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ORIGINAL ARTICLE

The effect of cholinesterase inhibition on liver dysfunction in experimental acute liver failure

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KEYWORDS

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Abstract *Introduction:* Acute liver failure (ALF), like sepsis, is associated with an overwhelming activation of the immune response in which hepatic and circulating inflammatory cytokines play a pivotal role. Cholinesterase inhibition has been shown to have anti-inflammatory properties in experimental sepsis. We investigated the role of neostigmine in attenuating D-galactosamine (D-GalN)-induced ALF.

Methods: Thirty-six female wistar rats were randomly allocated to three groups: a control group, a D-GalN group receiving a single i.p. injection of D-galactosamine (400 mg kg^{-1} BW) and a neostigmine-treated D-GalN group receiving a single i.p. injection of D-galactosamine followed 24 h later by i.p. injection of neostigmine methylsulfate 0.25% (80 μg kg^{-1} BW) three times daily for 3 successive days. Rats were sacrificed 24 h after the last injection. Plasma levels of liver transaminases, total proteins, albumin, prothrombin, total bilirubin and hepatic levels of superoxide dismutase and malondialdehyde were measured. Liver expression of cytokines (HMGB-1, TNF-α and IL-10) and histopathology were evaluated.

Results: Neostigmine attenuated liver dysfunction and improved liver synthetic and excretory functions, reduced proinflammatory cytokine HMGB1 (95% CI 0.33–1.09) and TNF-α (95% CI

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1.26–2.06) expression compared to D-GalN group (95% CI 2.67–4.73 and 7.33–14.53, respectively, $P < 0.001$) and increased expression of the anti-inflammatory cytokine IL-10 in liver tissue (95% CI 2.49–4.17 vs 0.04–0.21 in D-GalN group, $P < 0.001$). Neostigmine also significantly increased antioxidant level, and decreased oxidative burden caused by D-GaIN.

Conclusion: Neostigmine improved liver function in a rat ALF model through an anti-inflammatory activity.

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Acute liver failure (ALF) may lead to massive liver cell death and severe liver dysfunction, multiorgan failure and death, and thus represents a major therapeutic challenge [1]. It necessitates intensive care and the close cooperation of liver surgeons, hepatologists, intensivists and anesthesiologists specialized in liver disease. The use of emergency liver transplantation in ALF is limited by organ availability; newer therapeutic options are required to bridge to transplantation or to treat patients unsuitable for liver transplantation.

Hepatic and circulating inflammatory cytokines are central in the pathophysiology of ALF, being involved in hepatocyte necrosis, extrahepatic complications and hepatocyte regeneration [2]. Hepatocellular apoptosis is a crucial step in acute liver injury, functioning as a signal for leukocyte migration and attack on parenchymal cells, which establishes a vicious circle of aggravated leukocytic inflammation and cell death [3]. Although it is not yet fully understood whether apoptosis or necrosis predominates in acute liver injury [4], it is clear that treatment should target the downstream consequences of inflammatory activation. It may therefore be important to identify the signals that modulate the subsequent cellular and molecular mechanisms responsible for liver cell death, in order to develop treatments to encourage regeneration, rather than cell death [4–6].

Besides the known cholinergic pathways involved in autonomic regulation, the 'cholinergic anti-inflammatory pathway' that inhibits macrophage cytokine production has been discovered [7–9]. Acetylcholine (ACh) can interact with α-7 subunits of nicotinic ACh receptors (α-7 nACh R), leading to inhibition of cytokine release [7–9]. Cholinergic activation has been shown to have anti-inflammatory effects [10–14]. Hepatic stellate cells have been recently shown to express functional nACh R subunits including an α-7 subtype [15].

As with sepsis, ALF is associated with overwhelming activation of the immune system. In a murine sepsis model, Hofer et al. demonstrated the protective effects of the cholinesterase inhibitor neostigmine, which reduces the production of proinflammatory cytokines, TNF-α, IL-1β and IL-6, and improves survival [11]. The present study was designed to test the hypothesis that neostigmine, a cholinesterase inhibitor, can modulate liver functions, pro inflammatory cytokines HMGB-1, TNF-α and the anti-inflammatory cytokine IL-10 in an ALF model.

Materials and methods

Experimental animals

Thirty-six adult female Wistar rats weighing 200–250 g were purchased from the experimental animal farm (Giza, Egypt) and maintained in the Physiology Department Animal House under standard conditions of boarding. They were given free access to regular diet and water. The care of animals and all experimental procedures were approved by the Research Ethics Committee at the Faculty of Medicine, Ain-Shams University, Egypt (FMASU REC No. 1032/2011).

