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Nimesulide effects on the blood pro-oxidant–antioxidant status in lipopolysaccharide-challenged mice

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Abstract

The maintenance of the pro-oxidant–antioxidant equilibrium between ROS production and antioxidant protection systems is an important element of systemic defence and requires efficient control. The aim of the study was to monitor the dynamics of antioxidants and lipid peroxidation in mice challenged intraperitoneally with *Escherichia coli* (O111:B4) lipopolysaccharide (LPS) and to evaluate the antioxidant potential of the non-steroidal anti-inflammatory drug nimesulide. Albino mice were divided into three groups ($n = 36$). Group I received a single intraperitoneal (i.p.) injection with 25 µg/0.5 mL LPS. Thirty minutes before LPS, group II received orally (p.o.) 100 mg/kg nimesulide. The preparation was administered for 4 days. Group III received only nimesulide at the indicated dose for 4 days. The blood parameters were analysed at hour 0 (prior to treatment applied to each group), post treatment hours 6 and 24, and days 3, 5 and 9. Assayed parameters included catalase, reduced glutathione, albumin, glucose, ferric reducing ability of plasma (FRAP), malondialdehyde and oxidative stress index. LPS induced continuous hypoglycaemia, decreased catalase activity and reduced glutathione, but FRAP and albumin were preserved. The application of nimesulide alone did not alter oxidative stress index and enhanced FRAP. Its co-administration with LPS normalised reduced glutathione, decreased catalase and increased malondialdehyde concentrations and oxidative stress index. The application of nimesulide as antioxidant requires objective evaluation of associated benefits and risks.

Keywords Antioxidant defence · Lipopolysaccharide · Lipid peroxidation · Nimesulide · Mice

Introduction

Intra- and extracellular antioxidant systems are an essential part of systemic defence, helping to eliminate the endogenous and exogenous free radicals (Noori 2012). Reactive oxygen species (ROS), produced during redox reactions, and phagocytic oxidative killing mechanisms at high concentrations react with various organic compounds inducing cell destruction, and structural modifications of albumin and serum proteins (Ahmad et al. 2016; Choudhary et al. 2016). Consequently, the metabolism, energy exchange and systemic regulatory systems are impaired, and cell functions and intracellular signalling pathways are altered (Fonseca et al. 2016). Therefore, the equilibrium between ROS production and antioxidant

protection systems is important, albeit vulnerable (Mishra et al. 2005).

Numerous studies have shown that lipopolysaccharide (LPS) of Gram-negative bacteria induces mitochondrial dysfunction and oxidative stress (Israelachvili 2010; Birben et al. 2012). Liu and Bing (2011) provide evidence that this virulence factor activates apoptosis, production of inflammatory mediators, cytokines (IL-1, TNF-α, IL-6) and nitric oxide (NO). Despite the detailed investigations on the pathogenetic nature of LPS, therapeutical approaches to its inhibition through polymyxin B haemoperfusion (Hayashi 2012) and through synthetic peptides (Arana et al. 2003) did not yield the expected results (Buttenschoen et al. 2010). Also, more than 90% of new drugs tested on mice and rats (biological species with low sensitivity to LPS) failed in tests with humans (highly sensitive to LPS) but there is still no consistent explanation for the relevant mechanism responsible for the different sensitivity of biological species to LPS (Ghosn et al. 2010; Rosenfeld et al. 2010; Wimley 2010; Kaconis et al. 2011; Kosovrasti et al. 2016). Not only species-related LPS sensitivity but also breed- and age-related features are

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important to characterise clinical manifestations of the challenge (Deitschel et al. 2012; Holowaychuk et al. 2012). In research on LPS effects, the selection of experimental model is highly dependent on the dose, route of application and bacterial strain that produced LPS, as different bacteria and their strains contain variable amounts of endotoxin. Among the options for control of LPS-induced systemic damage is the efficient management of inflammation allowing it to restore its role of a vital non-specific mechanism of tissue protection and repair (Callahan et al. 2014).

