N-acetylcysteine protects hepatocytes from hypoxia-related cell injury

Jan Heil1, Daniel Schultze2, Peter Schemmer2, Helge Bruns3

1Department of General and Visceral Surgery, Goethe-University Hospital Frankfurt, Frankfurt/M., Germany
2Department of General, Visceral and Transplantation Surgery, Heidelberg University Hospital, Heidelberg, Germany
3European Medical School, University Hospital for General and Visceral Surgery, Klinikum Oldenburg, Carl von Ossietzky University, Oldenburg, Germany

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.

Address for correspondence

Dr. Helge Bruns, Klinikum Oldenburg, Carl von Ossietzky University Oldenburg, 10 Rahel Straus St., 26133 Oldenburg, Germany, e-mail: helge.bruns@uni-oldenburg.de

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 Cri-gler-Najjar syndrome was treated by HT [13].
N-acetylcysteine protects hepatocytes

To achieve a clinically relevant effect, $2.8 \times 10^7$ to $3.9 \times 10^{10}$ 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, $80 \times 10^5$ 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 $37^\circ C$ 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 $37^\circ C$ and 5% CO$_2$. Groups 3 (Hypo) and 4 (NAC/hypo) were cultured using a modular incubator chamber (Billups-Rothenberg, Del Mar, USA) at $37^\circ C$ 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...
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 × 10⁵ ±24 249) and hypoxic (260 × 10⁵ ±77 382) conditions, NAC significantly increased cell counts to 696 × 10⁵ ±100 936 (\( p = 0.01 \)) and 442 × 10⁵ ±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).
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
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 oxygen 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.