Experimental protocol

The rats were randomly allocated to three groups of twelve: Control group (C) rats received normal saline as a single intraperitoneal (i.p.) injection (1.5 ml kg^{-1}, (as solvent of D-galactosamine), followed 24 h later by i.p. injection of normal saline three times daily for 3 successive days (1 ml kg^{-1}, as solvent of neostigmine). Group D-GalN rats received a single i.p. injection of D-galactosamine (Sigma, St. Louis, MO, USA) at a dose of 400 mg kg^{-1} body weight (BW) [16] followed 24 h later by i.p. injection of normal saline three times daily for 3 successive days. Neostigmine-treated D-galactosamine (group N) rats received a single i.p. injection of D-galactosamine at a dose of 400 mg kg^{-1} BW, followed 24 h later by i.p. injection of neostigmine methylsulfate 0.25% (EIPICO, Egypt) at a dose of 80 μg kg^{-1} BW three times daily for 3 successive days [11]. All animals were sacrificed 24 h after the last injection i.e. at day 5. A graphical depiction of the study design is presented in Figure 1.

Experimental procedures

The rats, having had free access to water, were weighed on the day of experiment after an overnight fast and then anesthetized by i.p. injection of thiopental sodium (EIPICO, Egypt), 40 mg kg^{-1} BW. The abdominal aorta was then exposed and two blood samples collected: one into a tube containing 3.2% sodium citrate for determination of prothrombin time (PT) and the other into a heparinised tube for determination of plasma alanine transferase (ALT), aspartate transferase (AST), alkaline phosphatase (ALP), total proteins, albumin (ALB) and total bilirubin (TBIL).

The liver was removed from the abdominal cavity. The left lobe was flushed with physiological saline and stored at −80 °C for subsequent measurement of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. The right lobe was divided into two portions; one was stored at −80 °C for later determination of gene expression of HMGB-1, TNF-α and IL-10 and the other was used for histological study.

Liver function assay

Plasma ALT and AST activities were estimated by Reitman-Frankel colorimetric methods whereas TBIL analysis was car-