The selective COX-2 inhibitor nimesulide has marked anti-inflammatory properties (Bennet and Villa 2000; Bennet 2001; Rainsford et al. 2005), attributed to effect on key inflammatory signalling pathways (Bennet 1999; Rainsford 2006)—inhibition of prostaglandin synthesis (especially that of PGE2); pro-inflammatory cytokines (TNF-α, IL-1, IL-6); inhibition of release of platelet-activating factor, and of histamine produced by basophils and tissue mast cells (Rainsford et al. 2005); impairment of chemotaxis and transendothelial migration of blood cells. Some authors reported high hepatotoxicity of nimesulide (Krishanappa 2010; Das and Roy 2011; Sozer et al. 2011), while others recommended its use in lung inflammation and osteoarthritis (Zheng et al. 2000; Sohi and Khanduja 2003; Al-Abd et al. 2014). Antioxidant activity of nimesulide is also reported (Khalil et al. 2012). The existing controversies require detailed in vivo studies in animal models to evaluate the properties of the drug and its potential for optimisation and control of LPS-induced oxidative stress.

The aim of the present study was to monitor the dynamics of blood antioxidants and lipid peroxidation in mice challenged intraperitoneally with *Escherichia coli* (O111:B4) lipopolysaccharide and to evaluate the antioxidant potential of the non-steroidal anti-inflammatory drug nimesulide.

Materials and methods

Chemicals

Lipopolysaccharide from *E. coli*, *Serotype* O111:B4 (Sigma Chemical, USA Co, L-3024, Lot 11H4087) and nimesulide (Heisinn Birex Phamaceutical Ltd., Damastown, Mulhuddart, Dublin 15, Ireland) were used in the experiments.

Animals

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

One hundred and eight albino mice, weighing 23–32 g, about 3 months of age, from both sexes were obtained from the Experimental Laboratory Animals Breeding Base, Slivnitsa, Bulgaria. Mice were housed indoor at a temperature of 21 ± 2 °C, humidity ($60 \pm 10\%$), and light regimen, and had free access to water and pelleted feed suitable for the species. All animals underwent a 2-week period of adaptation before the start of the trial.

Experimental design

Mice were allotted into 3 groups with 36 mice each. Twelve hours before the experiment's start, food was withheld. Mice from experimental group I received a single intraperitoneal (i.p.) injection with 25 μg/0.5 mL LPS. Thirty minutes prior to LPS injection, animals from experimental group II received orally (p.o.) via an oesophageal probe 100 mg/kg nimesulide. The preparation was administered for 4 consecutive days, once daily at 8.30 AM before feeding. The mice from experimental group III received only nimesulide at the indicated dose via an oesophageal probe for 4 consecutive days. The monitoring of blood parameters included hour 0 (prior to treatment applied to each group), post treatment hours 6 and 24, and post treatment days 3, 5 and 9.

Sample collection and laboratory parameters

Blood samples were collected after sedation and euthanasia of mice. Parameters were assayed in erythrocyte lysate, serum and plasma. The following parameters were determined: catalase (CAT, kU/L)—by the method of Goth (1991); reduced glutathione (rGSH, mmol/L)—by the method of Beutler et al. (1963); albumin (g/L)—with a commercial bromocresol green assay kit (Giesse Diagnostics, Italy); glucose (mmol/L)—with a commercial enzymatic assay kit (Human Diagnostica, GmbH Germany). The ferric reducing ability of plasma (FRAP, mmol/L) was determined by the method of Benzie and Strain (1996), and serum malondialdehyde (MDA, μmol/L)—by the thiobarbituric acid method (Uchiyama and Michara 1978) modified by Andreeva et al. (1988). The oxidative stress index (OSI) was calculated as

$$OSI = 100 \times MDA \div 1000 \times FRAP.$$

Statistical analysis

Results are presented as mean ± SD. Comparisons were made both within each group as well as among the three groups at each time interval by means of one-way ANOVA test and Tukey's post hoc test (Graph Pad InStat3). Differences were considered statistically significant at the $p < 0.05$ level.

Results

Changes in blood antioxidants

During the entire period of the study, catalase activity in blood remained lower than baseline in all groups (Fig. 1). In group I, its levels remained lower both at day 5 ($p < 0.01$) and 9 ($p < 0.01$) compared to hour 0. Nimesulide applied 30 min prior to LPS injection (group II) did not succeed to change the trend to catalase reduction with statistically significantly lower concentration at day 3 ($p < 0.01$) and lowest levels by day 5 compared to baseline ($p < 0.001$) as well as vs hour 6 ($p < 0.01$) and hour 24 ($p < 0.05$). By the 9th day, blood catalase began to increase in this group but initial levels were not attained ($p < 0.01$). In mice from group III, catalase decreased gradually, even after nimesulide intake was interrupted with substantial differences vs baseline by post treatment days 5 ($p < 0.05$) and 9 ($p < 0.01$). No statistically significant intergroup differences were found out.

