose of allopurinol, with or without fetal treatment with the NO clamp, maintained the increased maternal pHa. In contrast, all other variables across the groups returned to pre-infusion values.

### Fetal arterial blood gas, acid base and metabolic status

Pre-infusion values for fetal arterial blood gas, acid base and metabolic status were similar in all fetuses and were within the normal range for the Welsh Mountain sheep fetus at this stage of gestation (Table 5-2). Infusion with vehicle or allopurinol had no effect on basal arterial blood gas or acid base status. In all fetuses, acute hypoxia induced significant falls of similar magnitude in fetal $PaO_2$ and Sat Hb without any alteration to $PaCO_2$ (Table 5-2). Acute hypoxia induced a significant decrease in pHa and ABE by the end of the hypoxic challenge in control fetuses only (Table 5-2). In all fetuses, acute hypoxia led to a significant increase in blood lactate. In contrast, a significant increase from baseline in blood glucose during hypoxia only reached significance in the control fetuses and fetuses from mothers treated with the low dose of allopurinol. When blood glucose and lactate were calculated as a change from normoxic baseline, the increments from baseline in blood glucose and lactate were significantly depressed in fetuses from mothers treated with the high

**Table 5-1. Maternal arterial blood gas and acid base status**

| | | N | | | H | R |
|---|---|---|---|---|---|---|
| | | Pre i | During i | Post i | | |
| $pH_2$ | Vehicle | 7.53±0.01 | 7.51±0.01 | 7.54±0.01 | 7.52±0.01 | 7.52±0.01 |
| | Low Allopurinol | 7.53±0.01 | 7.53±0.01 | 7.52±0.02 | 7.52±0.03 | 7.53±0.02 |
| | High Allopurinol | 7.50±0.02 | 7.61±0.02*,† | 7.57±0.01* | 7.56±0.01* | 7.56±0.01* |
| | High Allopurinol + NO Clamp | 7.49±0.01 | 7.61±0.02*,† | 7.57±0.01* | 7.57±0.02* | 7.57±0.01* |
| $P_aCO_2$ (mmHg) | Vehicle | 34.5±0.8 | 35.3±1.3 | 33.5±0.9 | 34.8±0.8 | 34.2±0.8 |
| | Low Allopurinol | 35.8±1.6 | 35.8±1.1 | 36.8±0.4 | 35.7±0.8 | 35.5±1.3 |
| | High Allopurinol | 36.1±1.7 | 33.5±1.1 | 33.8±0.8 | 34.6±0.8 | 34.2±0.7 |
| | High Allopurinol + NO Clamp | 37.7±0.8 | 34.7±0.8 | 37.0±1.0 | 34.9±0.9 | 35.5±1.0 |
| $P_8O_2$ (mmHg) | Vehicle | 106±3 | 100±3 | 109±4 | 33±1* | 101±2 |
| | Low Allopurinol | 104±3 | 101±4 | 101±3 | 35±2* | 105±4 |
| | High Allopurinol | 105±2 | 86±5*,† | 107±2 | 37±1* | 107±3 |
| | High Allopurinol + NO Clamp | 104±2 | 77±3*,† | 100±2 | 37±3* | 102±3 |
| Sat Hb (%) | Vehicle | 97.3±0.7 | 96.5±0.8 | 96.7±0.7 | 54.1±2.5* | 95.9±0.6 |
| | Low Allopurinol | 95.6±0.4 | 95.5±0.6 | 95.8±0.5 | 60.4±3.9* | 96.1±0.4 |
| | High Allopurinol | 98.5±1.5 | 92.6±1.3 | 99.0±1.3 | 59.4±2.8* | 99.1±1.3 |
| | High Allopurinol + NO Clamp | 99.2±0.6 | 97.2±1.2 | 98.8±1.0 | 57.8±4.9* | 99.1±0.5 |
| ABE ($meq.l^{-1}$) | Vehicle | 5.8±0.5 | 5.7±0.4 | 5.8±0.6 | 6.0±0.5 | 5.4±0.5 |
| | Low Allopurinol | 6.4±1.3 | 7.2±1.3 | 6.8±1.4 | 7.2±1.4 | 6.6±1.3 |
| | High Allopurinol | 5.9±0.7 | 11.7±1.3*,† | 9.5±1.0*,† | 8.8±1.2 | 8.6±1.1 |
| | High Allopurinol + NO Clamp | 6.2±1.4 | 12.7±1.3*,† | 8.8±1.7 | 8.3±1.6 | 8.7±1.4 |
| [Glucose] ($mmol.l^{-1}$) | Vehicle | 2.95±0.24 | 3.08±0.25 | 3.08±0.24 | 3.10±0.20 | 3.19±0.29 |
| | Low Allopurinol | 3.38±0.30 | 3.69±0.35 | 3.73±0.41 | 3.43±0.36 | 3.54±0.42 |
| | High Allopurinol | 2.49±0.18 | 2.56±0.20 | 2.53±0.23 | 2.63±0.24 | 2.80±0.23 |
| | High Allopurinol + NO Clamp | 2.89±0.13 | 2.91±0.15 | 2.89±0.16 | 2.89±0.17 | 3.31±0.21 |
| [Lactate] ($mmol.l^{-1}$) | Vehicle | 0.39±0.07 | 0.45±0.08 | 0.47±0.10 | 0.56±0.09 | 0.44±0.05 |
| | Low Allopurinol | 0.50±0.11 | 0.52±0.11 | 0.54±0.11 | 0.55±0.11 | 0.52±0.13 |
| | High Allopurinol | 0.44±0.04 | 0.69±0.12 | 0.68±0.16 | 0.95±0.20 | 0.66±0.15 |
| | High Allopurinol + NO Clamp | 0.40±0.03 | 0.57±0.03 | 0.56±0.06 | 0.97±0.23 | 0.75±0.18 |

Values represent the means ± S.E.M. at 0 (Pre i), 50 (During i) and 115 (Post i) min of normoxia, at 30 min of hypoxia (H) and at 60 min of recovery (R) for mothers exposed to 0.5 hour of hypoxia either during saline vehicle infusion (n=11), treatment with the low dose of allopurinol (30 $mg.kg^{-1}$; n=5), treatment with the high dose allopurinol (150 $mg.kg^{-1}$; n=9) or treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp (n=6). Significant difference ($P<0.05$) are: *, within group with respect to time period Pre i. †, between groups with respect to saline vehicle infusion (Two-way ANOVA with post hoc Tukey test).

**Table 5-2. Fetal arterial blood gas and acid base status**

| | | N | | | H | R |
|---|---|---|---|---|---|---|
| | | Pre i | During i | Post i | | |
| $pH_2$ | Vehicle | 7.36±0.01 | 7.37±0.01 | 7.37±0.01 | 7.30±0.02* | 7.26±0.02* |
| | Low Allopurinol | 7.36±0.00 | 7.36±0.00 | 7.36±0.01 | 7.32±0.01 | 7.30±0.01* |
| | High Allopurinol | 7.36±0.01 | 7.37±0.01 | 7.36±0.01 | 7.32±0.02 | 7.30±0.02* |
| | High Allopurinol + NO Clamp | 7.33±0.02 | 7.34±0.01 | 7.35±0.01 | 7.35±0.01 | 7.27±0.03* |
| $P_aCO_2$ (mmHg) | Vehicle | 53.1±1.5 | 51.5±1.2 | 51.5±1.0 | 53.8±1.2 | 52.9±1.3 |
| | Low Allopurinol | 57.0±1.2 | 54.3±1.8 | 55.8±0.9 | 55.0±1.0 | 53.5±1.4 |
| | High Allopurinol | 53.1±0.8 | 51.3±1.2 | 52.8±1.2 | 52.9±1.0 | 52.7±0.8 |
| | High Allopurinol + NO Clamp | 55.8±0.7 | 53.0±0.8 | 53.5±0.9 | 52.6±1.2 | 52.6±0.9 |
| $P_8O_2$ (mmHg) | Vehicle | 20±1 | 20±1 | 20±1 | 9±1* | 20±1 |
| | Low Allopurinol | 19±1 | 19±1 | 19±1 | 9±0* | 18±1 |
| | High Allopurinol | 21±1 | 19±1 | 20±1 | 10±1* | 20±1 |
| | High Allopurinol + NO Clamp | 22±1 | 20±1 | 21±1 | 11±1* | 22±1 |
| Sat Hb (%) | Vehicle | 56.4±3.0 | 52.9±3.3 | 51.5±2.3 | 17.3±1.5* | 50.8±3.3 |
| | Low Allopurinol | 51.3±1.4 | 50.7±2.5 | 52.9±2.1 | 19.1±1.4* | 49.4±2.1 |
| | High Allopurinol | 55.0±3.2 | 49.5±3.5 | 52.5±3.1 | 20.3±2.0* | 52.9±3.1 |
| | High Allopurinol + NO Clamp | 58.8±2.9 | 52.5±3.1 | 54.0±3.7 | 21.5±3.6* | 54.3±4.4 |
| ABE ($meq.l^{-1}$) | Vehicle | 3.6±0.5 | 3.4±0.4 | 3.6±0.5 | -1.4±1.2* | -3.8±1.3* |
| | Low Allopurinol | 4.6±0.7 | 4.2±0.6 | 4.2±0.6 | 0.7±1.2 | -1.5±1.1* |
| | High Allopurinol | 3.0±0.6 | 0.9±2.6 | 3.4±0.5 | 0.1±1.3 | -0.9±1.4* |
| | High Allopurinol + NO Clamp | 1.7±1.0 | 1.3±0.8 | 1.7±0.6 | -1.3±1.3 | -3.0±1.8* |
| [Glucose] ($mmol.l^{-1}$) | Vehicle | 0.91±0.10 | 0.97±0.25 | 0.96±0.09 | 1.40±0.18* | 1.38±0.18* |
| | Low Allopurinol | 1.15±0.12 | 1.17±0.35 | 1.16±0.14 | 1.52±0.23 | 1.40±0.24 |
| | High Allopurinol | 0.97±0.09 | 0.84±0.20 | 0.82±0.08 | 1.09±0.12 | 1.08±0.16 |
| | High Allopurinol + NO Clamp | 0.91±0.08 | 0.89±0.15 | 0.88±0.04 | 1.09±0.12 | 1.21±0.07 |
| [Lactate] ($mmol.l^{-1}$) | Vehicle | 0.87±0.06 | 0.87±0.08 | 1.10±0.06 | 3.46±0.51* | 4.97±0.49* |
| | Low Allopurinol | 1.02±0.11 | 1.02±0.12 | 1.07±0.17 | 2.88±0.59* | 3.80±0.51*,† |
| | High Allopurinol | 0.85±0.06 | 0.98±0.09 | 1.00±0.06 | 2.63±0.50* | 3.30±0.51*,† |
| | High Allopurinol + NO Clamp | 0.95±0.10 | 1.16±0.11 | 1.36±0.10 | 2.79±0.62* | 3.95±0.72* |

Values represent the means ± S.E.M. at 0 (Pre i), 50 (During i) and 115 (Post i) min of normoxia, at 30 min of hypoxia (H) and at 60 min of recovery (R) for fetuses exposed to 0.5 hour of hypoxia either during maternal saline vehicle infusion (n=11), maternal treatment with the low dose of allopurinol (30 $mg.kg^{-1}$; n=5), maternal treatment with the high dose allopurinol (150 $mg.kg^{-1}$; n=9) or maternal treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp (n=6). Significant difference ($P<0.05$) are: *, within group with respect to time period Pre i. †, between groups with respect to saline vehicle infusion (Two-way ANOVA with post hoc Tukey test).

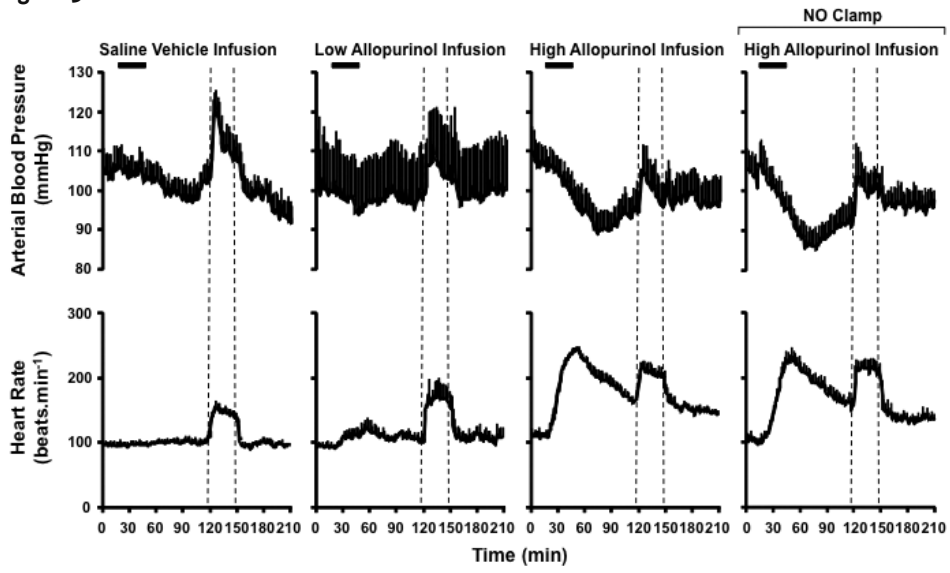
dose of allopurinol relative to control (Δ[glucose]: 0.49±0.15 vs. 0.12±0.13 mmol.l$^{-1}$, Δ[lactate]: 2.59±0.51 vs. 1.59±0.48 mmol.l$^{-1}$, P<0.05 for saline vs. high allopurinol). Fetal treatment with the NO clamp during maternal infusion with the high dose of allopurinol restored the increment in fetal blood glucose and lactate towards control levels (Δ[glucose]: 0.29±0.11 mmol.l$^{-1}$, Δ[lactate]: 1.85±0.61 mmol.l$^{-1}$).

During recovery, PaO2 and Sat Hb returned to pre-hypoxic levels in all fetuses whilst PaCO2 remained unaltered (Table 5-2). There was a significant decrease in pHa and ABE in all fetuses (Table 5-2). All fetuses continued to show a significant increase in blood lactate during recovery and blood glucose remained significantly elevated from normoxic baseline only in control fetuses (Table 5-2). The increments from baseline in blood glucose and lactate during recovery were again significantly depressed in fetuses from mothers treated with the high dose of allopurinol relative to control (Δ[glucose]: 0.47±0.15 vs. 0.10±0.16mmol.l$^{-1}$, Δ[lactate]: 4.10±0.50 vs. 2.45±0.48 mmol.l$^{-1}$, P<0.05 for saline vs. high allopurinol). Fetal treatment with the NO clamp during maternal infusion with the high dose of allopurinol restored the increment in fetal blood glucose but not lactate towards control levels during recovery (Δ[glucose]: 0.31±0.10 mmol.l$^{-1}$, Δ[lactate]: 3.01±0.71 mmol.l$^{-1}$, P<0.05 for saline vs. high allopurinol + NO clamp).

**Effects of allopurinol on maternal and fetal basal cardiovascular function**

Pre-infusion values for maternal arterial blood

**Figure 5-2. Maternal cardiovascular function**




Values represent the means ± S.E.M. calculated every minute for arterial blood pressure and heart rate during 2 hours of normoxia, 0.5 hour of hypoxia (dashed lines) and 1 hour of recovery for mothers either during saline vehicle infusion (n=11), treatment with the low dose of allopurinol (30 mg.kg$^{-1}$; n=5), treatment with the high dose allopurinol (150 mg.kg$^{-1}$; n=9) or treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp (n=6).

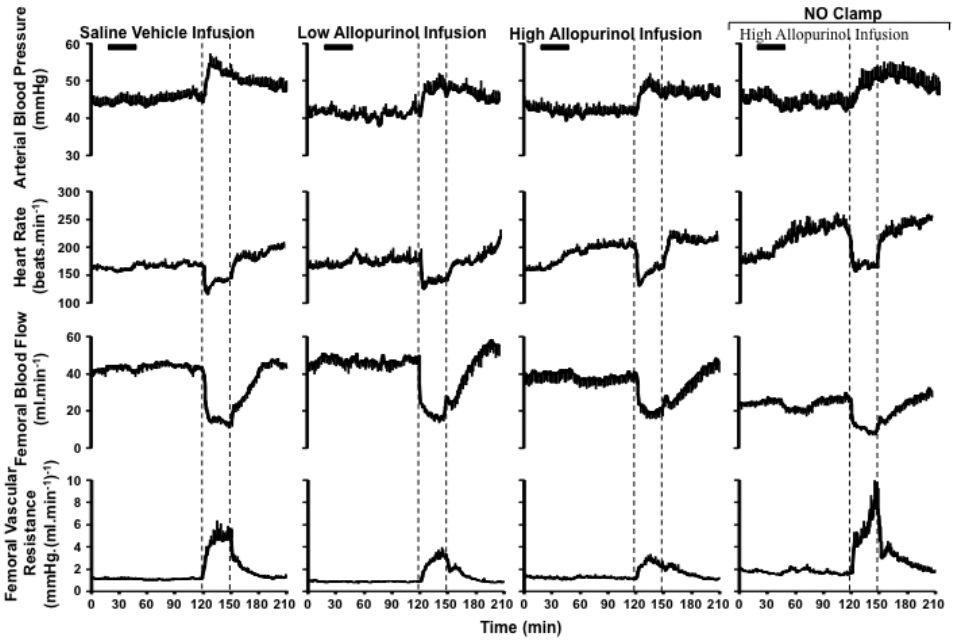
pressure and heart rate were similar in all ewes (Figure 5-2). Infusion with saline or the low dose of allopurinol had no effect on basal maternal cardiovascular function. In contrast, infusion with the high dose of allopurinol led to a significant decrease in maternal basal arterial blood pressure and a significant increase in maternal basal heart rate (Figure 5-2, 5-4A).

Pre-infusion values for fetal arterial blood pressure, heart rate and femoral vascular resistance were similar in all fetuses (Figure 5-3). Maternal infusion with the low or high dose of allopurinol, with or without the NO clamp, significantly decreased basal fetal arterial blood pressure but only infusion with the high dose of allopurinol, with or without fetal treatment with the NO clamp, significantly increased basal fetal heart rate. Allopurinol treatment at either dose did not affect basal fetal femoral blood flow or fetal femoral vascular resistance (Figure 5-3, 5-4B).

**Effects of allopurinol on maternal and fetal cardiovascular function during hypoxia**

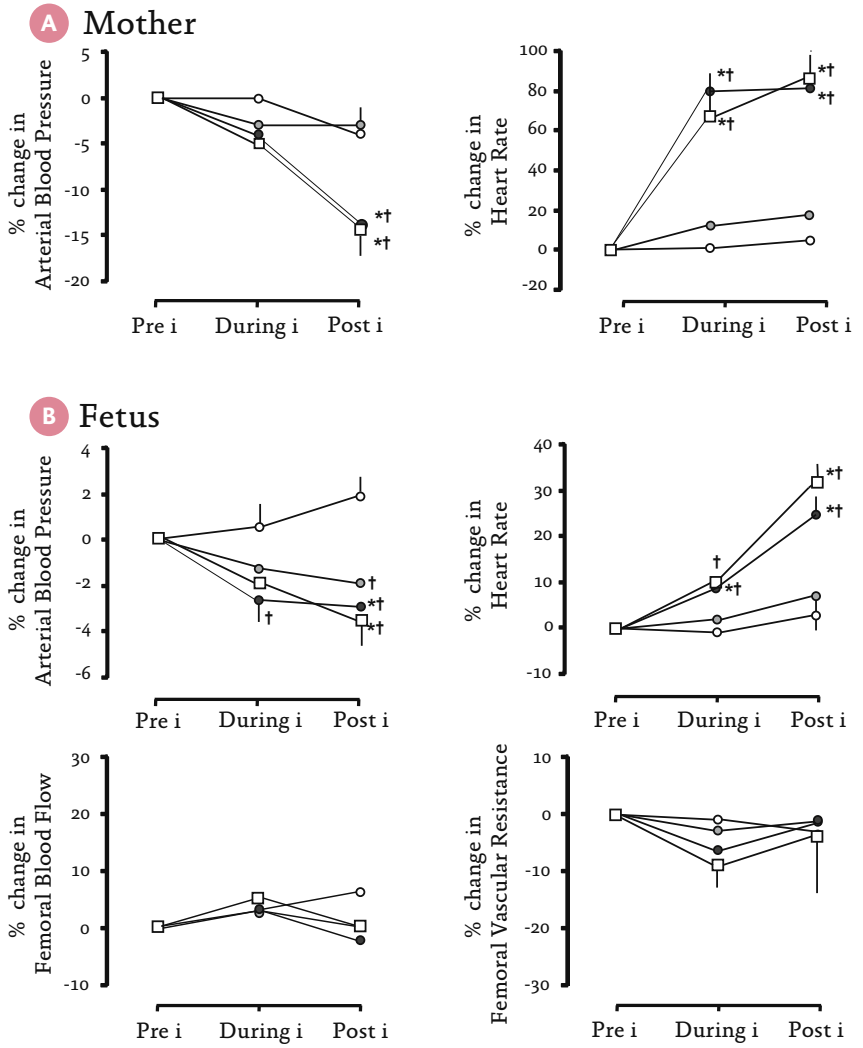
Maternal arterial blood pressure and heart rate increased significantly during acute hypoxia following maternal infusion with vehicle (Fig-

**Figure 5-3. Fetal cardiovascular function**



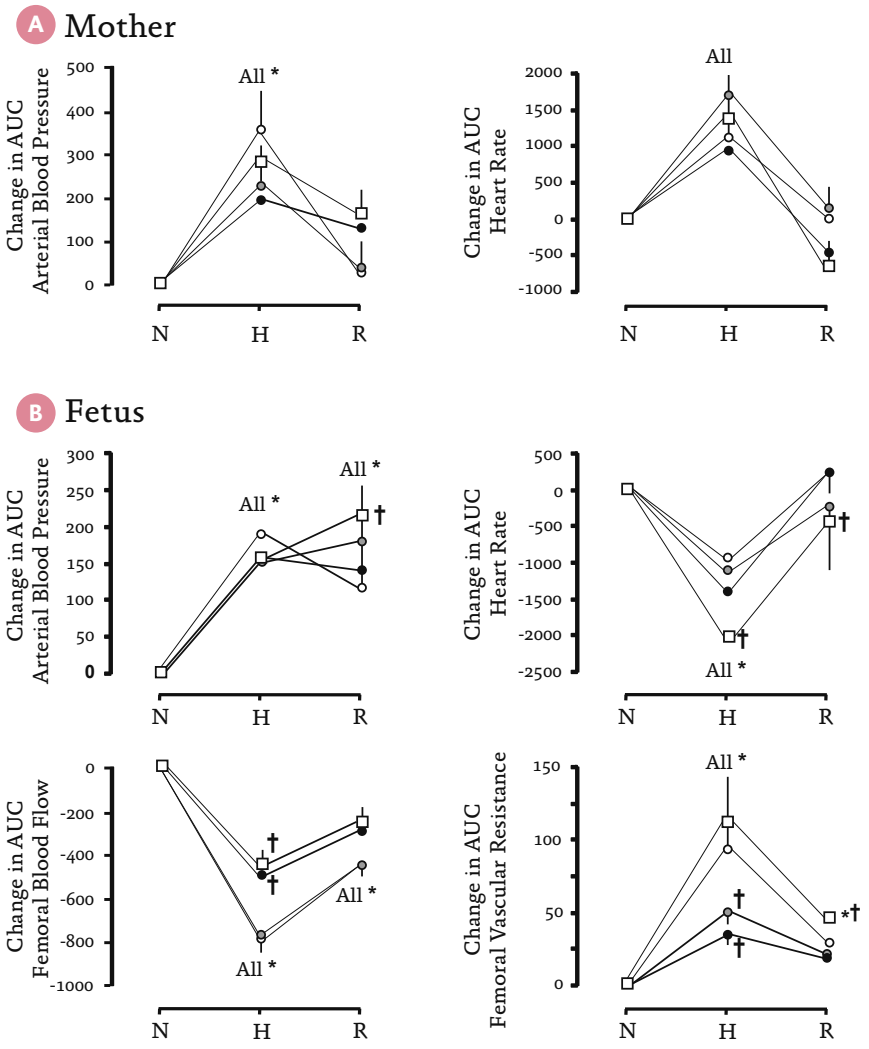

Values represent the means ± S.E.M. calculated every minute for fetal arterial blood pressure, fetal heart rate, fetal femoral blood flow and fetal femoral vascular resistance during 2 hours of normoxia, 0.5 hour of hypoxia (dashed lines) and 1 hour of recovery for fetuses either during maternal saline vehicle infusion (n=11), maternal treatment with the low dose of allopurinol (30 mg.kg$^{-1}$; n=5), maternal treatment with the high dose allopurinol (150 mg.kg$^{-1}$; n=9) or maternal treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp (n=6).