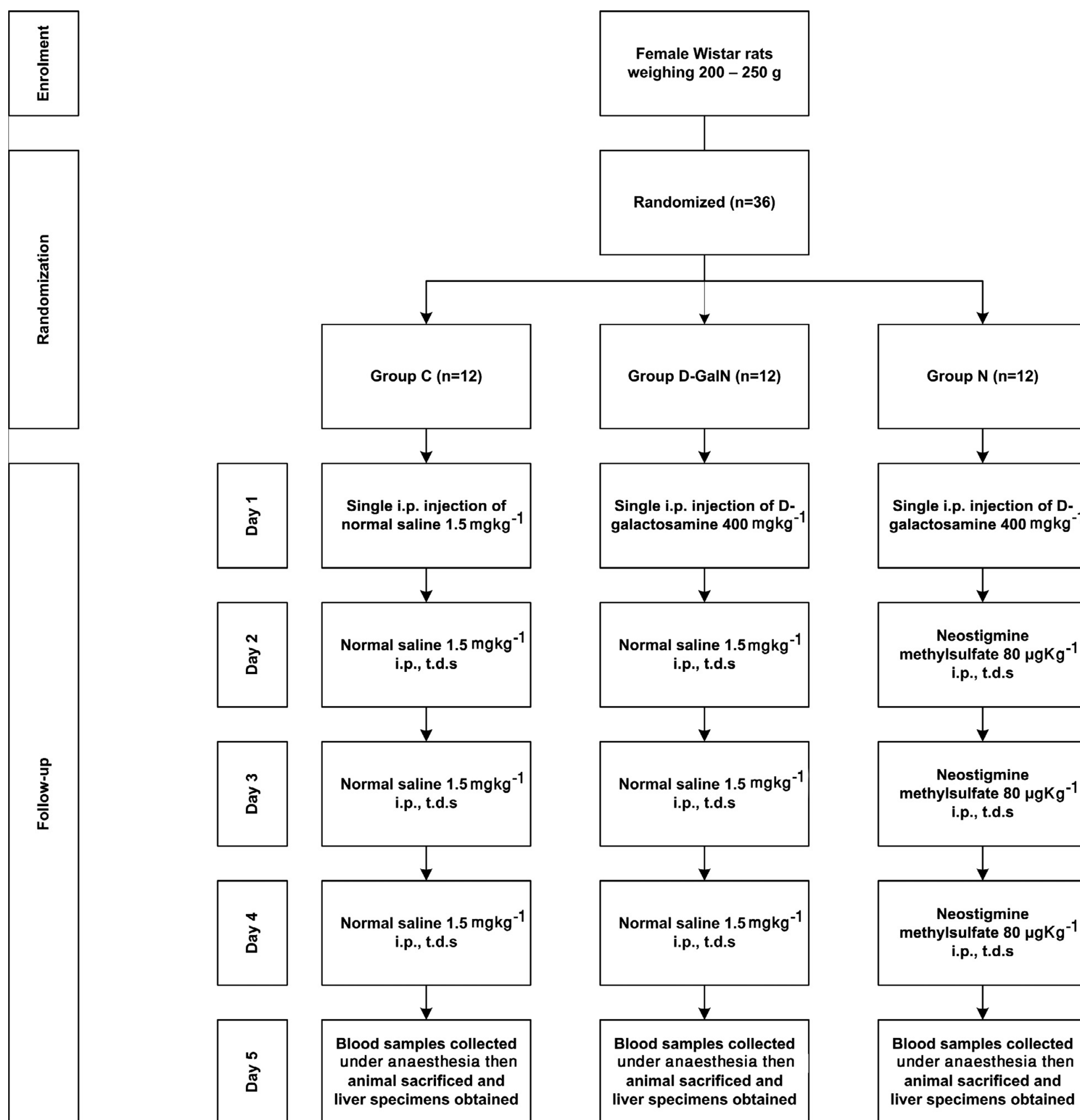

Figure 1 Flowchart illustrating animal enrollment, randomization, experimental protocol, and analysis of data.

ried out by Jendrassik-Grof method using kits supplied by Quimica Clinica Aplicada S.A., Spain. Plasma ALP activity, total proteins and ALB were determined by quantitative colorimetric method using kits supplied by Biolabo SA, France and Stanbio-laboratory, U.S.A. PT was determined according to the method of Caen et al., using Neoplastine Cl plus kits supplied by Diagnostica Stago, France [17].

Oxidant/antioxidant markers

Antioxidant enzyme SOD activity and MDA concentration, as indices of lipid peroxidation products, were assayed in the liver. Liver homogenates were prepared by homogenizing liver tissues (200 mg ml^{-1}) in cold buffer (50 mM potassium phosphate, pH 7.5, and 1 mM EDTA) for SOD and liver tissues (100 mg ml^{-1}) in 1.15% KCl buffer for MDA, using tissue homogonizer (IKA-WERK, Ultra-Turrax, West Germany). Tissue homogenates were centrifuged at 4000 rpm for 15 min and the supernatants stored at −80 °C. SOD was measured by its inhibition of phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye [18]. MDA was determined by spectrophotometric measurement of the color occurring during the reaction of MDA with thiobarbituric acid [19]. Kits were supplied by Bio-diagnostic, Egypt.

Measurement of HMGB-1, TNF-α and IL-10 gene expression in the liver by quantitative real-time PCR (RT-qPCR)

Specimens of liver were subjected to tissue disruption and homogenization with a rotor–stator homogenizer. Total

RNA was extracted immediately according to the manufacturer's instructions of MagNA Pure Compact Nucleic Acid Isolation Kit I (Cat. No. 03730964001-Roche, Germany) using the MagNa Pure Compact Instrument (Roche-Germany) which is a fully automated system. The total yield of RNA was determined spectrophotometrically. The extracted purified RNA was converted into complementary DNA (cDNA) by reverse transcriptase and amplified by Light Cycler-RNA Amplification Kit SYBR Green I (Cat. No. 2015137).The kit is for a one-step RT-PCR using the Light Cycler 2.0 System (Roche, Germany). RT-qPCR was performed with specific primers corresponding to the proinflammatory cytokine genes: HMGB-1, TNF-α, and IL-10. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used to normalize the amount of RNA in each sample. The forward and reverse primers for: HMGB-1 were: 5′-TGTCCACACACCCTGCATATTG-3′ and 5′- AATCCCATGGTGTGACAGAATTGA-3′; IL-10 were: 5′-CTTGCACTACCAAAGCCACA-3′ and 5′-AAGTG TGGCCAGCCTTAGAA-3′; TNF-α were: 5′-ATCTTCTC AAAATTCGAGTGACAA-3′ and 5′-TGGGAGTAGACAA GGTACAACCC-3′; and GAPDH were: 5′-TTCACCACC ATGGAGAAGGC-3′ and 5′-GGCATGGACTGTGGTCAT GA-3′.

The specificity of the amplification products was controlled using a melting curve analysis. The thermal cycling profile consisted of: initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and final elongation at 72 °C for 30 s for 40 cycles. The melting temperature profile was assessed for each PCR run to confirm the specificity of PCR products.

The levels of HMGB-1, TNF-α and IL-10 transcripts were normalized to the housekeeping gene GAPDH. mRNA expression was calculated using the standard curve.

Histological examinations

Coded histological slides of rat livers were examined by two independent assessors in the research group, blinded with respect to group allocation.