Erythrocyte content of reduced glutathione (rGSH) in group I was lower than initial one as early as the 6th hour after LPS challenge ($p < 0.05$) and persisted low until the end of the study (Fig. 2). In group II, no statistically significant deviations were recorded. On the contrary, mice from group III showed strongly reduced rGSH levels on hours 6 and 24 ($p < 0.001$ vs hour 0). The subsequent trend towards rGSH elevation was however constant and by the 5th and 9th days of the experiment, the levels even exceeded those in groups I and II (Fig. 2).

In group III, nimesulide administration did not lead to relevant changes in blood albumin concentrations which varied around baseline ones (Fig. 3). The LPS injected to mice from group I however resulted in statistically significant decrease in albumin on hour 6 ($p < 0.05$ vs hour 0; $p < 0.001$ vs group III). Afterwards, the parameter increased considerably by the 24th

hour ($p < 0.001$ vs hour 6), 3rd day ($p < 0.001$ vs hour 6) and 5th day ($p < 0.001$ vs hour 6) to attain levels higher than baseline ones by the 9th day ($p < 0.05$). In mice from group II, time course of albumin was similar to that in group I. Its levels were considerably lower than initial ones on hour 6 ($p < 0.01$), hour 24 ($p < 0.01$) and day 5 ($p < 0.05$) (Fig. 3).

Blood glucose in group I was low as early as the 6th post treatment hour ($p < 0.001$ vs hour 0; $p < 0.01$ vs group III). It remained low also on day 5 with statistically significant differences vs hour 0 ($p < 0.01$), and group III ($p < 0.05$). Then, blood glucose rose to final concentrations (9th day) significantly higher compared to those on hour 6 ($p < 0.001$) and day 5 ($p < 0.05$) (Fig. 4). In group II, blood glucose also decreased on hour 6 ($p < 0.05$ compared to hour 0; $p < 0.001$ compared to group III), hour 24 and day 5 ($p < 0.05$). However, on days 3 and 9, its concentrations exceeded those at hours 6 and 24 ($p < 0.01$). Nimesulide, applied independently to group III did not result in any statistically significant deviations.

The total plasma antioxidant capacity as seen from FRAP levels was increased on hour in all groups (Fig. 5). The greatest increase was noted in group III ($p < 0.001$ vs baseline) followed by group II ($p < 0.01$ vs group III), and finally by group I ($p < 0.001$ vs group III). In LPS-treated mice however, FRAP were significantly higher than initial ones on day 3 ($p < 0.01$), day 5 ($p < 0.001$) and day 9 ($p < 0.001$), prevailing over those in groups II and III at the same time intervals. In the group treated with nimesulide + LPS, FRAP varied around initial levels except for hour 24 when it was substantially lower than levels by hour 6 ($p < 0.05$).

Oxidative stress markers

Serum MDA concentrations on hour 6 were significantly higher than initial values in all groups, with the highest elevation in LPS-challenged group ($p < 0.001$) and nimesulide-