**Figure 5-4. Statistical summary of the basal effects of allopurinol**



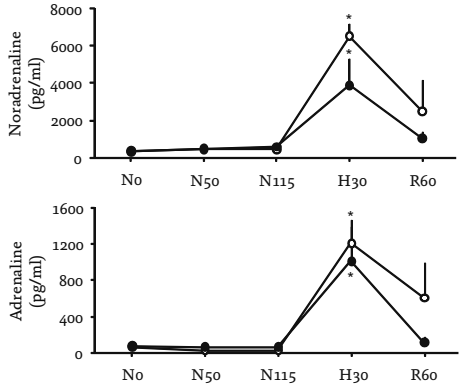

Values are the means ± S.E.M. for the percent change in mean cardiovascular variables in the mother (A) and in the fetus (B). These were calculated before (Pre i, 0-20 min), during (During i, 21-50 min) and after (Post i, 51-120 min) maternal infusion with saline vehicle (○; n=11), maternal treatment with the low dose of allopurinol (30 mg.kg$^{-1}$;●; n=5), maternal treatment with the high dose allopurinol (150 mg.kg$^{-1}$;●; n=9) or maternal treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp (□; n=6). Significant difference ($P<0.05$) are: *, within group with respect to time period Pre i. †, between groups with respect to saline vehicle infusion (Two-way ANOVA with *post hoc* Tukey test).

**Figure 5-5. Statistical summary of the effects of allopurinol during hypoxia**




Values are the means ± S.E.M. for the absolute change in the area under the curve (AUC) from normoxic baseline in cardiovascular variables in the mother (A) and in the fetus (B). These were calculated as 30 minute epochs during normoxia (N, 90-120 min), hypoxia (H, 121-150 min) and recovery (R, 151-180 min) after maternal infusion with saline vehicle (○; n=11), maternal treatment with the low dose of allopurinol (30 $mg.kg^{-1}$;●; n=5), maternal treatment with the high dose allopurinol (150 $mg.kg^{-1}$;●; n=9) or maternal treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp (□; n=6). Significant difference ($P<0.05$) are: *, within group with respect to time period Pre i. †, between groups with respect to saline vehicle infusion (Two-way ANOVA with *post hoc* Tukey test).

**Figure 5-6. Fetal plasma catecholamine responses to acute hypoxia**

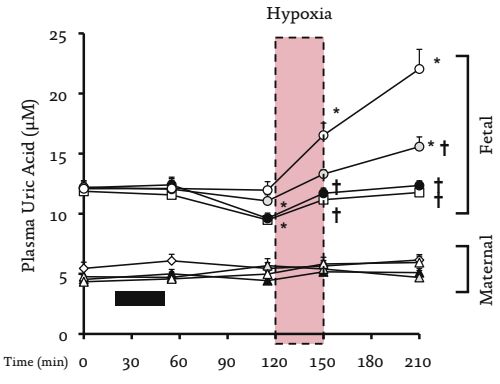



Values represent the means ± S.E.M. for fetal plasma adrenaline and noradrenaline at 0 (Pre i), 50 (During i) and 115 (Post i) min of normoxia (N), at 30 min of hypoxia (H) and at 60 min of recovery (R) for fetuses exposed to 0.5 hour of hypoxia either during maternal saline vehicle infusion (white dots, n=5) or maternal treatment with the high dose allopurinol (150 mg.kg$^{-1}$; black dots, n=5).

ure 5-2, 5-5A). Although treatment with either dose of allopurinol tended to diminish the increment in maternal arterial blood pressure during hypoxia, this failed to reach significance (Figure 5-5A). Both maternal arterial blood pressure and heart rate returned to pre-hypoxic levels during recovery across all groups.

In all groups, acute hypoxia led to a significant increase in fetal arterial blood pressure and femoral vascular resistance and a significant decrease in fetal heart rate and fetal femoral blood flow (Figure 5-3, 5-5B). The increase in arterial blood pressure and the decrease in heart rate were similar across all groups whilst the increase in femoral vascular resistance was markedly diminished in fetuses treated with maternal infusion with allopurinol (Figure 5-3, 5-5B). Fetal treatment with the NO clamp returned the femoral vascular resistance response to control levels in fetuses from mothers infused with the high dose of allopurinol. The increase

**Figure 5-7. Maternal and fetal plasma uric acid concentrations during the hypoxia protocol**




Values are the means ± S.E.M. for maternal and fetal plasma uric acid during the hypoxia protocol after maternal infusion with saline vehicle (○; n=5), maternal treatment with the low dose of allopurinol (30 mg.kg$^{-1}$;●; n=5), maternal treatment with the high dose allopurinol (150 mg.kg$^{-1}$;●; n=5) or maternal treatment with the high dose of allopurinol during fetal blockade of NO synthase (NOS) with the NO clamp ( □; n=5). Significant difference (P<0.05) are: *, within group; †, between groups with respect to saline vehicle infusion (Two-way ANOVA with *post hoc* Tukey test).

in fetal arterial blood pressure was sustained in all groups during the recovery period (Figure 5-3, 5-5B). In contrast, fetal heart rate, femoral blood flow and femoral vascular resistance returned to pre-hypoxic values.

**Fetal plasma catecholamines**

Since increases in fetal plasma catecholamines contribute to the femoral vasoconstrictor response to acute hypoxia [17], it was of interest to determine if maternal allopurinol affected this response in the fetal circulation. Plasma noradrenaline and adrenaline showed a significant increase from baseline concentrations during acute hypoxia. These elevations in fetal plasma catecholamines were not affected by maternal treatment with the high dose of allopurinol (Figure 5-6).

**Maternal and fetal plasma uric acid**

Pre-infusion maternal uric acid levels were similar in all groups and were not significantly altered by allopurinol infusion or hypoxia. Fetal plasma uric acid levels were higher than maternal levels during the pre-infusion period. Hypoxia increased fetal plasma uric acid levels and there was a continued increase during the recovery period. In contrast, maternal plasma uric acid remained unaltered from baseline during acute hypoxia. Maternal allopurinol treatment led to a decrease in fetal plasma basal uric acid levels when given at 150 mg.kg$^{-1}$, with and without the fetal NO clamp. Both low and high doses of maternal allopurinol led to a dose-dependent inhibition of the increase in fetal plasma uric acid measured during acute hypoxia and recovery (Figure 5- 7).

## Discussion

This study tested the hypothesis that xanthine oxidase has a role in the regulation of fetal cardiovascular function during acute hypoxia. The principle findings of the study show that maternal treatment with allopurinol significantly diminished the rise in fetal plasma uric acid and the fetal femoral vasoconstrictor, hyperglycemic and lactic acidemic responses to acute hypoxia. The effects of maternal allopurinol on fetal femoral vascular resistance, glucose and lactate concentrations in fetal blood were prevented by fetal in vivo NO blockade. Therefore, the data support the hypothesis tested to imply that enhanced NO bioavailability is an involved mechanism mediating the effects of maternal allopurinol treatment on fetal cardiovascular function during acute hypoxia.

The fetal defense to acute hypoxia is largely contingent on fetal cardiovascular responses, which have been well characterized. This fetal cardiovascular defense includes bradycardia and peripheral vasoconstriction [23,24]. The latter aids the redistribution of the fetal combined ventricular output away from less essential vascular beds to maintain oxygen and nutrient delivery to the brain; the so-called brain sparing effect [25,26]. The physiology underlying this response is also well delineated. The fetal bradycardia and peripheral vasoconstriction are triggered exclusively by a carotid body chemoreflex [24,27]. Release of hormones, such as catecholamines, into the fetal circulation maintain the neurally-triggered peripheral vasoconstriction [17] and return fetal heart rate back to basal levels, opposing the enhanced vagal tone [24]. The neural and endocrine peripheral vasoconstriction is further fine-tuned by an oxidant tone, created by the interaction between $\bullet O^{2-}$ and NO during acute hypoxia, whereby a fall in the ratio favors dilatation and an increase enhances constriction [10-13,15]. Data in the present study show that maternal treatment with allopurinol markedly diminished the fetal peripheral vasoconstrictor response to acute hypoxia without affecting fetal bradycardia and that this hemodynamic effect was prevented by fetal in vivo NO blockade. Therefore, the data in this study support that activation of xanthine oxidase contributes to the femoral vasoconstrictor response during acute hypoxia by altering the peripheral vascular oxidant tone. Hence, maternal treatment with al-

lopurinol shifts the ratio between $\bullet O_2^-$ and NO towards dilatation, opposing chemoreflex and endocrine vasoconstrictor influences on the fetal femoral vascular bed. The normalization of the magnitude of the femoral vasoconstrictor response to acute hypoxia in the presence of fetal in vivo NO blockade during maternal treatment with allopurinol confirms this as an involved mechanism.

The increase in fetal blood glucose concentrations during acute hypoxia results from an inhibition in glucose uptake and utilization by peripheral tissues coupled with stimulation of hepatic glucose production [28,29]. The fetal lactic acidemia response to acute hypoxia principally arises from the anaerobic metabolism of glucose in hypoxic fetal tissues, in particular the hind limbs in which blood flow is markedly reduced [30]. As well as helping in the redistribution of blood flow away from less essential vascular beds towards the fetal brain, the peripheral vasoconstriction also markedly decreases oxygen consumption in the fetus, as the latter is exquisitely coupled to oxygen delivery [30]. Since fetal treatment with phentolamine prevented the glycemic response but enhanced insulin secretion during acute hypoxia [31], and since infusion of catecholamines increased glucose output in the sheep fetus [32], both the reduction in insulin-dependent glucose uptake and the increase in glucose production by the fetal tissues may be mediated via neural and endocrine adrenergic pathways. Depression of the glycemic response to acute hypoxia following fetal exposure to allopurinol in this study may therefore represent an effect on insulin release and/or on the glucogenic pathways mediated either via the neural sympathetic or plasma amine activities. Indeed, allopurinol has been reported to depresses the hyperglycemia of hemorrhagic shock via similar mechanisms [33]. The depressed circulating lactate concentrations during acute hypoxia in the fetus following exposure to allopurinol in the present study may have resulted from the diminished increased in blood glucose availability [34] and/or from the decreased production of lactate by the fetal hind limbs [30]. Reversal of the depressive effects of allopurinol on the fetal lactic acidemic response to acute hypoxia following fetal NO blockade may thus result from the restoration towards control levels of the hyperglycemic response and/or the femoral vasoconstrictor response during acute hypoxia in the fetus.

One could argue that inhibition of the fetal peripheral vasoconstrictor response to acute hypoxia following exposure to XO inhibition may be mediated via depressed chemo-transduction mechanisms within the carotid body and/or due to reduced activation of endocrine constrictor responses, such as the increase in plasma catecholamine levels in the fetus during acute hypoxia. However, in the present study, we further show that maternal treatment with even very high doses of allopurinol did not affect the magnitude of the increase in fetal plasma catecholamine concentrations during acute hypoxia. Inhibition of the fetal femoral vasoconstriction and depression of the fetal glycemic response during acute hypoxia as a consequence of an effect of allopurinol on plasma amine activities is therefore not supported. Similarly, dissociation between a lack of an effect of allopurinol on the fetal bradycardia, which persists during acute hypoxia, and inhibition of the fetal femoral vasoconstriction during the same time period does not support an effect of allopurinol at the level of the carotid chemoreflex, since both fetal bradycardia and fetal femoral vasoconstriction are triggered by the same carotid body chemoreflex [24,27]. Rather, these additional findings further support an affect of allopurinol acting to alter the local oxidant tone at the level of the fetal peripheral vasculature. Accordingly, we have reported that fetal treatment with other antioxidants, such as vitamin C, or other agents that increase NO bioavailability, such as statins, has a similar effect on the fetal peripheral vascular oxidant tone, shifting the ratio towards dilatation via NO-dependent pathways, and impair-

ing the redistribution of blood flow away from peripheral circulations during acute hypoxia in the fetus [11,13].

A hypotensive effect of allopurinol during basal conditions supports the idea that the cellular oxidant milieu also plays a tonic contribution to peripheral vascular resistance and that, under basal conditions, XO-derived $\bullet O_2^-$ is involved in arterial blood pressure maintenance. The mechanisms mediating the tachycardic responses to allopurinol in either the mother or fetus are less clear. The dissociation between the magnitude and timing of the depressor and cardiac responses both suggest that baroreflex activation is an unlikely contributing mechanism increasing heart rate. A more likely explanation is that allopurinol has direct chronotropic effects. Studies have reported that allopurinol increases myocardial contractility [35-37]. We have also reported that the tachycardic response to allopurinol during basal conditions in late gestation fetal sheep can be prevented by fetal treatment with the ß1-adrenergic antagonist atenolol, suggesting that allopurinol may enhance sympathetic influences on the heart.

In summary, data in the present study show that maternal treatment with low and high doses of allopurinol induces significant effects on maternal and fetal cardiovascular function not only during basal conditions but also in response to acute hypoxia. The data are not only of significance to the understanding of the physiological control of the fetal cardiovascular system during acute hypoxic stress, but they are also of particular clinical relevance in the context of ongoing trials in which allopurinol is being administered to pregnant women when their unborn child shows signs of hypoxic distress [38,39] as highlighted in a recent editorial [40]. Collectively, past and present evidence on the effects of allopurinol on the fetus are of mixed clinical implications. Beneficial effects of maternal treatment with allopurinol are supported by its protective effects on umbilical blood flow [10,20], and on the fetal heart [20] and fetal brain [41] during and following periods of ischemia and reperfusion. Detrimental consequences on the fetus of maternal treatment with allopurinol are supported by its effects in impairing fetal peripheral vascular reactivity to constrictor agonists [10] and on the fetal cardiovascular defense to acute hypoxia (present work). Clearly, further work is warranted. Until then, the therapeutic use of allopurinol in clinical obstetric practice should be approached with caution.

## ACKNOWLEDGMENTS

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# Chapter 6
# Antenatal allopurinol reduces hippocampal brain damage after acute birth asphyxia in late gestation fetal sheep

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## ABSTRACT

Free radical induced reperfusion injury is a recognized cause of brain damage in the newborn after birth asphyxia. The xanthine-oxidase inhibitor allopurinol reduces free radical synthesis and crosses the placenta easily. Therefore, allopurinol is a promising therapeutic candidate. This study tested the hypothesis that maternal treatment with allopurinol during fetal asphyxia limits ischemia-reperfusion (I/R) damage to the fetal brain in ovine pregnancy. The I/R challenge was induced by 5 repeated measured compressions of the umbilical cord each lasting 10 min, in chronically-instrumented fetal sheep at 0.8 of gestation. Relative to control fetal brains, the I/R challenge induced significant neuronal damage in the fetal hippocampal cornu ammonis zone 3 and 4. Maternal treatment with allopurinol during the I/R challenge restored the fetal neuronal damage towards control scores. Maternal treatment with allopurinol offers potential neuroprotection to the fetal brain in the clinical management of perinatal asphyxia.

During labor and delivery, the most common challenge to the fetus is repeated compression of the umbilical cord. If severe or prolonged enough, this may result in perinatal asphyxia, leading to marked fetal acidosis and cardiovascular compromise with subsequent hypoxic-ischemic encephalopathy (HIE), predictive for future cerebral palsy and cognitive disability [1]. The incidence of severe perinatal asphyxia is estimated 4-9 per 1000 full-term neonates, of whom 15-20% will die and about 25% of the survivors will permanently suffer from neuropsychological deficits [2].

To date, the only established treatment for HIE is therapeutic hypothermia of the neonate [3,4]. However, only mild to moderately asphyxiated infants seem to significantly benefit and only a small reduction of death or disability is seen [3,5,6]. Therefore, there is a great need for additional treatment options to help prevent or diminish cerebral damage in the infant due to perinatal asphyxia.

Repeated compressions of the umbilical cord not only induce fetal asphyxia and acidosis but also episodes of ischemia-reperfusion (I/R), promoting the excessive generation of reactive oxygen species (ROS) such as the superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$) anions [7-9]. Several potential antioxidant pharmacologic options to intervene in the putative neurotoxic cascade have already been studied [6,10]. Drugs like tetrahydrobiopterin, melatonin, nNOS inhibitors, xenon and vitamin C showed promising results in experimental studies, but have not yet been translated to clinical use [6,11].

One of the main pro-oxidant pathways stimulated by I/R is the xanthine oxidase pathway [12,13]. Allopurinol, a xanthine oxidase inhibitor and FDA-approved drug, inhibits the conversion of hypoxanthine into xanthine and uric acid, thereby limiting the toxic overproduction of ROS. In high doses, it also functions as a chelator of Non Protein Bound Iron (NPBI) and a direct scavenger of the hydroxyl radical [14]. Furthermore, substantial knowledge on the use of allopurinol in terms of dose and in vivo safety profiles is already available [15-21]. Collectively, these properties make allopurinol a strong potential candidate for cerebral neuroprotection arising from I/R damage.

A recent follow-up study of two prospective randomized controlled trials determining the effects of postnatally administered allopurinol in term asphyxiated human neonates showed a beneficial effect of treatment on mortality and severe disabilities at 4-8 years of age, but only in the moderately asphyxiated group [20]. It was further reported that treatment with allopurinol of the asphyxiated neonate improved neonatal outcome [15], however if the time-interval between I/R and treatment had been prolonged, or again when asphyxia had been too severe, no reduction in serious morbidity or mortality was observed [16]. Therefore, there has been accumulating interest in establishing whether perinatal cerebral outcome may be improved if the window of treatment with allopurinol is advanced, for instance via maternal treatment to cover the actual period of fetal asphyxia and of I/R in complicated labor. Maternal treatment with allopurinol crosses the placenta, it suppresses $O_2^{\cdot-}$ production in the fetus [22] and yields therapeutic levels in the neonatal circulation [23], justifying this route of administration for preventative therapy in obstetric practice.

Therefore, this study tested the hypothesis that maternal treatment with allopurinol during repeated episodes of fetal asphyxia would limit I/R damage to the fetal brain. The hypothesis was tested in chronically instrumented fetal sheep in late gestation subjected to an I/R challenge involving repeated, measured compression of the umbilical cord with or without maternal treatment with allopurinol.

## Materials and Methods

### Ethical Approval

The study was approved by the Cambridge University Ethical Review Committee. All procedures were performed under the UK Animals (Scientific Procedures) 1986 Act and conducted under the authority of the appropriate project and personal licenses.

### Animals and Surgical Procedures

Eleven Welsh Mountain Sheep were surgically instrumented for long-term recording at 124 days of gestation (term is ~ 145 days) using strict aseptic conditions, as previously described in detail [19,24,25]. Under general anesthesia (1.5-2.0% halothane in 50:50 $O_2$:$N_2O$), midline abdominal and uterine incisions were made, the fetal hind limbs were exteriorized and, on one side, fetal arterial (i.d., 0.86 mm; o.d., 1.52 mm; Critchly Electrical Products, NSW, Australia) and venous (i.d., 0.56 mm; o.d., 0.96 mm) catheters were inserted. Another catheter was anchored onto the fetal hind limb for recording of the reference amniotic pressure. In addition, a transit-time flow transducer was implanted around the left umbilical artery close to the common umbilical artery inside the fetal abdominal cavity (4SB; Transonic Systems Inc., Ithaca, NY, USA) [24,26]. An inflatable occluder cuff (In Vivo Metrics) was positioned around the proximal end of the umbilical cord, as described previously in detail. [19,26] Ewes were instrumented with arterial and venous catheters placed in the left femoral artery and vein, respectively. All incisions were closed in layers. The catheters, occluder cable and flow probe leads were then exteriorized via a keyhole incision in the maternal flank and kept inside a plastic pouch sewn onto the maternal skin.

### Post-Operative Care and Experimental Protocol

Antibiotics were administered daily to the ewe (0.20-0.25 $mg.kg^{-1}$ i.m. Depocillin; Mycofarm, Cambridge, UK) and fetus i.v. and into the amniotic cavity (150 $mg.kg^{-1}$ Penbritin; SmithKline Beecham Animal Health, Welwyn Garden City, Hertfordshire, UK). Following at least five days of post-operative recovery, all fetuses were submitted to an I/R challenge, produced by 5 x 10 minutes inflations of the cord occluder with sterile saline at 10-minute intervals. Each cord compression was designed to reduce umbilical blood flow by 80-90% from baseline, and to lead to a progressive fall in fetal arterial pH to 6.9 by the end of the fifth compression. In five fetuses, the I/R challenge was induced during maternal I.V. treatment with allopurinol (Sigma Ltd., 20 $mg.kg^{-1}$ maternal weight, dissolved in buffered saline and infused over a twenty minute period). In the remaining six fetuses, the I/R challenge was induced during maternal infusion with buffered saline at the same rate. Infusion of either allopurinol or vehicle started 10 minutes before the fourth umbilical cord compression and finished immediately after the end of it. The dosing regimen of allopurinol was adopted from the only study that used the drug in women undergoing uncomplicated labor [23].

