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Allopurinol attenuated the chemically-induced hypoxia (hypoxia-reoxygenation) injuries via down-regulation of the transcription factor HIF-1α in neuroblastoma cells

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ABSTRACT

Hypoxia and reoxygenation (H/R) conditions cause molecular injuries in neuronal tissues. This study was designed to validate an *in vitro* model of H/R conditions in Neuro-2A cells and the neuroprotective mechanism(s) of allopurinol on H/R-induced injuries. Hypoxia was induced by using 2-deoxy glucose and Antimycin A and cell viability, intracellular ATP content, reactive oxygen species and nitric oxide concentrations were determined. The expression of hypoxia inducible factor-1 α (HIF-1α) was evaluated by quantitative PCR. Hypoxia resulted in 80% ATP depletion, while more than 80% of the cells remained viable. Co-exposure to H/R and allopurinol protected cells from ATP depletion. Allopurinol treatment significantly ($p < 0.05$) reduced the H/R-induced reactive oxygen species (ROS) and nitric oxide (NO) production. Allopurinol (63.5 μM) lowered the hypoxia-induced upregulation of HIF-1α. Data suggest that chemically-induced hypoxia could be a useful research tool to evaluate neuroprotective agents and that the protective effects of allopurinol against H/R-induced molecular injuries are attributed to the regulation of HIF-1α.

1. Introduction

Hypoxia and ischemia lead to brain injuries and is one of the most frequently occurring causes of severe functional impairment injury and even death in human newborns [1,2]. A recent report indicated that perinatal asphyxia has an incidence of 1 to 6 per 1000 live full-term births, and therefore represents the third most common cause of neonatal death (23%) after preterm birth (28%) or severe infections (26%) [3]. The need to prevent perinatal hypoxia has recently reinforced in a multi-centre clinical trial [4]. Hypoxia is followed by reperfusion/reoxygenation injury, which significantly contributes to asphyxia-related brain damage [5]. Accumulating data indicate that the neuronal damage is related to the formation of ROS including superoxide, hydrogen peroxide, and hydroxyl radicals [6]. Additionally, it has been shown that NO production is also elevated during hypoxia [7]. Another important marker of hypoxia condition is the ATP depletion in affected cells due to its increased break down to adenosine diphosphate, adenosine monophosphate, adenosine, inosine, and hypoxanthine [8,9]. In addition to the neuronal system, other organs including the liver, heart, kidneys, and the lungs are also affected by hypoxia-reoxygenation (H/R)-induced cellular injuries [10–12].

Allopurinol (ALP), 1, 5-dihydro-4h-pyrazolo [3,4-d] pyrimidin-4-one is a xanthine oxidase (XO) inhibitor, which is traditionally used for the treatment of gout [13]. Several studies indicate a protective effect of ALP on H/R-induced injuries during birth asphyxia and the preventive effect on cerebral H/R injuries in adult humans, rats and lambs [14,15]. An extensive multi-centre clinical trial with pre-emptive application of ALP to women in labour at term with clinical indices of foetal hypoxia, showed some promising results, although no significant changes in the applied

Abbreviations: ALP, Allopurinol; AA, Antimycin A; 2-DG, 2-deoxy glucose; DMEM, Dulbeco's Modified Eagle's medium; H2DCF-DA, 2′, 7′, -dichlorodihydro-fluorescein diacetate; DTT, Dithiothreitol; FCS, foetal calf serum; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HIF-1α, hypoxia inducible factor-1 α; MTT, [3-(4 5-dimethylthiazol-2- yl)-2 5-diphenyltetrazolium bromide]; NEAA, non-essential amino acid; NMDA, N-methyl D-aspartate; NO, nitric oxide; ROS, reactive oxygen species.

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biomarker that is available for infants could be measured [4]. ALP has also been used in human and veterinary medicine to decrease H/R-related injuries in different organs including the heart, liver, kidneys, and the small intestine [16].

Previous investigations have demonstrated that ALP inhibits purine degradation under hypoxic condition thereby preventing the accumulation of adenosine and inosine in the brain, and acting as a neuroprotectant [17]. Moreover, it has been shown that ALP reduces the ROS production in the freshly isolated cardiomyocytes of neonatal rat and in the heart of rats that exposed to H/R conditions [18].

Understanding of the molecular mechanism(s) responsible for the regulation of H/R- induced injuries requires investigations into the profile of gene(s) expression under H/R conditions. Activation of transcription factors is the fastest cellular response, which occurs in situations such as ischemia and hypoxia [19]. It has been reported that hypoxia inducible factor-1α (HIF-1α) as a transcription factor, mediates the adaptive responses to low oxygen availability. In contrast to normal conditions, at which HIF-1α is unstable due to a cytosolic proteasome pathway, under hypoxic condition it becomes stable, accumulates, and translocates into the nucleus. There HIF-1α activates a variety of genes by binding to the DNA consensus sequence 5′-RCGTG-3′ in the promoter regions of these target genes [20].

Despite the fact that an increasing number of studies from human and veterinary medicine are suggesting a neuroprotective effect of ALP at H/R conditions, there are relatively little data available confirming these findings in *in vitro* models, that allow to examine the molecular mechanism(s) of the protective effect of ALP on H/R conditions. Particularly the expression pattern of the prominent transcriptional factor HIF-1α during these conditions has not been fully elucidated, yet. It was therefore the aim of this study to present a model using Neuro-2A cells to uncover the biochemical and molecular neuroprotective mechanism(s) of allopurinol.

2. Materials and methods

2.1. Chemicals

Allopurinol (ALP), 2-deoxy glucose (2-DG), Antimycin A (AA), luciferase/luciferin reagent, diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, and MTT [(3–4,5- dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] were purchased from Sigma Chemical Co. St Louis, MO, USA. Dichlorodihydrofluoroscein diacetate (H2DCF- DA) was obtained from Molecular probes (Leiden, the Netherlands). Dulbeco's Modified Eagle's medium (DMEM), non-essential amino acid (NEAA), foetal calf serum (FCS), and trypsin EDTA were supplied by Invitrogen (Breda, the Netherlands).

2.2. Cell culture and hypoxia induction

Neuro-2A cells were grown in DMEM supplemented with 10% FCS, 1% penicillin (100 units/ml), streptomycin (100 μg/ml), 1% NEAA, and 1% L-glutamine. Cells were sub-cultured every 3–4 days and remained at 37 °C in a humidified atmosphere of 5% CO2 in air. For cell viability, intracellular-ATP content, ROS and NO production assays, cells were seeded in 96-well tissue culture plates at density of 1 × 104 cells/well and in 0.2 ml medium. Cells were incubated for 24 h prior to exposure to test chemicals. To induce hypoxia and optimize the protocol, cells were exposed to the metabolic inhibitors 2-DG and AA in glucose-free medium [21]. Preliminary studies indicated that the concentrations of 5 mM 2-DG along with 5 μm AA would be practically suitable to produce a hypoxic condition in Neuro-2A cells.