Fig. 1 Time course of changes in blood catalase activity (kU/L) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs hour 0; ++$p < 0.01$ vs hour 6; v $p < 0.05$ vs hour 24 within each group
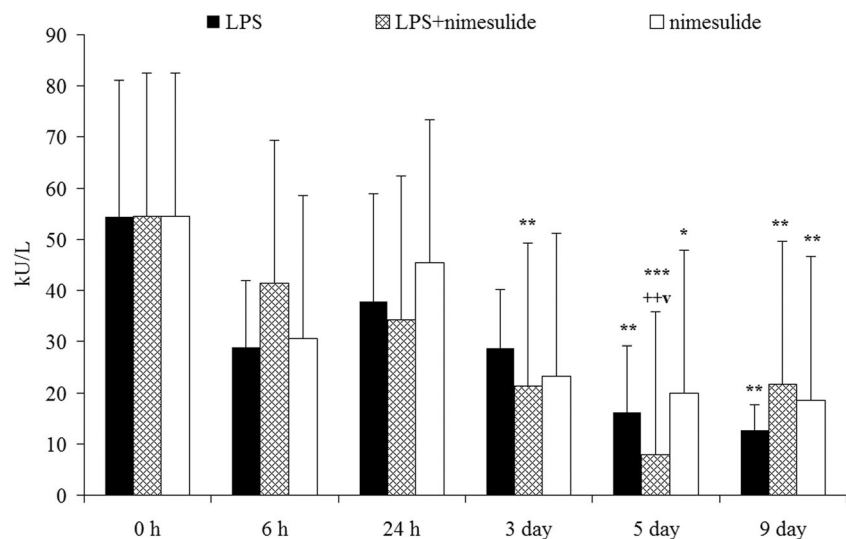
Fig. 2 Time course of changes in blood reduced glutathione (mmol/L) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant within-group differences: $*p < 0.05$, $***p < 0.001$ vs hour 0; $++p < 0.01$, $+++p < 0.001$ vs hour 6. Statistically significant between-group differences: c_1 $p < 0.05$ vs group III
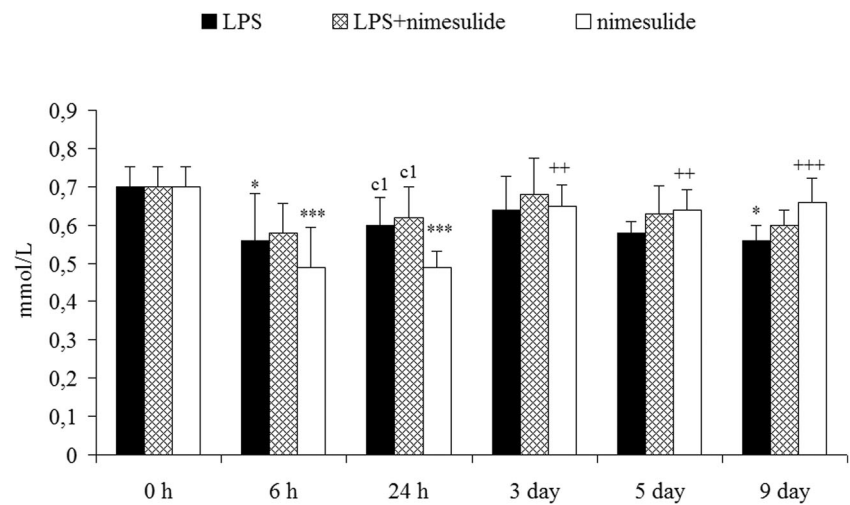
treated group ($p < 0.01$) (Fig. 6). At the end of the experiment, MDA levels in these groups remained higher than baseline ones. No significant changes were observed in mice treated with LPS + nimesulide except for day 9 when concentrations of the marker were lower than those in group III ($p < 0.01$).

Oxidative stress index (OSI) values between hour 24 and day 3 in mice from group II were the highest (Fig. 7). The peak on hour 24 corresponded to that on hour 6 in Group I. Then, OSI in LPS-challenged animals decreased considerably compared to baseline on hour 24 ($p < 0.01$), days 3, 5 and 9 ($p < 0.001$). In the group that received nimesulide only, OSI values did not change remarkably with slight variations except for day 9 ($p < 0.05$ vs day 5).

Discussion

The used experimental model allowed revealing disturbance in antioxidant status, interactions between enzyme and non-enzyme antioxidant systems in mice, treated either

with LPS or nimesulide alone, or with both substances. Data for group I showed a very early (hour 6) reduction of blood catalase activity, attributed to the fast destructive effect on LPS on cell detoxication potential. It could be suggested that changes in CAT antioxidant activity resulted from accumulation of considerable amounts of hydrogen peroxide after LPS challenge as the enzyme catalyses its degradation to water and oxygen (Held 2015). A large number of small non-enzyme molecules with detoxication function are also part of the antioxidant potential. Glutathione is an antioxidant tripeptide scavenging hydroxyl and peroxyl radicals (Held 2015) and it is indirectly involved as a co-factor of glutathione peroxidase in H_2O_2 elimination (Arthur 2000). rGSH levels in mice from group I remained lower than baseline throughout the experiment (Fig. 2). Watson et al. (2003) affirmed that disturbances in rGSH had an effect on extracellular antioxidants—vitamins E and C, as glutathione regenerates their active forms. According to Navarro et al. (1997) and Jones (2000), the ratio of oxidised (GSSG) to reduced (rGSH)