### Blood sampling regimen

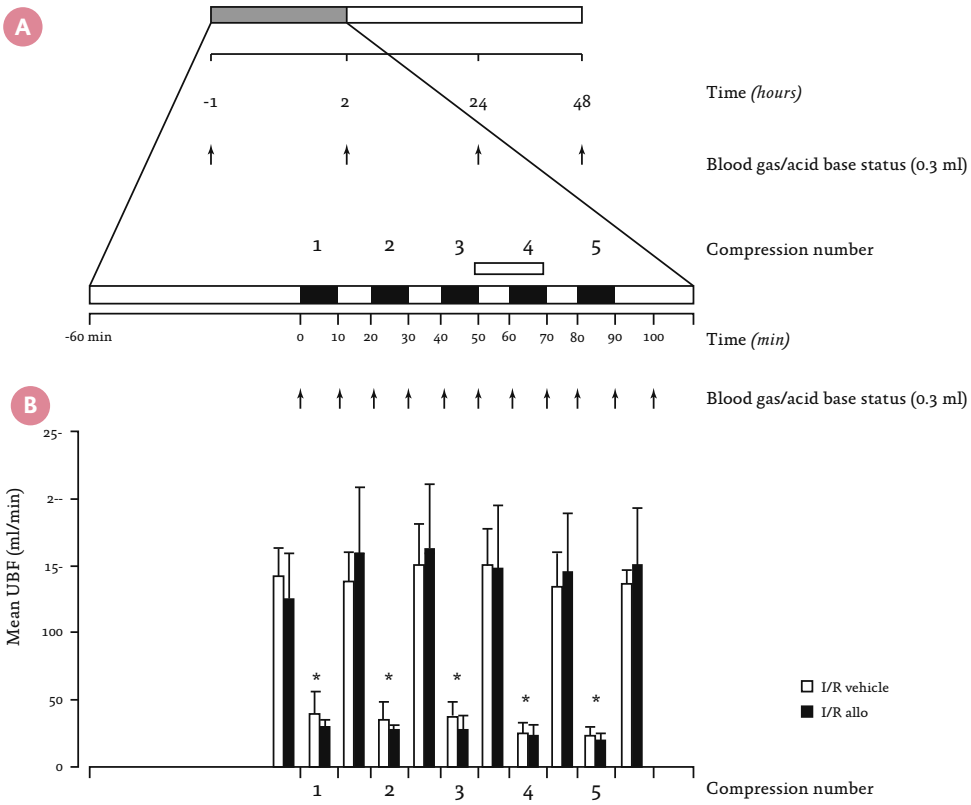
To determine arterial blood gas, acid base and metabolic status, maternal and fetal arterial blood samples (0.3 mL) were drawn into sterile syringes one hour prior to the I/R challenge, at 10 minute intervals during and for 48 hours following the I/R challenge (ABL5 Blood Gas Analyzer, Radiometer, Copenhagen, Denmark; see Figure 6-1). Values for percentage saturation of hemoglobin with oxygen (SatHb) were determined using a hemoximeter (OSM3; Radiometer). Blood glucose and lactate concentrations were measured by an automated analyser (Yellow Springs 2300 Stat Plus; YSI Ltd., Farnborough, UK). Additional paired maternal and fetal blood samples (1 mL) were taken in the allopurinol treated pregnancies at varying set intervals, starting at the onset of the infusion period and up to five hours following the end of infusion, to compile a comprehensive serial profile of maternal and fetal plasma concentrations of

allopurinol and oxypurinol without affecting materno-fetal concentrations of hemoglobin. Reversed-phase high-performance liquid chromatography (HPLC) with UV-detection at 254 nm was used for the quantification of allopurinol and oxypurinol in both the fetal and maternal plasma. The method was linear between 0.5 and 25 mg.L$^{-1}$ with a lower limit of detection of 0.2 mg.L$^{-1}$ for both compounds [27].

**Histological Evaluation**

Forty-eight hours after the end of the experi-

**Figure 6-1. Experimental protocol**




At least 5 days after surgery, all fetuses were subjected to an ischemia–reperfusion (I/R) challenge (gray bar). After 1 hour of basal recording, the I/R challenge consisted of 5 compressions of the umbilical cord, each of 10 minutes' duration (black bars) with a 10-minute interval. Maternal infusion with allopurinol or vehicle started 10 minutes before the fourth umbilical cord compression and finished immediately after the end of it (white bar). Fetal arterial blood samples (arrows) were taken for analysis of blood gas and metabolic status. B, Data are umbilical blood flow (UBF) mean+standard error of the mean (SEM), before and after each umbilical cord compression in 5 I/R allopurinol pregnancies (black bars) and in 6 I/R vehicle pregnancies (white bars). Significant differences *P < .05 versus baseline (2-way repeated measures analysis of variance with t Newman-Keuls test).

mental protocol, ewes and fetuses were subjected to humane euthanasia using a lethal dose of sodium pentobarbitone (200 mg.kg$^{-1}$ I.V. Pentoject; Animal Ltd., York, UK) for tissue collection. Tissues were also harvested from 6 un-instrumented fetal sheep at 0.8 gestation, which served as age-matched controls. Immediately after euthanasia, a Caesarean section was performed and fetal brains were perfusion-fixed with formaldehyde delivered through the carotid arteries under constant pressure. Fixed brains were embedded in paraffin. Four-micrometer coronal sections were cut at the level of the dorsal hippocampus, caudate nucleus and cerebellum and stained with H and E and a combined staining procedure using acid fuchsin-thionin (0.1% fuchsin, Gurr/BDH; 0.25% thionin, Chroma). The latter stain highlights (acidophilic) damaged neurons; these neurons have a bright pink cytoplasm on a blue Nissl background. Cells were considered to be necrotic if they had acidophilic cytoplasm, nuclear pyknosis or loss of nuclear detail. The proportion of necrotic cells was determined in the hippocampus (Cornu Ammonis (CA), areas 1+2 and 3+4, dentate gyrus and parahippocampal cortex), thalamus and caudate nucleus by light microscopy (Zeiss standard 25, Germany) at 100x and 400x magnification. Examination was performed using a 6-point scale: 0 (0%), 1 (>0-10%), 2 (>10-50%), 3

**Table 6-1. Fetal arterial blood gases and metabolic status**

| | Experimental Group | Baseline | At start infusion | After end of infusion | +48 h post |
|---|---|---|---|---|---|
| pHa | I/R vehicle | 7.36±0.01 | 7.11±0.03* | 6.97±0.03* | 7.34±0.01 |
| | I/R allo | 7.36±0.01 | 7.07±0.02* | 6.97±0.03* | 7.35±0.01 |
| ABE (mEq.L$^{-1}$) | I/R vehicle | 4.8±0.5 | -6.7±2.1* | -15.0±1.7* | 3.0±0.8 |
| | I/R allo | 3.6±0.8 | -9.0±0.98* | -15.0±1.3* | 2.8±0.5 |
| PaO2 (mmHg) | I/R vehicle | 21.3±2.0 | 14.2±1.9* | 14.0±1.4* | 23.8±2.0 |
| | I/R allo | 20.8±1.9 | 13.6±2.4* | 13.4±3.7* | 21.4±1.3 |
| PaCO2 (mmHg) | I/R vehicle | 58.0±1.2 | 81.7±3.4* | 90.8±5.5* | 57.0±0.91 |
| | I/R allo | 55.2±2.0 | 84.4±5.4* | 90.6±5.4* | 54.8±1.2 |
| SatHb (%) | I/R vehicle | 55.3±5.0 | 24.0±3.4* | 21.8±2.1* | 58.8±5.7 |
| | I/R allo | 57.9±7.4 | 24.6±5.0* | 22.8±5.9* | 58.1±5.5 |
| Lactate (mmol.L$^{-1}$) | I/R vehicle | 1.13±0.20 | 6.11±1.17* | 9.79±1.24* | 0.92±0.14 |
| | I/R allo | 1.05±0.20 | 6.09±0.94* | 9.09±0.99* | 1.19±0.27 |
| Glucose (mmol.L$^{-1}$) | I/R vehicle | 0.97±0.09 | 1.66±0.29* | 1.72±0.10* | 1.04±0.16 |
| | I/R allo | 0.80±0.11 | 1.65±0.38* | 1.18±0.30* | 1.05±0.23 |

Fetal physiologic parameters (arterial pH, blood gases, glucose and lactate values) 60 min before umbilical cord compression (baseline), immediately before and immediately after the end of infusion of allopurinol or buffered saline and at 48 hours after the I/R challenge (see Materials and Methods and Figure 6-1). Significant differences within groups are *$p < 0.05$ *vs* baseline (two-way RM ANOVA with post hoc *t*-Newman-Keuls test). There were no significant differences between the two treatment groups.

(>50-90%), 4 (>90-99%) and 5 (100%) [28-30]. Sections were randomly numbered and scored simultaneously using a dual viewing attachment by two investigators (JK and MH) who were unaware of the experimental groups.

To assess apoptosis, another set of four-micrometer coronal sections of the hippocampus, thalamus, basal nuclei, cortex and white matter was stained with a cleaved caspase-3 stain. The degree of apoptosis was determined using a 3-point scoring system as previously described [31]. Sections were randomly numbered and scored simultaneously using a dual viewing attachment by two investigators (HT and PN) who were unaware of the experimental groups.

**Data and statistical analyses**

Cardiovascular data were recorded continually at 1 s intervals using a computerized Data Acquisition System (Department of PDN, University of Cambridge, UK). Summary measures analysis was applied to the cardiovascular serial data to focus the number of comparisons and areas under the curve were calculated for statistical comparison, as previously described in detail [32]. For all variables, values are expressed as mean ± S.E.M. Comparisons between groups were assessed using one or two-way ANOVA with an appropriate post hoc test. The relationships between indices of fetal brain damage and values for fetal arterial blood gases or acid base and metabolic status were assessed using the Pearson Product Moment correlation. For all comparisons, $P < 0.05$ was considered significant.

## Results

**Plasma levels of allopurinol**

Administration of allopurinol to pregnant ewes led to elevations in fetal plasma levels of allopurinol between 4 and 7 $mg.L^{-1}$ within 90 minutes of the start of the infusion. These fetal plasma concentrations of allopurinol are within the human therapeutic range for xanthine oxidase inhibition [23]. Plasma levels of the active metabolite oxypurinol rose more gradually to levels between 1 and 1.5 $mg.L^{-1}$ at two hours after maternal administration and these elevations lasted much longer, returning towards baseline 5 hours after treatment.

**Fetal arterial blood gas, acid base and metabolic status**

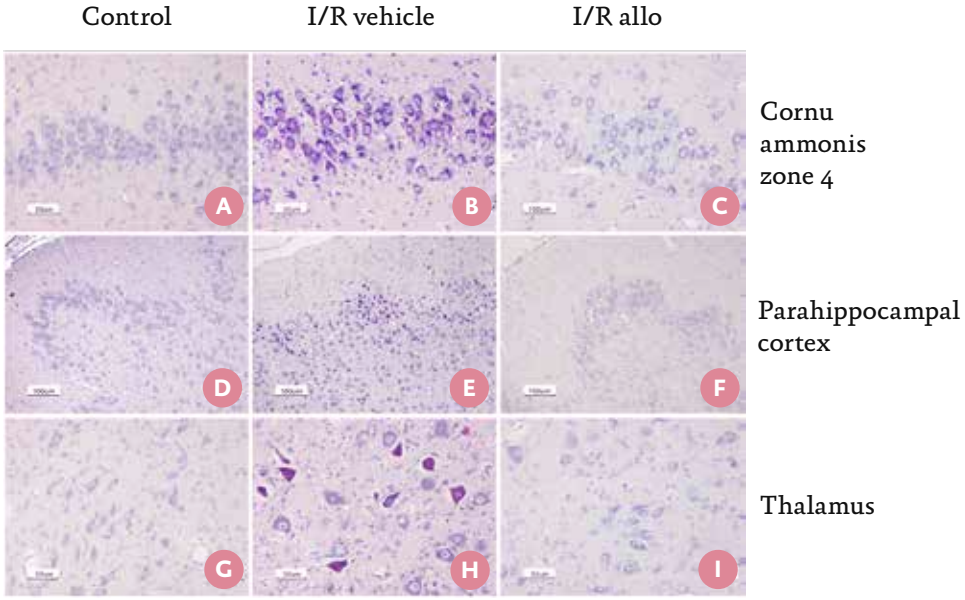
Repeated compressions of the umbilical cord transiently decreased fetal $PaO_2$, SatHb, pH, and arterial base excess (ABE) and increased $PaCO_2$, glucose and lactate concentrations (Table 6-1).

Changes in the vehicle and allopurinol treated groups, respectively, before and at the end of the I/R challenge are summarized as follows: fetal pH was reduced from 7.36 ± 0 at baseline to 6.97 ± 0.03 versus 7.36 ± 0 to 6.97 ± 0.03 by the end of the fifth compression (both groups $P < 0.05$), arterial base excess was reduced from 4.8 ± 0.5 to -15 ± 1.7 $mEq.L^{-1}$ versus 3.6 ± 0.8 to -15 ± 1.3 $mEq.L^{-1}$ (both groups $P < 0.05$), $PaO_2$ was reduced from 21 ± 2 to 14 ± 1 mmHg versus 21 ± 2 to 13 ± 2 mmHg (both groups $P<0.05$), SatHb was reduced from 55 ± 5 to 22 ± 2 % versus 58 ± 7 to 23 ± 6 % (both groups $P < 0.05$), $PaCO_2$ was increased from 58 ± 1 to 91 ± 6 mmHg versus 55 ± 2 to 91 ± 5 mmHg (both groups $P < 0.05$), lactate levels rose from 1.1 ± 0.2 to 9.8 ± 1.7 $mmol.L^{-1}$ versus 1.1 ± 0.2 to 9.9 ± 1.2 $mmol.L^{-1}$ (both groups $P < 0.05$) and glucose levels rose from 1.0 ± 0.1 to 1.7 ± 0.1 $mmol.L^{-1}$ versus 0.8 ± 0.1 to 1.2 ± 0.3 $mmol.L^{-1}$ (both groups $P < 0.05$). There were no differences in the magnitude of any change in arterial blood gas, acid base excess or metabolic status between groups.

**Assessment of Neuronal Necrosis**

Relative to controls, the I/R challenge induced significantly greater neuronal damage in the hippocampal cornu ammonis zone 3 and 4 of fetal sheep brains (Figures 6-2 and 6-3). Maternal treatment with allopurinol during the I/R challenge restored fetal cerebral neuronal dam-

**Figure 6-2. Histopathological images; acid fuchsin thionin staining**




Representative images of the histopathological appearance of fetal sheep brains at 0.8 of gestation in controls (A, D, and G), the ischemia–reperfusion (I/R) vehicle group (B, E, and H), and the I/R allopurinol group (C, F, and I). Panels A to C represent zone 4 of the cornu ammonis; panels D to F are images of the parahippocampal cortex; and panels G, H, and I represent the thalamus. An acid fuchsin and thionin staining was used to detect neuronal loss (acidophilic positive neurons).

age in these areas towards control scores (Figures 2 and 3). Differences in fetal brain neuronal damage between control and I/R groups, and relative protection following I/R by maternal allopurinol treatment, was also prominent, although outside statistical significance (P=0.08), in the fetal dentate gyrus and thalamic regions (Figure 6-3).

**Assessment of Neuronal Apoptosis**

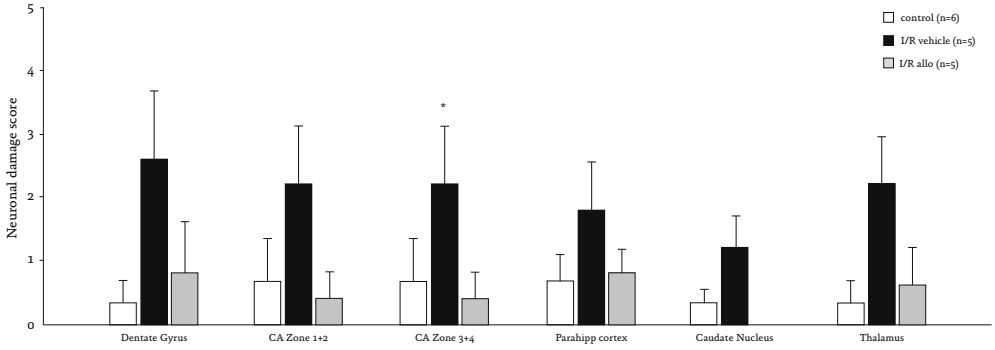
Relative to controls, significantly greater apoptosis in the basal nuclei and cortex was measured in fetuses subjected to the I/R challenge. However, the magnitude of apoptosis in these fetal brain regions was similar between vehicle and allopurinol treated groups (Figure 6-4). Differences in the extent of neuronal apoptosis between control and fetuses subjected to the I/R challenge, independent of allopurinol treatment, were also apparent in other fetal brain regions. However, these differences fell outside statistical significance (P=0.08, Figure 6-4).

When variables representing fetal arterial blood gases, acid base and metabolic status were correlated to indices of cerebral necrosis or apoptosis in all brain regions within individual animals for all groups, no significant relationships were found.

**Fetal Cardiovascular Variables**

Data concerning fetal cardiovascular variables

**Figure 6-3. Neuronal damage scores in fetal sheep brain**



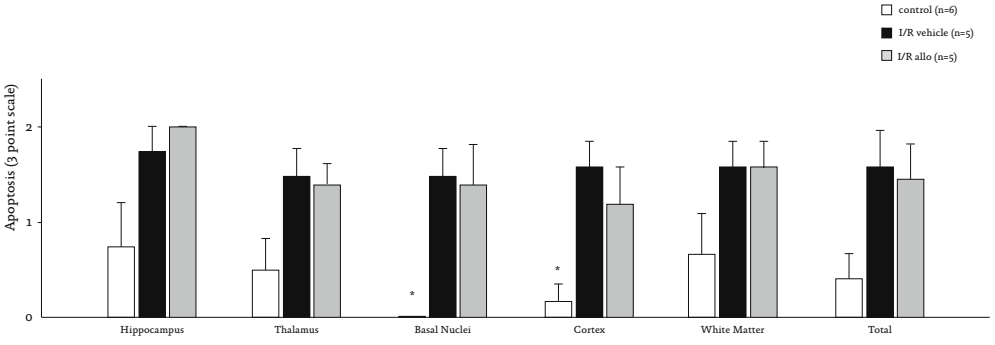

Values are mean+standard error of the mean (SEM). *P < .05 versus control (repeated measures analysis of variance & Bonferroni test).

are described in detail in a previous article by our group[19]. These data show that maternal treatment with allopurinol helped maintain umbilical blood flow and reduced fetal cardiac oxidative stress after I/R.

## Discussion

This study was designed to investigate the potential neuroprotective effects of the FDA-approved drug allopurinol, when administered to the mother during intra-uterine asphyxia at term. To test the hypothesis, we developed an experimental model to simulate perinatal asphyxia by using 5 intermittent umbilical cord occlusions leading to clinically relevant metabolic acidosis with subsequent brain damage in late gestation fetal sheep. At the start of maternal allopurinol infusion, mean pH was 7.07±0.02 and 7.11±0.03 in the I/R allopurinol and I/R vehicle groups respec-

**Figure 6-4. Apoptosis (assessed with cleaved caspase 3) in fetal sheep brain**




Values are mean+standard error of the mean (SEM). *P < .05 versus ischemia–reperfusion (I/R) vehicle and I/R allopurinol (1-way analysis of variance & Bonferroni).

tively, indicating severe fetal asphyxia. In the human clinical setting, this would be the time-point to start preparing an emergency Cesarean section or instrumented vaginal delivery, a window of time in which maternal infusion with allopurinol could be incorporated.
The data show that in the late gestation ovine fetus repeated, intermittent, measured compressions of the umbilical cord led to neuronal cell damage and apoptosis in most brain areas examined. Ischemia-reperfusion induced significantly greater neuronal damage in the hippocampal cornu ammonis zone 3 and 4, as compared to non-ischemic controls. Differences between groups were prominent, although outside statistical significance, in the dentate gyrus and thalamus regions. Maternal administration with allopurinol during I/R restored fetal neuronal damage towards control scores, confirming a possible role for xanthine oxidase in the pathway leading to neuronal damage and supporting a potential neuroprotective effect of antenatal antioxidant treatment.

Several experimental models to induce fetal asphyxia have previously been performed; either partial occlusion of the umbilical cord for 30 minutes to several hours, repetitive infrequent occlusions lasting 5 minutes, occlusions of increasing length and/or frequency and (nearly) total occlusion for 10 minutes, as in our model [28,29,33-40]. Although a broad variation of distribution patterns of neuronal damage and apoptosis have been identified after using these different techniques, the observed (para)hippocampal vulnerability has frequently been described [28,34,35,41]. In our study, the hippocampus and the parahippocampal cortex, areas involved in memory and spatial orientation and navigation, were also the most severely damaged brain areas, results in keeping with the characteristics of such studies.

Apparent differences in histological brain damage between the I/R allo and I/R vehicle groups that did not reach statistical significance in the preset study are most likely due to the small number of subjects used. Alternatively, a lack of an effect of allopurinol treatment could reflect brain damage triggered via pro-oxidant pathways other than via the activation of xanthine oxidase. Several other pathways may promote oxidative stress, such as the glutamate-induced excitotoxicity which proceeds via NMDA receptor activation, producing $Ca^{2+}$ influx and thereby activation of $Ca^{2+}$-dependent NOS, particularly nNOS. At high concentrations, NO- reacts with superoxide ($O_2^{\cdot -}$) to produce peroxynitrite (ONOO-), which in turn induces lipid peroxidation and mitochondrial nitrosylation. Consequently, mitochondrial dysfunction and membrane depolarization develop with further release of $O_2$.
An increased influx of calcium also leads to the activation of cytosolic phospholipases, increasing eicosanoid release and inflammation with concomitant accumulation of neutrophils. A combined therapeutic approach using drugs, which influence different parts of the neurotoxic cascade, like melatonin [42] or tetrahydrobiopterin, might therefore further improve the outcome of antioxidant therapy.

In the present study, analysis of indices of apoptosis also revealed significant programmed cell death in most brain areas of fetal sheep that underwent ischemia-reperfusion. However, maternal treatment with allopurinol during the I/R challenge did not have a significant effect on reducing the amount of apoptotic brain cells. This may be because histological assessment was performed at 48 hours after ischemia reperfusion, while the process of apoptosis normally reaches its maximum 72 hours after a hypoxic ischemic event [30].

Previous papers have reported on effects of allopurinol in post-asphyxial reperfusion brain damage in both animals and humans. Although allopurinol has been administered both before and after hypoxic-ischemic insults in these studies, most of the experiments have been car-

ried out postnatally. Only five other studies in the literature have reported on antenatally administered allopurinol, but none of them have described any effects on the histology of the brain [18,19,22,23,43].

In the present study, therapeutic ranges of allopurinol and its metabolite oxypurinol have shown to be attainable after antenatal maternal treatment with allopurinol, while no adverse side effects were observed. These findings are in line with previous studies in both animals and humans [18,22,23,43]. Masaoka *et al*. were the first to investigate antenatally administered allopurinol during intermittent umbilical cord occlusion in fetal sheep, and described a significantly reduced production of superoxide in the allopurinol treated animals [22]. This indicates either a direct effect of allopurinol in preventing the production of free radicals, in scavenging free radicals once formed, or both. These results were confirmed in the ovine fetuses used in the present study, in which we found reduced indices of oxidative stress in the cardiovascular system after maternal treatment with allopurinol [19]. A randomized controlled human clinical pilot study performed by our group has also shown a significant reduction of Non Protein Bound Iron (NPBI) in the cord blood of allopurinol treated infants, confirming the oxidative stress-reducing effect of allopurinol in humans. Furthermore, an inverse correlation between levels of allopurinol and levels of S-100ß, a marker of brain damage, in umbilical cord blood was seen, further indicating a potential neuroprotective effect [18].

In summary, this is the first report investigating the protective value of antenatal administration of allopurinol during birth asphyxia on brain damage in the late gestation sheep fetus. The data support the hypothesis tested that maternal treatment with allopurinol during repeated episodes of fetal asphyxia would limit I/R damage to the fetal brain. Maternal treatment with allopurinol therefore remains a promising therapeutic option to complement neonatal head cooling. A large prospective multicenter placebo controlled trial in the Netherlands investigating the effect of maternal administration of allopurinol on markers of brain damage and neonatal outcome in humans has recently completed recruitment [44].

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# Chapter 7

# Antenatal allopurinol for reduction of birth asphyxia induced brain damage (ALLO-Trial); a randomized double blind placebo controlled multicenter study (study protocol)

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*BMC Pregnancy Childbirth. 2010 Feb 18;10:8*

## ABSTRACT

**Background**
Hypoxic-ischemic encephalopathy is associated with the development of cerebral palsy and cognitive disability later in life and is therefore one of the fundamental problems in perinatal medicine. The xanthine-oxidase inhibitor allopurinol reduces the formation of free radicals, thereby limiting the amount of hypoxia-reperfusion damage. In case of suspected intra-uterine hypoxia, both animal and human studies suggest that maternal administration of allopurinol immediately prior to delivery reduces hypoxic-ischemic encephalopathy.

