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Original paper

N-acetylcysteine protects hepatocytes from hypoxia-related cell injury

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Abstract

Aim of the study: Hepatocyte transplantation has been discussed as an alternative to liver transplantation in selected cases of acute and chronic liver failure and metabolic diseases. Immediately after infusion of hepatocytes, hypoxia-related cell injury is inevitable. N-acetylcysteine (NAC) has been suggested to attenuate hypoxic damage. This study's objective was to evaluate NAC's protective effect in a model of hypoxia-related hepatocyte injury.

Material and methods: HepG2 cells were used as a model for hepatocytes and were cultured under standardized hypoxia or normoxia for 24 hours with or without NAC. Growth kinetics were monitored using trypan blue staining. The activation of apoptotic pathways was measured using quantitative real-time PCR for Bcl-2/Bax and p53. The proportions of vital, apoptotic and necrotic cells were verified by fluorescence activated cell sorting using annexin V-labelling. The expression of hypoxia inducible factor 1 (HIF-1) was measured indirectly using its downstream target vascular endothelial growth factor A (VEGF-A).

Results: After NAC, cell proliferation increased under both hypoxia and normoxia by 528% and 320% ($p < 0.05$), while VEGF-A expression decreased under normoxia by 67% and 37% ($p < 0.05$). Compared to cells treated without NAC under hypoxia, the Bcl-2/Bax ratio increased significantly in cells treated with NAC. This finding was confirmed by an increased number of vital cells in FACS analysis.

Conclusions: NAC protects hepatocytes from hypoxic injury and ultimately activates anti-apoptotic pathways.

Key words: hypoxia, N-acetylcysteine, HepG2, hepatocyte transplantation.

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Introduction

In acute and chronic liver failure, liver transplantation (LT) is the only curative treatment available. Since there is a lack of donor organs, there is an ever-growing discrepancy between patients on the waiting list and the number of LTs performed, consequently leading to a high waiting-list mortality [1, 2].

Initially, hepatocyte transplantation (HT) was explored in animal studies. Since the 1960s there have been attempts to perform hepatocyte transplantation as an alternative to LT and to use it as a bridging technique [3].

Cells isolated from rejected donor organs can be used for HT, but may be of poor quality, which can lead to decreased function and viability. Furthermore, cells from split livers or reduced-size liver procedures can be used [4]. Other sources of hepatocytes have been explored, but to date the differentiation of mesenchymal stromal cells (MSC), embryonic stem cells (ES) and induced pluripotent stem cells (iPS) to hepatocytes has only been used in *in vitro* models [5-10].

Groth *et al*. treated hyperbilirubinemia in glucuronyltransferase-deficient rats by intraportal transplantation of hepatocytes [11]. Besides intraportal application of hepatocytes, the spleen, peritoneum and the renal capsule have been investigated as potential sites for HT [12]. In 1998, a 10-year-old girl with Crigler-Najjar syndrome was treated by HT [13].

To achieve a clinically relevant effect, 2.8×10^7 to 3.9×10^{10} cells have to be transplanted [1, 14, 15]. Due to the risk of thromboembolism and portal hypertension, the total number of cells that can be transplanted in a single session is limited [16]. Moreover, the engraftment rates of transplanted hepatocytes are as low as 0% to 12%. In most reported cases, HT was not fully curative and patients had to be bridged towards LT [1].

An increased rate of viable and engrafted hepatocytes could be considered as a major improvement. At least in part, the underlying problem can be attributed to hypoxia during cell transplantation, but several other factors limit the amount of cells that integrate and function as hepatocytes after transplantation [17]. Cryopreservation of hepatocytes is a main contributor to decreased viability before cell transplantation and can impair hepatocyte function [18].

Another limiting factor is that only a small proportion of cells is able to pass the endothelial lining as well as the fact that the portal venous blood is already desaturated and therefore sufficient oxygen supply cannot be guaranteed [1, 19]. Since the number of available cells is limited, the viability of these cells needs to be optimized.