Fig. 3 Time course of changes in blood albumin (g/L) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant within-group differences: $*p < 0.05$ vs hour 0; $+++p < 0.001$ vs hour 6; v $p < 0.05$ vs hour 24. Statistically significant between-group differences: c_1 $p < 0.05$, c_2 $p < 0.01$, c_3 $p < 0.001$
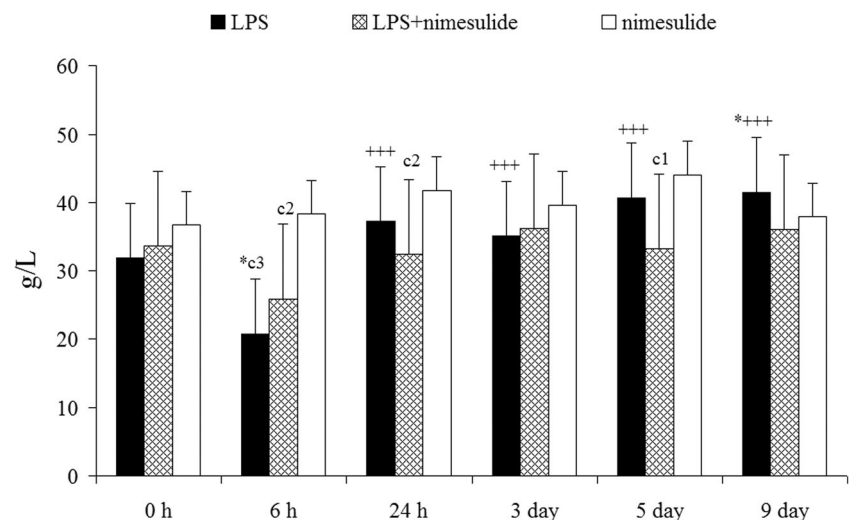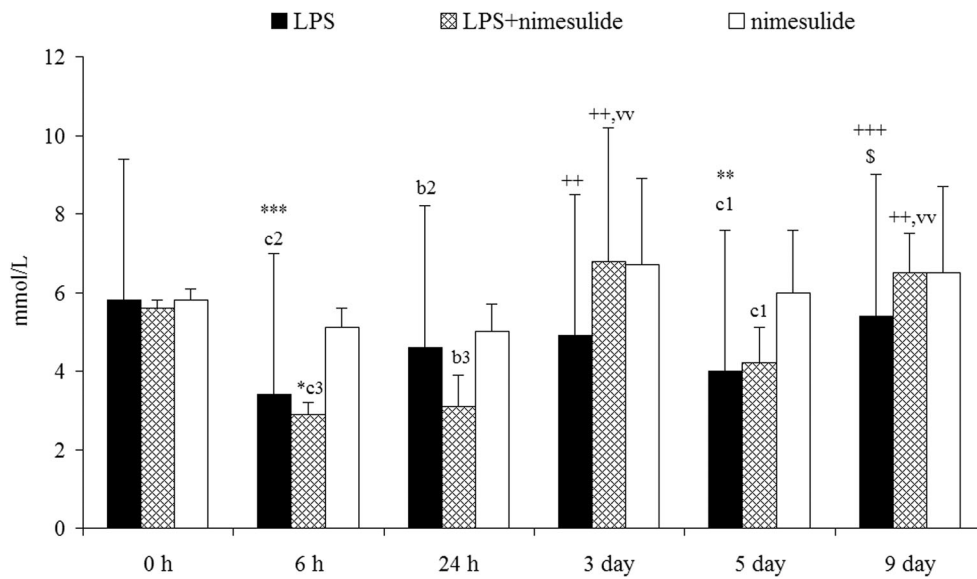
Fig. 4 Time course of changes in blood glucose (mmol/L) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant within-group differences: $*p < 0.05$, $**p < 0.01$,

$***p < 0.001$ vs hour 0; $+p < 0.05$, $++p < 0.01$, $+++p < 0.001$ vs hour 6; $v\ p < 0.05$, $vv\ p < 0.01$, $vvv\ p < 0.001$ vs hour 24; $\$$ vs day 5. Statistically significant between-group differences: $a_1\ p < 0.05$, $a_2\ p < 0.01$, $a_3\ p < 0.001$ vs group I; $b_1\ p < 0.05$, $b_2\ p < 0.01$, $b_3\ p < 0.001$ vs Group II; $c_1\ p < 0.05$, $c_2\ p < 0.01$, $c_3\ p < 0.001$ vs group III

glutathione is a dynamic marker of oxidative stress which is not commonly determined due to laborious GSSH assay. At the background of low concentrations of CAT and rGSH in mice from group I, the kinetics of non-enzymatic blood antioxidants, albumin and glucose, deserves to be discussed. By its thiol groups, albumin binds to erythrocytes and inhibits iron-dependent production of free radicals (Roche et al. 2008). Its concentrations on hour