**Methods/Design**
The proposed trial is a randomized double blind placebo controlled multicenter study in pregnant women at term in whom the fetus is suspected of intra-uterine hypoxia.
Allopurinol 500 mg IV or placebo will be administered antenatally to the pregnant woman when fetal hypoxia is suspected. Fetal distress is being diagnosed by the clinician as an abnormal or non-reassuring fetal heart rate trace, preferably accompanied by either significant ST-wave abnormalities (as detected by the STAN-monitor) or an abnormal fetal blood scalp sampling (pH < 7.20). Primary outcome measures are the amount of S100ß (a marker for brain tissue damage) and the severity of oxidative stress (measured by isoprostane, neuroprostane, non protein bound iron and hypoxanthine), both measured in umbilical cord blood. Secondary outcome measures are neonatal mortality, serious composite neonatal morbidity and long-term neurological outcome. Furthermore pharmacokinetics and pharmacodynamics will be investigated. We expect an inclusion of 220 patients (110 per group) to be feasible in an inclusion period of two years. Given a suspected mean value of S100ß of 1.05 ug/L (SD 0.37 ug/L) in the placebo group this trial has a power of 90% (alpha 0.05) to detect a mean value of S100ß of 0.89 ug/L (SD 0.37 ug/L) in the 'allopurinol-treated' group (z-test2-sided). Analysis will be by intention to treat and it allows for one interim analysis.

**Discussion**
In this trial we aim to answer the question whether antenatal allopurinol administration reduces hypoxic-ischemic encephalopathy in neonates exposed to fetal hypoxia.

Hypoxic-ischemic encephalopathy is associated with development of cerebral palsy and cognitive disability later in life [1]. The recognition, prevention and treatment of intra-uterine hypoxia is therefore one of the main priorities in perinatal medicine. Animal and human studies have shown that brain damage not only occurs during the hypoxic-ischemic event, but continues for hours up to days upon and after reoxygenation and reperfusion and is caused by production of free radicals [2].

Free radical formation due to conversion of hypoxanthine into xanthine by xanthine-oxidase is very important during this process [3]. Administration of the xanthine-oxidase inhibitor allopurinol (ALLO) reduces the production of free radicals, thereby limiting the amount of hypoxia-reperfusion damage [4,5]. Furthermore, ALLO also has a non-protein bound iron (pro-radical) chelating and direct free radical (hydroxyl) scavenging effect. Animal research in asphyxiated pigs demonstrated beneficial effects of postnatally administrated ALLO on cerebral energy status and cytotoxic edema [6].

A prospective randomized study in human neonates, examining the effects of ALLO in term asphyxiated neonates, showed an improvement of electrocortical brain activity and a reduction in free radical formation after neonatal ALLO administration [7]. A more recent paper by Gunes *et al.* [8] reports an improved neurological outcome after postnatal ALLO administration compared to a placebo in term asphyxiated neonates. Benders *et al* however demonstrated that ALLO was not effective if administrated 3 to 4 hours after the hypoxic incident to severely asphyxiated neonates [9]. However, when the most severely asphyxiated children were excluded from the study, a beneficial effect of ALLO was seen on neurological development. This is in line with previous studies by Gluckman *et al.* on neonatal head cooling [10]. They also demonstrated a beneficial effect of their treatment after exclusion of the most severely asphyxiated neonates. Apparently, no advantage of neonatal treatment is seen anymore, when the interval to the initiation of treatment is too long or when the brain damage is too severe. This has probably been the major disadvantage of late post neonatal treatment with ALLO on the Neonatal Intensive Care Unit (NICU). ALLO administrated at the NICU is likely to be given too late to provide adequate neuroprotection during the early period of reoxygenation in which the vast amount of free radicals is being produced.

Apparently, when the asphyxia has been too severe, the inflicted brain damage can no longer be reversed. It is conceivable that earlier ALLO treatment, i.e. the use of ALLO during labor in case of suspected fetal hypoxia, provides the opportunity to start earlier with the treatment, thereby limiting the amount of hypoxia-reperfusion injury and improving neurological outcome.

Pharmacological data have been published by Boda *et al*, who showed that pharmacological plasma levels were reached in the human fetus after ALLO administration to the mother [11]. A systematic review in Pubmed, searching for [ allopurinol, fetus, neonate, asphyxia, hypoxia ] did not provide us with any additional studies performed in humans. Some animal studies have been performed using piglets (neonatal administration), sheep and sows (maternal administration) [6,12-14]. These studies showed improved outcome after allopurinol treatment on cerebral energy status and recovery of umbilical blood flow respectively.

We recently performed a prospective randomized placebo controlled pilot study, in which we administered ALLO to the pregnant woman when fetal asphyxia was imminent. Data from this pilot study show an inverse correlation between levels of ALLO and the amount of S100ß, a biomarker for brain tissue damage, in cord blood [15]. In addition, we performed a study

in the chronically instrumented fetal sheep, in which we showed evidence of cardio- and neuroprotection after antenatal ALLO administration to the pregnant ewe during repeated periods of ischemia [13,16].

**Incidence and financial impact of birth asphyxia**

Birth asphyxia carries a high incidence, 4-9 per 1000 live born neonates. On estimate 1-4 of these neonates will die or develop a severe handicap [17,18]. With almost 200.000 deliveries in the Netherlands annually, this implicates that each year approximately 800 neonates will die or suffer from a handicap due to birth asphyxia. Moreover, there is evidence that individuals suffering from moderate birth asphyxia, develop behavioral problems later in life [19].

The health care costs of asphyxia are enormous. Many of these children are admitted to a neonatal intensive care unit at a cost of € 1.500, - per day, with an average duration of admission of 10 days. Estimated costs of disabled children are € 80.000, - and € 20.000, - yearly for severely and moderately handicapped children respectively. The costs of neonatal care are annually 20 million Euros, whereas the costs of care for disabled children born in one year are more than one hundred million Euros.

As the costs of ALLO and its administration are relatively low (100 Euros per pregnant woman), a small treatment effect will already make the intervention cost-effective.

**Safety of allopurinol**

Allopurinol has been used in internal medicine for many years for the treatment of gout. Side effects of ALLO are rare and most commonly include symptoms of hypersensitivity like skin rashes [20]. Stevens-Johnson syndrome has been reported, but only after prolonged treatment (> 10 days) in case of gout [21]. During previously performed studies in pregnant women and neonates, no adverse reactions were seen [11,15,22,23].

## Methods / Design

**Aims**

The objective of the study is to test the hypothesis that intra-uterine treatment with ALLO in case of suspected fetal hypoxia will reduce brain damage and with that may improve neonatal outcome.

**Participants/eligibility criteria**

Pregnant women with a gestational age of at least 36 weeks and suspected intra-uterine hypoxia (fetal distress) during labor can be included in the trial. Fetal distress is being diagnosed by the clinician as an abnormal or non-reassuring fetal heart rate trace, preferably accompanied by either significant ST-wave abnormalities (as detected by the STAN-monitor) or an abnormal fetal blood scalp sampling (pH < 7.20). Neonates suspected of chromosomal or congenital anomalies will be excluded.

**Procedures, recruitment, randomization and collection of baseline data**

The study will be a randomized double blind placebo controlled multicenter study. Hospitals which participate in the Dutch Consortium for Studies in Women's health and reproductivity will participate in the trial. The study will be staffed by research midwives and -nurses, who will counsel patients at the outpatient clinic or at the obstetric ward.

Before entry into the study, subjects will be informed about the aims, methods, reasonably anticipated benefits and potential hazards of the study. Subjects will be informed that their participation is voluntary and that they may withdraw consent to participate at any time during the study. They will be informed that choosing not to participate will not affect their care. In every center an independent gynecologist will be available for more detailed information both for patients and colleagues if required. After giving sufficient information, written informed consent will be obtained. The consent form must be signed before performance of any

study-related activity.
In case of suspected fetal distress and written informed consent, the patient will be randomized immediately prior to delivery (Figure 7-1).

All details of delivery are recorded in the case record form that is accessible through a website (www.studies-obsgyn.nl/allo). In case of admittance of one or more children to the neonatal intensive care unit, details of this admittance are also recorded.
Data will be collected using Oracle Clinical Remote Data Capture (RDC), which is a new generation of application systems that enable collection and cleanup of clinical trial data using the Internet. For detailed information on Oracle RDC see http://www.ctc-g.co.jp/~CTCLS/opa/en/. The expertise for this technology is already used in the study group and applied in the ZonMw funded consortium studies that are currently running.

**Interventions**
All women who are identified as having suspected fetal distress and have given informed consent prior to delivery will be randomized in two groups: One group of pregnant women will receive one IV dosage of 500 mg ALLO. The other group will receive one IV dosage of placebo antenatally.

**Outcome measures**
Primary outcome measures are related to biochemical brain damage markers (S100ß, enolase) and the severity of oxidative stress as measured by isoprostane, neuroprostane, non protein bound iron and hypoxanthine in umbilical cord blood.
The main primary outcome measure will be the protein S100ß as this is, at present, the most validated biochemical marker for brain tissue damage. Secondary outcome parameters are neonatal mortality and short term neonatal morbidity, such as hypoglycemia, convulsions and post-hypoxic neonatal encephalopathy, length of admission at the neonatal intensive care unit and placental transfer, pharmacodynamics and –kinetics of ALLO. Neonatal encephalopathy will be assessed by Sarnat- and Thompson scores [24,25].

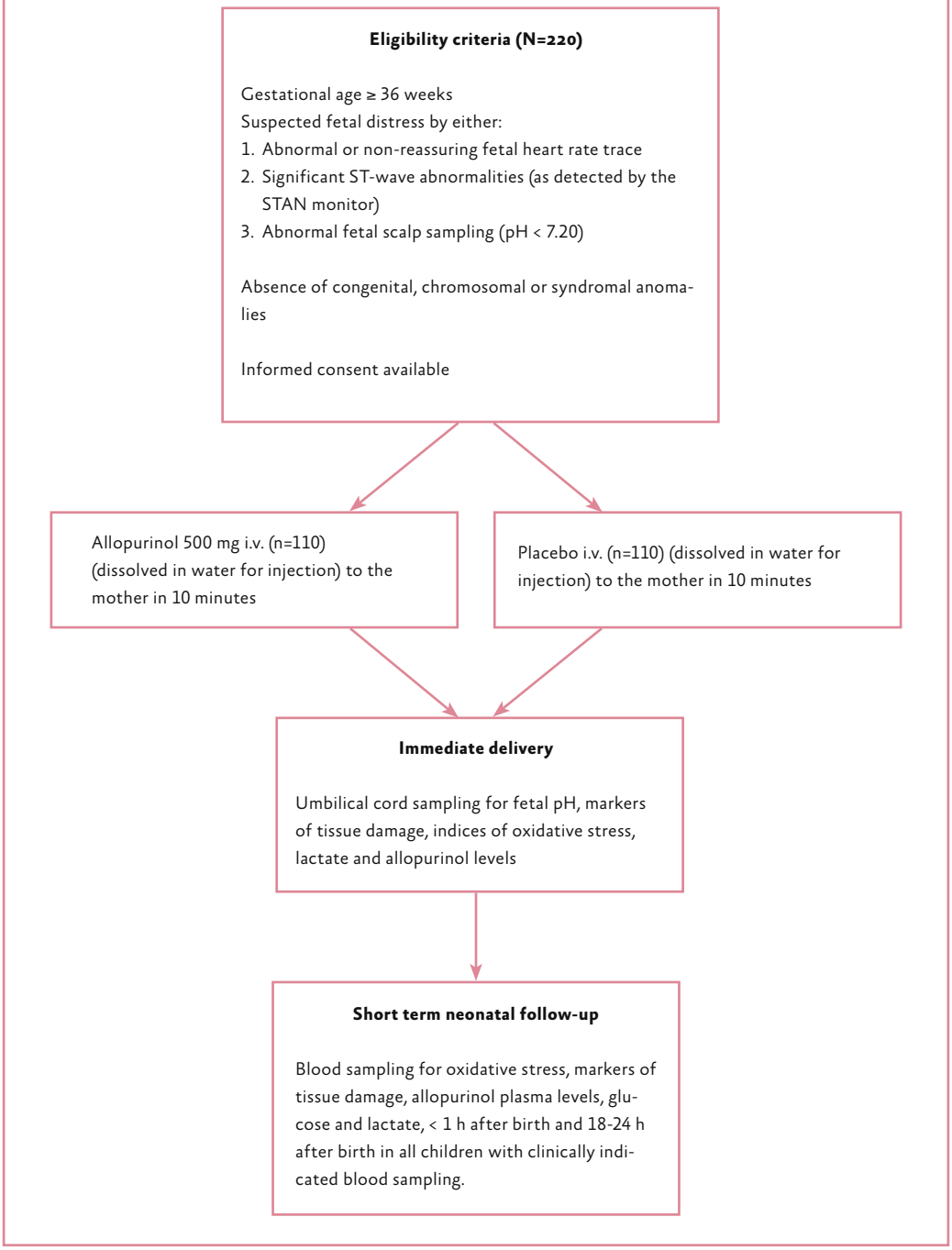
**Follow up of women and infants**
Blood sampling will be performed to assess the amount of tissue damage (i.e. S100ß, enolase, troponin), oxidative stress (i.e hypoxanthine, isoprostane, neuroprostane and non protein bound iron) and ALLO plasma levels. Immediately after delivery, samples of cord blood and maternal blood are obtained. One hour and 18-24 hours after birth neonatal blood samples are obtained in all children with clinically indicated blood sampling.

Long-term follow-up using neuropsychological tests and validated neurodevelopmental questionnaires at the age of 4-5 and 8 years and additional MRI at the age of 8 years is desirable, but is depending on future funding.

## Statistical methods

**Sample size**
The sample size is calculated based on the primary outcome measure cord-S100ß as a marker of brain damage. Our own pilot study showed a mean value of S100ß of 1.05 ug/L (SD 0.37 ug/L) in the 'non-treated' group with suspected intra-uterine hypoxia. Based on this same pilot study we expect the mean value of S100ß to be lower in the 'allopurinol-treated' group compared to the 'non-treated group'.
Based on the accrual of patients in our pilot study we consider an inclusion of 220 patients (110 ALLO, 110 placebo) to be feasible in an inclusion period of 2 years. Given a suspected mean value of S100ß of 1.05 ug/L (SD 0.37 ug/L) in the placebo group this trial has a power of 90% with an alpha of 0.05 to detect a mean value of S100ß of 0.89 ug/L (SD 0.37 ug/L) in the 'allopurinol-treated' group (z-test2-sided) [15].

**Figure 7-1. Flowchart ALLO-trial**



**Data analysis**
Data will be analyzed according to the intention to treat principle. Outcome measures will be analyzed with (non)parametric tests where continuous variables are concerned. Proportional data will be reported as relative risks with 95% confidence intervals. Multivariable regression techniques will be applied to correct for any important differences in prognostic baseline characteristics, despite of randomization, by adding all prognostic variables as independent variables. Both corrected and uncorrected group differences will be reported. Numbers needed to treat (NNT) will be reported for both continuous and discrete data.

**Interim analysis**
An interim analysis will be performed at t=0.5 using O'Brien –Fleming alpha spending function. An interim analysis will be performed after the inclusion of 110 women. This analysis will be done by an independent person that will be unaware of the allocation of treatment or placebo when they judge data on effectiveness.

**Data safety monitoring committee**
Serious Adverse Events (SAEs) and Suspected Unexpected Serious Adverse Reactions (SUSARs) will be reported to a Data Safety Monitoring Committee (DSMC). The DSMC can order to perform an extra interim analysis and, if indicated, terminate the trial prematurely.

**Ethical considerations**
This study is approved by the National Central Committee on Research involving Human Subjects (CCMO – NL26516.000.09).

## Discussion

In conclusion, hypoxic-ischemic encephalopathy is associated with development of cerebral palsy and cognitive disability later in life and is therefore one of the fundamental problems in perinatal medicine. Prevention of brain damage and developing adequate therapy is therefore of big importance. The xanthine-oxidase inhibitor allopurinol (ALLO) reduces free radical formation, thereby limiting the amount of hypoxia-reperfusion damage. Animal and human studies suggest that administration of ALLO immediately prior to delivery in case of suspected fetal asphyxia might reduce hypoxic-ischemic encephalopathy. We designed a randomized placebo controlled multicenter trial to determine whether intra-uterine treatment with allopurinol reduces hypoxic-ischemic encephalopathy in neonates exposed to fetal hypoxia.
To our knowledge no similar studies are or will shortly be performed in the Netherlands or abroad.

## ACKNOWLEDGEMENTS

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**Trial registration number**
Clinical Trials, protocol registration system: NCT00189007

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# Chapter 8
# Rapid target allopurinol concentrations in the hypoxic fetus after maternal administration during labor

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## ABSTRACT

**Design**
We used data from a randomized double blind multicenter trial comparing maternal allopurinol versus placebo in case of imminent fetal hypoxia (NCT00189007).

**Patients**
We studied 58 women in labor at term, with suspected fetal hypoxia prompting immediate delivery, in the intervention arm of the study.

**Setting**
Delivery rooms of 11 Dutch hospitals.

**Intervention**
500 mg allopurinol, intravenously to the mother, immediately prior to delivery.

**Main outcome measures**
Drug disposition (maternal plasma concentrations, cord blood concentrations) and drug safety (maternal and fetal adverse events).

**Results**
Within 5 minutes after the end of maternal allopurinol infusion target plasma concentrations of allopurinol of ≥2 mg/L were present in cord blood. Of all analyzed cord blood samples, 95% (52/55) had a target allopurinol plasma concentration at the moment of delivery. No adverse events were observed in the neonates. Two mothers had a red and/or painful arm during infusion.

**Conclusions**
A dose of 500 mg intravenous allopurinol rapidly crosses the placenta, and provides target concentrations in 95% of the fetuses at the moment of delivery, which makes it potentially useful as a neuroprotective agent in perinatology with very little side-effects.

Despite advances in maternal and neonatal care, limited progress has been achieved in reducing the incidence of fetal asphyxia and subsequent neurodevelopmental disabilities [1]. Neuronal damage following birth asphyxia is most likely caused by the formation of free radicals in the reperfusion phase starting directly after birth [2]. Currently, the only established treatment for free radical induced hypoxic-ischemic encephalopathy is postnatal mild hypothermia, often started several hours after the ischemic and reperfusion phases [3,4]. Therefore, there is need to optimize therapeutic options to further prevent or reduce hypoxia-induced brain damage during labor, preferably in an earlier stage in the process of (or even before) free radical production.

Several potential antioxidant pharmacologic options to intervene in the putative neurotoxic cascade have already been studied [5,6]. Drugs like tetrahydrobiopterin, melatonin, nNOS inhibitors, xenon and vitamin C showed promising results in experimental studies, but have not yet been translated to clinical use [7,8].

Several pathways may promote oxidative stress during labor, such as the glutamate-induced excitotoxicity which proceeds via N-methyl-D-aspartate (NMDA) receptor activation, producing $Ca^{2+}$ influx and thereby activation of $Ca^{2+}$-dependent nitric oxide synthase (NOS), particularly neuronal NOS [9-12]. At high concentrations, nitric oxide ($NO^{\cdot}$) reacts with superoxide ($O_2^{-\cdot}$) to produce peroxynitrite ($ONOO^-$), which in turn induces lipid peroxidation and mitochondrial nitrosylation [13]. Consequently, mitochondrial dysfunction and membrane depolarization develop with further release of ($O_2^{-\cdot}$). An increased influx of calcium also leads to the activation of cytosolic phospholipases, increasing eicosanoid release and inflammation with concomitant accumulation of neutrophils [13,14].

One of the main pro-oxidant pathways stimulated by ischemia and reperfusion is the xanthine oxidase pathway [15,16]. Allopurinol, a FDA-approved xanthine oxidase inhibitor for lowering uric acid concentrations in patients with gout and neoplastic diseases, inhibits the conversion of hypoxanthine into xanthine and uric acid, thereby inhibiting the production of the reactive oxygen species hydrogen peroxide ($H_2O_2$) and superoxide [17-20]. Furthermore, in high dosages, it functions as a chelator of non-protein bound iron and as a direct scavenger of hydroxyl radicals. All these mechanisms could potentially result in neuroprotective effects. Allopurinol is metabolised into the active metabolite oxypurinol, which blocks uric acid production. Its plasma concentrations are therefore used in the monitoring of gout patients [21,22].

A recent follow-up study of two prospective randomised controlled trials in human neonates in which the effects of *postnatally* administered allopurinol in term asphyxiated neonates were studied, showed a beneficial effect of allopurinol administration on mortality and severe disabilities at 4-8 years of age, but only in the moderately asphyxiated group [23]. These beneficial effects are in line with previous findings on neonatal head cooling after acute fetal hypoxia at term [3]. Most likely no advantage of treatment occurs anymore when the interval to the initiation of treatment is too long or when brain damage is already too severe. It is conceivable that earlier allopurinol treatment, i.e. maternally administered allopurinol during labor in case of suspected fetal hypoxia, may earlier limit the amount of free radical production and subsequent hypoxia-reperfusion injury.

For successful intrauterine neuroprotection of the hypoxic fetus during the perinatal reperfusion and reoxygenation phases, a 'candidate' drug such as allopurinol should, besides being effective in the fetus, readily cross the placenta and should not be harmful to mother and fetus.

The aim of our study was to investigate the

pharmacological applicability of allopurinol for intrauterine neuroprotection after maternal administration, by studying drug disposition (maternal plasma concentrations, cord blood concentrations) as well as drug safety (maternal and neonatal adverse events). We studied the required minimal time that was needed to obtain intrauterine target allopurinol concentrations after the end of maternal allopurinol administration, to identify the possible therapeutic time window for administration of allopurinol to the mother in labor in case hypoxia occurs.

## Methods

### Setting and study population

The study population was extracted from women participating in a randomized double blind placebo controlled multicenter trial performed by our group (NCT00189007) [5]. The trial focused on the effect of maternally administered allopurinol during fetal hypoxia on lowering brain specific biomarkers of neonatal brain damage. The study was conducted in 11 hospitals participating in the Dutch Consortium for Studies in Women's Health and Reproductivity. Pregnant women with a gestational age of at least 36 weeks and suspected intra-uterine fetal hypoxia, prompting immediate delivery, were eligible to participate in the trial. Fetal hypoxia was suspected, being an abnormal or non-reassuring fetal heart rate trace (according to FIGO-criteria), significant ST-wave abnormalities (detected by the STAN-monitor) or abnormal fetal blood scalp sampling (pH < 7.20) [24]. Women bearing a child suspected of chromosomal or congenital anomalies were not eligible [12]. For this study, only singletons were used in the assessment of the transplacental pharmacokinetics.

### Intervention

Women were randomly assigned in a double-blind fashion to receive either a single intravenous dose of 500 mg allopurinol (ALLO) in 50mL water for injection (Acepurin, AcePharmaceuticals, Zeewolde, the Netherlands) or placebo (CONT, 500mg Mannitol/50mL water for injection) administered in 10 minutes immediately prior to delivery [5]. The dose of allopurinol was based on a study in healthy pregnancies, performed by Boda *et al*. [25], and on a previously pilot-study performed by our group [26]. Currently the maximum allowed dose of allopurinol in a single dose in clinical use is 500 mg (as stated in the SPC of the product) [27].

Study drugs were stored at 20-25°C and dissolved immediately before administration. Allopurinol and placebo had exactly the same appearance; freeze dried white powder in glass vials with a pH of 11 after dilution with water for injection.