2.3. Cell treatment in the chemically-induced hypoxia conditions

Neuro-2A cells were seeded in 96-well culture plates as described previously and were incubated for 24 h prior to exposure to test chemicals. The cells were rinsed with phosphate buffer solution (PBS) and treated with hypoxic medium and/or hypoxic medium containing different concentrations of ALP (12.7, 63.5 and 127 μM). ALP concentrations were selected based on previously published studies [22]. Measurement of the individual parameters were conducted after exposing cells to 30 min hypoxia, and 2 and 4 h reoxygenation in the absence and presence of ALP.

2.4. Cell viability and morphology

Cell viability was quantified by the colorimetric MTT [3-(4, 5-dimethylthiazol-2- yl)-2, 5-diphenyltetrazolium bromide] assay. This assay measures the reduction of dimethylthiazol diphenyl tetrazolium bromide (MTT) as a measure of cellular succinate-dehydrogenase activity. In brief: Following 30 min hypoxia, and subsequent 2 and 4 h reoxygenation with and without ALP treatment, the medium was discarded and 0.2 ml MTT solution (3 mg/ml) was added to the cells. After 3 h incubation at 37 °C the MTT solution was discarded and the intracellular purple insoluble formazan was solubilized by adding 100 μl/well of lysis buffer (0.5% sodium dodecyl sulphate, 36 mM HCl, and isopropanol acid). Following shaking on orbital shaker the optical density (OD) was measured at 594 nm using a micro plate reader (BIORAD Model 3550). Cell viability was expressed as percentage of non-treated controls calculated as (A595 treated cells/A595 of control) × 100.

The morphological changes of Neuro-2A cells were observed under a phase-contrast Olympus IX51 microscope (Olympus IX51, Olympus Corporation, Tokyo, Japan) at various conditions including control, hypoxic, ALP-treated cells and photographs were taken at a 200 × magnification.

2.5. Intracellular ATP-content assay

The medium was removed and cells washed with pre-warmed PBS and thereafter the lysis reagent (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2 diaminocyclohexane- *N*, *N*, *N*', *N*'-tetra acetic acid, 10% glycerol, 1% Trito X-100) was added to the cells and mixed. Luciferase/Luciferin (L/L) reagent was added and luminescence was measured at 520 nm (Fluostar Optima, BMG Labtechnologies GmbH, Germany). The relative light unite (RLU) was expressed as (A520- treated cells/A520-control cells) × 100.

2.6. Determination of ROS generation

The ROS generation by hypoxia was measured in treated and non-treated cells by using 2′,7′, −dichlorodihydrofluorescein diacetate (H2DCF-DA) as a maker according to Hemple *et al.* [23]. Briefly, Neuro-2A cells were seeded in 96-well culture plates as described above. After 24 h incubation, the cells were washed with pre- warmed PBS and pre-incubated for 60–80 min with 20 μM H2DCF-DA in 50 μl krebs ringer phosphate glucose solution (KRPG; 10 mM glucose, 10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgSO4, pH 7.4). After this pre-incubation time, the H2DCF-DA solution was removed and the cells rinsed with PBS and treated with hypoxic medium and/or hypoxic medium containing different concentrations of ALP. Following 30 min hypoxia, 2 and 4 h' reoxygenation conditions, ROS production was measured using a spectrofluorometer microplate reader at an emission wavelength of 538 nm and an excitation wavelength of 485 nm. Relative ROS production was expressed as an increase in fluorescence compared to fluorescence of the appropriate control (100%).

2.7. NO measurement

The total NO production was determined in cell culture supernatant by measuring the amount of generated NO according to the Griess reaction [24].

2.8. RNA isolation and RT-PCR

Total RNA was isolated from Neuro-2A cells using standard TRIZOL method. RNA amounts were determined spectrophotometrically, and RNA purity was measured by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) as A260/A280 ratio with expected values between 1.8 and 2.

For RT-PCR, cDNA was synthesized in a 20 μl reaction mixture containing 4 μl of 5 x iScript reaction mix, 1 μl iScript reverse transcriptase and 15 μl nuclease-free water containing 1 μg RNA template, according to protocol of the manufacturer (iScript TM cDNA Synthesis Kit, BIO RAD). The synthesized cDNA was amplified by a standard PCR mixture. Cycling protocol for 20 μl reaction mix were 5 min at 25 °C, followed by 30 min at 45 °C, and 5 min at 85 °C.

2.9. Quantitative-PCR analysis of HIF-1α

Using the Icycler MyIQ system (Bio-Rad Laboratories), cDNA from control, hypoxic, and H/R Neuro-2A cells, which were treated with ALP for 30 min, 2 and 4 h were subjected to qPCR in 25 μl reaction mix, containing 10 μl cDNA template, 0.75 μl forward primer, 0.75 μl reverse primer, 12.5 μl IQ SYBR Green super-mix solution, and 1 μl DNA-free water. Q-PCR conditions were as follows: enzyme activation at 95 °C for 3 min, 1 cycle, followed by 40 cycles of denaturation: 95 °C for 20 s; annealing: 64.2 °C for HIF-1α and 65 °C for GAPDH for 30 s; elongation: 72 °C for 30 s. The specific primers for HIF-1α and GAPDH genes were designed and manufactured by Invitrogen (Invitrogen, Life technologies, Paisley, Scotland, UK). Melt-curve analysis was performed on each primer set to ensure that no primer dimmer or nonspecific amplification was present under the optimized cycling condition. Pair primers for qPCR along with annealing temperatures and references are presented in Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.biopha.2017.01.143. The presence of any genomic DNA in synthesized cDNA samples was checked by addition of a RNA sample that was not reverse transcribed to Q-PCR reactions. No product was synthesized in the control sample. Individual values for HIF-1α mRNA expression were obtained after normalization to the housekeeping gene Glyceraldehydes 3-Phosphate Dehydrogenase (GAPDH), which was stable during the treatment period.

2.10. Statistical analysis

Each experiment was repeated three times. Data in bar graphs are given as the means ± standard deviation (SD). Significant differences between samples were determined by one-way ANOVA followed by Bonferroni *post hoc* test using GraphPad prism version 4.00 for windows (GraphPad Software, San Diego, California, USA). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Cell viability and intracellular ATP level measurements

The first perquisite in present study was to optimize the chemically-induced hypoxia in Neuro-2A cells. To optimize the experimental protocol two known cytotoxicity endpoints, i.e. Cell viability measurement by the MTT assay and intracellular ATP-content assay were evaluated. Fig. 1-A and B shows the effect of