N-acetylcysteine (NAC) is a well-known antioxidative agent which protects multiple organs and cells from ischemic and other damage. Hepatoprotective, anti-inflammatory, immunomodulatory and kidney-protective effects have been demonstrated [20-26]. Due to its chemical structure, it can act as a glutathione (GSH) precursor and substitute [27]. In acetaminophen-induced liver failure, the intravenous application of NAC is the treatment of choice [28]. The protective effect is based on the promotion of hepatic GSH synthesis and reduced protein binding [29]. In a prospective, double blind trial with 173 patients with acute liver failure, patients were treated with NAC or a placebo [30]. Transplant-free survival increased from 27% in controls to 40% in patients treated with NAC ($p = 0.043$). A recent randomized controlled trial found an attenuated increase in liver enzymes, lower serum CRP levels and a significantly decreased hospital stay after liver resection in cirrhotic patients after NAC [31]. In a recent retrospective analysis of patients with idiosyncratic liver injury after flupirtine, a combined treatment with NAC and prednisolone was shown to decrease AST, ALT and INR levels [32]. Taken together, there is considerable evidence from clinical data underlining the clinical efficacy and importance of NAC as a liver protective agent.

At least in part, the hepatoprotective effect of NAC is related to GSH replenishment. Furthermore, NAC improves mitochondrial energy metabolism through induction of mitochondrial enzymes including increased levels of pyruvate dehydrogenase [29, 33, 34]. Moreover, NAC can inhibit NF-κB and pro-inflammatory cytokines such as interleukin-1 and tumour necrosis factors, which play a pivotal role in the regulation and induction of apoptosis [27, 35, 36]. In addition, NAC has a positive impact on the microcirculatory blood flow and tissue oxygenation [37]. Some authors have hypothesized that NAC leads to an improved outcome in terms of decreased postoperative transaminases and improved liver function after LT [38].

In regard to HT, the physiological role of NAC has not been fully investigated yet [33]. Therefore, this study aims to evaluate the protective effect of NAC in a model of hypoxic HT.

Material and methods

Cell culture

Hep G2-cells were used as a model for human hepatocytes and were cultured in normoxic and hypoxic conditions on 24-well plates. In each well, 80×10^5 cells were used. Cells were cultured for 48 hours using high glucose DMEM (PAA Laboratories GmbH, Pasching, Austria), supplemented with 10% FBS (PAA Laboratories GmbH, Pasching, Austria) and 10% penicillin/streptomycin (Biochrom AG, Berlin, Germany) at 37°C in 5% CO_2 humidified atmosphere. In a pilot study preceding the described experiments, ascending concentrations of NAC (0.1-10 mM) were used. An effect was detected when a concentration of 2 mM was used. With higher concentrations, there was no statistically significant increase of the detected effect. Thus, a concentration of 2 mM was employed for the experiments presented here. After 48 hours, the culture medium was changed to DMEM supplemented with (groups 2 and 4) or without (groups 1 and 3) 2 mmol/l NAC (Carl Roth GmbH, Karlsruhe). Groups 1 (Norm) and 2 (NAC/norm) were cultured at standard conditions in a humidified atmosphere at 37°C and 5% CO_2. Groups 3 (Hypo) and 4 (NAC/hypo) were cultured using a modular incubator chamber (Billups-Rothenberg, Del Mar, USA) at 37°C in 5% O_2 for 24 hours. In each group triplets were used for each assay.

Growth kinetics

To monitor growth kinetics trypan blue staining was used as described elsewhere [39]. Briefly, the cell solution was diluted using an equal volume of Trypan blue 0.5% (w/v) (Biochrom AG, Berlin, Germany) and 10 µl were transferred to a Neubauer improved cell

Table 1. Primers used for qRT-PCR analysis

Name	Primer
GAPDH forward	ATGACTCTACCCACGGCAAG
GAPDH reverse	GGAAGATGGTGATGGGTTTC
Bax forward	GCAGATCATGAAGACAGGGG
Bax reverse	ACACTCGCTCAGCTTCTTGG
Bcl-2 forward	GAACATTTCGGTGACTTCCG
Bcl-2 reverse	CCTGTTGATCATCCCTGGAG
p53 forward	CCCAAGCAATGGATGATTTGA
p53 reverse	GGCATTCTGGGAGCTTCATCT
β-actin forward	GAAATCGTGCGTGACATTAAGG
β-actin reverse	TCAGGCAGCTCGTAGCTTCT
VEGF-A forward	CTTGCCTTGCTGCTCTACC
VEGF-A reverse	CACACAGGATGGCTTGAAG