### Bioanalysis

Venous cord blood and maternal blood samples were obtained immediately after birth according to protocol. Allopurinol and oxypurinol plasma concentrations were determined by the Clinical Pharmaceutical and Toxicological laboratory of the University Medical Center Utrecht. This method is used for research and routinely monitoring of patients with gout on allopurinol therapy [28]. Blood samples were collected in heparinized tubes and centrifuged at 3000 rpm for10 minutes. Plasma concentrations were determined using reversed- phase high-performance liquid chromatography (RP-HPLC) with UV-detection at 254 nm. The method was linear between 0.5 and 25 mg/l with a lower limit of quantification (LLoQ) of 0.2 mg/l for both compounds.

### Transplacental transport

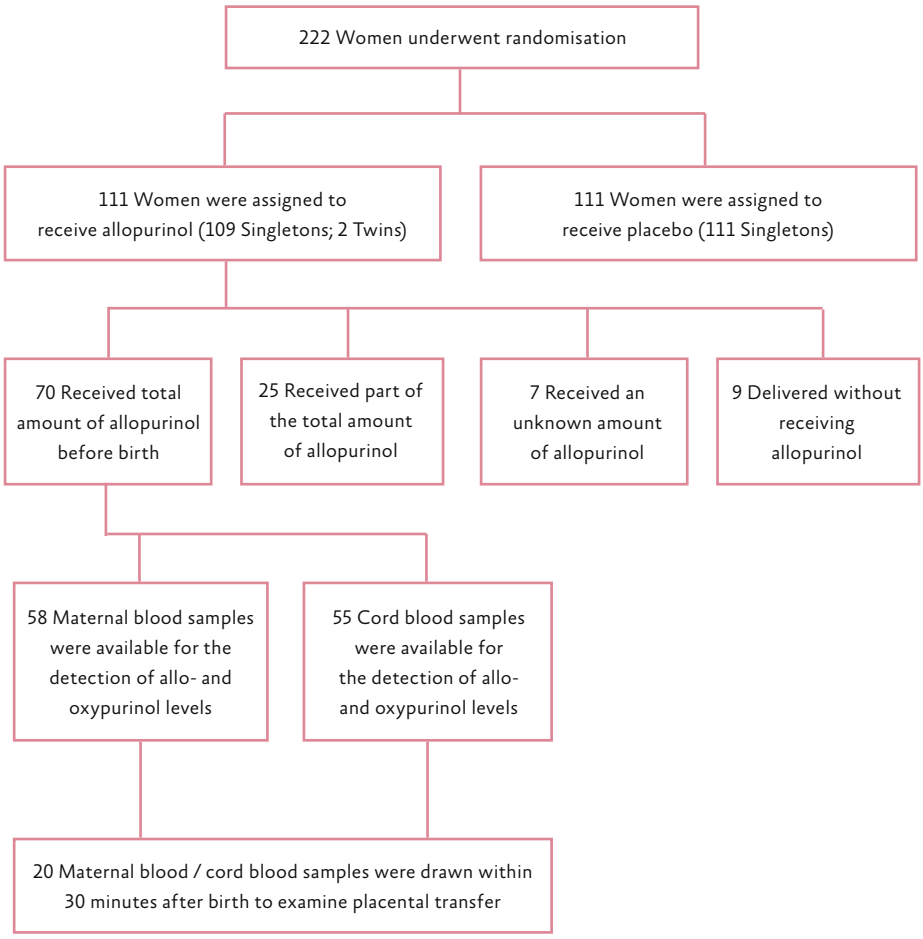
Transplacental transport of allopurinol was studied by comparing obtained maternal and cord plasma concentrations immediately after birth. The transplacental ratio is expressed as the plasma concentration in the fetus divided by the plasma concentration in the mother. Only maternal and cord blood samples that were drawn within a 30 minute interval were used to calculate the transplacental ratio, oth-

erwise the ratio could be distorted due to ongoing drug clearance in the mothers blood after collection of the cord blood. Transplacental transport can occur via passive (e.g. diffusion), active (e.g. ATP-requiring transporters) or combined transport mechanisms (e.g. facilitated diffusion) [29]. Only in presence of active transport, fetal drug concentration can exceed the concentrations in the mother (transplacental ratio >1), since diffusion depends on the concentration-gradient. Without active transport, transplacental ratios can only reach a maximum of 1 (e.g. equal concentration in fetus and mother).

**Safety**

The occurrence of (serious) adverse events and obtained plasma concentrations of allopurinol and oxypurinol were used to study maternal and fetal safety. For maternal safety of the drug only oxypurinol plasma concentrations were assessed, since only for oxypurinol a target window for uric acid lowering therapy in patients

**Figure 8-1. Flowchart of patient enrollment and follow-up**



with gout (target >5 mg/L) is known [30].

**Ethical considerations**

This study was approved by the National Central Committee on Research involving Human Subjects (CCMO – NL26516.000.09) and by Scientific Boards and Ethical Committees of the 11 participating hospitals (the University Medical Center Utrecht, the Máxima Medical Center Veldhoven, the University Medical Center Groningen, the VU University Medical Center Amsterdam, the Academic Medical Center Amsterdam, the Diakonessenhuis Utrecht, the Jeroen Bosch Hospital 's Hertogenbosch, the Maastricht University Medical Center, the Leiden University Medical Center, the Groene Hart Hospital Gouda, the Gelre Hospital Apeldoorn).

Informed consent was obtained from both future parents, antenatally at the outpatient clinic or at the labor ward. Randomization only occurred if fetal hypoxia was suspected.

**Trial registration**

The study is registered in the Dutch Trial Register (NTR1383) and the Clinical Trials protocol registration system (NCT00189007).

## Results

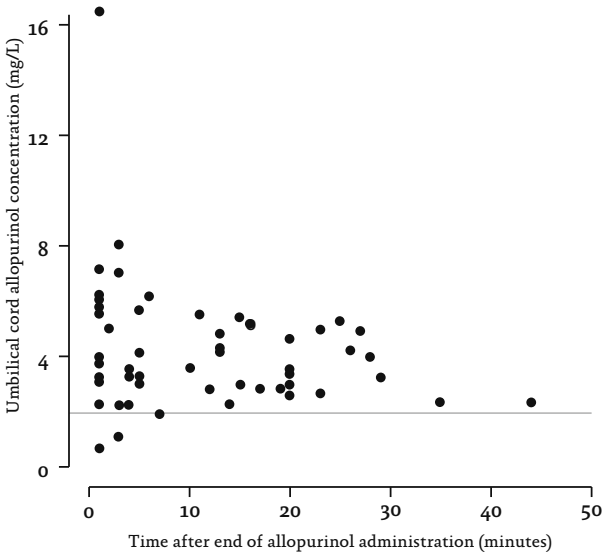
From October 2009 until December 2011, 222 eligible mothers and their 224 infants were enrolled in the randomized trial. We randomly allocated 111 women (two were pregnant of a dichorionic twin) to the group that received 500 mg allopurinol; 111 women received a placebo drug (figure 8-1). In total 70 mothers received the full allopurinol dose of 500 mg. Forty-one of the 111 women did not receive the full dose because the child was born before the whole dose was administered.

**Table 8-1. Characteristics of the analyzed pregnant women and neonates**

| Characteristic | Value (range)[a] |
|---|---|
| **Mothers (n=58)** | |
| Age (years) | 33.1 (19.0 – 41.0) |
| Gravida | 2 (1 – 8) |
| Parity | 0 (0 – 3) |
| Time between end of allopurinol administration and blood withdrawal (minutes) | 57 (5 – 309) |
| Measured allopurinol plasma concentration (mg/L) | 4.3 (0.9 – 14.0) |
| Measured oxypurinol plasma concentration (mg/L) | 3.7 (0.8 – 8.0) |
| **Neonates (n=55)** | |
| Birth weight (kg) | 3.22 (1.66 – 4.38) |
| Gestational age (weeks+days) | 40+1 (36+1 - 42+0) |
| Time between end of allopurinol administration and blood withdrawal (minutes) | 6 (1 – 44) |
| Measured allopurinol umbilical cord concentration (mg/L) | 3.7 (0.7 – 16.5) |
| Measured oxypurinol umbilical cord concentration (mg/L) | 0.9 (<LLQ[b] – 3.91) |

[a] values are expressed as median unless specified otherwise
[b]<LLQ = below lower limit of quantification, < 0.2 mg/L for allopurinol and oxypurinol

**Figure 8-2. Allopurinol concentrations in umbilical cord samples after intravenous maternal allopurinol administration (n = 55)**




In 58 mothers who received the full dose blood samples for the analysis of allopurinol and oxypurinol plasma concentrations were available as well as all relevant information (administration times, blood withdrawal times) (figure 8-1). Allopurinol and oxypurinol plasma concentrations in cord blood were available, and all relevant information was known in 55 neonates of the mothers that received the full dose. Characteristics of the studied women and neonates are displayed in table 8-1.

**Fetal drug disposition and transplacental transport**

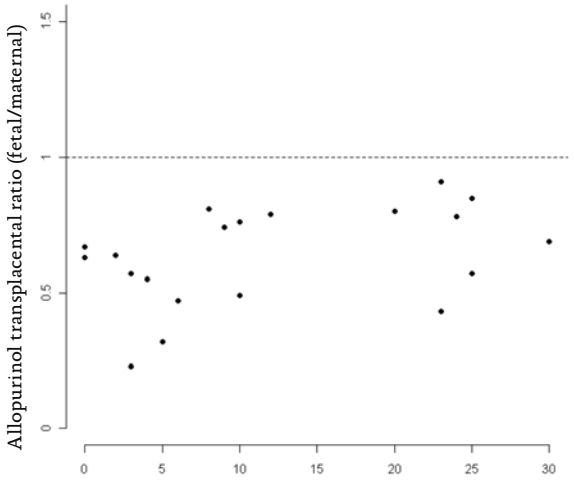
Within 5 minutes after the end of maternal allopurinol infusion target plasma concentrations of allopurinol were present in cord blood. Of all analyzed cord blood samples, 95% (52/55) had a target allopurinol plasma concentration of ≥2 mg/L at the time of delivery. No concentrations below the target concentration were found if the time interval between the end of allopurinol infusion and delivery was at least 7 minutes. Umbilical cord allopurinol plasma concentrations are illustrated in figure 8-2.

In 20 cases maternal and cord blood samples were drawn within half an hour of each other. The observed transplacental ratio (cord blood / mother) for allopurinol ranged from 0.23 to 0.91. In none of the cases the allopurinol concentration in cord blood exceeded the maternal allopurinol plasma concentration (for all cases the transplacental ratio was < 1). Transplacental ratios are shown in figure 8-3.

**Maternal and neonatal safety**

No adverse events were observed in the neonates in the allopurinol group and no serious adverse events or suspected unexpected serious adverse reactions occurred in the mothers who had received allopurinol. This is in line with the detected oxypurinol plasma levels in the maternal blood samples. Of the mothers who received allopurinol, 14% (8/58) had a target oxypurinol plasma concentration (≥5 mg/L for lowering

**Figure 8-3. Allopurinol transplacental ratio (fetal/maternal) after maternal allopurinol administration (n = 20)**




uric acid concentrations) corresponding with a target level for obtaining a hypo-uricemic effect (figure 8-4). These target concentrations were observed between 27 and 155 minutes after the end of the allopurinol administration. Five of the 111 women (4.5%) had a red and/or painful arm after the infusion of allopurinol, which recovered within several hours after the end of administration.

## Discussion

After administration of a single intravenous dose of 500 mg allopurinol to the mother, target plasma concentrations were observed in 95% of the umbilical cord samples at the moment of delivery. Since target plasma concentrations in umbilical cord samples were already observed within 5 minutes after the end of the allopurinol administration, maternal distribution and transplacental transport occur rapidly.
Allopurinol is most likely transferred to the fetus via passive transport since transplacental ratios of >1 were not found in our studied cases. An active transplacental transport seems unlikely given the consistency of this ratio.
A dose of 500 mg allopurinol seems to be a safe dose for both mother and fetus, since no adverse events were observed except some transient redness and pain around the IV cannula in 4.5% of the participating mothers. Allopurinol hypersensitivity was not observed in our patients.
In preclinical and clinical studies, allopurinol has shown to have neuroprotective properties. An animal study in chronically instrumented fetal sheep has shown evidence of cardio- and neuroprotection after antenatal allopurinol administration to the pregnant ewe during repeated periods of ischemia [31,32]. In a prospective randomized placebo controlled pilot study in which allopurinol was administered to the pregnant woman when fetal asphyxia was imminent, we found an inverse correlation between levels of allopurinol and the amount of S100ß, a biomarker for brain tissue damage, in cord blood [25].
In an animal model of asphyxia it has been shown that the production of reactive oxygen

species after ischemia and reperfusion peaks within 10-20 minutes after the end of the ischemia-reperfusion period, depending on the duration of the ischemic period [2]. Pre-ischemia administration of allopurinol to rats resulted in a decreased amount of superoxide anion radicals production during the reperfusion phase and suppressed the free radical peak [16]. These observations emphasize that allopurinol is immediately effective as a neuroprotectant and target levels should ideally be present right at the start of ischemia and reperfusion. The fact that allopurinol has a rapid transplacental passage favors its administration as soon as signs of fetal hypoxia occur.

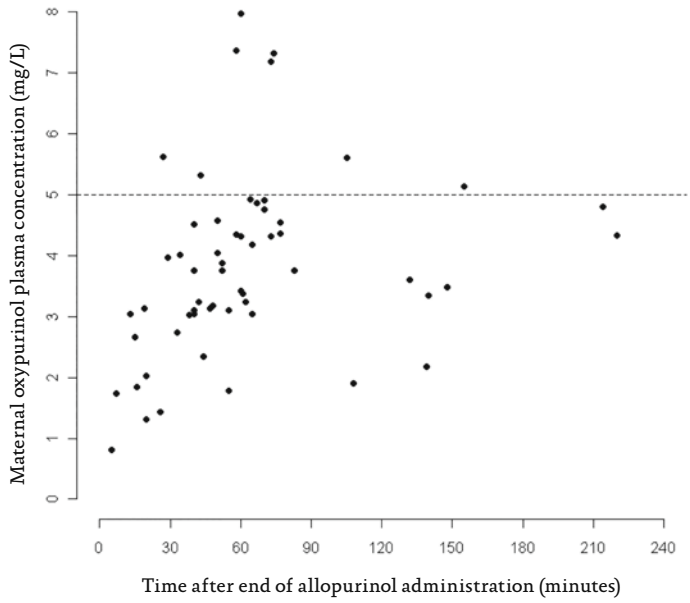
A previous study examining transplacental transport of 600 mg of allopurinol administered orally to women after the onset of labor showed target allopurinol levels in the cord blood of all 68 participating women, with a minimum time of 23 minutes after administration [26]. Corrected for bioavailability, an oral dose of 600 mg allopurinol is equal to about 500 mg of an intravenous dose. During labor gastric emptying is known to be delayed and laboring women are prone to vomiting, so oral administration of allopurinol may result in a delayed and unpredictable concentration in the fetus [33].

It seems, therefore, preferable to choose the intravenous administration route. The fact that in the present study target allopurinol levels were detected in cord blood within 5 minutes after the end of administration further emphasizes this point.

In one third of the participating women we did not succeed to administer the whole 500 mg dose of allopurinol before birth. In cases like these, an "escape" dose of allopurinol, administered via the umbilical cord at the resuscitation table, might be considered in future studies.

In conclusion, our study showed that intravenously administered allopurinol to the moth-

**Figure 8-4. Maternal oxypurinol plasma concentration after intravenous maternal allopurinol administration (n = 58)**



er, rapidly crosses the placenta, resulting in target neonatal allopurinol concentrations at birth and is safe to both mother and neonate, making this a promising approach for early fetal neuroprotective therapy during labor.

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# Chapter 9
# Maternal allopurinol administration during suspected fetal hypoxia; a novel neuroprotective intervention? A multicenter randomized placebo controlled trial (the ALLO-trial)

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*Submitted*

## ABSTRACT

**Objective**

To determine whether maternal allopurinol treatment during suspected fetal hypoxia would reduce the release of biomarkers associated with neonatal brain damage.

**Study design**

We performed a randomized double blind placebo controlled multicenter trial among laboring women with clinical indices of fetal hypoxia in 11 participating hospitals. Fetal hypoxia was diagnosed by an abnormal fetal heart rate, significant ST-changes on fetal ECG and/or fetal scalp pH<7.20. When immediate delivery was foreseen based on these symptoms, women were allocated to receive allopurinol 500 mg IV (ALLO) or placebo IV (CONT). Primary endpoint was S100ß in cord blood, a tissue-specific biomarker for brain damage. Analyses were performed according to the intention-to-treat principle. The difference in median (DiM) S100ß between the treatment groups was estimated with quantile regression. We also examined the difference in infants with values >75$^{th}$ percentile (>p75) between the groups, which is reported as Risk Ratios (RR).

**Results**

222 women were randomized to receive allopurinol (ALLO, n=111) or placebo (CONT, n=111). Cord S100ß was not significantly different between the two groups; 44.5 pg/mL (20.2 to 71.4) in the ALLO-group vs 54.9 pg/mL (26.8 to 94.7) in the CONT-group (DiM -7.69 (95%CI -24.9 to 9.52). The distribution of infants with an S100ß value >p75 was not significantly different either (ALLO, n=23 (23%)) vs CONT, n=25 (27%); $RR_{S100ß>p75}$ 0.85 (95%CI 0.53 to 1.4)). Post-hoc subgroup-analysis showed a marked gender difference in treatment effect of allopurinol in favor of girls. There were significantly less girls with an S100ß value >p75 in the ALLO-group (ALLO, n=5 (12%)) as compared to the placebo-treated group (CONT, n=10 (31%); $RR_{S100ß>p75}$ 0.37 (95%CI 0.14 to 0.99)). This difference was not found in boys (ALLO, n= 18 (32%) vs CONT, n=15 (25%); $RR_{S100ß>p75}$ 1.4 (95%CI 0.84 to 2.3)).

**Conclusions**

Maternal treatment with allopurinol during fetal hypoxia did not significantly lower neuronal damage markers in cord blood. Post-hoc analysis revealed a potential beneficial treatment effect in girls.

Hypoxic-ischemic encephalopathy is strongly associated with cerebral palsy and cognitive disability [1]. The prevention and treatment of fetal hypoxia is therefore an important priority in perinatal medicine. Animal and human studies have shown that brain damage not only occurs during hypoxic-ischemic events, but continues after reoxygenation and reperfusion due to production of free radicals [2].

At present, the only established treatment for hypoxic-ischemic encephalopathy in the clinical setting mild hypothermia, but only moderately asphyxiated infants seem to benefit with a small reduction of death and disability [3]. There is, therefore, a need for additional treatment options to prevent or reduce cerebral damage after perinatal asphyxia.

Allopurinol, a xanthine oxidase inhibitor and FDA-approved drug for gout, inhibits conversion of hypoxanthine into xanthine and uric acid, thereby limiting the excessive production of reactive oxygen species. Furthermore, at high dosages, the combined functions of chelating Non Protein Bound Iron (NPBI) and scavenging hydroxyl radicals might collectively result in a neuroprotective effect [4]. Substantial knowledge on the use, dosage, safety profile and placental transfer of allopurinol is already available, making it very interesting for translation to clinical use in perinatology. When administered postnatally to asphyxiated rats, allopurinol preserved cerebral metabolism and reduced both cytotoxic edema and perinatal hypoxic-ischemic brain damage [5]. Clinical trials in neonates at the NICU (neonatal intensive care unit), however, could not entirely confirm these results [6,7]. No advantage of neonatal treatment was seen in human infants when the interval to initiation of treatment had been too long or when brain damage was too severe [6,7]. It is therefore conceivable that advancing the window of treatment forward, by administering allopurinol to the mother and fetus during labor in case of suspected fetal hypoxia, may provide the opportunity to limit the generation of excess free radical formation and subsequent reperfusion injury in the infant.

A recent study in chronically instrumented fetal sheep provided evidence of cardiac and neuroprotection following antenatal allopurinol administration to pregnant ewes during repeated periods of fetal ischemia [8,9]. Furthermore, a randomized placebo controlled pilot study in which we administered allopurinol to pregnant women with imminent fetal asphyxia, showed an inverse correlation between allopurinol levels and S100ß, a clinically used biomarker for brain tissue damage, in cord blood [10].

In the present multicenter randomized placebo controlled clinical trial we evaluated our hypothesis that maternal allopurinol treatment during fetal hypoxia reduces markers of neonatal brain damage in cord blood.

## Methods

### Recruitment / Eligibility criteria

This study was conducted at the delivery wards of 11 hospitals participating in the Dutch Consortium for Studies in Women's health and Reproductivity, as a double blind placebo controlled randomized trial. Pregnant women with a gestational age ≥ 36 weeks and suspected intra-uterine fetal hypoxia, prompting immediate delivery, were eligible to participate in the trial. Women bearing a child suspected of chromosomal or congenital anomalies were not eligible. Informed consent was obtained antenatally at the outpatient clinic or at the labor ward. Randomization only occurred if the clinician decided to end labor and deliver the child immediately because of the suspicion of fetal hypoxia (e.g. an abnormal or non-reassuring fetal heart rate trace (according to FIGO-criteria) [11], significant ST-wave abnormalities (detected by the STAN-monitor) [12] or abnormal fetal blood scalp sampling (pH < 7.20).

### Intervention

Women were randomly assigned in a double-blind fashion to receive either a single in-

travenous dose of 500 mg allopurinol (ALLO) in 50mL water for injection (Acepurin®, AcePharmaceuticals) or placebo (CONT, 500mg Mannitol/50mL water for injection) administered, in 10 minutes, immediately prior to delivery. The dose of allopurinol was based on a study in healthy pregnancies [13] and on our pilot-study [10]. This dose of 500 mg is the maximum dosage approved in adults as a single intravenous injection, as stated in the SPC of the product [14].
AcePharmaceuticals (Zeewolde, the Netherlands) prepared the study drugs and generated the random allocation sequence in blocks of four (2:2), using a computer program ("RandList", version 1.2 (Windows XP), clinical trial version) with stratification according to participating hospitals. Every site was supplied with sequentially numbered study drug packages, labeled in a blind manner. Allocation was performed by an attending nurse who administered the contents of the first in line sequentially numbered study drug package, as soon as a consenting patient met the inclusion criteria. The nurse carrying out the study-protocol was not directly involved in the care of the woman in labor to ensure no delay in delivering the child.
Study drugs were stored at 20-25°C and dissolved immediately before administration. Allopurinol and placebo had exactly the same appearance; both were freeze-dried as white powder in glass vials with a pH of 11 after dilution. Participants, care providers and those who assessed outcomes remained blinded for allocation until the statistical analysis of the primary outcome parameter.

**Outcome parameters**

Primary outcome measure was S100ß in cord blood, an extensively studied biochemical marker for brain-tissue damage and subsequent neurodevelopmental disabilities [15-17]. S100ß is present in high concentrations in glial cells and Schwann cells. During a hypoxic-ischemic insult, this protein is released from damaged tissue in the systemic circulation. Several studies have shown that elevated plasma levels of S100ß are directly related to the development of hypoxic-ischemic encephalopathy [15-17].

Secondary outcome parameters were indirect markers of free radical production (8-isoprostane, neuroketal), neonatal mortality and short-term morbidity. Isoprostanes and neuroprostanes are prostaglandin-like compounds produced by free radical-induced peroxidation of arachidonic acid and docosahexaenoic acid respectively, which are highly enriched in the brain [18,19]. As a next step highly reactive αketoaldehydes (neuroketals) are formed as products of free radical-induced peroxidation of neuroprostanes [18,20].