Light microscopic study (LM)

Small liver slices were fixed in 10% formalin and processed to form paraffin sections of 5 μm thickness and stained with Hematoxylin and Eosin (H&E). These sections were graded numerically to assess the histological features in acute hepatic injury. They were semi-quantitatively evaluated in terms of vacuolar degeneration in hepatocytes, eosinophility of hepatocytes and mononuclear cellular infiltration. Two independent assessors with experience in histological assessment of liver injury were asked to rate the appearance of 5 high power fields per 5 different sections in each rat under study. The morphological changes were assessed independently and graded as follows: 0 = no observed changes, 1–2 = mild changes, 3–4 = moderate changes and 5–6 = severe changes [20]. Each assessor was asked to view and score the same set of slides on a second occasion following recoding (re-blinding). Sections were randomly coded, by a third individual, on both occasions. Randomization codes were broken after each assessment session. Scores reported are the mean of the two scores.

Electron microscopic study (TEM)

Small liver specimens (1 mm^3) were fixed in phosphate-buffered gluteraldehyde and processed to form capsules. Ultra-thin sections (50–60 nm thickness) were cut using an ultra-microtom. Sections were mounted on copper grids and stained with a saturated solution of uranyl acetate followed by lead citrate. Ultra-thin sections were examined and photographed by JEM-1200EXII transmission electron microscope in Faculty of Science, Ain-Shams University.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS®) version 20 (SPSS® Inc., Chicago, IL). Normality of quantitative data distribution was tested using the Shapiro Wilk test. Normally distributed numerical data were presented as mean ± SD with 95% Confidence Interval (CI) and between-group differences were compared parametrically using one-way analysis of variance (ANOVA). The Scheffé *post hoc* test was used for pairwise comparisons whenever the one way ANOVA revealed a significant difference. For inter-group comparisons of numerical data exhibiting skewed distribution, the Kruskal Wallis test was used, and the Mann–Whitney *U* test was employed *post hoc* whenever a statistically significant difference was detected with the Kruskal Wallis test. To preserve the α-error at 0.05 as set initially, the Bonferroni method was used to correct for multiple pairwise comparisons with the Mann–Whitney *U*-test.

All reported *P* values are two-tailed. A corrected *P* value of <0.017 for pairwise comparisons conducted with the *U*-test was considered statistically significant. Otherwise, $P < 0.05$ was regarded as statistically significant.

Results

Liver function assay

Table 1 shows that D-GaIN increased plasma ALT, AST, ALP, TBIL and prolonged PT compared to controls. In rats treated with neostigmine 24 h after D-GaIN injection, there was lower ALT, AST, ALP, TBIL and PT levels compared to D-GaIN group ($P < 0.001$).

Oxidant and antioxidant markers

D-GaIN increased MDA as compared to control (1.88 ± 0.61 Ug^{-1}; 95% CI 1.49–2.26 Ug^{-1}; vs 0.79 ± 0.36 Ug^{-1}, $P < 0.001$). Neostigmine protected against peroxidative damage caused by D-GaIN, reducing hepatic MDA (0.81 ± 0.43 Ug^{-1}; 95% CI 0.54–1.08 Ug^{-1}; $P < 0.001$). Conversely, D-GaIN reduced SOD levels (346.11 ± 166.24 μmol g^{-1}) (95% CI 240.49–451.73 μmol g^{-1}) as compared to control (1685.27 ± 674.84 μmol g^{-1}) ($P < 0.001$). Whereas, neostigmine increased liver SOD (798.14 ± 458.66 μmol g^{-1}; 95% CI 506.72–1089.56 μmol g^{-1}; $P < 0.001$) as compared to group D-GalN (Table 1).

Liver HMGB-1, TNF-α and IL-10 expression

D-GalN-induced ALF led to an increase in proinflammatory cytokines. HMGB-1expression increased (95% CI 2.67–4.73;

Table 1 Liver function assays, body weight and oxidant and antioxidant markers.

Group	Control	D-galactosamine	Neostigmine-treated D-galactosamine
Body weight (g)	217.8 ± 39.9	225.7 ± 25.9	220.8 ± 26.5
PT (sec)	19.4 ± 2.35	88.7 ± 32.3[a]	27.0 ± 13.9[b]
ALT (U ml^{-1})	10.6 ± 1.02	92.2 ± 10.21[a]	11.3 ± 1.06[b]
AST (U ml^{-1})	14.9 ± 7.41	84.5 ± 11.29[a]	21.6 ± 9.44[b]
ALP (IU L^{-1})	66.5 ± 12.74	117.4 ± 49.14[a]	83.9 ± 23.40[b]
Total proteins(g dL^{-1})	7.43 ± 0.29	5.59 ± 0.65[a]	6.65 ± 0.53[b]
ALB (g dL^{-1})	4.45 ± 0.62	3.03 ± 0.62[a]	4.37 ± 0.48[b]
TBIL (mg dL^{-1})	0.397 ± 0.23	1.546 ± 0.62[a]	0.637 ± 0.38[b]
MDA (U g^{-1})	0.79 ± 0.36	1.88 ± 0.61 [a]	0.81 ± 0.43 [b]
SOD (μmol g^{-1})	1685.27 ± 674.84	346.11 ± 166.24[a]	798.14 ± 458.66[b]