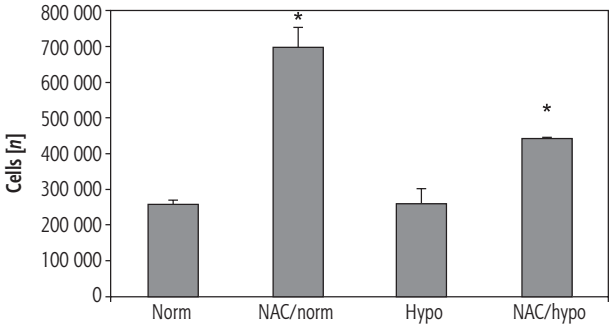

Fig. 1. Cell proliferation. Number of cells after 72 hours. Norm: 256 × 10^3 ±24 249. NAC/norm: 696 × 10^3 ±100 936 (p = 0.01). Hypo: 260 × 10^3 ±77 382. NAC/hypo: 442 × 10^3 ±6928 (p = 0.05)

counting chamber and mean values and standard deviations were calculated.

Fluorescence activated cell sorting

To measure the proportion of vital, apoptotic and necrotic cells annexin V/propidium iodide labelling was performed and a FACSCalibur Cell Analyzer (Becton Dickinson, Heidelberg, Germany) was used. Cells were washed and incubated in FITC-annexin V (1 mg/ml) solution for 10 minutes. Subsequently, propidium iodide solution (1 mg/ml) was added. The assessment of cells was done using CellQuest Software (Becton and Dickinson, Heidelberg, Germany).

Quantitative reverse transcriptase quantitative polymerase chain reaction

The mRNA expression of pro- and anti-apoptotic markers was analyzed using qRT-PCR. The expression of p53, Bax and Bcl-2 was measured. Moreover, VEGF-A expression was analysed to evaluate the cells' reaction to conditions.

The RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) was used to isolate mRNA from the samples and the First Strand cDNA Synthesis Kit (Roche Diagnostic GmbH, Mannheim, Germany) was used for the transcription into cDNA. Quantitative PCR was done using the StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). The analysis was done by the ΔΔCT method using the StepOne Software 2.1 (Applied Biosystems, Foster City, USA).

Custom primers were used for Bax, Bcl-2, p53 and VEGF-A. GAPDH was used as an endogenous control for Bax, BCL-2 and p53, and β-actin was used as an endogenous control for VEGF-A (Table 1).

Statistics

For descriptive presentation of data, mean values and standard error are used. Analysis of variance (ANOVA) was used for statistical testing and $p < 0.05$ was considered statistically significant.

Results

Growth kinetics

Initially 80 × 105 cells were seeded in each well. After incubation for 72 hours in a humidified atmosphere the cell count increased in all groups. While there was not a significantly different cell count between normoxic (256 × 10^3 ±24 249) and hypoxic (260 × 10^3 ±77 382) conditions, NAC significantly increased cell counts to 696 × 10^3 ±100 936 (p = 0.01) and 442 × 10^3 ±6928 (p = 0.05) under normoxic and hypoxic conditions (Fig. 1).

Fluorescence activated cell sorting

Under normoxic conditions, the proportion of vital cells increased from 82% to 94%, while the amount of necrotic and apoptotic cells decreased from 15% to 5% and from 3% to 1%. Under hypoxic conditions the number of vital cells decreased from 82% to 68% compared to normoxic conditions while the proportion of apoptotic and necrotic cells increased from 3% to 5% and from 15% to 27%. NAC increased the number of vital cells under hypoxic conditions from 68% to 80%. While there was no difference for apoptotic cells, the proportion of necrotic cells decreased from 27% to 15% (Fig. 2).