6 after the LPS challenge were reduced but thereafter increased significantly and remained higher than baseline during the rest of the study period (Fig. 3). Considering the hepatotoxic effect of LPS (Buttenschoen et al. 2010; Ronco et al. 2010), changes could not be attributed to enhanced liver synthesis of albumin. It could be therefore suggested that high blood albumin levels either resulted from LPS-induced damage of renal nephrons or it
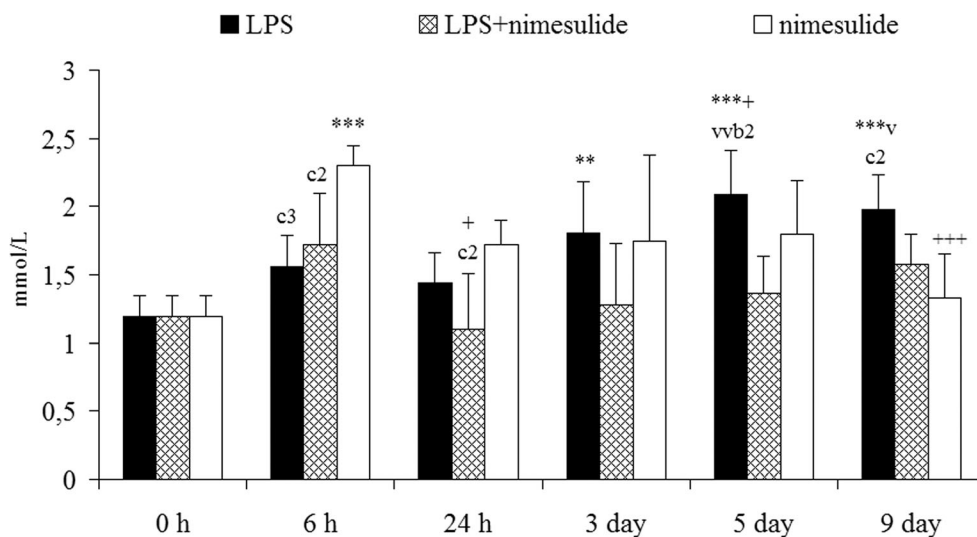

Fig. 5 Time course of changes in ferric reducing ability of plasma (mmol/L) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant within-group differences: $**p < 0.01$,

$***p < 0.001$ vs hour 0; $+p < 0.05$, $+++p < 0.001$ vs hour 6; $v\ p < 0.05$, $vv\ p < 0.01$, $vvv\ p < 0.001$ vs hour 24; Statistically significant between-group differences: $b_2\ p < 0.01$ vs group II; $c_2\ p < 0.01$, $c_3\ p < 0.001$ vs Group III

Fig. 6 Time course of changes in serum malondialdehyde (MDA) (µmol/L) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant within-group differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs hour 0; ++$p < 0.01$, +++$p < 0.001$ vs hour 6. Statistically significant between-group differences: b_1 $p < 0.05$, vs group II; c_2 $p < 0.01$ vs group III
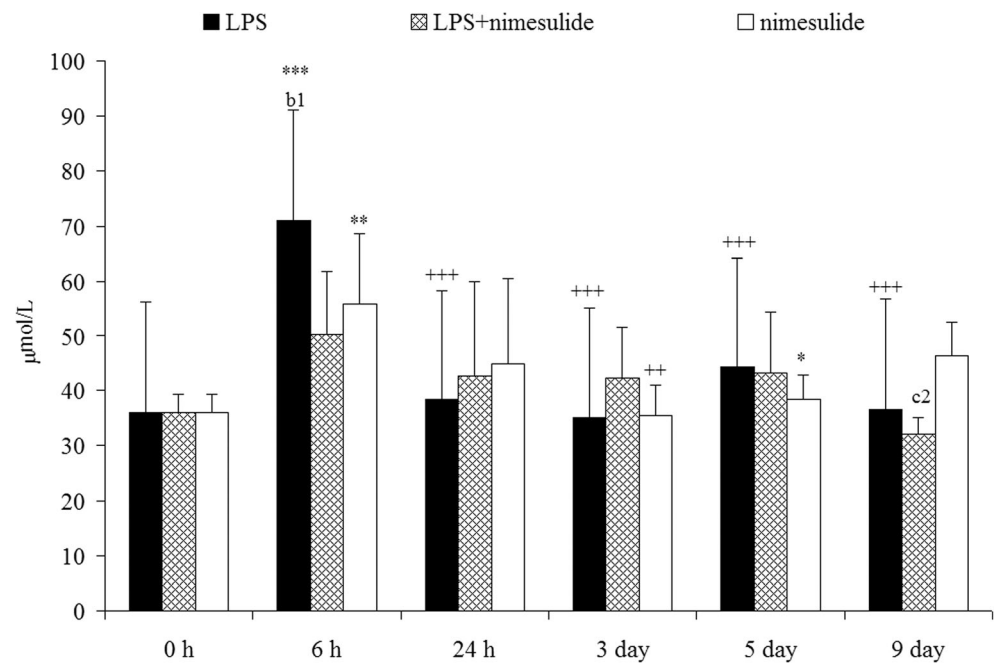
originated from depots, e.g. from the skin—an important reserve of extravascular albumin which could pass in blood vessels if necessary. Albumin has an important role in hydrogen peroxide inactivation and ROS damage prevention (Zhu and Crouch 1992). The precise control of albumin content is important as, being a primary serum protein, it maintains a number of homeostatic parameters, oncotic pressure, and is involved in transport of electrolytes and hormones (Infusino and Panteghini 2013). LPS impaired glucose homeostasis inducing long-term hypoglycaemia in mice from group I, well pronounced by hour 6 and day 5 (Fig. 4). Our data are in line with those of Raetzsch et al.