**Data collection**

Fetal venous cord blood and maternal blood samples were obtained immediately after birth. To assess the severity of fetal hypoxia, venous cord lactate and arterial cord pH were measured at the participating site. Blood samples for assessment of brain damage markers, free radical markers and plasma concentrations of allopurinol and its active metabolite oxypurinol were collected in heparinized tubes and centrifuged at 3000 rpm/10 minutes within 30 minutes after collection. The supernatant was stored at a temperature of -70°C until batch analysis at the end of the recruitment period.
All details of delivery and neonatal admission were recorded by trained research nurses and midwives at the participating site in a case record form that was accessible through a website (www.studies-obsgyn.nl/allo). Data were collected using Oracle Clinical Remote Data Capture (RDC, http://www.ctc-g.co.jp/~CTCLS/opa/en/).

**Laboratory techniques**

*Brain damage markers*

Brain damage markers were analyzed by Haemoscan (Groningen, the Netherlands). The test for detecting S100ß is based on sandwich formation of immobilized anti-S-100 antibody, S100ß from the test sample and biotin-labeled

S100ß antibody. Label is measured after streptavidin-HRP binding and subsequent conversion of *o*-phenylenediamine (OPD) (Abnova, Taipei, Taiwan). 8-Isoprostane was determined by means of a competitive enzyme immunoassay (Cayman, Ann Arbor, MI). Neuroketals were measured by immunoassay (EIA) by competition with a specific antibody for binding to neuroketal coated microtiter plates (Haemoscan, Groningen, The Netherlands).

*Allopurinol / oxypurinol*

Allopurinol (ALLO) and oxypurinol (OXY) plasma concentrations were determined by the laboratory of clinical pharmacology in the University Medical Center Utrecht using reversed-phase high-performance liquid chromatography with UV-detection at 254 nm for quantification of ALLO and OXY in plasma. The method was linear between 0.5 and 25 mg/l with a lower limit of quantification of 0.2 mg/l for both compounds [21].

**Statistical methods**

*Sample size*

The sample size was calculated based on the primary outcome measure cord S100ß as a marker of brain damage. Based on our pilot study we expected mean values of S100ß to be lower in the 'ALLO-treated' group compared to the 'non-treated group'. Given a suspected mean value of S100ß of 1.05 ug/L (SD 0.37 ug/L) in the placebo group [10], this trial needed 220 patients (110 ALLO, 110 placebo) to detect a mean value of S100ß of 0.89 ug/L (SD 0.37 ug/L) in the 'ALLO-treated' group (z- $test_{2\text{-sided}}$) with a power of 90% and an alpha of 0.05.

*Data analysis*

An independent data safety monitoring committee monitored the trial and reviewed interim results. An interim analysis was performed after including 110 women using O'Brien –Fleming alpha spending function [22]. This analysis was done by an independent person who was unaware of the allocation of treatment or placebo when judging the data on significant differences in neonatal mortality, neonatal NICU-admission and serious adverse events. At interim analysis a nominal value p-value < 0.005, and at the final analysis a nominal p-value < 0.049 was considered to be statistically significant.

All analyses were based on the intention-to-treat principle. We compared the primary outcome, in women exposed to allopurinol as compared with placebo. Since the distribution of S100ß was right-skewed, we calculated a difference in medians (DiM) with 95% confidence intervals (95% CIs) by using linear quantile mixed models. To account for the stratified randomization, the analysis was adjusted for center by fitting a random intercept for each center.

For continuous outcomes that were normally distributed with or without log-transformation, linear mixed models were used to calculate difference in means or difference in geometric means, respectively. Differences in dichotomous outcomes were assessed using a log-binomial mixed model to estimate a risk ratio (RR) with 95% CI, while accounting for the stratified randomization. All statistical analyses were conducted in R for Windows, version 2.15.0.

*Post-hoc analyses*

Three post-hoc analyses were performed. Because both S100ß and neuroketal approached physiological values, we examined the effect of allopurinol in infants with S100ß- and neuroketal values > p75. We also performed a subgroup analysis in infants with a cord pHa < 7.15. Finally, because of gender-specific treatment effects in previous studies investigating neuroprotective strategies [23-26], we performed a post-hoc subgroup analysis based on the sex of the infant. The treatment effect was investigated using an interaction term between the treatment and gender in the regression model. When the interaction term was significant (p < 0.05), the treatment effect was also estimated in boys and girls separately.

## Results

From October 2009 through December 2011, 222 eligible mothers and their 224 infants were enrolled in the trial. We randomly allocated 111 women (two had a dichorionic twin pregnancy) to the ALLO-group and 111 to the placebo (CONT) group (figure 9-1). One mother with a twin pregnancy received the medication before birth of both children. In this case, no blood sampling was performed due to logistic problems. The other mother bearing a twin pregnancy received the drug after the delivery of the first child. Only cord blood of the second child was obtained for analysis of the primary outcome.

Of all participants 98 (87%) and 92 (83%) cord blood samples were collected in the ALLO-group and CONT-group respectively to assess the primary (S100ß) and secondary outcome parameters (neuroketal, 8-isoprostane and allopurinol levels) (figure 9-1). Missing cord blood samples,

**Figure 9-1. Enrollment, Randomization and Follow-up of Study Participants**

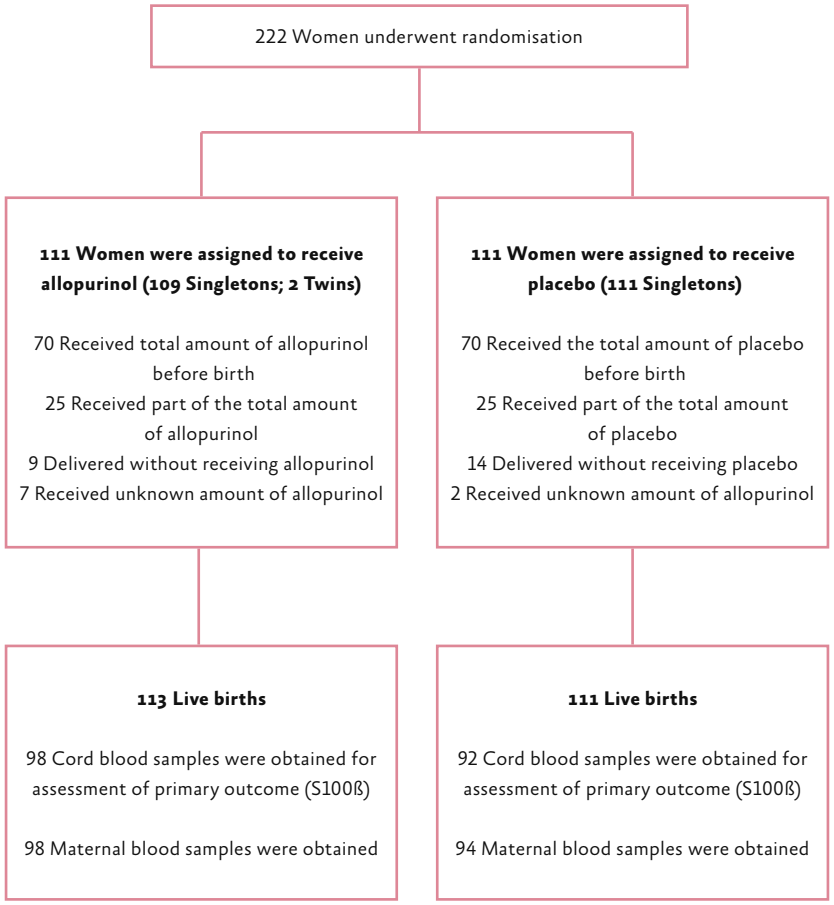


**Table 9-1. Baseline Characteristics**

| | ALLO N=111 | CONT N=111 |
|---|---|---|
| Maternal Age, mean ± SD | 32.6 ± 4.9 | 32.7 ± 4.3 |
| Gestational Age, median (IQR) | 40.1 (38.6-41.0) | 40.6 (39.1-41.3) |
| Nulliparity, n (%) | 84 (76) | 82 (74) |
| Multiple gestation, n (%) | 2 (1.8) | 0 (0) |
| Diabetes Mellitus / Gestational n (%) | 12 (11) | 9 (8) |
| PIH / PE, n (%) | 33 (30) | 22 (20) |
| Smoking, n (%) | 10 (9) | 10 (9) |
| Termination of labor based on* | | |
| Abnormal fetal heart rate trace, n (%) | 103 (93) | 103 (93) |
| Significant ST-wave abnormalities, n (%) | 24 (22) | 33 (30) |
| Fetal blood scalp sampling with pH < 7.20, n (%) | 15 (14) | 22 (20) |
| Time between infusion and delivery, median (IQR) | 14.0 (7.3-26.8) | 11.5 (7.0-28.5) |
| Tocolytics administered, n (%) | 76 (68) | 84 (76) |
| | | |
| Infants† | 113 | 111 |
| Mode of delivery | | |
| Spontaneous, n (%) | 18 (16) | 11 (10) |
| Instrumented vaginal delivery, n (%) | 44 (39) | 57 (52) |
| Cesarean Section, n (%) | 49 (43) | 42 (38) |
| Male gender child, n (%) | 66 (58) | 72 (65) |
| Birth weight (gram), mean ± SD | 3217 ± 567 | 3329 ± 536 |

ALLO: Allopurinol group; CONT: Placebo group; SD: Standard Deviation; IRQ: interquartile range; PIH: Pregnancy Induced Hypertension; PE: Preeclampsia. * Numbers for this variable exceed the total number of included women, since some patients had more than one reason to terminate labor. † The ALLO-group contained two dichorionic twin pregnancies.

and subsequent missing values of brain damage markers, were mainly due to logistic problems (no appropriate blood sampling material present or a prolonged (> 30 min) time between blood sampling and centrifuging the blood).

**Clinical outcome measures**

Baseline characteristics of the groups were comparable (table 9-1). Overall more boys (61.6%) than girls (38.4%) were included in the trial. Boys and girls were equally distributed between the two groups.

There were no significant differences in Apgar score, umbilical artery pH and Base Excess, or hypoglycemia between the groups (table 9-2). The mean umbilical artery pH in the ALLO- and CONT-group was 7.19 ± 0.084 and 7.19±0.080 respectively. None of the children developed convulsions or hypoxic-ischemic encephalopathy, and there were no differences between the two groups in the amount of NICU-admissions (ALLO n=11 (10%) vs CONT n= 9 (8%) (table 9-2)).

**Table 9-2. Neonatal Data as a Function of Treatment Group**

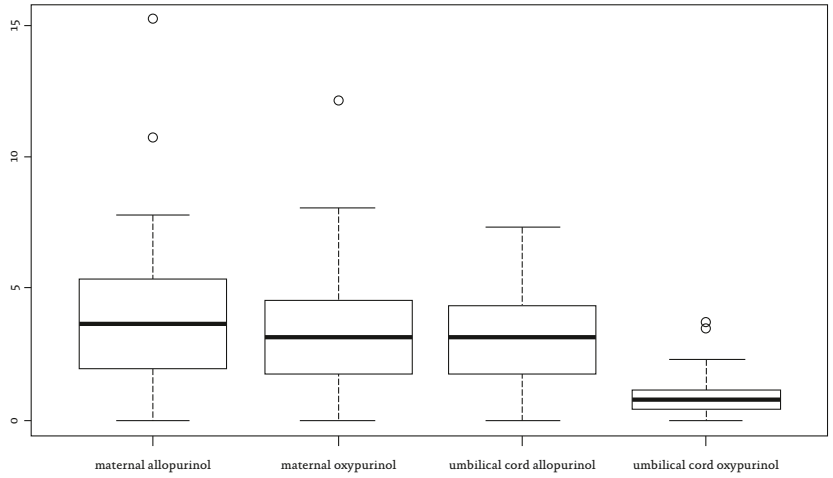
| | ALLO N=113* | CONT N=111 | Risk Ratio (95% CI)† ALLO vs CONT |
|---|---|---|---|
| Apgar score < 7 at 5 min, n (%) | 8 (7.2) | 6 (5.4) | 1.33 (0.48 to 3.73) |
| Umbilical artery pH, mean ± SD | 7.19 ± 0.084 | 7.19 ± 0.080 | -0.002 (-0.025 to 0.021)§ |
| Umbilical artery BE, mean ± SD | -6.7 ± 3.9 | -6.8 ± 3.8 | 0.043 (-0.95 to 1.03)§ |
| Umbilical artery pH (< 7.05) and BE (< -12) , n (%) | 3 (2.7) | 1 (0.9) | 3.00 (0.31 to 28.7) |
| NICU admission, n (%) | 11 (10) | 9 (8) | 1.22 (0.52 to 2.86) |
| Length of stay > 2 days, n (%) | 3 (3) | 4 (4) | 0.75 (0.17 to 3.30) |
| Hypoglycemia, n (%) | 18 (16) | 16 (15) | 1.13 (0.60 to 2.10) |
| Convulsions, n (%) | 0 (0) | 0 (0) | NA |
| Hypoxic Ischemic Encephalopathy, n (%) | 0 (0) | 0 (0) | NA |

ALLO: Allopurinol group; CONT: Placebo group; CI: Confidence Interval; SD: Standard Deviation; IRQ: interquartile range; BE: Base Excess; NICU: Neonatal Intensive Care Unit. * Two dichorionic twin pregnancies. † Associations are reported as risk ratios, unless stated otherwise; § Mean difference (95% CI).

**Maternal and cord allopurinol and oxypurinol concentrations**

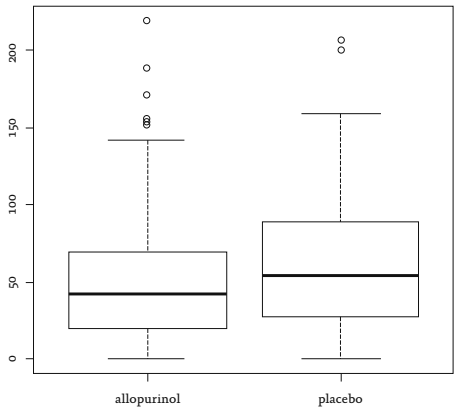
The median total dose of the study drug received by women before delivery in the ALLO-group was 500 mg (interquartile range (IQR) 370 to 500 mg). Time from start of maternal administration of ALLO or placebo to birth ranged from 0 to 59 minutes (median 14 minutes (IQR

**Figure 9-2. Maternal allopurinol/oxypurinol and cord allopurinol/oxypurinol concentrations (mg/L) at birth**




Data shown as box-whisker plots (presented as median and IQR).

**Figure 9-3. Umbilical Cord S100ß levels**




Cord concentrations of the primary outcome measure S100ß (pg/mL) in the allopurinol-treated and placebo-treated groups shown as box-whisker plots (presented as median and IQR).

7.3 to 27 minutes)) and 0 to 99 minutes (median 12 minutes (IQR 7.0 to 29 minutes)) respectively. There were 73 (33%) cases where the mother did not receive the total amount of study medication, mostly due to fast delivery of the child. Maternal and umbilical cord ALLO and OXY concentrations are shown in figure 9-2. Maternal ALLO and OXY concentrations ranged between 0 µg/mL and 15 µg/mL (median: 3.9 (IQR 2.1 to 5.5) µg/mL) and between 0 µg/mL and 8.0 µg/mL (median: 3.2 (IQR 2.1 to 4.5) µg/mL), respectively. Umbilical cord concentrations of ALLO and OXY ranged between 0 µg/mL and 16 µg/mL (median: 3.3 µg/mL) and between 0 µg/mL and 6.8 µg/mL (median: 0.82 µg/mL) respectively. Target blood levels of ALLO (≥ 2.0 µg/ml) in cord blood were reached in 64 (72%) of the measured samples.

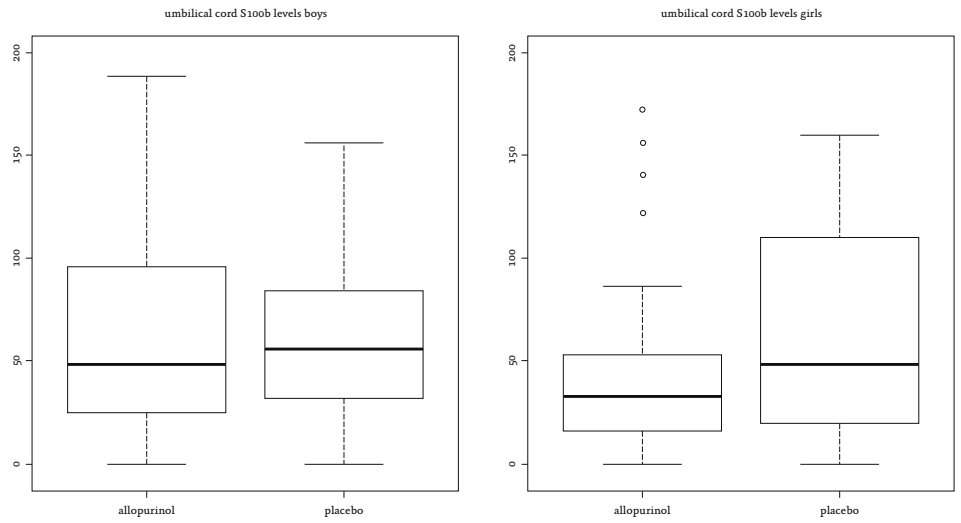
**Cord S100ß, Neuroketal and 8-isoprostane concentrations**

The primary outcome measure S100ß did not significantly differ between the two groups; 44.5 pg/mL (20.2 to 71.4) in the ALLO-group vs 54.9 pg/mL (26.8 to 94.7) in the CONT-group (DiM -7.69 (95%CI -24.9 to 9.52, figure 9-3), nor did 8-isoprostane and neuroketal (table 9-3). Since S100ß and neuroketal approached physiological values, we also examined the distribution of infants with values >p75 between the two groups (table 9-3). The number of infants with an S100ß-value >p75 did not differ between the ALLO- and placebo group (ALLO, n=23 (23%) vs CONT, n=25 (27%); ($RR_{S100ß>p75}$ 0.85 (95%CI 0.53 to 1.4)). This also applied for neuroketal (ALLO, n= 22 (23%) vs CONT, n=24 (27%); ($RR_{neuroketal>p75}$ 0.85 (0.51 to 1.4)). Post-hoc subgroup-analysis indicated a significant treatment-interaction for infants with an arterial cord pH < 7.15 for neuroketal-values >p75. Stratified analysis showed a treatment effect in this group, in which significantly fewer infants had neuroketal-values >p75 in the ALLO-group (n=3 (14%)) as compared to the placebo-group (n=11 (46%); p=0.033). No interactions were found for S100ß or 8-isoprostane.

**Post-hoc analysis of gender differences**

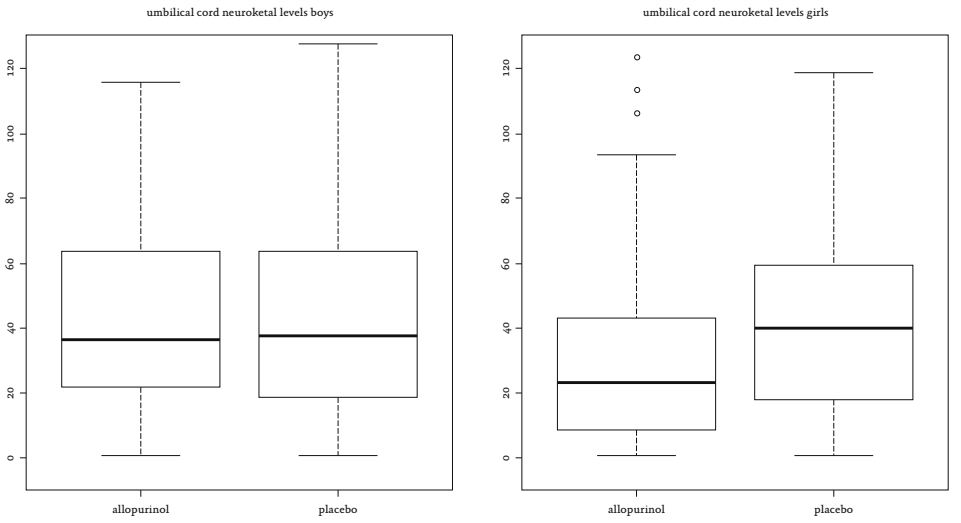
Post-hoc subgroup-analysis showed a significant treatment-gender interaction for S100ß >p75 (p=0.049) and neuroketal (p=0.027). Stratified analysis indicated a marked gender difference in treatment effect of allopurinol in favor of girls for both S100ß and neuroketal (table 9-3, figure 9-5). There were significantly less girls with an S100ß value >p75 in the ALLO-group as compared to the CONT-group (n=5 (12%) vs n=10 (31%); $RR_{S100ß>p75}$ 0.37 (95%CI 0.14 to 0.99), table 9-3). This difference was not found in boys (ALLO, n=18 (32%) vs CONT, n=15 (25%); $RR_{S100ß>p75}$ 1.4 (95%CI 0.84 to 2.3)). Furthermore, the mean neuroketal value in cord blood of allopurinol-treated girls was significantly lower than in placebo-treated girls (ALLO, 18.0 pg/mL (12.1 to 26.9) vs CONT, 32.2 pg/mL (22.7 to 45.7); GMD -16.4 (95%CI -24.6 to -1.64), table 9-3, figure 9-5). Again, this difference was not found in boys (ALLO, 32.4 pg/mL (24.4 to 43.1) vs CONT, 32.1 pg/mL (24.7 to 41.8); GMD 2.38 (95%CI -10.3 to 21.3))

**Figure 9-4. Gender Specific Cord Concentrations of S100ß (pg/mL)**




Data are shown as box-whisker plots (presented as median and IQR).

**Figure 9-5. Gender Specific Cord Concentrations of Neuroketal (pg/mL)**




Data are shown as box-whisker plots (presented as median and IQR).

**Serious Adverse Events and Suspected Unexpected Severe Adverse Reactions**

One Serious Adverse Event (severe postpartum hemorrhage resulting in IC-admission of the mother) and one Suspected Unexpected Severe Adverse Reaction (anaphylactic reaction after the administration of study medication and preoperative medication prior emergency Cesarean section) were reported during the study, both for patients in the placebo-group.

## Discussion

In this trial, maternal administration of allopurinol during suspected fetal hypoxia did not significantly lower brain damage markers (S100ß, neuroketal and 8-isoprostane) in cord blood. Post-hoc analysis, however, revealed a potential neuroprotective effect in girls as indicated by the lower S100ß and neuroketal values in the treatment group. There is increasing evidence that gender difference plays a role in the effectiveness of pharmacologic neuroprotection after perinatal ischemia-reperfusion [27]. This gender difference in treatment effect might be explained by different pathways for programmed cell-death or by hormonal influences [23-26].

This was the first multicenter randomized clinical trial that evaluated whether a maternally administered neuroprotective drug during *acute* fetal hypoxia could reduce neuronal damage markers in cord blood. The primary outcome, S100ß, is a surrogate measure of brain damage. Whereas clinical outcome parameters would have provided us with more optimal answers to our hypothesis, the used biomarker has extensively been investigated and has proven to be directly related to the amount of neuronal damage and clinical outcome [15-17,28]. A follow-up study on neurodevelopmental outcome at 2 years of age using ASQ- en CBCL-questionnaires is currently running.

Despite the complexity of the study, we were able to obtain 86% of all cord samples. Levels of cord S100ß measured in this trial were slightly lower than those obtained in the pilot study [10]. This can be explained by the fact that perinatal hypoxia was less severe in our multicenter trial as compared to the pilot study (mean pHa Pilot: 7.16 and mean lactate Pilot 8.0 mmol/L; mean pHa RCT 7.19 and mean lactate RCT 6.9 mmol/L) [10]. The total group of infants with imminent fetal hypoxia during labor, including the non-randomized patients, in participating hospitals had a mean cord pHa of 7.15, confirming the relatively mild hypoxia in our studied population. The treatment effect of allopurinol might potentially be larger among fetuses with more severe fetal hypoxia. This is partly confirmed by the post-hoc analysis we performed in infants with an arterial pH lower than 7.15, in which we found significantly less infants with high neuroketal values in the ALLO group, compared to the placebo group.