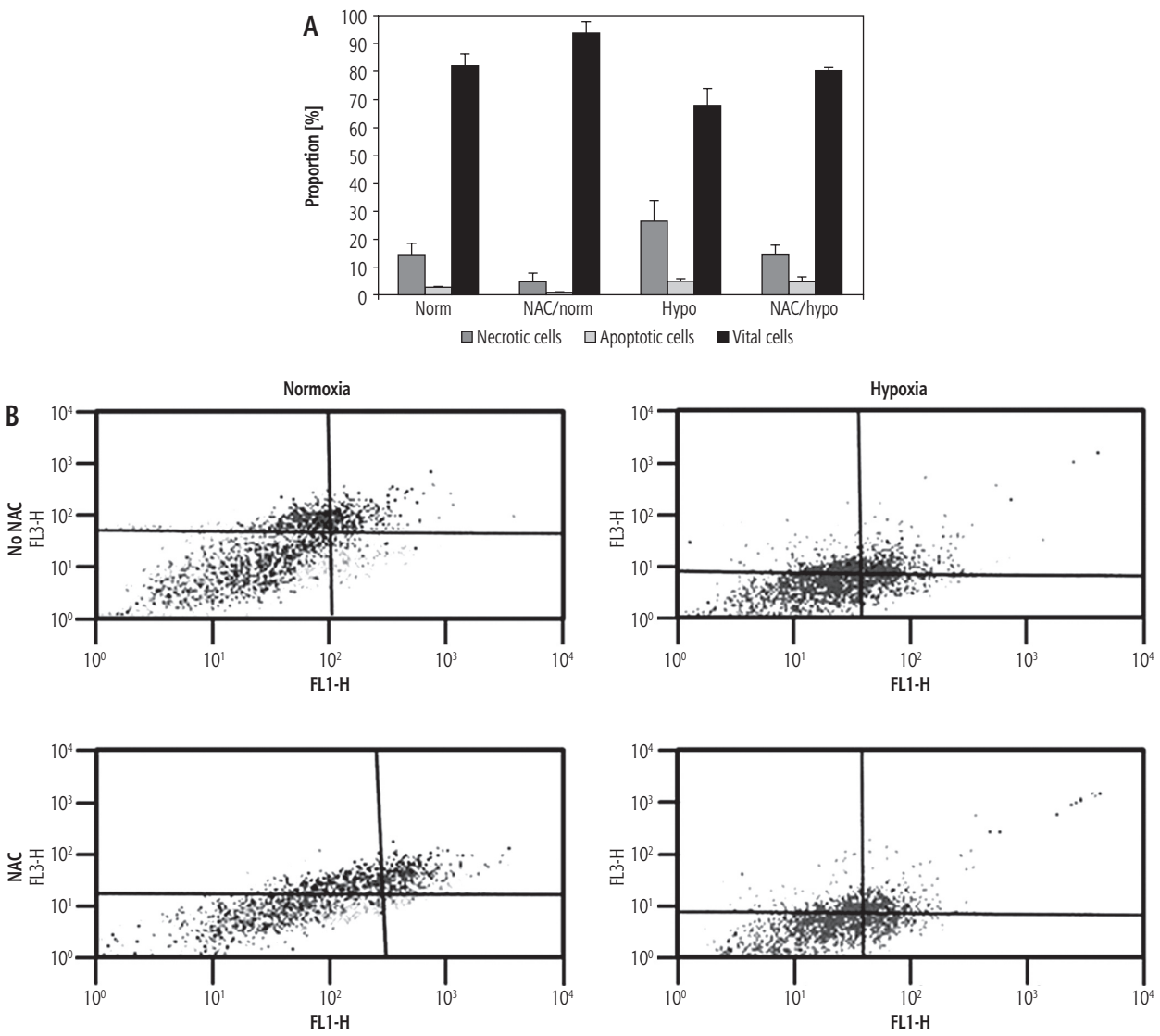

Fig. 2. FACS. **A**) Proportion of necrotic, apoptotic and vital cells measured using annexin V labelling. Norm: vital cells: 82%; apoptotic cells: 3%; necrotic cells: 15%. NAC/norm: vital cells: 94%; apoptotic cells: 1%; necrotic cells: 5%. Hypo: vital cells: 68%; apoptotic cells: 5%; necrotic cells: 27%. NAC/hypo: vital cells: 82%; apoptotic cells: 3%; necrotic cells: 15%. **B**) Representative images of FACS analysis using annexin V (FL1-H) and PI (FL3-H) labelling for cells treated with or without NAC under normoxic and hypoxic conditions

Quantitative reverse transcriptase quantitative polymerase chain reaction

In NAC-treated cells under normoxia, the expression of the apoptotic markers decreased significantly. Bax expression decreased by the factor 0.34 (p = 0.0017), Bcl-2 expression decreased by 0.31 (p = 0.0031) and p53 expression decreased by 0.58 (p = 0.0094). There were no significant changes in the Bcl-2/Bax ratio. Under hypoxia, the expression of all markers increased significantly while the Bcl-2/Bax ratio decreased significantly from 1 to 0.98 (p = 0.01) in comparison to normoxia. After the application of NAC all apoptotic markers increased significantly [Bax: 4.06 (p = 0.0003); Bcl-2: 4.34 (p = 0.0007); p53: 2.57 (p = 0.005)] and the Bcl-2/Bax ratio increased from 0.98 to 0.99 (p = 0.003) (Fig. 3).