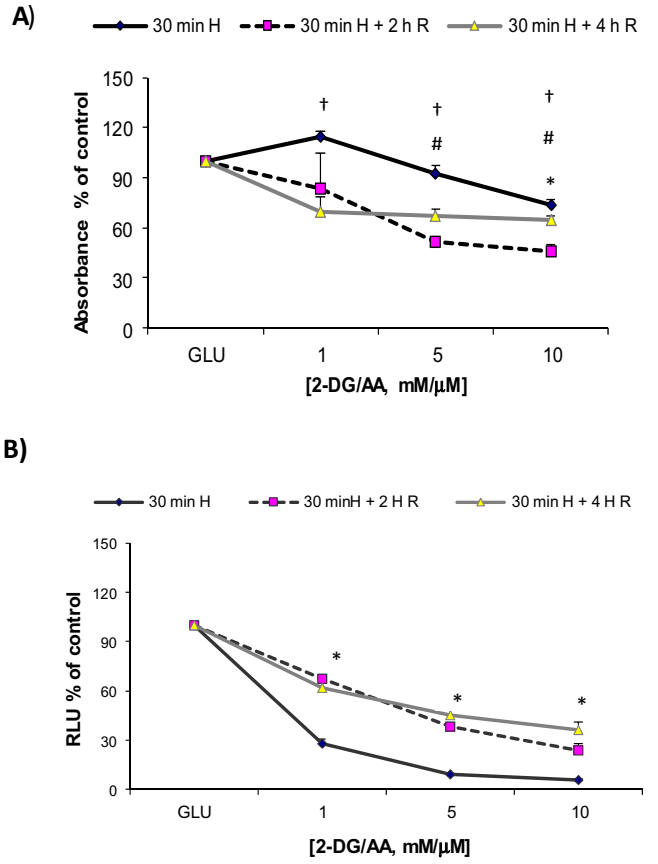

Fig. 1. Cell viability (A) was affected significantly only after exposure to the highest given concentrations of 2- DG-AA is represented * difference in comparisons with the controls, # and † significant differences between control cells and cells exposed 30 min to hypoxia followed by 2 and 4 h reoxygenation, respectively.;(B) Intracellular ATP content under the same condition; a significant difference ($p < 0.05$) between the control cells and hypoxic and H/R cells was observed. Values represent mean ± SD from three independent experiments.

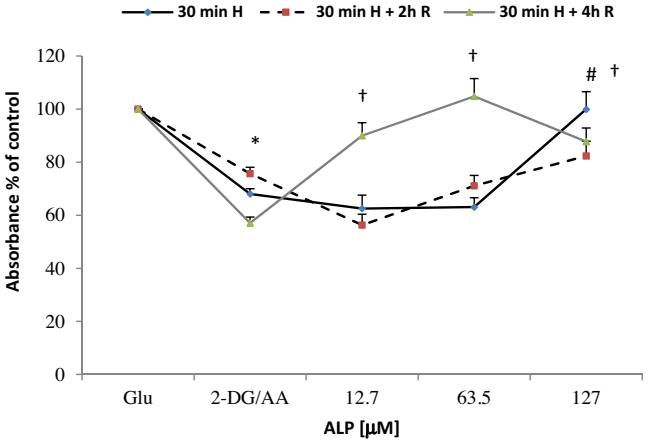

Fig. 2. Effect of ALP on cell viability under H/R conditions. A significant difference between the control and hypoxic (30 min hypoxia) cells is represented by an asterisk and # indicates a significant difference ($p < 0.05$) between treated and non-treated hypoxic cells after 30 min hypoxia and † represents significant differences between treated and non-treated hypoxic cells after 4 h reoxygenation. GLU; indicates the normal cell culture medium and 2-DG/AA represent the chemically-induced hypoxia. Values represent mean ± SD from three independent experiments.

various concentrations of 2-DG-AA on cell viability and ATP-content in Neuro-2A cells, respectively. Despite the remarkable ATP depletion after 30 min hypoxia, MTT results showed that almost 80% of the cells were still viable. Moreover, ATP depletion following 30 min hypoxia was found in a concentration-dependent manner. On the basis of this results, a concentrations of 5 mM 2-DG along with 5 μm AA was selected as a standard procedure to establish hypoxic conditions in Neuro-2A cells in the forthcoming experiments. The subsequently performed reoxygenation step on hypoxic cells for 2 and 4 h, demonstrated no significant differences on cell viability, but ATP levels re-established ($p < 0.05$).

3.2. ALP attenuated cell death during the hypoxia and reoxygenation and improved the H/R-induced morphological changes

Neuroprotective effects of ALP at different concentrations during hypoxia and also during 2 and 4 h reoxygenation periods were examined. We found that ALP only recovered cell viability at the highest given concentration (127 μM), when the Neuro-2A cells were exposed to 30 min hypoxia. However, reoxygenation of the hypoxic cells for 2 and 4 h along with the ALP treatment resulted even at the lowest concentration of ALP (12.7 μM) in a significantly ($p < 0.05$) improved cell viability indicating not only a