References

1. Jorns C, Ellis EC, Nowak G, et al. Hepatocyte transplantation for inherited metabolic diseases of the liver. J Intern Med 2012; 272: 201-223.
2. Jochmans I, van Rosmalen M, Pirenne J, Samuel U. Adult liver allocation in Eurotransplant. Transplantation 2017; 101: 1542-1550.
3. Gupta S, Wilson JM, Chowdhury JR. Hepatocyte transplantation: development of new systems for liver repopulation and gene therapy. Semin Liver Dis 1992; 12: 321-331.
4. Mitry RR, Hughes RD, Aw MM, et al. Human hepatocyte isolation and relationship of cell viability to early graft function. Cell Transplant 2003; 12: 69-74.
5. Lange C, Bassler P, Lioznov MV, et al. Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. World J Gastroenterol 2005; 11: 4497-4504.
6. Lange C, Bassler P, Lioznov MV, et al. Hepatic gene expression in cultured rat mesenchymal stem cells. Transplant Proc 2005; 37: 276-279.
7. Lange C, Schroder J, Stute N, et al. High-potential human mesenchymal stem cells. Stem Cells Dev 2005; 14: 70-80.
8. Groth A, Ottinger S, Kleist C, et al. Evaluation of porcine mesenchymal stem cells for therapeutic use in human liver cancer. Int J Oncol 2012; 40: 391-401.
9. Mallanna SK, Duncan SA. Differentiation of hepatocytes from pluripotent stem cells. Curr Protoc Stem Cell Biol 2013; 26: Unit 1G 4.
10. Hansel MC, Granignoli R, Skvorak KL, et al. The history and use of human hepatocytes for the treatment of liver diseases: the first 100 patients. Curr Protoc Toxicol 2014; 62: 14.12.1-14.12.23.
11. Groth CG, Arborg B, Bjorken C, et al. Correction of hyperbilirubinemia in the glucuronotransferase-deficient rat by intraportal hepatocyte transplantation. Transplant Proc 1977; 9: 313-316.
12. Gupta S, Bhargava KK, Novikoff PM. Mechanisms of cell engraftment during liver repopulation with hepatocyte transplantation. Semin Liver Dis 1999; 19: 15-26.
13. Fox JI, Chowdhury JR, Kaufman SS, et al. Treatment of the Cri-gler-Najjar syndrome type 1 with hepatocyte transplantation. N Engl J Med 1998; 338: 1422-1426.
14. Fisher RA, Strom SC. Human hepatocyte transplantation: worldwide results. Transplantation 2006; 82: 441-449.
15. Vacanti JP, Kulig KM. Liver cell therapy and tissue engineering for transplantation. Semin Pediatr Surg 2014; 23: 150-155.
16. Ho CM, Chen YH, Chien CS, et al. Transplantation speed offers early hepatocyte engraftment in acute liver injured rats: A translational study with clinical implications. Liver Transpl 2015; 21: 652-661.
17. Ibars EP, Cortes M, Tolosa L, et al. Hepatocyte transplantation program: Lessons learned and future strategies. World J Gastroenterol 2016; 22: 874-886.
18. Stephenne X, Najimi M, Sokal EM. Hepatocyte cryopreservation: is it time to change the strategy? World J Gastroenterol 2010; 16: 1-14.
19. Smith MK, Peters MC, Richardson TP, et al. Locally enhanced angiogenesis promotes transplanted cell survival. Tissue Eng 2004; 10: 63-71.
20. van Tonder JJ, Gulumian M, Cromarty AD, Steenkamp V. In vitro effect of N-acetylcysteine on hepatocyte injury caused by dichlorodiphenyltrichloroethane and its metabolites. Hum Exp Toxicol 2014; 33: 41-53.
21. Shimizu MH, Gois PH, Volpini RA, et al. N-acetylcysteine protects against star fruit-induced acute kidney injury. Ren Fail 2017; 39: 193-202.
22. Yu W, Guo F, Song X. Effects and mechanisms of pirfenidone, prednisone and acetylcysteine on pulmonary fibrosis in rat idiopathic pulmonary fibrosis models. Pharm Biol 2017; 55: 450-455.
23. da Costa M, Bernardi J, Costa L, et al. N-acetylcysteine treatment attenuates the cognitive impairment and synaptic plasticity loss induced by streptozocin. Chem Biol Interact 2017; 272: 37-46.
24. Liao CY, Chung CH, Wu CC, et al. Protective effect of N-acetylcysteine on progression to end-stage renal disease: Necessity for prospective clinical trial. Eur J Intern Med 2017; 44: 67-73.
25. Čolović MB, Vasić VM, Djuric DM, Krstić DZ. Sulphur-containing amino acids: protective role against free radicals and heavy metals. Curr Med Chem 2018; 25: 324-335.
26. Bartekova M, Baranick M, ferencyzova K, Dhallan NS. Beneficial effects of N-acetylcysteine and N-mercaptoPropionylglycine on ischemia reperfusion injury in the heart. Curr Med Chem 2018; 25: 355-366.
27. Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci 2003; 60: 37-46.
28. Polson J, Lee WM, American Association for the Study of Liver D. AASLD position paper: the management of acute liver failure. Hepatology 2005; 41: 1179-1197.
29. Saito C, Zwingmann C, Jaeschke H. Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine. Hepatology 2010; 51: 246-254.
30. Lee WM, Hynan LS, Rossaro L, et al. Intravenous N-acetylcysteine improves transplant-free survival in early stage non-acetaminophen acute liver failure. Gastroenterology 2009; 137: 856-864.
31. Sayed E, Gaballah K, Younis E, et al. The effect of intravenous infusion of N-acetyl cysteine in cirrhotic patients undergoing liver resection: A randomized controlled trial. J Anaesthesiol Clin Pharmacol 2017; 33: 450-456.
32. Borlak J, van Bömmel F, Berg T. N-acetylcysteine and prednisolone treatment improved serum biochemistries in suspected flupirtine cases of severe idiosyncratic liver injury. Liver Int 2018; 38: 365-376.
33. Zwingmann C, Bilodeau M. Metabolic insights into the hepatoprotective role of N-acetylcysteine in mouse liver. Hepatology 2006; 43: 454-463.
34. Koch A, Trautwein C. N-acetylcysteine on its way to a broader application in patients with acute liver failure. Hepatology 2010; 51: 338-340.
35. Jones AL. Mechanism of action and value of N-acetylcysteine in the treatment of early and late acetaminophen poisoning: a critical review. J Toxicol Clin Toxicol 1998; 36: 277-285.
36. Hogquist KA, Nett MA, Unanue ER, Chaplin DD. Interleukin 1 is processed and released during apoptosis. Proc Natl Acad Sci U S A 1991; 88: 8485-8489.
37. Harrison PM, Wendon JA, Gimson AE, et al. Improvement by acetylcysteine of hemodynamics and oxygen transport in fulminant hepatic failure. N Engl J Med 1991; 324: 1852-1857.
38. Jegatheeswaran S, Siriwardena AK. Experimental and clinical evidence for modification of hepatic ischaemia-reperfusion injury by N-acetylcysteine during major liver surgery. HPB (Oxford) 2011; 13: 71-78.
39. Fiegel HC, Bruns H, Hoper C, et al. Cell growth and differentiation of different hepatic cells isolated from fetal rat liver in vitro. Tissue Eng 2006; 12: 123-130.
40. Oh SH, Lim SC. A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. Toxicol Appl Pharmacol 2006; 212: 212-223.
41. Wu XY, Luo AY, Zhou YR, Ren JH. N-acetylcysteine reduces oxidative stress, nuclear factorkappaB activity and cardiomyocyte apoptosis in heart failure. Mol Med Rep 2014; 10: 615-624.
42. Roy MJ, Vom A, Czabotar PE, Lessene G. Cell death and the mitochondria: therapeutic targeting of the BCL-2 family-driven pathway. Br J Pharmacol 2014; 171: 1973-1987.
43. Koch S, Claesson-Welsh L. Signal transduction by vascular endothelial growth factor receptors. Cold Spring Harb Perspect Med 2012; 2: a006502.
44. Sceneay J, Liu MC, Chen A, et al. The antioxidant N-acetylcysteine prevents HIF-1 stabilization under hypoxia in vitro but does not affect tumorigenesis in multiple breast cancer models in vivo. PLoS One 2013; 8: e66388.
45. Roy H, Bhardwaj S, Yla-Herttuala S. Biology of vascular endothelial growth factors. FEBS Lett 2006; 580: 2879-2887.