In contrast to postnatal neuroprotection of infants with proven asphyxia, the present trial recruited mothers with *suspected* fetal hypoxia. Fetal surveillance during labor remains difficult and is often false positive in detecting fetal hypoxia, which might result in substantial over-treatment. However, although the arterial pH of the infants was within the normal range at birth, elevated lactate levels did suggest there had been earlier periods of fetal hypoxia. A finding that is supported by data from Derks *et al*, in which arterial cord pH of fetal sheep restored earlier than lactate levels in cord blood, after a period of controlled asphyxia [8]. Finally, our study was not designed to examine the difference in treatment effect between genders, which limits the strength of our conclusions.

Allopurinol remains promising as a neuroprotective drug in perinatology. Our recent follow-up study of two small prospective randomized controlled trials in neonates, investigating effects of postnatal allopurinol in asphyxiated neonates, showed beneficial effects of allopurinol on mortality and severe disabilities at 4-8 years of age in moderately asphyxiated neonates [29]. Furthermore, maternal allopurinol treatment helped to maintain umbilical blood

**Table 9-3. Important Chemical Markers Umbilical Blood**

| | ALLO (n=98) | CONT (n=92) |
|---|---|---|
| General blood measures, umbilical cord | | |
| Uric Acid, mean ± SD | 0.34 ± 0.076 | 0.35 ± 0.096 |
| Lactate, mean ± SD | 6.55 ± 2.45 | 7.14 ± 2.91 |
| Troponin, geometric mean (IQR) | 0.017 (0.013 to 0.021) | 0.018 (0.014 to 0.024) |
| Biomarkers brain damage, umbilical cord | | |
| S100ß in pg/ml, median (IQR) | 44.5 (20.2 to 71.4) | 54.9 (26.8 to 94.7) |
| S100ß girls | 32.8 (15.3 to 53.3) | 48.4 (20.3 to 109.1) |
| S100ß boys | 48.6 (25.2 to 96.6) | 31.8 (14.4 to 83.0) |
| S100ß > 75th percentile (85 pg/ml), n (%) | 23 (23) | 25 (27) |
| S100ß girls, n (%) | 5 (12) | 10 (31) |
| S100ß boys, n (%) | 18 (32) | 15 (25) |
| Neuroketal in pg/ml, geometric mean (95% CI) | 25.2 (19.8 to 32.1) | 32.1 (26.1 to 39.6) |
| Neuroketal girls | 18.0 (12.1 to 26.9) | 32.2 (22.7 to 45.7) |
| Neuroketal boys | 32.4 (24.4 to 43.1) | 32.1 (24.7 to 41.8) |
| Neuroketal > 75th percentile (62.5 pg/ml), n (%) | 22 (23) | 24 (27) |
| Neuroketal girls, n (%) | 7 (17) | 8 (26) |
| Neuroketal boys, n (%) | 15 (27) | 16 (28) |
| Isoprostane in pg/ml, geometric mean (95% CI) | 128.4 (108.0 to 152.7) | 117.1 (93.8 to 146.3) |

ALLO: Allopurinol group; CONT: Placebo group; CI: Confidence Interval; SD: Standard Deviation; IRQ: interquartile range.
* The difference in medians was estimated using linear quantile mixed models. Quantile regression was used because for S100ß the distribution of S100ß was right-skewed and could not be normalized using e.g. a log-transformation. † P < 0.05.

flow and reduced fetal cardiac oxidative stress after ischemia-reperfusion in fetal sheep [8]. Fetal sheep treated with allopurinol also had significantly less neuronal damage in the hippocampal brain area [9]. All results from previous research indicate cardio- and neuroprotective effects of allopurinol administered to neonates or fetuses during or after perinatal hypoxia [5,6,8-10,29,30]. Although the present trial could only confirm (potential) neuroprotection in girls, it is remarkable that even in these very mildly hypoxic fetuses a treatment effect of allopurinol might have been revealed. Whether the neuroprotective effect is really gender specific with a direct effect of allopurinol on the brain or secondary to a cardioprotective effect remains to be elucidated.

In conclusion, our study indicates that allopurinol is safe and potentially effective in preventing hypoxic damage to the brain of newborn girls. The latter, however, is based on post-hoc

| Difference in mean (95%CI) ALLO vs CONT | Difference in geometric mean (95% CI) ALLO vs CONT | Difference in median (95% CI)* ALLO vs CONT | Risk Ratio (95% CI) ALLO vs CONT |
|---|---|---|---|
| | | | |
| -0.003 (-0.026 to 0.021) | | | |
| -0.60 (-1.35 to 0.16) | | | |
| | -0.001 (-0.004 to 0.003) | | |
| | | | |
| | | | |
| | | -7.69 (-24.9 to 9.52) | |
| | | -12.5 (-50.0 to 25.0) | |
| | | -3.10 (-21.1 to 14.9) | |
| | | | 0.85 (0.53 to 1.36) |
| | | | 0.37 (0.14 to 0.99) † |
| | | | 1.38 (0.84 to 2.28) |
| | | | |
| | -7.53 (-15.5 to 3.62) | | |
| | -16.4 (-24.6 to -1.64) † | | |
| | 2.38 (-10.3 to 21.3) | | |
| | | | 0.85 (0.51 to 1.41) |
| | | | 0.66 (0.27 to 1.63) |
| | | | 0.99 (0.54 to 1.81) |
| | | | |
| | 6.5 (-23.7 to 47.1) | | |

analyses and should be more intensively investigated before being implemented in perinatal medicine. Furthermore, future research should combine fetal allopurinol treatment with postnatal neuroprotective interventions if the newborn infant appears to be really asphyxiated at birth (i.e. Apgar score < 7 at 5 minutes, pH umbilical artery < 7.05). Alternatively, in case of unexpected birth asphyxia, allopurinol treatment via the umbilical cord at the resuscitation table might result in a more optimal selection of infants needing treatment. This approach bypasses any placental problems, which may result in suboptimal placental transfer of allopurinol. And finally, any future research focusing on perinatal neuroprotection should always take gender differences into account.

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**Trial registration**
Clinical Trials protocol registration system (NCT00189007), Dutch Trial Register (NTR1383).

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**Study Protocol**
The study protocol was published [31].

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# Chapter 10
General discussion

In this thesis we have investigated the use of the xanthine oxidase inhibitor allopurinol as a neuroprotective and cardioprotective agent during acute fetal hypoxia in three different species. This current chapter will discuss and summarize the results of our studies that focused on the pharmacological, neuroprotective and cardiovascular effects of allopurinol on the fetus in the late gestational rat, sheep and human pregnancy.

As explained in more detail in **Chapter 1 to 3**, current postnatal (pharmacologic) treatment of birth asphyxia-related damage to the newborn brain has a limited neuroprotective effect, even when therapy has been started within the "window of opportunity", being within 6 hours after birth. This might be due to the fact that maximal formation of reactive oxygen species (ROS) occurs within the first 30-to-60 minutes after reperfusion and reoxygenation. Moreover, already during fetal hypoxia substantial amounts of ROS are being produced.

The FDA-approved drug allopurinol is known to inhibit the formation of ROS, thereby potentially limiting free radical induced hypoxic ischemic encephalopathy. Our randomized clinical trial (ALLO trial), described in **Chapter 7** and **Chapter 9,** investigated whether biomarkers for brain damage would be reduced after complicated hypoxic labor if the window of treatment with allopurinol would be initiated earlier, via maternal treatment, to cover the actual period of fetal hypoxia and reperfusion. Further research conducted in this thesis investigated the mechanisms of action of allopurinol in terms of xanthine oxidase inhibition (**Chapter 4** and **5**), placental transfer (**Chapter 8**) and perinatal outcome after maternal (**Chapter 6** and **9**) or postnatal (**Chapter 3**) allopurinol treatment.

Adequate placental passage of allopurinol is necessary to obtain therapeutic fetal plasma levels and subsequent potential beneficial effects on the fetus. Furthermore, the route and timing of administration, and dosage of the drug are important to obtain therapeutic levels of allopurinol at the start of reperfusion and reoxygenation, without having major negative side effects on both mother and fetus. Since allopurinol is not registered for neuroprotection during perinatal hypoxia it is difficult to define therapeutic levels of allopurinol for this specific indication. It is however known that certain plasma levels of allopurinol are able to inhibit xanthine oxidase (XO), which may result in lower levels of circulating ROS and free radicals, and subsequently less tissue damage [1-4].

Previous reports have confirmed that maternal treatment with allopurinol crosses the ovine [1,2], porcine [5] and human [6,7] placenta to achieve fetal plasma concentrations of allopurinol and its active metabolite oxypurinol. Our rat model showed that treatment with 30 $mg.kg^{-1}$ allopurinol significantly suppressed XO-activity two hours after oral administration for at least 24 hours in all tissues examined with fetal allopurinol and oxypurinol plasma levels of 2.1 $\mu g\ ml^{-1}$ and 4.0 $\mu g\ mg^{-1}$ respectively (**Chapter 4**). Although higher doses of oral allopurinol did cause higher blood levels of both allopurinol and oxypurinol, they did not result in significantly more XO inhibition but only make the occurrence of adverse side effects of the drug more likely (**Chapter 5**). Peak plasma-levels of allopurinol between 4 and 7 $\mu g\ ml^{-1}$ were found in the fetal sheep in our sheep model after the intravenous administration of 20 $mg.kg^{-1}$ allopurinol to the pregnant ewe and were maintained past 1 h after the start of maternal administration [1]. These allopurinol levels led to improved cardiovascular function (less fetal cardiac workload during and immediately after asphyxia) [1] and reduced neuronal damage in the hippocampal area in the treated sheep fetuses (**Chapter 6**).

Placental transfer in pregnant women was first examined by Boda *et al*, who found allopurinol levels of more than 7 $\mu g\ ml^{-1}$ in cord blood al-

ready 23 minutes after oral allopurinol administration of 600 mg to women going through uncomplicated labor [6]. In a clinical pilot study, performed by our group, median allopurinol levels of 2.0 μg $ml^{-1}$ (range 0.2-7.3 μg $ml^{-1}$) were seen in the cord blood of infants, 56 minutes (range 18 to 190 minutes) after a single intravenous dose of 500 mg allopurinol to the mother when suspicion of fetal hypoxia prompted immediate delivery [7]. Furthermore, an inverse correlation between the levels of allopurinol and oxypurinol and S100ß (an important tissue marker for neuronal damage) in cord blood was found. Infants with an allopurinol level ≥ 2 μg $ml^{-1}$ in their cord blood had significantly lower S100ß values than placebo treated infants. In our subsequently performed randomized clinical trial (RCT), in 95% of all measured cord blood samples an allopurinol plasma concentration of ≥ 2 μg $ml^{-1}$ was measured at the time of delivery after maternal allopurinol administration of 500mg intravenously (~6mg.$kg^{-1}$) (**Chapter 8**). No allopurinol concentrations lower than 2 μg $ml^{-1}$ were found if the time interval between the end of allopurinol infusion and delivery was at least 7 minutes. In this multicenter RCT, however, these values did not significantly lower brain damage markers (S100ß, neuroketal and 8-isoprostane) in cord blood (**Chapter 9**), probably due to the fact that the extent of hypoxia was relatively mild in the recruited group of patients.

In summary all performed studies investigating maternally administered allopurinol show a high correlation between paired maternal and fetal concentrations of allopurinol and oxypurinol, independent of time of administration or dose. These data indicate that placental transfer of allopurinol is fast, reliable and consistent in all species examined. Fetal levels of allopurinol and oxypurinol were always lower than maternal levels, reflecting a potential passive transfer across the placenta.

Collectively, allopurinol remains of interest as a neuroprotective drug in perinatology. Our follow-up study of two small prospective randomized controlled trials in neonates (described in **chapter 3**) investigating effects of postnatal allopurinol in asphyxiated neonates, showed beneficial effects of allopurinol on mortality and severe disabilities at 4-8 years of age in moderately asphyxiated neonates [8]. Beneficial effects of maternal treatment with allopurinol are supported by its protective effects on umbilical blood flow [1,9], and on the fetal heart [1] and fetal brain [7,10] (**Chapter 6**) during and following periods of ischemia and reperfusion. No adverse effects were seen in the treated women or human infants after allopurinol administration in all performed studies (**Chapter 3**, **8** and **9**) [8,10].

There are, however, also some drawbacks to the use of allopurinol in clinical perinatology. In our randomized clinical trial, maternal administration of allopurinol during suspected fetal hypoxia did not significantly lower brain damage markers (S100ß, neuroketal and 8-isoprostane) in cord blood (**Chapter 9**). Fetal surveillance during labor remains difficult and is often false positive in detecting fetal hypoxia, which might result in substantial over-treatment. Furthermore, although no adverse effects were seen in the mothers and infants in all clinically performed research on allopurinol, there are some potential adverse effects. Maternal treatment with high doses of allopurinol resulted in impairing fetal peripheral vascular reactivity to constrictor agonists [9] and on the fetal cardiovascular defense to acute hypoxia in fetal sheep (**Chapter 5**). These effects, however, were only seen after the administration of very high doses of allopurinol (100 mg/kg), which is 5 times higher than the highest clinically used dose. Finally the xanthine oxidase pathway is not the only pathway responsible for the production of reactive oxygen species. Several other pathways may promote oxidative stress, such as described in **Chapter 2**. A combined therapeutic approach using drugs that influence different parts of the neurotoxic cascade might therefore further improve the outcome of antioxidant therapy [11].

## Gender differences

Post-hoc analysis of the multicenter RCT did reveal a potential neuroprotective effect in girls as indicated by the lower S100ß and neuroketal values in the treatment group. There is increasing evidence that gender differences play a role in the effectiveness of pharmacologic neuroprotection after perinatal ischemia-reperfusion [12]. A wide variety of clinical and experimental studies suggest gender differences in sensitivity and response to cerebral injury. In perinatology male fetuses seem to be more prone in developing (a suspicion of) fetal hypoxia than female fetuses [13], which is also reflected in our clinical trial in which we included significantly more male than female fetuses (62% vs 38% respectively). Not only are males more susceptible to perinatal insults, they also suffer more long-term cognitive deficits as compared to females with comparable injury [12]. This gender difference might be explained by different pathways for programmed cell-death or by hormonal influences [14-17]. Animal studies using induced neonatal hypoxia-ischemia suggest that this sex discrepancy may be caused by the presence of certain sex-specific hormones, such as circulating estradiol [18] and testosterone [19,20]. Substantially elevated testosterone present in human male fetuses may enhance neuronal excitotoxicity following a hypoxic-ischemic insult. Further evidence suggests that following such injury, male and female cells diverge in the proportional activation of caspase-dependent and caspase-independent pathways leading to apoptotic death [14,16,21,22]. Interestingly, research has revealed the sexes to differentially favor one of these two pathways, with females largely utilizing the caspase-dependent pathway and males relying more heavily on the caspase-independent pathway of cell death following an hypoxic-ischemic insult [14,16,21,22]. Finally, data indicate that females may possess a gene-linked advantage through a family of inhibitors of apoptosis, the most potent being X-linked IAP (XIAP) [23-25]. XIAP is known to act on the caspase-dependent apoptotic pathway, and it is possible that increased expression of XIAP in females may contribute to an advantage for females following perinatal hypoxia-ischemia. Taken together, this evidence suggests interplay of hormonal differences and genetically determined apoptotic mechanisms, through which perinatal females may be provided with a level of protection against hypoxic-ischemic injury that is greater than for perinatal males [12].

## Suggestions for future research and implications for clinical practice

In conclusion, the studies presented in this thesis indicate that maternally administered allopurinol rapidly crosses the placenta, is effective in suppressing xanthine oxidase activity, has little adverse side effects (as long as clinically compatible dosages are used) and is potentially effective in preventing hypoxic ischemic damage to the heart and brain in fetal sheep and human female neonates. The latter, however, is based on post-hoc analyses and should be more intensively investigated before being implemented in perinatal medicine.

Maternally administered allopurinol seems to have too little beneficial effects when used as a solemn therapeutic approach for perinatal asphyxia in a clinical setting, probably because fetal surveillance during labor is still too insensitive to optimally detect imminent fetal asphyxia. This may result in a suboptimal selection of patients really needing the treatment.

Future research could therefore combine fetal allopurinol (pre)treatment (prevention) with another promising fetal neuroprotective agent like melatonin [26], and postnatal neuroprotective interventions (repair), such as hypothermia, postnatally administered allopurinol, 2-IB [14], erythropoietin [27,28] or stem cells [29] if the newborn infant appears to be really asphyxiated at birth (i.e. Apgar score < 7 at 5 minutes, pH umbilical artery < 7.05). Alternatively, in case of un-

expected birth asphyxia, allopurinol treatment via the umbilical cord at the resuscitation table might result in a more optimal selection of infants needing treatment. This approach bypasses any placental problems, which may result in suboptimal placental transfer of allopurinol. And finally, any future research focusing on perinatal neuroprotective strategies should always take gender differences into account.

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# Chapter 11
Summary
Nederlandse samenvatting

## SUMMARY

Despite advances in obstetric practice, acute intra-partum fetal hypoxia-ischemia (HI) or birth asphyxia remains one of the most common forms of fetal stress. Hypoxia-ischemia damages the susceptible fetal brain and especially reperfusion of previously ischemic brain tissue is recognized as an important mechanism for substantial additional brain injury due to the excessive formation of free radicals known as reactive oxygen species (ROS). The resulting hypoxic-ischemic encephalopathy (HIE) is associated with the development of cerebral palsy and cognitive disabilities later in life.

As explained in more detail in **Chapter 1 to 3**, current postnatal (pharmacologic) treatment of birth asphyxia-related damage to the newborn brain has a limited neuroprotective effect, even when therapy has been started within the "window of opportunity", being within 6 hours after birth. This might be due to the fact that maximal formation of ROS occurs within the first 30-to-60 minutes after reperfusion and reoxygenation. Moreover, already during fetal hypoxia, substantial amounts of ROS are being produced. We therefore postulated that antenatal (i.e. maternal) pharmacological neuroprotection of the fetus, combined with postnatal neuroprotective strategies (such as hypothermia), might be a more optimal approach to prevent this free radical induced brain damage.

**Chapter 2** summarizes the molecular mechanisms underlying early reperfusion-reoxygenation damage and focuses on the most investigated pharmacological agents (phenobarbital, vitamin C and E, allopurinol, melatonin and xenon) to be given antenatally to the mother to neuroprotect the hypoxic fetus. One potential strategy to diminish the detrimental effects of fetal hypoxia would be to decrease the associated excessive generation of ROS from stimulated pro-oxidant mechanisms within the cell, such as the xanthine oxidase pathway. Administration of the xanthine oxidase inhibitor allopurinol reduces the production of superoxide free radical formation, thereby limiting the amount of hypoxia-reperfusion damage. Furthermore, allopurinol also has a non-protein bound iron (pro-radical) chelating and direct free radical (hydroxyl) scavenging effect, especially when high dosages are administered.

**Chapter 3** describes the long-term effects of *neonatal* allopurinol-treatment in infants suffering from moderate to severe birth asphyxia. Fifty-four term infants were included when suffering from moderate to severe birth asphyxia in two previously performed trials. Infants received either two doses of 20 mg/kg allopurinol intravenously starting within 4 hours after birth (with an interval of 12 hours) or served as controls. Children, who survived and could be tested, were assessed with the Wechsler Preschool and Primary Scales of Intelligence test (WPPSI) or Wechsler Intelligence Scale for Children (WISC) and underwent a neurological examination. The effect of allopurinol on severe adverse outcome (defined as mortality or severe disability at the age of 4-8 years) was examined in the total group of asphyxiated infants and in a predefined subgroup of moderately asphyxiated infants (based on the amplitude integrated electroencephalogram). The mean age during follow-up (n = 23) was 5 years and 5 months (SD 1y 2mo). There were no differences in long-term outcome between the allopurinol treated infants and controls. However, subgroup analysis of the moderately asphyxiated group showed significantly less severe adverse outcome in the allopurinol treated infants compared to controls (25% vs 65%; RR 0.40 (95%CI 0.17 to 0.94)). The reported data may suggest a (neuro)protective effect of neonatal allopurinol-treatment, but only in moderately asphyxiated infants. These beneficial effects are in line with previous findings on neonatal head cool-

ing after acute fetal hypoxia at term by Gluckman *et al*. Most likely no advantage of treatment occurs anymore when the interval to the initiation of treatment is too long or when brain damage is already too severe. It is conceivable that earlier allopurinol treatment, i.e. maternally administered allopurinol during labor in case of suspected fetal hypoxia, may earlier limit the amount of free radical production and subsequent hypoxia-reperfusion injury.

The aim of this thesis was to investigate the applicability of maternally administered allopurinol for fetal neuroprotection and to define strategies for future practice.

## PART I: ANIMAL STUDIES

In **Chapter 4** the capacity of allopurinol to be taken up by the mother, cross the placenta, rise to therapeutic levels and suppress xanthine oxidase activity in the fetus was established in a rodent model. On day 20 of pregnancy, Wistar dams were given 30 or 100 mg.kg$^{-1}$ allopurinol orally. Maternal and fetal plasma allopurinol and oxypurinol concentrations were measured, and xanthine oxidase activity in the placenta and maternal and fetal tissues determined. There were significant strong positive correlations between maternal and fetal plasma allopurinol ($r=0.97$, $P<0.05$) and oxypurinol ($r=0.88$, $P<0.05$) levels. Under baseline conditions, maternal heart (2.18±0.62mU.mg$^{-1}$), maternal liver (0.29±0.08mU.mg$^{-1}$), placenta (1.36±0.42mU.mg$^{-1}$), fetal heart (1.64±0.59mU.mg$^{-1}$) and fetal liver (0.14±0.08mU.mg$^{-1}$) samples all showed significant xanthine oxidase activity. This activity was suppressed in all tissues 2h after allopurinol administration and remained suppressed 24h later ($P<0.05$), despite allopurinol and oxypurinol levels returning towards baseline. The data provide a rodent model of xanthine oxidase inhibition in the mother, placenta and fetus, allowing investigation of the role of xanthine oxidase-derived reactive oxygen species in the maternal, placental and fetal physiology during healthy and complicated pregnancy.

Hypoxia is a common challenge to the fetus, promoting a physiological defense to redistribute blood flow towards the brain and away from peripheral circulation. During acute hypoxia, reactive oxygen species (ROS) interact with nitric oxide (NO) to provide an oxidant tone, which contributes to the mechanisms redistributing the fetal cardiac output, however the source of ROS responsible for this defence is unknown. In **Chapter 5** we investigated whether ROS derived from xanthine oxidase (XO) contribute to the fetal peripheral vasoconstrictor response to hypoxia via interaction with NO-dependent mechanisms. Fifteen pregnant ewes and their fetuses were surgically prepared for long-term recording at 118 days of gestation (term is approximately 145 days). After 5 days recovery, mothers were infused i.v. for 30 min with either vehicle, low (30 mg.kg$^{-1}$) or high (150mg.kg$^{-1}$) allopurinol with or without fetal NO blockade. Allopurinol inhibited the increase in fetal plasma uric acid and suppressed the fetal femoral vasoconstrictor, glycemic and lactate acidemic responses during hypoxia (all $P<0.05$), effects that were restored to control levels with fetal NO blockade. The data provide evidence for the activation of fetal XO in vivo during hypoxia and for XO-derived ROS in contributing to the fetal peripheral vasoconstriction, part of the fetal defense to hypoxia. The data in this study are of significance to the understanding of the physiological control of the fetal cardiovascular system during hypoxic stress. The findings are also of clinical relevance in the context of obstetric trials, if high doses of allopurinol are being administered to pregnant women when the fetus shows signs of hypoxic distress.