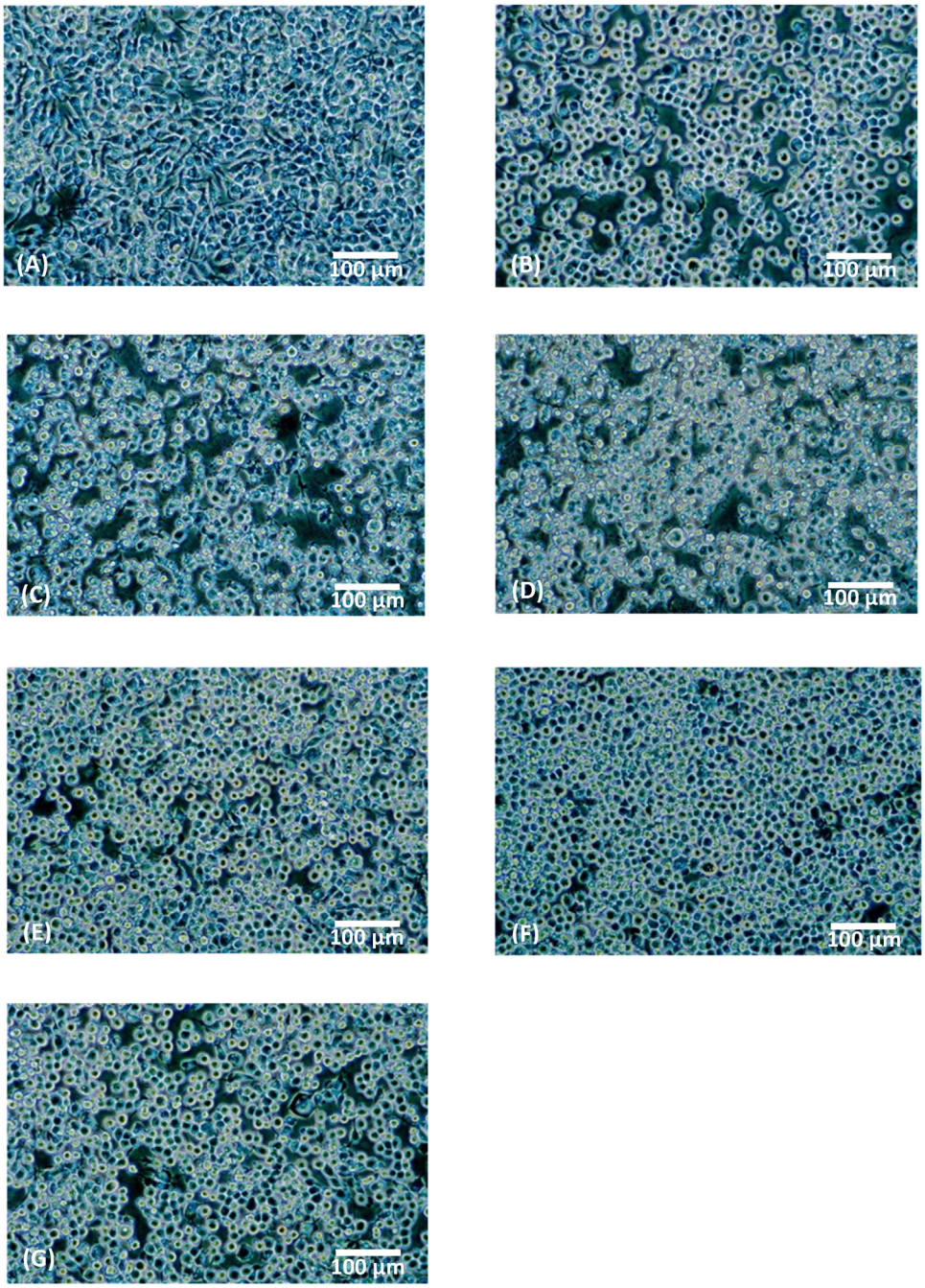

Fig. 3. Morphological changes in Neuro-2A cells under various H/R conditions: (A) normal cells non-treated, (B) 30 min hypoxia, 2 h and 4 h reoxygenation (C and D), and H/R cells (4 h), which were concomitantly treated with various concentrations of allopurinol (E = 12.7, F = 63.5 and G = 127 μM ALP, respectively). The photographs were taken at a 200× magnification.

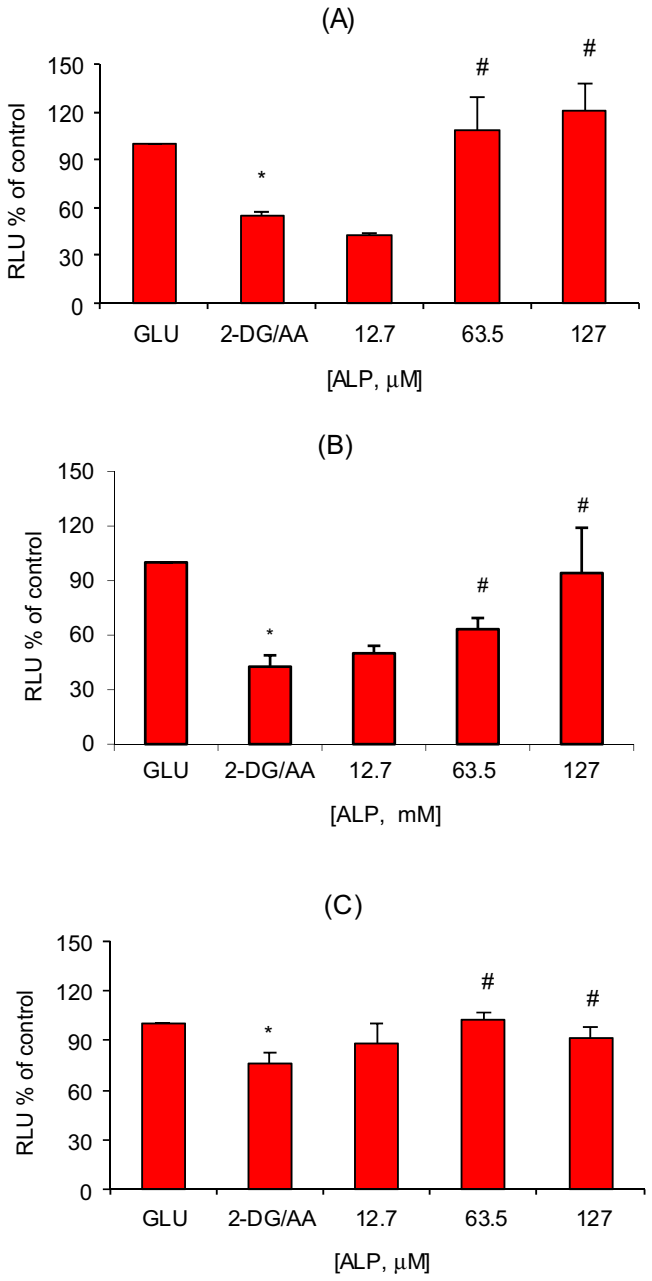

Fig. 4. ALP concentration-dependently prevented the intra-cellular ATP depletion during (A) hypoxic and (B and C) 2 and 4 h reoxygenated Neuro-2A cells. Significant differences between the control and hypoxic cells are represented by an * and # indicates a significant difference ($p < 0.05$) between treated and non-treated hypoxic cells. GLU; indicates the normal cell culture medium with all supplements and 2-DG-AA represent the chemically-induced hypoxia. Values (relative light unite, RLU) represent mean ± SD from three independent experiments.

concentration-dependent but also time-dependent effect of ALP on cell viability (Fig. 2).

The morphological alterations were studied with phase contrast microscopy (Fig. 3-A and B). Hypoxic non-treated Neuro-2A cells exhibited some morphological differences in comparison to control cells such as reduced confluence, spindle shape and branching. While no substantial difference between the hypoxic untreated cells and those re-oxygenated for 2 or 4 h are visible, cell membrane protrusions resembling membrane blebs were clearly documented (Fig. 3-C and D). ALP-treatment not completely but concentration-dependently protected cells from such morphological alterations (Fig. 3-E–G).

3.3. ALP prevents ATP depletion during the hypoxia and reoxygenation in

3.3.1. Neuro-2A cells

The hypoxia-induced ATP depletion could be reversed after exposing Neuro-2A cells simultaneously to hypoxia and the ALP treatment (Fig. 4-A–C). In contrast to cell viability, ATP levels recovered already significantly at a concentration of 63.5 μM ALP. In addition, ALP treatment in the reoxygenation period resulted in a remarkable recovery of hypoxia-induced ATP-depletion.

3.4. ALP reduced the ROS production

As shown in Fig. 5-A, a significant increase in the ROS generation following 30 min hypoxia was recorded and only a high concentration of ALP (127 μM) could lower it slightly. However, 30 min hypoxia followed by 2 and 4 h reoxygenation caused remarkable increase of the ROS generation, indicating that not only hypoxia but also reoxygenation leads to the production of ROS. No significant difference was observed between 2 and 4 h reoxygenation in terms of the ROS production. However, treatment of H/R cells with different concentrations of ALP resulted in a marked reduction of the ROS generation in a concentration-dependent manner (Fig. 5-B, and 5-C).

3.5. ALP lowered the H/R-induced NO production

A 30 min hypoxia caused a significant ($p < 0.05$) elevation of the NO generation in Neuro-2A cells. We found that not only hypoxia but also reoxygenation promoted the NO production (Fig. 6-A). Exposing the cells to hypoxia and simultaneously various concentrations of ALP reduced the NO generation (Fig. 6-B). Effect of the ALP treatment during reoxygenation period was more pronounced and ALP attenuated significantly ($P < 0.05$) the NO generation when compared with non-treated hypoxic cells (Fig. 6-C, and D).