(2009). Possibly, LPS-mediated changes in glucose homeostasis are complex but their presence as early as the 6th hour suggests a hormonal control. The research of Nguyen et al. (2014) and Nohr et al. (2016) using gene expression analysis proves without doubt cytokine-mediated activation of insulin secretion by LPS. Vogel et al. (1991) discussed the role of IL-1 and TNF in LPS-induced hypoglycaemia and showed decrease liver glucose production and food intake associated with the effect of these cytokines on the appetite centre. In rats with endotoxaemia, Cornell (1989) found out increase in IL-1, while Ellingsgaard et al. (2011) demonstrated that IL-6

Fig. 7 Time course of changes in oxidative stress index (OSI) in mice, treated i.p. with pure lipopolysaccharide (LPS) (group I), with the combination p.o. nimesulide + i.p. LPS (group II) and with p.o. nimesulide only (group III) at 100 mg/kg for 4 days. Statistically significant within-group differences: **$p < 0.01$, vs hour 0; ++$p < 0.01$, +++$p < 0.001$ vs hour 6; v $p < 0.05$, vv $p < 0.01$, vvv $p < 0.001$ vs hour 24; $ vs day 5. Statistically significant between-group differences: b_1 $p < 0.05$, b_3 $p < 0.001$ vs group II; c_1 $p < 0.05$, c_3 $p < 0.001$ vs group III
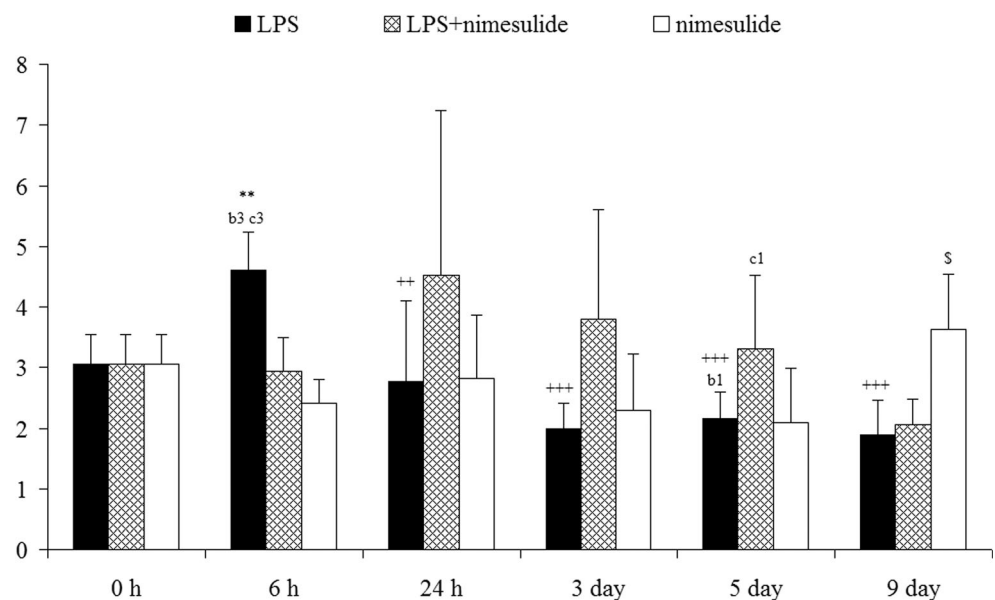
increased insulin secretion through glucagon-like peptide 1 (GLP-1) by L-cells and alpha cells. LPS-induced changes in antioxidant status influence oxidative stress markers. On post treatment hour 6, OSI values in Group I exceeded those in the other two groups (Fig. 7). This corresponded with established high serum MDA concentrations at that time (Fig. 6). Then, oxidative stress parameters MDA and OSI in this group decreased, although Ronco et al. (2010) affirmed that LPS-induced oxidative stress was among the leading pathogenetic mechanisms of tissue damage in endotoxaemia.