Values presented are mean + SD.
[a] Significant difference to control group $P < 0.001$.
[b] Significant difference to D-galactosamine group $P < 0.001$. ALT = alanine transferase, AST = aspartate transferase, ALP = alkaline phosphatase, ALB = albumin, TBIL = total bilirubin, MDA = malondialdehyde, and SOD = superoxide dismutase.

$P < 0.001$) as did TNF-α expression in liver tissue (95% CI 7.33–14.53; $P < 0.001$ compared to control) (Figure 2). Treatment with neostigmine reduced these increases (95% CI 0.33–1.09; $P < 0.001$ and 95% CI 1.26–2.06; $P < 0.001$, HMGB-1 and TNF-α respectively) (Figure 2). Expression of the anti-inflammatory cytokine IL-10 was unchanged in D-GalN (95% CI 0.04–0.21; $P = 0.853$) (Figure 2), but neostigmine treatment markedly increased its expression in liver tissue (95% CI 2.49–4.17; $P < 0.001$) (Figure 2).

Liver Histopathology

Examination of H & E stained sections of D-GalN group showed focal and bridging hepatic necrosis accompanied by dilated blood sinusoids and congested portal venules and central veins. Vacuolated and degenerated hepatocytes appeared mainly in zones I and II of the hepatic lobule, with increased eosinophility of hepatocytes with pyknotic or karyolitic nuclei

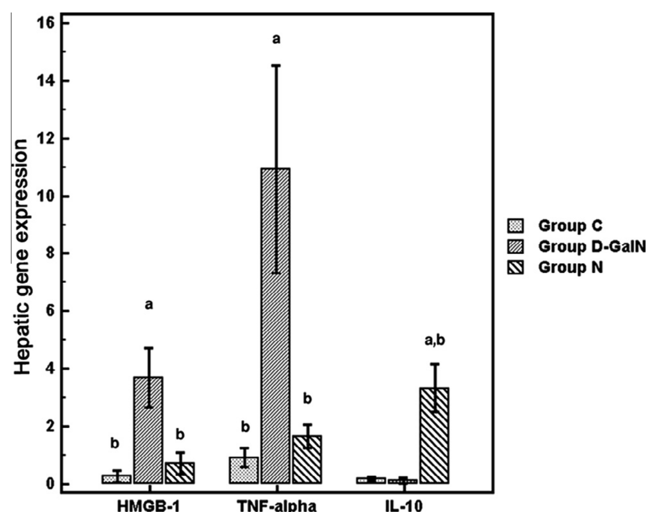

Figure 2 Hepatic gene expression of high mobility group box-1 (HMGB-1), tumor necrosis-α (TNF-α) and interleukin (IL)-10. Error bars represent 95% Confidence Interval. (a) Significantly different from control group $P < 0.001$. (b) Significantly different from D-galactosamine group $P < 0.001$.

($P < 0.001$) (Table 2). Further, mononuclear cellular infiltration was observed around portal areas, blood sinusoids and the central vein ($P < 0.001$), compared to controls (Figure 3). TEM examination of liver sections in group D-GalN showed many lipid vacuoles in the hepatocytes, some of which encroached on the nuclei, causing nuclear membrane irregularities. The cytoplasm showed dilatation of the rough and smooth endoplasmic reticulum (rER, and sER), dilatation of the Golgi apparatus and disfigured swollen mitochondria with poorly distinguishable cristae. In addition, apparently wide spaces of Disse were noticed containing collagen fibers with loss of normal hepatocytic microvilli (Figure 4).

Treatment with neostigmine clearly improved D-GalN-induced liver damage. Figure 3 and Table 2 show the reduction in vacuolar degeneration and necrosis, eosinophility of hepatocytes and mononuclear cellular infiltration ($P < 0.001$ compared to group D-GalN).

Neostigmine treatment improved hepatocyte architecture, showing fewer lipid vacuoles, reduced electron-density of mitochondria and less dilation of rER, sER and Golgi apparatus in TEM examination compared to group D-GalN (Figure 4). Also, nuclear configuration and intercellular spaces with interdigitating microvilli of the hepatocytes appeared comparable to the control group (Figure 4).