3.6. ALP suppressed the hypoxia-induced upregulation of the HIF1-1α expression

To understand the transcriptional pathway of H/R and ALP effects in Neuro-2A cells, the expression of HIF-1α was analysed under H/R conditions. A 30 min hypoxia caused a significant increase in the HIF-1α expression ($p < 0.05$), while co-exposure of cells to hypoxia and 63.5 μM ALP (as an effective dose level) for 30 min, and 2 and 4 h reoxygenation periods resulted in a significant ($p < 0.05$) suppression of the HIF-1α induction. However, after 4 h reoxygenation HIF-1α expression was slightly higher when compared to 30 min and 2 h reoxygenation. To confirm the hypothesis that the reduction in HIF-1α expression is due to the ALP treatment, we analysed mRNA levels expression after hypoxia followed by 2 h reoxygenation without the ALP treatment and found that 2 h reoxygenation alone already resulted in a significant reduction in HIF-1α expression. However, there was still a remarkable difference between the ALP-treated and non-treated groups (Fig. 7).

4. Discussion

Cerebral ischemia, prenatal cerebral hypoxia-ischemia, preterm birth, congenital malformations and stroke are the major causes of mortality in neonates [25–28]. Moreover, cerebral hypoxia and ischemia are the most common disorders that damage neurons [29]. An extensive multi-centre clinical trial supported the hypothesis that the administration of allopurinol to pregnant

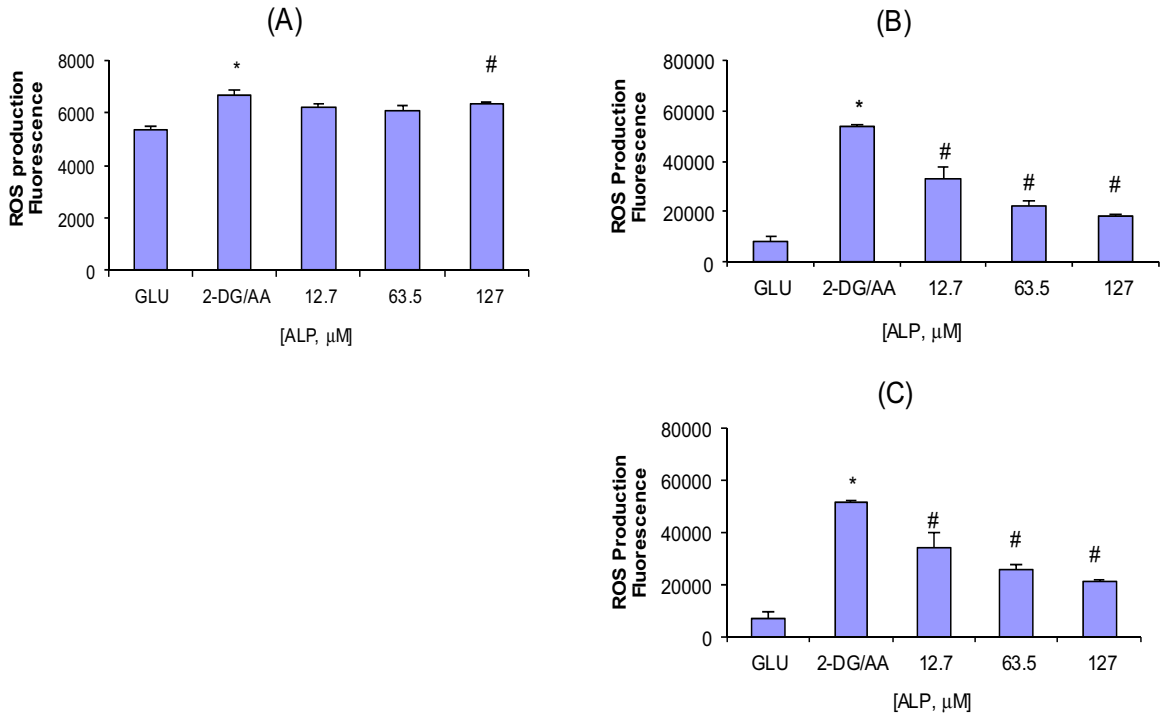

Fig. 5. Effect of ALP on ROS generation in hypoxia (A) and reoxygenation (B and C) conditions; A significant difference between the control and hypoxic cells is represented by an * and # indicates a significant difference ($p < 0.05$) between treated and non-treated hypoxic cells. GLU; indicates the normal cell culture medium with all supplementations and 2-DG-AA represent the chemically-induced hypoxia. Values represent mean ± SD from three independent experiments.

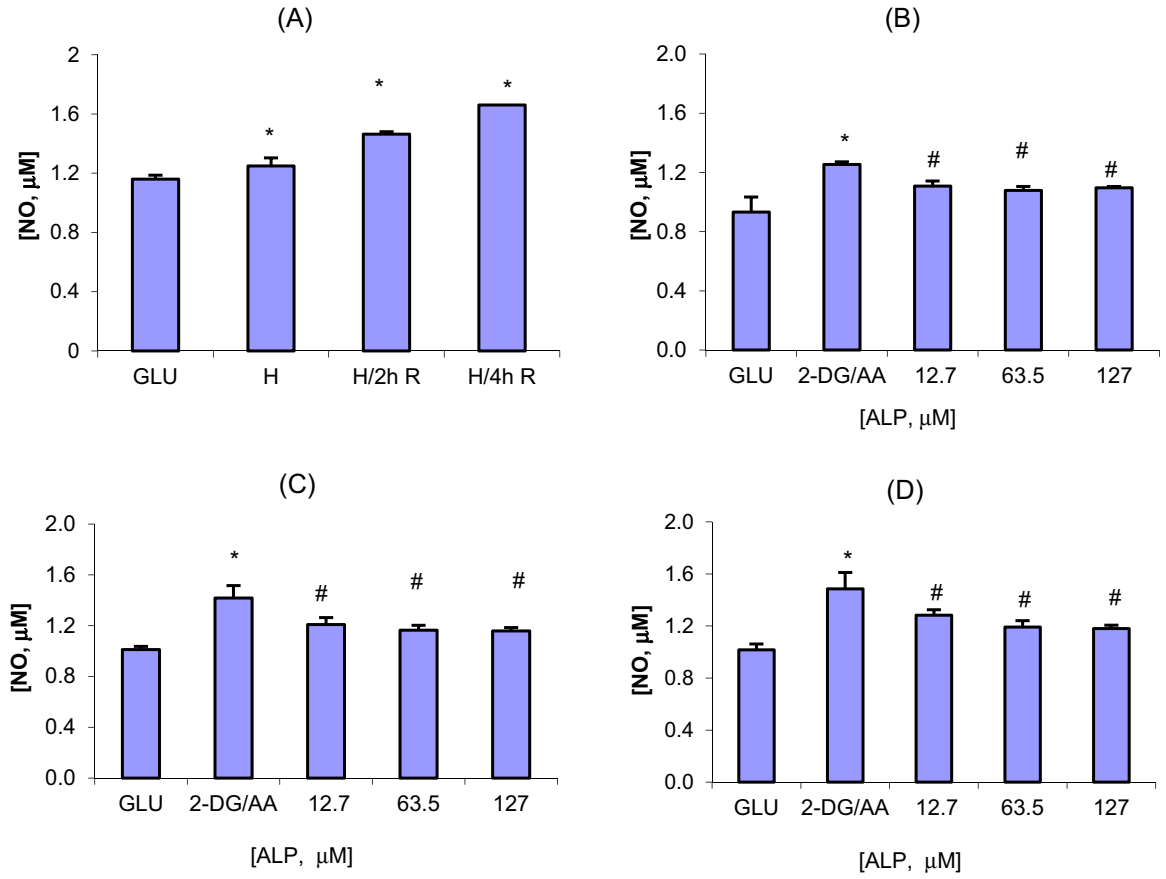

Fig. 6. Effect of (A): A 30 min hypoxia, (B) A 30 min hypoxia and concurrently ALP treatment, (C) A 30 min hypoxia followed by 2 h reoxygenation and ALP treatment and (D) A 30 min hypoxia followed by 4 h reoxygenation and ALP treatment, on the NO production in Neuro-2A cells; A significant difference between the control and hypoxic cells is represented by an asterisk and # indicates a significant difference ($p < 0.05$) between treated and non-treated hypoxic cells. GLU; indicates the normal cell culture medium with all supplementations and 2-DG-AA represent the chemically-induced hypoxia. Values represent mean ± SD from three independent experiments.