VEGF-A increased significantly under hypoxic conditions compared to normoxic cell cultures by 726% (p = 0.00018). There was no further effect by NAC. Under normoxic conditions, NAC reduced the expression of VEGF-A time-dependently. After 24 hours the expression decreased to 0.67 (p = 0.04) and after 48 hours to 0.37 (p = 0.0017) (Fig. 4).

Discussion

In HT, hypoxia is unavoidable and may lead to poor cell engraftment and subsequent cell death. After

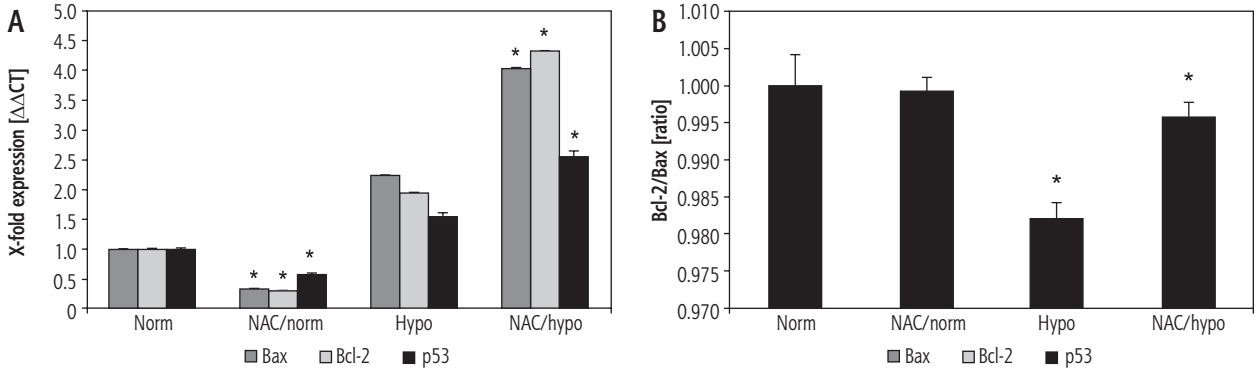

Fig. 3. Activation of pro- and anti-apoptotic markers by PCR. **A**) Expression of Bax, Bcl-2 and p53. Norm: Expression under normoxic conditions as reference. NAC/norm: Bax: 0.34 (p = 0.0017); Bcl-2: 0.31 (p = 0.0031); p53: 0.58 (p = 0.0094). Hypo: Bax: 2.25 (p = 0.0034); Bcl-2: 1.95 (p = 0.0095); p53: 1.56 (p = 0.02). NAC/hypo: Bax: 4.06 (p = 0.0003); Bcl-2: 4.34 (p = 0.0007); p53: 2.57 (p = 0.005). **B**: Bcl-2/Bax ratio. Norm: Bcl-2/Bax ratio under normoxic conditions as reference. NAC/norm: 0.999 (p = 0.83). Hypo: 0.982 (p = 0.01). NAC/hypo: 0.996 (p = 0.0025)