Discussion

This study examined the effect of the cholinesterase inhibitor neostigmine on D-GalN-induced ALF in rats. ALF was verified chemically by raised levels of liver enzymes, prolonged PT, increased plasma TBIL, decreased plasma levels of total proteins and ALB. Histological and ultrastructural examination of the liver also confirmed D-GalN-hepatotoxicity. Neostigmine attenuated liver dysfunction and improved liver synthetic and excretory functions, reduced proinflammatory cytokine TNF-α and HMGB1 expression and increased expression of the anti-inflammatory cytokine IL-10 in liver tissue.

As treatment of human acute liver failure cannot usually be initiated at the time of onset, even successful experimental treatments initiated immediately after or prior the onset of ALF provide little rationale for clinical development [16,21,22]. Liver-borne inflammation occurs at about 6–12 h

Table 2 Histopathological scores in rat liver sections.

Group	Control	D-galactosamine	Neostigmine treated D-galactosamine
Degeneration and necrosis	0 (0–0)	4.5 (4–5)[a]	1.5 (1–2)[a,b]
Increase eosinophility	0 (0–0.75)	5 (4–5)[a]	1(1–2)[a,b]
Inflammatory cell infiltration	0 (0–1)	3 (3–4)[a]	1.5 (1–2)[a,b]

Data are presented as median (interquartile range).
[a] Significant difference to control group $P < 0.001$.
[b] Significant difference to D-galactosamine group $P < 0.001$.

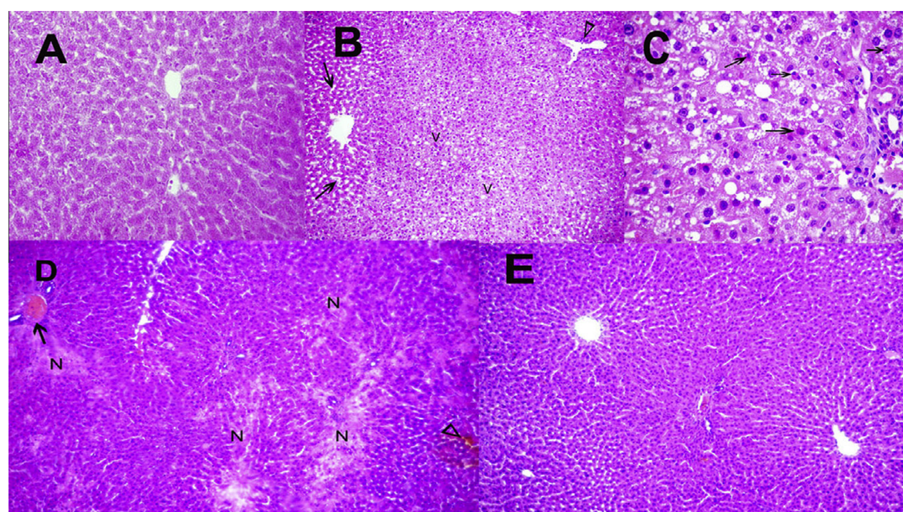

Figure 3 Photomicrographs of rat liver sections: (A) control group, showing normal hepatic lobule. D-galactosamine group showing: (B) vacuolated hepatocytes (V) dilated blood sinusoids in zone I (Δ) and mononuclear cellular infiltration in the portal area (↑); (C) eosinophilic degeneration of hepatocytes (↑) and (D) confluent necrosis of hepatocytic plates (N), congested portal vein (↑) and congested blood sinusoids (Δ). (E) Neostigmine-treated group showing improvement of liver architecture. (H&E: A, B, D, E 200× – C 400×).

after the administration of D-GalN [6,16,23]. High mobility group box 1 (HMGB1) protein has been identified as a late mediator of endotoxin lethality, HMGB1 protein itself can cause an acute inflammatory response manifested by increased production of proinflammatory cytokines and neutrophil accumulation. The delayed kinetics of HMGB1 protein release indicate that this protein is a distal mediator of acute inflammation, it can be detected 24 h after monocyte activation [24]. In our model, to study the effect of neostigmine in a clinically relevant time frame, we started drug therapy 24 h after D-GalN intake.

TNF-α and late pro-inflammatory cytokine HMGB-1 are critical acute liver failure mediators, and interventions that suppress their activity can improve survival [6,16,22,23,25]. HMGB-1 is released several hours later than other proinflammatory cytokines [24]. A reciprocal functional relationship was found between the activities of the early TNF-α and late HMGB-1 cytokines. HMGB-1 is a potent activator of monocyte cytokine release, and its release is promoted by TNF-α [26]. The anti-inflammatory activity of neostigmine in an ALF model is shown by its inhibition of TNF-α and HMGB-1 expression and enhanced release of the anti-inflammatory cytokine IL-10.