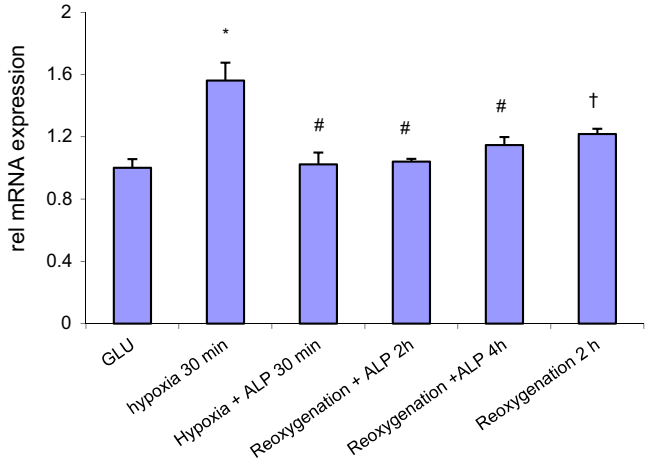

Fig. 7. Effect of hypoxia, reoxygenation and the allopurinol treatment on HIF-1α expression in Neuro-2A cells; A significant difference between the control and hypoxic cells is represented by an * and # indicates a significant difference ($p < 0.05$) between hypoxic cells and other groups which received ALP (63.6 μM) and † shows a significant difference between 2 h reoxygenation with and without ALP treatment. H = hypoxia, R 2h = 2 h reoxygenation, R 4h = 4 h reoxygenation. Values represent mean ± SD from three independent experiments.

woman might reduce the risk of hypoxia-induced neuronal damage. However, results in this study remained partly inconclusive as only one biomarker of neuronal tissue damage could be measured in the neonates [30]. Neuroprotective effects of ALP in infants and in rabbits undergoing heart surgery have also been documented [31,32].

In the present study we established a chemically-induced hypoxia model, which was validated in Neuro-2A cells, a mouse neural crest-derived cell line, commonly used in neurotoxicity testing. This cell line is considered to be suitable for neurotoxicity studies due to its high sensitivity allowing an accurate estimate of neurotoxicity in functional assays [33]. This model was used to unravel the protective mechanisms of ALP on hypoxia and reoxygenation conditions.

It is well known that the chemically-induced hypoxia by inhibition of mitochondrial oxidative phosphorylation and inhibition of glycolysis causes a significant reduction in intracellular ATP level [21]. We demonstrated that the 2-DG/AA-chemically-induced hypoxia for 30 min caused a significant ATP reduction in Neuro-2A cells (to 5% of control). However, results from cell viability assessment following chemically-induced hypoxia-reoxygenation at the highest given concentration (2- DG/AA), resulted only in a loss of cell viability by 20% under hypoxic conditions and 35 and 45% loss in cell viability after 2 and 4 h reoxygenation, respectively. These results are in line with previous findings in primary cultured cortical neurons, in which the hypoxia was induced by low oxygen levels (less than 1 mol% O_2). Also in these experiments, a reduced intracellular ATP production and loss of cell viability was observed [34].

Taken together, these first experiments indicated that the established model is suitable to study the effect of allopurinol on hypoxia/reperfusion injury.

Therefore the second part of the current study was devoted to evaluate the mechanisms that are involved in the neuroprotective effects of ALP on H/R-induced damages in Neuro-2A cells. The ALP treatment could mitigate the loss of cell viability when it was used at the highest concentration for 30 min under hypoxia condition and during the 2 h reoxygenation period. However there was a significant positive effect at the low and high concentrations after 4 h reoxygenation, indicating concentration- and time-dependent effects of ALP on cell viability. Morphological observations indicated that hypoxia and substantial reoxygenation resulted, cell membrane protrusions and loos of the normal shape of neuronal cells, which was presented as branching and spindle shape of the cells. These morphological alterations resemble the apoptotic pathway of cell death and, as mentioned earlier, ALP was able to show its anti-apoptotic effects on H/R cells [35,36].

The initial experiments have also indicated that the intracellular levels of ATP declined significantly during hypoxia and to a lesser extent during the reoxygenation period. Treatment of H/R cells with various concentrations of ALP prevented this ATP depletion suggesting that ALP may reactivate mitochondria to produce more ATP. It has been demonstrated before that during hypoxia, intracellular ATP is catabolized to hypoxanthine [8]. As ALP is an inhibitor of xanthine oxidase it decreases the production of hypoxanthine and hence prevents excessive ATP depletion. Our findings are also in accordance with previous reports demonstrating that ALP exerts its therapeutic effects on ischemic myocardium by enhancing ATP levels [32].

Although recovering ATP level may partly explain the improved cell viability, other mechanisms may contribute to the protective effects of ALP. Measurement of ROS and NO production during both hypoxia and reoxygenation conditions and subsequently with and without the ALP treatment, demonstrated an increased radical formation under H/R conditions. ALP quite effectively could reduce the ROS and NO production particularly in reoxygenation period. These results can be again partly explained by the inhibition of xanthine oxidase (XO) by ALP, as XO plays a pivotal role in the ROS generation during H/R. Moreover, it had been suggested earlier that ALP is capable to scavenge ROS directly [37]. At the same time, there is evidence indicating that XO activation can also enhance the NO production particularly under acidic condition such as hypoxia [38,39].Thus our finding that during hypoxia and more notably during reoxygenation period the NO production is increased, confirm these previous reports. More recently it has been demonstrated also that ALP significantly lowered the NO production in human RBCs at both physiologic and acidic pH, representing normal and ischemic conditions, respectively [7].