The possibility for ROS control by antioxidants is a reasonable approach used in the present experiment. The selection of nimesulide as a drug in LPS-challenged animals is motivated both by its anti-inflammatory properties and its antioxidant effects (Khalil et al. 2012). Our studies showed that when nimesulide was applied independently (group III), FRAP levels increased substantially and until the 3rd day they remained higher than those in groups I and II and compared to baseline levels (Fig. 5). Furthermore, mice from group III did not exhibit any statistically significant deviations in OSI values. This confirms the antioxidant properties of the drug. Bennet (1999) discussed the ability of nimesulide to inhibit the production of superoxide anions from neutrophils, whereas Bevilacqua et al. (1994) indicated that its effects are achieved through suppression of type IV phosphodiesterase. This enzyme increased intracellular cAMP levels and exerted a negative feedback on synthesis of pro-inflammatory cytokines, leukotrienes and histamine (Rainsford 2006). Al-Abd et al. (2014) established that applied independently nimesulide altered myeloperoxidase activity of neutrophils and reduced considerably their infiltration. Our studies showed that it did not affect blood non-enzymatic antioxidants (albumin, glucose) as seen from the lack of significant changes in group III. Despite the beneficial effects of nimesulide on extracellular antioxidants, the analysis of blood CAT and rGSH showed lower levels than baseline throughout the study which agrees with the results of Singh et al. (2012). The authors focused their attention on mitochondria and demonstrated that nimesulide increased nitric oxide concentrations. The result is mitochondrial dysfunction (Aamir et al. 2014). Possibly, the mechanisms of action of nimesulide on antioxidant and redox enzyme systems are rather complex and varying, displaying the modulating potential of the drug. This is confirmed by changes in lipid peroxidation (Fig. 6) in group III with MDA levels higher than initial ones. Compared to other non-steroid anti-inflammatory drugs, nimesulide has a different chemical structure, and pharmacological and pharmacokinetic properties (Bennet 1999; Rainsford 2006; Krishnappa 2010). Its anti-inflammatory effects are well investigated and rely on blockade of key signalling pathways of inflammation (Krishnappa 2010). Several studies however have documented its adverse effects (Bessone 2010; Modi et al. 2012; Aamir et al. 2014; Al-Abd et al. 2014; Bhattacharya et al. 2015). According

to Rainsford (2006), the bioconversion of nimesulide in the liver by cytochrome P450 (CYP-2C9, CYP-2C19 and CYP-1A2) yielded a reactive metabolite. Bhattacharya et al. (2015) reported vasculitis and hepatitis, while Singh et al. (2012) provided evidence for reduced mitochondrial activity, increased membrane permeability and activation of caspase-9 and caspase-3, indicating triggering of apoptosis.

Applied to mice challenged intraperitoneally with LPS, nimesulide succeeded to normalise rGSH concentrations but blood catalase activity remained lower than baseline one. In this group, OSI was high until the 5th day consequently both to low FRAP and high serum MDA levels during that period. Albumin values in the co-administered group remained lower than baseline ones during the entire period of observation unlike those in group I when albumin was higher between post treatment days 5 and 9. Although nimesulide alone (group III) did not influence this parameter, its toxicity increased when combined with LPS evidenced by commented changes in albumin, synthesised in the liver. In mice from group II, nimesulide corrected LPS-induced hypoglycaemia (between the 3rd and 9th days) (Fig. 4).

In conclusion, the supplementation of LPS-challenged mice with nimesulide had a beneficial effect expressed with normalisation of blood rGSH concentration and regulation of LPS-induced hypoglycaemia. The benefit/risk ratio should be, however, thoroughly evaluated to differentiate also adverse effects, low catalase activity and total antioxidant capacity of plasma, high oxidative stress index and serum MDA concentration.

Compliance with ethical standards

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