The rate of albumin synthesis may be significantly altered in the critically ill. In the acute-phase response to trauma, inflammation or sepsis, there is a decrease in the rate of transcription of albumin mRNA and the synthesis of albumin [27]. Proinflammatory cytokines, IL-6 and TNF-α both act to reduce gene transcription and thereby the synthesis of albumin [28]. Induced inflammation in rats decreased the concentration of albumin mRNA and the rate of albumin synthesis [29]. As well, a sustained inflammatory response in critical illness may lead to prolonged inhibition of albumin synthesis [27]. Neostigmine improved total protein and albumin levels in D GalN-induced ALF rats, this could be implicated by its anti-inflammatory activity through the reduction of the proinflammatory cytokines TNF-α and HMGB-1.

Neostigmine inhibits acetylcholine inactivation and thus prolongs the excitation of cholinergic synapses, potentially enhancing cholinergic activity. The "cholinergic anti-inflammatory pathway" has been described as a mechanism for neuronal control of inflammation via the efferent vagus nerve [7,9]. Wang et al. showed that α-7nACh R is the molecular link between the brain and the immune system in the cholinergic anti-inflammatory pathway [8]. The activation of immune cell α-7 nACh R regulates the intracellular signals that control cytokine transcription and translation [8,25].

Cholinergic anti-inflammatory pathway activation has been reported in animal models of systemic inflammatory reactions and cardiac dysfunction [11,12]. The expression of functional

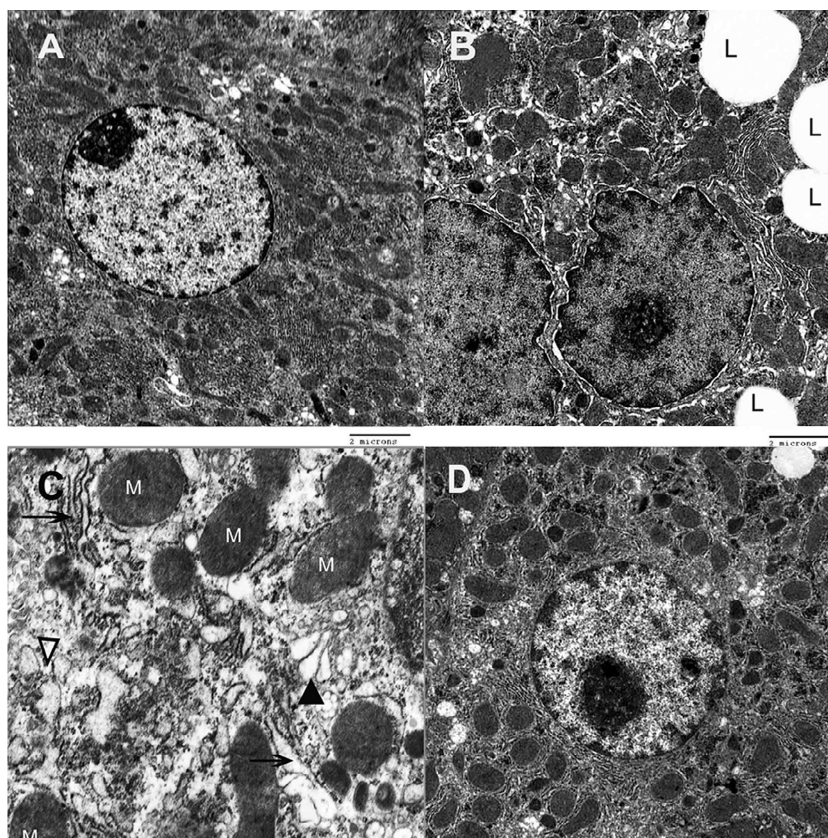

Figure 4 Electron micrographs of rat liver sections of: (A) Control group. D-galactosamine group: (B) irregular nuclear membrane and multiple lipid vacuoles (L); (C) dilated rER (↑), dilated sER (Δ), dilated Golgi apparatus (▲) and disfigured swollen mitochondria (M). (D) Neostigmine-treated group showing improvement of hepatocyte structure. (TEM: A, B, D 10,000×, scale bar = 2 μ – C 7500×, scale bar = 500 nm).

nACh R subunits on hepatic stellate cells in human hepatocytes, including the α-7 subtype, was recently discovered by Soeda et al. [15].