Under H/R conditions the transcription factor HIF-1α is activated and plays a crucial role in the regulation of the adaptive responses of cells [40]. We could demonstrate that also in our model of chemically-induced hypoxia a significant increase in the mRNA levels of HIF-1α could be measured. Further analyses showed that this transcriptional response is altered not only by oxygen deprivation but also by ALP treatment. There is similar report that supports this finding as it has been shown that vitexin, a natural flavonoid with antioxidant and anti-inflammatory properties, down-regulates the HIF-1α expression in rat pheochromacytoma (PC12) cells [41]. The down-regulation of HIF-1α in ALP treated cells suggests a direct effect of ALP on the hydroxylase enzymes that regulate the activity of HIF-1α [42]. It is therefore assumed that the protective effect of ALP on the ROS and NO generation is associated with the regulation of HO-1 and iNOS by HIF-1α. Recent *in vivo* studies using a rodent model support this hypothesis as it has been shown that the early inhibition of HIF-1α reduced the ischemic/reperfusion-induced brain injury [43]. Another interesting finding in the current study was the slightly but significantly ($p < 0.05$) more pronounced up-regulation of HIF-1α in ALP-treated cells, which were exposed to 4 h reoxygenation in comparison to the cells at 2 h reoxygenated. Although the given ALP concentration was the same, HIF-1α mRNA level varied. To explain this difference it should be recalled that the level of reactive oxygen species (ROS) during the reperfusion period is higher than during hypoxia conditions and obviously the level of ROS produced in 4 h was higher than that in 2 h. The cellular ROS concentration is one of the key factors, which regulate the HIF-1α

expression, therefore the high concentration of ROS would result in higher mRNA levels of HIF-1α [44,45].

In summary, we demonstrated that the chemically-induced hypoxia can be considered a novel model of hypoxia induction in neuronal cells. In turn this model provides the opportunity to investigate various preventive, protective and therapeutic effects of different compounds under hypoxic conditions. In addition our study showed that the cellular damages during H/R conditions are attributable at least partly to the increase in ROS and NO production and that ALP attenuated this effect. Moreover, it could be demonstrated that the neuroprotective effect of ALP on H/R injuries is associated with decreased activation of the transcription factor HIF-1α.

Conflict of interest

The authors declare that there is no conflict of interests.

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Author contributions

J. A-A. and H.M. designed and performed the experiments. H. M., J. A-A., N.S. and J. F-G analysed the data. H. M., N. S., and J. F-G., wrote the paper.