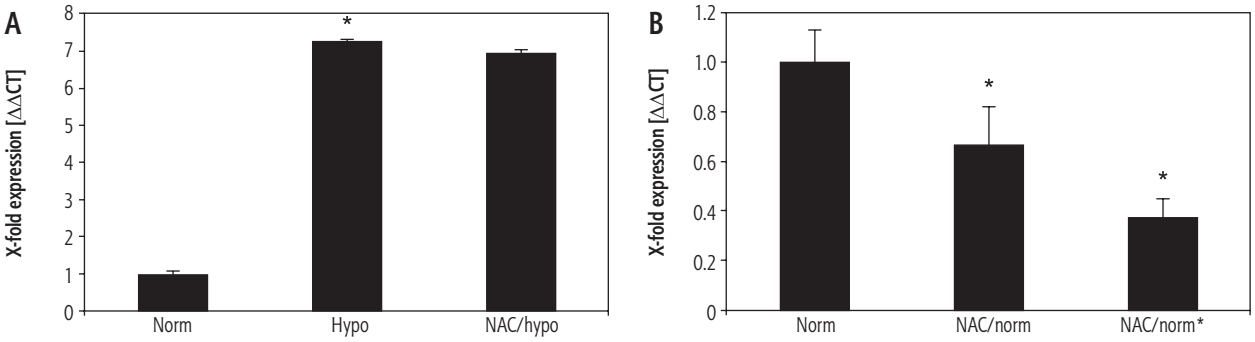

Fig. 4. A) VEGF-A expression. Norm: VEGF-A expression under normoxic conditions as reference. Hypo: 7.26 (p = 0.0002). NAC/hypo: 6.93 (p = 0.6). **B**) VEGF-A expression measured by PCR under normoxic conditions. Norm: VEGF-A expression under normoxic conditions. NAC/norm: VEGF-A expression after 24 hours in normoxic conditions + NAC: 0.67 (p = 0.04). NAC/norm*: VEGF-A expression after 48 hours in normoxic conditions + NAC: 0.37 (p = 0.0017).

infusion of cells via the portal vein or into the spleen, cells engraft in the sinusoids and have to survive under hypoxic conditions [19]. While *in vivo* hypoxia may be as low as 0.1-1% oxygen, we chose to use a 5% oxygen model for our *in vitro* experiments. In contrast to cell culture, *in vivo* oxygen transport cannot be linked exclusively to the level of oxygen, but to hemoglobin-bound oxygen and diffusion in a three dimensional structure. This can be considered to be a general limitation of *in vitro* studies related to the effects of hypoxia. Since our study's focus was not related to monitoring effects linked to the level of hypoxia but aimed at detecting an effect of NAC on hypoxia-related changes, we chose a moderate level of hypoxia. In this study, NAC has been evaluated as a possible supportive drug. There was a significant effect of NAC on cell proliferation under both normoxic and hypoxic conditions.

An influence of NAC on apoptosis has already been shown. Oh *et al.* demonstrated an inhibitory effect of NAC on apoptosis in a HepG2 model with cadmium-induced apoptosis triggered by caspases 3, 8 and 9 as well as Bax [40]. Interestingly, in this model the effect of NAC was independent of reactive oxygen species (ROS) production. Using FACS analysis, our study confirmed an influence on the proportion of vital, apoptotic and necrotic cells.

Under both hypoxic and normoxic conditions, NAC regulated the expression of all apoptotic markers. Notably, when supplementing NAC in hypoxic conditions the Bcl-2/Bax ratio increased compared to hypoxic controls without supplementation of NAC. A similar effect in terms of an increased Bcl-2/Bax ratio was demonstrated by Wu *et al.* in myocardial cells in an animal heart failure model when doxorubicin-induced apoptosis was reduced significantly after supplementation of NAC [41]. NAC seems to have more than just an antioxidative effect. Other authors have demonstrated a positive effect on the mitochondrial stability due to an influence on mitochondrial enzymes [29, 33, 34]. These observations are in accordance with the effect that NAC has on expression of Bcl-2 and Bax, which are promoters of mitochondrial apoptosis [42].

VEGF-A, which is a downstream target marker of hypoxia inducible factor 1 (HIF-1), increases under hypoxic conditions. NAC blocks HIF-1 stabilization and therefore the activation of VEGF-A, which is activated by HIF-1 [43, 44]. In our study, no significant effect of VEGF-A was observed when NAC was added under hypoxic conditions. However, NAC time-dependently reduced VEGF-A expression significantly under normoxic conditions, while cell proliferation and survival were independent of VEGF-A due to the application of NAC. It should be noted that VEGF-A is a cellular marker for hypoxic stress and a mediator for cell adaptation to a harmful environment [45]. Even if the cells were not exposed to real oxidative stress, it seems that NAC has a stronger influence on cell proliferation and apoptosis than VEGF-A.

In conclusion, NAC significantly increases cell proliferation while apoptosis is decreased. NAC could be a worthwhile addition to carrier solutions in HT since the cells needed are limited, may be of poor quality and may be exposed to desaturated portal venous blood.

Nonetheless, further research is required in order to verify that NAC has beneficial effects on hepatocyte transplantation.

Disclosure

Authors report no conflict of interest.

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