We chose a dose of 80 μg Kg^{-1}, three times daily, which Hofer et al. have shown to have remarkable anti-inflammatory effects in a murine sepsis model [11]. Freeling et al. showed similar effects [12], whereas Kox et al. showed no benefit of neostigmine in a ventilator-induced lung inflammation model [30]. This could be attributed to the relatively mild inflammatory response in their model (compared to fulminant sepsis or ALF), which probably was not severe enough to activate the cholinergic anti-inflammatory pathway [30] – this pathway is regarded as a reflex response to control excessive inflammation [9]. Other possible explanations might be their use of atropine as a part of the anesthetic regime, which by blocking the nicotinic ACh receptor response reduces the mitigating effects of acetylcholine on TNF-α expression [31,32], or compartmentalization. Kox et al. evaluated effects on mechanical ventilation-induced lung inflammation [30], the present study assessed liver inflammation while Hofer et al. investigated sepsis-induced systemic inflammation [11].

The different timings and organs may explain the results; the capacity of cells from different organs to produce inflammatory cytokines can be either enhanced, unchanged, or decreased depending upon the experimental models [33]. Akinci et al. also failed to detect any protective effects of neostigmine at doses of 100 μg Kg^{-1} in a mouse model of endotoxin-induced septic-shock. Interestingly, at higher dosages (300 μg Kg^{-1}), a decrease in interstitial inflammation was observed but mortality was increased, possibly because of non-specific parasympathetic effects such as those on the cardiovascular system [34].

Inhibition of cytokine biosynthesis by the cholinergic anti-inflammatory pathway is caused by cholinergic neurotransmission acting on α-7nACh R [7–9]. Both direct electrical stimulation of the vagus nerve [10,35] and application of α-7nACh R agonists [11,13,14,30] inhibit the synthesis of TNF-α and HMGB-1. Neostigmine also exerts profound cholinomimetic effects via inhibition of peripheral anticholinesterase. Although in our ALF model, neostigmine was anti-inflammatory, the actual mode of action is unclear.

The increased hepatic MDA, the end product of lipid peroxidation, and decreased hepatic SOD, an antioxidant, are convenient markers of oxidative stress. Reactive oxygen species (ROS) play a critical role in the induction and propagation of liver disease [16]. Massive ROS production in response to ischemia or toxic injury to the liver causes lipid peroxidation of cellular membranes, and protein and DNA oxidation [16,36]. The main sources of ROS are hepatocyte mitochondria, activated macrophages (Kupffer cells) and infiltrating neutrophils [16,37]. ROS can trigger the translocation of nuclear factor-kB (NF-κB) to the nucleus and activate inflammatory

cytokines that in turn contribute to further production of ROS [16,37]. Hepatocytes can release HMGB-1 in response to oxidative stress [26]. HMGB-1 also stimulates neutrophil ROS production [38], and increases the activation of NF-κB resulting in an increased cytokine release [39,40]. Mitochondria are both a target and a source of ROS, which play an important role in physiologic signaling mechanisms and in the regulation of the apoptotic pathway. The latter triggers intracellular free radical production during the induction of cell death [16,41]. Our electron microscopy findings included disfigured mitochondrial structure, dilatation of sER and rER, and nuclear membrane irregularities, suggesting increased free radical activity in D-GalN-induced ALF. Treatment with neostigmine reduced hepatic MDA and increased hepatic SOD, reflecting an upregulation of antioxidant defense mechanisms. Further, focal and bridging necrosis, increased eosinophility of hepatocytes and inflammatory cell infiltration induced by D-GalN were significantly less with neostigmine. The toxic fatty infiltration we observed in the liver parenchyma is especially significant. Oxidative stress originating from increased intracellular levels of fatty acids is implicated in hepatocellular injury in steatosis [16,42].

We found that D-GalN-induced hepatocyte lipid accumulation was markedly reduced by neostigmine, which we assume to ameliorate hepatic steatosis through an anti-inflammatory activity which is likely to reduce oxidative stress-induced inflammation. The beneficial effect of neostigmine on this ALF model is also emphasized by the improvement of hepatic architecture and restoration of hepatic integrity. Cholinergic activation by means of cholinesterase inhibition is already being used clinically, as in the treatment of the central anticholinergic syndrome or as an adjuvant in pain therapy [43].

To our knowledge this is the first study that has explored this topic. In this study we pharmacologically activated the cholinergic anti-inflammatory pathway in a way that could also be used in patients.

Limitations of the study

Whether neostigmine effects are partially or totally mediated via the α-7nACh receptor, this is considered a limitation of the study and would require further experimental studies using a selective α-7nAChR agonist. Another limitation was the difficulty in obtaining the initial baseline laboratory data from the rats.

Clinical implications

Cholinesterase inhibition via neostigmine might be considered as a therapeutic concept in patients suffering acute hepatic failure which may provide a rationale for further confirmation and exploration and may have important implications in the development of new treatment strategies to bridge selected patients to transplantation or to treat those not considered candidates for liver transplantation.

Conclusion

We have demonstrated that cholinesterase inhibition with neostigmine counteracts D-GalN-induced ALF through an anti-inflammatory activity.

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