References

[1] D.C. Yesilirmak, A. Kumral, K. Tugyan, S. Cilaker, H. Baskin, O. Yilmaz, N. Duman, H. Ozkan, Effects of activated protein C on neonatal hypoxic ischemic brain injury, Brain Res. 19 (2008) 56–62.
[2] C.C. Leonardo, K.R. Pennypacker, Neuroinflammation and MMPs: potential therapeutic targets in neonatal hypoxic-ischemic injury, J. Neuroinflamm. 6 (2009) 13.
[3] R. Antonucci, A. Porcella, M.D. Pilloni, Perinatal asphyxia in the term newborn, JPNIM 3 (2014) e030269.
[4] J.J. Kaandorp, F. van Bel, S. Veen, J.B. Derks, F. Groenendaal, M. Rijken, E. Rose, M.M. Venema, C.M. Rademaker, A.F. Bos, M.J. Benders, Long-term neuroprotective effects of allopurinol after moderate perinatal asphyxia: follow-up of two randomized controlled trials, Arch. Dis. Child Fetal Neonatal Ed. 97 (2012) F162–166.
[5] F. Capani, C.F. Loidl, F. Aguirre, L. Piehl, G. Facorro, A. Hager, Changes in reactive oxygen species (ROS) production in rat brain during global perinatal asphyxia: an ESR study, Brain Res. 914 (2001) 204–207.
[6] C. Peeters, F. van Bel, Pharmacotherapeutical reduction of posthypoxic-ischemic brain injury in the newborn, Biol. Newnate 79 (2001) 274–280.
[7] A.J. Webb, A.B. Milsom, K.S. Rathod, W.L. Chu, S. Qureshi, M.J. Lovell, F.M. Lecomte, D. Perrett, C. Raimondo, E. Khoshbin, Z. Ahmed, R. Uppal, N. Benjamin, A.J. Hobbs, A. Ahluwalia, Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase, Circ. Res. 103 (2008) 957–964.
[8] M.S. Turner, G.A. Haywood, P. Andreka, L. You, P.E. Martin, W.H. Evans, K.A. Webster, N.H. Bishopric, Reversible connexin 43 dephosphorylation during hypoxia and reoxygenation is linked to cellular ATP levels, Circ. Res. 95 (2004) 726–733.
[9] P.J. Marroa, O.P. Misharb, M. Delivoria-Papadopoulos, Effect of Allopurinol on brain adenosine levels during hypoxia in newborn piglets, Brain Res. 16 (2006) 444–450.
[10] T. Kaneda, K. Ku, T. Inoue, M. Onoe, H. Oku, Postischemic reperfusion injury can be attenuated by oxygen tension control, Jpn. Circ. J. 65 (2001) 213–218.
[11] Y. Kinugasa, K. Ogino, Y. Furuse, T. Shiomi, H. Tsutsui, T. Yamamoto, O. Iqawa, I. Hisatome, C. Shigemasa, Allopurinol improves cardiac dysfunction after ischemia-reperfusion via reduction of oxidative stress in isolated perfused rat hearts, Circ. J. 67 (2003) 781–787.
[12] W.Y. Lee, S.M. Lee, Synergistic protective effect of ischemic preconditioning and allopurinol on ischemia/reperfusion injury in rat liver, Biochem. Biophys. Res. Commun. 349 (2006) 1087–1093.
[13] P. Pacher, A. Nivorozhkin, C. Szabo, Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol, Pharmacol. Rev. 58 (2006) 87–114.
[14] M. Shadid, R. Moison, P. Steendijk, L. Hiltermann, H.M. Berger, F. van Bel, The effect of antioxidative combination therapy on post hypoxic-ischemic perfusion metabolism, and electrical activity of the newborn brain, Pediatr. Res. 44 (1998) 119–124.
[15] F. Van Bel, M. Shadid, R.M. Moison, C.A. Dorrepaal, J. Fontijn, L. Monteiro, M. Van De Bor, H.M. Berger, Effect of allopurinol on postasphyxial free radical formation cerebral hemodynamics, and electrical brain activity, Pediatrics 101 (1998) 185–193.
[16] C.A. Farquharson, R. Butler, A. Hill, J.J. Belch, A.D. Struthers, Allopurinol improves endothelial dysfunction in chronic heart failure, Circulation 106 (2002) 221–226.
[17] N. Karageorgos, N. Patsoukis, E. Chroni, D. Konstantinou, S.F. Assimakopoulos, C. Georgiou, Effect of N-acetylcysteine: allopurinol and vitamin E on jaundice-induced brain oxidative stress in rats, Brain Res. 1111 (2006) 203–212.
[18] M.G. Angelos, V.K. Kutala, C.A. Torres, G. He, J.D. Stoner, M. Mohammad, Hypoxic reperfusion of the ischemic heart and oxygen radial generation, Am. J. Physiol. 290 (2006) 341–347.
[19] U. Boonyaprakob, J.E. Gadsby, V. Hedgpeth, P.A. Routh, G.W. Almond, Expression and localization of hypoxia inducible factor-1alpha mRNA in the porcine ovary, Can. J. Vet. Res. 69 (2005) 215–222.
[20] P.H. Maxwell, Hypoxia-inducible factor as a physiological regulator, Exp. Physiol. 90 (2005) 791–797.
[21] N. Unno, S. Baba, M.P. Fink, Cytosolic ionized Ca2+ modulates chemical hypoxia-induced hyperpermeability in intestinal epithelial monolayers, Am. J. Physiol. 274 (1998) 700–708.
[22] S.S. Essawy, A.A. Elbaz, Role of adenosine receptor in the anti-nociceptive effects of allopurinol in mice, Eur. Rev. Med. Pharmacol. Sci. 17 (2013) 1857–1863.
[23] S.L. Hempel, G.R. Buettner, Y.Q. O'Malley, D.A. Wessels, D.M. Flaherty, Dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2',7'-dichlorodihydrofluorescein diacetate, 5(and 6)-carboxy- 2',7'-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123, Free Radic. Biol. Med. 27 (1999) 146–159.
[24] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Analysis of nitrate nitrite, and [15N] nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[25] E. Graham, K. Ruis, A. Hartman, F. Northington, H.E. Fox, A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy, Am. J. Obstet. Gynecol. 199 (2008) 587–595.
[26] A. Kumral, F. Tuzun, D. Yesilirmak, N. Duman, H. Ozkan, Role of epigenetic regulatory mechanisms in neonatal hypoxic-ischemic brain injury, Med. Hypotheses 72 (2009) 692–693.
[27] E. Maloney-Wilensky, V. Gracias, A. Itkin, K. Hoffman, S. Bloom, W. Yang, S. Christian, P.D. LeRoux, Brain tissue oxygen and outcome after severe traumatic brain injury: a systematic review, Crit. Care Med. 37 (2009) 2057–2063.
[28] A. Egbe, S. Uppu, S. Lee, A. Stroustrup, D. Ho, S. Srivastava, Congenital malformations in the newborn population: a population study and analysis of the effect of sex and prematurity, Pediatr. Neonatal. 56 (2015) 25–30.
[29] Z. Wang, D. Wu, H.V. Vinters, Hypoxia and reoxygenation of brain microvascular smooth muscle cells *in vitro*: cellular responses and expression of cerebral amyloid angiopathy-associated proteins, APMIS 110 (2002) 423–434.
[30] J.J. Kaandorp, M.J. Benders, E. Schuit, C.M. Rademaker, M.A. Oudijk, M.M. Porath, S.B. Oetomo, M.G. Wouters, R.M. van Elburg, M.T. Franssen, A.F. Bos, T.R. de Haan, J. Boon, I.P. de Boer, R.J. Rijnders, C.J. Jacobs, L.H. Scheepers, D.A. Gavilanes, K.W. Bloemenkamp, M. Rijken, C.A. van Meir, J.S. von Lindern, A.J. Huisjes, S.C. Bakker, B.W. Mol, G.H. Visser, F. Van Bel, J.B. Derks, Maternal allopurinol administration during suspected fetal hypoxia: a novel neuroprotective intervention? A multicentre randomised placebo controlled trial, Arch. Dis. Child Fetal Neonatal Ed. 100 (2015) F216–F223.
[31] H. Akdemir, Z. Asik, H. Pasaoglu, I. Karakucuk, I.S. Oktem, R.K. Koc, The effect of allopurinol on focal cerebral ischemia: an experimental study in rabbits, Neurosurg. Rev. 24 (2001) 131–135.
[32] R.R. Clancy, S.A. McGaurn, J.E. Goin, D.G. Hirtz, W.I. Norwood, J.W. Gaynor, M.L. Jacobs, G. Wernovsky, W.T. Mahle, J.D. Murphy, S.C. Nicolson, J.M. Steven, T.L. Spray, Allopurinol Neurocardiac protection trial in infants undergoing heart surgery using deep hypothermic circulatory arrest, Pediatrics 108 (2001) 61–70.
[33] K.T. LePage, R.W. Dickey, W.H. Gerwick, E.L. Jester, T.F. Murray, On the use of Neuro-2A neuroblastoma cells versus intact neurons in primary culture in neurotoxicity studies, Crit. Rev. Neurobiol. 17 (2005) 27–50.
[34] Y.M. Noh, Y.S. Kim, Y.Y. Lee, Y.J. Lee, S.H. Kim, H.J. Yu, S.H. Koh, The early activation of PI3 K strongly enhances the resistance of cortical neurons to hypoxic injury via the activation of downstream targets of the PI3 K pathway and the normalization of the levels of PARP activity ATP, and NAD^+, Mol. Neurobiol. 47 (2013) 757–769.
[35] F.J. Geske, R. Lieberman, R. Strange, L.E. Gerschenson, Early stages of p53-induced apoptosis are reversible, Cell Death Differ. 8 (2001) 182–191.
[36] R. Go'mez-Sintes, F. Herna'ndez, A. Bortolozzi, F. Artigas, J. Avila, P. Zaratin, J.P. Gotteland, J.J. Lucas, Neuronal apoptosis and reversible motor deficit in dominant-negative GSK-3 conditional transgenic mice, EMBO J. 26 (2007) 2743–2754.
[37] A.J. Augustin, F.H. Grus, S. Hunt, Effects of allopurinol on free-radical- induced reduction of the proliferation of retinal pigment epithelial cells, Doc. Ophthalmol. 93 (1996) 231–236.
[38] T.M. Millar, J.M. Kanczler, T. Bodamyali, D.R. Blake, C.R. Stevens, Xanthine oxidase is a peroxynitrite synthase: newly identified roles for a very old enzyme, Redox Rep. 7 (2002) 65–70.

[39] P. Sanchez-Cruz, A.E. Alegrı'a, Quinone-enhanced reduction of nitric oxide by xanthine/xanthine oxidase, Chem. Res. Toxicol. 22 (2009) 818–823.
[40] G.L. Semenza, Expression of hypoxia-inducible factor 1: mechanisms and consequences, Biochem. Pharmacol. 59 (2000) 47–53.
[41] H.J. Choi, J.S. Fun, B.G. Kim, S.A. Kim, H. Jeon, Y. Soh, Vitexin an HIF- 1alpha inhibitor, has anti-metastatic potential in PC12 cells, Mol. Cells 22 (2006) 291–299.
[42] A. Siddiq, L.R. Aminova, R.R. Ratan, Prolyl 4-hydroxylase activity-responsive transcription factors: from hydroxylation to gene expression and neuroprotection, Front. Biosci. 13 (2008) 2875–2887.
[43] C. Chen, Q. Hu, J. Yan, X. Yang, X. Shi, J. Lei, L. Chen, H. Huang, J. Han, J.H. Zhang, C. Zhou, Early inhibition of HIF-1α with small interfering RNA reduces ischemic–reperfused brain injury in rats, Neurobiol. Dis. 33 (2009) 509–517.
[44] S. Bonello, C. Zähringer, R.S. BelAiba, T. Djordjevic, J. Hess, C. Michiels, T. Kietzmann, A. Gorlach, Reactive oxygen species activate the HIF-1 promoter via a functional NFkB site, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 755–761.
[45] A.A. Qutub, A.S. Popel, Reactive oxygen species regulate hypoxia- inducible factor- 1α differentially in cancer and ischemia, Mol. Cell. Biol. 28 (2008) 5106–5119.