In **Chapter 6** we tested the hypothesis that maternal treatment with allopurinol during fetal asphyxia limits ischemia-reperfusion (I/R) damage to the fetal brain in ovine pregnancy. The I/R challenge was induced by 5 repeated measured compressions of the umbilical cord each lasting 10 min, in chronically-instrumented fetal sheep at 0.8 of gestation. Relative to

control fetal brains, the I/R challenge induced significant neuronal damage in the fetal hippocampal cornu ammonis zone 3 and 4. Maternal treatment with allopurinol during the I/R challenge restored the fetal neuronal damage towards control scores. The results of this study suggest that maternal treatment with allopurinol offers potential neuroprotection to the fetal brain in the clinical management of perinatal asphyxia.

## PART II: CLINICAL TRIAL

**Chapter 7** provides a detailed description of the study protocol of our clinical trial (the ALLO-trial). The ALLO-trial was conducted as a randomized double blind placebo controlled multicenter trial among laboring women with suspected fetal hypoxia in 11 participating hospitals. Fetal hypoxia was suspected in case of an abnormal fetal heart rate, significant ST-changes on fetal ECG or fetal scalp pH<7.20. When immediate delivery was foreseen based on these symptoms, women were allocated to receive allopurinol 500 mg IV or placebo IV. Primary outcome measures were the amount of S100ß (a marker for brain tissue damage) and the severity of oxidative stress (measured by isoprostane, neuroprostane), both measured in umbilical cord blood. Secondary outcome measures were neonatal mortality, serious composite neonatal morbidity and long-term neurological outcome. Furthermore pharmacokinetics and pharmacodynamics were investigated.

**Chapter 8** describes drug disposition (maternal plasma concentrations, cord blood concentrations) and drug safety (maternal and fetal adverse events). Data from the randomized double blind multicenter trial were used. In 55 of 111 women in the intervention arm of the study the total dose of allopurinol was administered to the mother before delivery of the child, and a cord blood sample was available. Within 5 minutes after the end of maternal allopurinol infusion target plasma concentrations of allopurinol of ≥2 mg/L were present in cord blood. Of all analyzed cord blood samples, 95% (52/55) had a target allopurinol plasma concentration at the moment of delivery. No adverse events were observed in the neonates. Two mothers had a red and/or painful arm during infusion.

In **Chapter 9** the main outcomes of the clinical ALLO-trial are described. In the ALLO-trial 222 women were randomized to receive allopurinol (ALLO, n=111) or placebo (CONT, n=111). Cord S100ß was not significantly different between the two groups; 44.5 pg/mL (20.2 to 71.4) in the ALLO-group vs 54.9 pg/mL (26.8 to 94.7) in the CONT-group (DiM -7.69 (95%CI -24.9 to 9.52)). The distribution of infants with an S100ß value >p75 was not significantly different either (ALLO, n=23 (23%) vs CONT, n=25 (27%); $RR_{S100ß>p75}$ 0.85 (95%CI 0.53 to 1.4)). Post-hoc subgroup-analysis showed a marked gender difference in treatment effect of allopurinol in favor of girls. There were significantly less girls with an S100ß value >p75 in the ALLO-group (ALLO, n=5 (12%)) as compared to the placebo-treated group (CONT, n=10 (31%); $RR_{S100ß>p75}$ 0.37 (95%CI 0.14 to 0.99)). This difference was not found in boys (ALLO, n=18 (32%) vs CONT, n=15 (25%); $RR_{S100ß>p75}$ 1.4 (95%CI 0.84 to 2.3)). In conclusion, maternal treatment with allopurinol during fetal hypoxia did not significantly lower neuronal damage markers in cord blood, probably due to the fact that fetal hypoxia was relatively mild in the investigated population. Post-hoc analysis however revealed a potential beneficial treatment effect in girls.

In conclusion (as stated in the general discussion in **Chapter 10**), the studies presented in this thesis indicate that maternally administered allopurinol rapidly crosses the placenta, is effective in suppressing xanthine oxidase activity, has little adverse side effects (as long as clinically compatible dosages are used) and is potentially effective in preventing hypoxic ischemic damage to the heart and brain in fetal sheep and human female neonates. The latter, however, is based on post-hoc analyses and should be more intensively investi-

gated before being implemented in perinatal medicine.
Maternally administered allopurinol seems to have too little beneficial effects when used as a solemn therapeutic approach for perinatal asphyxia in a clinical setting, probably because fetal surveillance during labor is still too insensitive to optimally detect imminent fetal asphyxia. This may result in a suboptimal selection of patients really needing the treatment.

Future research could therefore combine fetal allopurinol (pre)treatment (prevention) with another promising fetal neuroprotective agent like melatonin, and postnatal neuroprotective interventions (repair), such as hypothermia, postnatally administered allopurinol, 2-IB, erythropoietin or stem cells if the newborn infant appears to be really asphyxiated at birth (i.e. Apgar score < 7 at 5 minutes, pH umbilical artery < 7.05). Alternatively, in case of unexpected birth asphyxia, allopurinol treatment via the umbilical cord at the resuscitation table might result in a more optimal selection of infants needing treatment. This approach bypasses any placental problems, which may result in suboptimal placental transfer of allopurinol. And finally, any future research focusing on perinatal neuroprotective strategies should always take gender differences into account.

# NEDERLANDSE SAMENVATTING

Hersenschade ten gevolge van asfyxie (zuurstoftekort) bij de pasgeborene is een ernstige complicatie binnen de verloskunde. Pasgeborenen die blootgesteld zijn aan asfyxie kunnen direct of op latere leeftijd ernstige problemen ervaren in hun neurologische ontwikkeling. In milde gevallen kunnen zich leerproblemen, gedragsproblemen of geheugenproblemen voordoen. In ernstigere gevallen kan er zelfs sprake zijn van sterfte, spasticiteit of mentale retardatie. Herkennen, voorkomen en behandelen van zuurstofproblemen tijdens de bevalling is daarom van groot belang.

## Het ontstaan van hersenschade

Op het moment van asfyxie wordt een reeks chemische reacties in gang gezet in het lichaam welke in **hoofdstuk 2** meer in detail worden besproken. Kort gezegd komt er ten gevolge van asfyxie een grote hoeveelheid aan schadelijke stoffen vrij, vrije radicalen genaamd. Vrije radicalen zorgen voor schade aan alles wat ze tegenkomen, zoals celmembranen, celkernen en DNA. Dit kan overal in het lichaam gebeuren, maar vooral het hart en de hersenen van pasgeborenen zijn hiervoor erg gevoelig. Op het moment van zuurstofgebrek zelf kan er al hersenschade ontstaan, maar als er daarna weer zuurstofrijk bloed het hart of hersenen instoomt (reperfusie) wordt de grootste hoeveelheid aan deze vrije radicalen gevormd. Logischerwijs geldt; hoe meer schade er ontstaat, hoe ernstiger de neurologische gevolgen op latere leeftijd waarschijnlijk zullen zijn.

Tot op heden is koeling van de pasgeborene op de Neonatale Intensive Care Unit (NICU) de enige therapie waarvan wetenschappelijk bewezen is dat het de mate van hersenschade na asfyxie kan beperken. Deze behandeling wordt echter pas ingezet na de bevalling, wanneer het grootste deel van de schadelijke stoffen al is gevormd.

## Allopurinol

Allopurinol is een geneesmiddel dat al sinds lange tijd gegeven wordt voor jicht, een gewrichtsaandoening die wordt veroorzaakt door verhoogde urinezuurspiegels in het bloed. Naast het verlagen van urinezuurspiegels kan allopurinol ook de vorming van vrije radicalen voorkómen door de remming van een enzym; genaamd xantine-oxidase, en op die manier mogelijk hersenschade bij de pasgeborene beperken.

In de afgelopen 15-20 jaar komen uit verschillende onderzoeken steeds meer aanwijzingen dat allopurinol inderdaad hersenschade ten gevolge van asfyxie kan verminderen. Bij de mens werd het middel voor deze indicatie in de jaren negentig van de vorige eeuw als eerste onderzocht door het na de geboorte te geven aan ernstig asfyctische kinderen. Bij dit onderzoek werd een verminderde radicalenproductie en behoud van hersendoorbloeding gezien bij de kinderen die na de geboorte allopurinol kregen toegediend. Toen deze studie in 2003 met meer patienten werd herhaald bleken er echter geen significante verschillen zichtbaar op korte termijn uitkomsten. Een mogelijke verklaring hiervoor is dat het gemiddelde tijdstip van toediening (3 uur na de geboorte) te laat was om een significante daling in vrije radicalen productie te kunnen bewerkstelligen. In **hoofdstuk 3** worden de resultaten beschreven van het vervolgonderzoek bij deze kinderen op 4-8 jarige leeftijd. De kinderen die nog leefden en konden worden getest werden getest met de Wechsler Preschool and Primary Scales of Intelligence test (WPPSI) of Wechsler Intelligence Scale for Children (WISC), afhankelijk van hun leeftijd op dat moment. Het effect van allopurinol op ernstige morbiditeit (sterfte of ernstige neurologische beperkingen op 4-8 jarige leeftijd) werd onderzocht in de gehele studiegroep (n=54) en in een voorgespecificeerde groep van matig ernstig asfyctische kinderen. De gemiddelde leeftijd tij-

dens follow-up (n=23) was 5 jaar en 5 maanden (SD 1 jaar, 2 maanden). Er waren geen verschillen in lange termijn uitkomsten tussen de kinderen die allopurinol hadden gekregen en de kinderen die het medicijn niet hadden gehad. In de subgroep van matig ernstig asfyctische kinderen werd wel een significant effect van allopurinol gevonden op het verminderen van sterfte of ernstige neurologische beperkingen (25% vs 65%; RR 0.40, 95%CI 0.17-0.94). Deze uitkomsten suggereren een mogelijk neuroprotectief effect van neonatale behandeling met allopurinol, maar alleen in matig ernstig asfyctische kinderen. Deze uitkomsten zijn vergelijkbaar met de studies naar neonatale koeling van de onderzoeksgroep van Dr. Gluckman. Waarschijnlijk heeft de ingezette therapie onvoldoende effect als de behandeling te laat wordt ingezet of de hersenschade al te ver gevorderd is.

Om de allopurinol zo vroeg mogelijk bij het kind te kunnen krijgen ontstond de gedachte om het aan de moeder te geven op het moment van een verdenking op foetale nood. Op deze manier komt het middel, via de placenta, al bij het kind nog voordat het geboren is.

Met dit idee werd van 2005 tot 2007 een pilotonderzoek verricht. Hierbij werden 53 vrouwen onderzocht bij wie tijdens de bevalling een dusdanige verdenking op foetale nood bestond dat acuut ingrijpen noodzakelijk was door middel van een keizersnede danwel vacuumpomp- of tangverlossing. De helft van deze vrouwen kreeg op dat moment 500 mg allopurinol i.v. en de andere helft kreeg een niet werkzaam medicijn (placebo). Uit dit pilotonderzoek kwam ten eerste naar voren dat allopurinol zeer snel de placenta passeert. Ten tweede bleek er een significant verband te bestaan tussen de hoogte van de spiegels van allopurinol en de waarde van een marker voor hersenweefselschade (S100ß) in het navelstrengbloed. Hoe hoger de allopurinol-spiegels waren, hoe lager de waarde van S100ß.

## Dierexperimentele studies

**Hoofdstuk 4 tot en met 6** beschrijven dierexperimentele studies die zijn uitgevoerd aan de Universiteit van Cambridge, Engeland. In **hoofdstuk 4** werden verschillende eigenschappen van allopurinol onderzocht bij zwangere ratten. Zowel de mate van opname van allopurinol door de moeder (wanneer oraal toegediend), de mate van placenta-passage, het bereiken van therapeutische spiegels in de foetus als de mate van remming van xanthine-oxidase bij de foetus werden onderzocht. Op de 20$^{e}$ dag van de zwangerschap (normale zwangerschapsduur bij raten is 21 dagen), kregen zwangere ratten 30 of 100 mg.kg$^{-1}$ allopurinol oraal toegediend. Maternale en foetale plasma allopurinol en oxypurinol spiegels werden gemeten en xanthine oxidase activiteit werd bepaald in de placenta en in maternale en foetale weefsels. Er waren significant positieve correlaties tussen maternale en foetale allopurinol ($r=0.97$, $P<0.05$) waarden. Onder normale omstandigheden was er sprake van verhoogde xanthine oxidase activiteit in het maternale hart (2.18±0.62mU.mg$^{-1}$), de maternale lever (0.29±0.08mU.mg$^{-1}$), de placenta (1.36±0.42mU.mg$^{-1}$), het foetale hart (1.64±0.59mU.mg$^{-1}$) en de foetale lever (0.14±0.08mU.mg$^{-1}$). Deze activiteit was onderdrukt in alle weefsels 2 uur na allopurinol toediening (zowel bij 30 als bij 100 mg.kg$^{-1}$ allopurinol) en bleef onderdrukt tot minstens 24 uur later ($P<0.05$), ondanks dalende allopurinol spiegels.

Hypoxie is een veel voorkomend probleem voor het ongeboren kind, waarbij er een fysiologisch compensatie mechanisme is door bloeddoorstroming van verschillende organen te verplaatsen (redistribueren) van de perifere circulatie (bijvoorbeeld huid en spieren) naar meer belangrijke organen, zoals de hersenen. Tijdens acute hypoxie zorgen vrije zuurstofradicalen tezamen met stikstof oxide voor een veranderde vaatwandtonus die bijdraagt aan het onderliggende mechanisme van redistributie van het bloed. De bron van deze vrije zuurstof radicalen

is echter nog grotendeels onbekend. **Hoofdstuk 5** laat zien dat vrije radicalen die vrijkomen bij de omzetting van hypoxanthine in xanthine door xanthine oxidase (XO), bijdragen aan de foetale perifere vasoconstrictie (samenknijpen van vaten) bij hypoxie via stikstofoxide-afhankelijke mechanismen. Vijftien zwangere schapen en hun ongeboren lammeren werden operatief voorzien van meetapparatuur op dag 118 van de zwangerschap (normale zwangerschapsduur bij schapen is ongeveer 145 dagen). Na een herstelperiode van 5 dagen, kregen de moeders ofwel een placebo medicijn, ofwel een "lage" dosis allopurinol (30 mg.kg-1) ofwel een "hoge" dosis allopurinol (150mg.kg-1) intraveneus toegediend, met of zonder blokkade van stikstofoxide. Allopurinol remde de stijging van foetale urinezuurspiegels en onderdrukte foetale femorale vasoconstrictie, glycemische en zuur-base reacties tijdens hypoxie (allen $P<0.05$). Deze effecten werden teniet gedaan wanneer foetaal stikstof oxide werd geblokkeerd. De data in deze studie leveren bewijs voor de activatie van foetale XO tijdens hypoxie bij schapen. Het toont tevens aan dat de door XO veroorzaakte vrije radicalen productie mede verantwoordelijk is voor de foetale perifere vasoconstrictie die onderdeel is van de foetale beschermingsmechanismen bij hypoxie. De data zijn van groot belang in het begrijpen van de fysiologische controle mechanismen van het foetale cardiovasculaire systeem tijdens hypoxie en zijn daarnaast belangrijk bij het verrichten van klinische trials wanneer hoge doses van allopurinol worden toegediend aan de zwangere vrouw als de foetus tekenen van hypoxie vertoont.

In **hoofdstuk 6** werd, opnieuw bij zwangere schapen, de hypothese getoetst dat maternale behandeling met allopurinol tijdens foetale asfyxie reperfusieschade aan het foetale brein kan verminderen. Episodes van ischemie en reperfusie werden bewerkstelligd door 10 foetale lammeren op dag 118 van de zwangerschap, vijf opeenvolgende navelstrengcompressies van 10 minuten te laten ondergaan. Ten opzichte van 6 controle-lammeren veroorzaakte ischemie en reperfusie significant meer histologische neuronale schade in cornu ammonis zone 3 en 4 van de foetale hippocampus (een deel van het brein dat o.a. verantwoordelijk is voor het geheugen). Maternale behandeling met allopurinol tijdens de episodes van ischemie en reperfusie brachten de gevonden histologische scores voor neuronale schade weer terug naar controle waarden. De resultaten van deze studie suggereren wederom dat maternale behandeling met allopurinol een potentieel neuroprotectief effect heeft op het foetale brein ten tijde van perinatale asfyxie.

### De humane klinische studie – de ALLO-trial

Met de hoopgevende uitkomsten van het dierexperimentele onderzoek en het eerder uitgevoerde humane pilotonderzoek werd besloten het humane pilotonderzoek op landelijk niveau te herhalen, zodat er met grotere aantallen patienten en in verschillende patiëntengroepen uitspraken kunnen worden gedaan.

De ALLO-trial, een dubbelblind gerandomiseerde placebo gecontroleerde multicenter trial includeerde patiënten van oktober 2009 tot december 2011 in 11 verschillende ziekenhuizen in Nederland. De onderzoeksmethoden waren hetzelfde als bij het pilotonderzoek en worden in detail beschreven in **hoofdstuk 7**. Op het moment dat een patiënte de inclusiecriteria bereikte was er sprake van een dusdanige verdenking op foetale hypoxia dat werd gestreefd naar de direct geboorte van het kind (abnormaal cardiotocogram (CTG), significant STAN-event of een micro bloed onderzoek (MBO) met een pH < 7.20), en werd 500 mg allopurinol of een placebo medicijn i.v. toegediend via een spuitenpomp. Nadat het kind geboren was, werd navelstrengbloed afgenomen voor het meten van de S100ß -waarde (een bekende biochemische marker voor hersenweefselschade), de mate van vrije radicalenproductie (neuroprostaan en isoprostaan) en de allopurinol-spiegels.

In **hoofdstuk 8** onderzochten we de placenta-passage van allopurinol bij de mens door middel van het bepalen van maternale plasma concentraties van allopurinol en allopurinol concentraties in navelstrengbloed. Daarnaast beoordeelden we de veiligheid van het middel door te kijken naar ongewenste effecten bij moeder en/of kind. Data uit de ALLO-trial werden hiervoor gebruikt: in de trial kregen 70 van de 111 vrouwen de totale hoeveelheid allopurinol toegediend vóór de geboorte van het kind. Van 55 van deze 111 moeder-kind paren die allopurinol kregen toegediend was navelstrengbloed beschikbaar voor het bepalen van de allopurinol spiegels. In deze groep werden binnen 5 minuten na het einde van de maternale allopurinol infusie streef-spiegels van allopurinol in het navelstrengbloed gevonden van ≥2 mg/L. Van allopurinolspiegels ≥2 mg/L is aangetoond dat het XO-activiteit kan remmen. Van al de geanalyseerde samples had 95% (52/55) een waarde boven deze streef-spiegels ten tijde van de geboorte van het kind. Omdat allopurinol al geruime tijd wordt gegeven voor jicht weten we veel over de bijwerkingen van het medicijn. De belangrijkste bijwerkingen die op kunnen treden zijn gewoonlijk zeldzaam en meestal van lichte aard. De meest voorkomende reacties zijn huidreacties, zoals jeuk en roodheid. Nooit eerder werden ernstige bijwerkingen beschreven bij een éénmalige dosis en ook in deze trial werden geen nadelige effecten waargenomen bij de neonaten. Twee moeders hadden een rode en/of pijnlijke arm tijdens de infusie van allopurinol.

In **hoofdstuk 9** worden de uitkomsten van de ALLO-trial beschreven. In totaal werden 222 vrouwen geincludeerd, van wie 111 allopurinol kregen toegediend en 111 vrouwen een placebo-medicijn. De waarde van de hersenschade marker S100ß was niet significant verschillend tussen de twee groepen (ALLO, 44.5 pg/mL (20.2 tot 71.4) vs placebo, 54.9 pg/mL (26.8 tot 94.7); DiM -7.69 (95%CI -24.9 tot 9.52)). Ook het aantal kinderen met een S100ß waarden >p75 was niet significante verschillend tussen de twee behandelgroepen (ALLO, n=23 (23%) vs placebo, n=25 (27%); $RR_{S100ß>p75}$ 0.85 (95%CI 0.53 tot 1.4)). Post-hoc subgroep analyse toonde een opvallend sexe verschil in behandeleffect in het voordeel van meisjes. Er waren significant minder meisjes met een S100ß waarde >p75 in de ALLO-groep (ALLO, n=5 (12%)) ten opzichte van de placebo-groep (placebo, n=10 (31%); $RR_{S100ß>p75}$ 0.37 (95%CI 0.14 to 0.99)). Dit verschil werd niet terug gezien bij jongens ($RR_{S100ß>p75}$ 1.4 (95%CI 0.84 to 2.3)). Samengevat verlaagde maternale behandeling met allopurinol ten tijde van een verdenking op foetale hypoxie niet significant neuronale hersenschade markers in het navelstrengbloed. Post-hoc analyse toonde echter wel een mogelijk voordelig effect van maternale behandeling van allopurinol bij meisjes.

Zoals wordt beschreven in de "general discussion" in **hoofdstuk 10** laten de studies in dit proefschrift zien dat maternale toediening van allopurinol snel de placenta passeert, effectief is in het remmen van xanthine oxidase en weinig nadelige effecten heeft (zolang klinisch compatibele doseringen worden aangehouden). Ook lijkt het middel potentieel effectief in het verminderen van schade aan het hart en de hersenen bij foetale schapen en vrouwelijke neonaten. Dit laatste is echter gebaseerd op post-hoc analyses en zal eerst meer uitgebreid moeten worden onderzocht alvorens het toe te passen in de perinatologische zorg.

Maternale allopurinol lijkt te weinig significant voordelige effecten te hebben wanneer het wordt toegepast als op zichzelf staande therapeutische benadering voor perinatale asfyxie in de klinische setting. Mogelijk is dit het gevolg van de nog altijd relatief insensitieve foetale bewakingsmethoden die resulteren in een suboptimale selectie van kinderen die daadwerkelijk de behandeling nodig hebben.

Toekomstig onderzoek zou zich daarom kunnen richten op het combineren van foetale (voor-)behandeling op het moment van een verdenking

op foetale nood met bijvoorbeeld allopurinol en een ander veelbelovend foetaal neuroprotectief middel als melatonine (preventie), in combinatie met neonatale interventies (herstel), zoals hypothermie, postnataal toegediende allopurinol, 2-IB, erythropoietine of stamcellen op het moment dat de pasgeborene daadwerkelijk tekenen van ernstige asfyxie vertoont (bijvoorbeeld een Apgar score < 7 na 5 minuten en een arteriele navelstreng pH < 7.05). In het geval van onverwachte asfyxie na de geboorte van het kind kan worden overwogen directe behandeling met allopurinol toe te passen op de resuscitatie-tafel door het toedienen van allopurinol via de navelstreng. Dit optimaliseert de selectie van de juiste patiëntengroep en vermijdt mogelijke placentaire problemen (abruptio placentae, placenta-insufficiëntie) die kunnen resulteren in een suboptimale passage van allopurinol door de placenta. Tot slot dient toekomstig onderzoek op het gebied van perinatale hypoxie altijd rekening te houden met mogelijke gender-verschillen bij het analyseren van de gevonden uitkomsten.

**Chapter 12**

List of publications

List of co-authors

Dankwoord

Over de auteur

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