# Preventive effect of rosiglitazone on liver injury in a mouse model of decompression sickness

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**Abstract**

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**Background and aims:** Severe decompression sickness (DCS) is a multi-organ injury. This study investigated the preventive effects of rosiglitazone on liver injury following rapid decompression in mice and examined the underlying mechanisms.

**Methods:** Mice were randomly divided into four groups: a control group, vehicle group, and rosiglitazone (5 and 10 $mg \cdot kg^{-1}$) groups, the latter three being exposed to a pressure of 911 kPa. Haematoxylin and eosin staining, plasma levels of alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase and blood cell counts were used to evaluate liver injury at 30 min after rapid decompression. The expression of endothelial and inducible nitric oxide synthase (iNOS) and its phosphorylation were measured to uncover the underlying molecular mechanisms.

**Results:** A significant increase in plasma ALT, red blood cells and platelets, and a decrease in neutrophils were observed in the vehicle group. Furthermore, the expression of iNOS, E-selectin and the total level of NO in hepatic tissue, and soluble E-selectin in the plasma were significantly elevated in the vehicle group. Rosiglitazone pre-treatment prevented the increases in ALT (and AST), soluble E-selectin concentration, red blood cells and platelet counts. Moreover, rosiglitazone reduced over-expression of iNOS and the NO level, prevented the fall in neutrophil count and promoted the phosphorylation of iNOS in the liver.

**Conclusions:** Pre-treatment with rosiglitazone ameliorated liver injury from severe DCS. This preventive effect may be partly mediated by stimulating endothelial NO production, improving endothelial function and limiting inflammatory processes.

**Key words**

Animal model; Diving research; Injuries; Nitric oxide; Pharmacology

## Introduction

Decompression sickness (DCS) is caused by bubbles formed in the blood and tissues during or after a rapid reduction in environmental pressure and is a multi-organ injury.[1] Although use of an effective decompression plan can prevent DCS, it is difficult to completely avoid it happening especially in emergencies. Thus, other preventive methods targeting the pathophysiological processes have become an important strategy to reduce the severity of DCS. Research has mainly focused on the central nervous system injury,[1,2] but other injuries such as to liver and endothelial tissue cannot be ignored;[3–6] thus we investigated how injury to the liver could be reduced.

Previous research found that promoting nitric oxide (NO) generation and release by various methods, such as agent-mediation and appropriately timed exercise pre-diving, could reduce bubble generation and prevent DCS injury especially in the CNS.[7–11] Whereas administration of the nitric oxide synthase (iNOS) inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) increased bubble formation and mortality in rats,[12] the effects of L-NAME treatment on the susceptibility to DCS in male and female rats were different.[13] These reports suggest that stimulated production of endothelium-derived NO via the activation of endothelial nitric oxide synthase (eNOS) and preservation of endothelial integrity might decrease liver injury induced by rapid decompression.

Rosiglitazone is an hypoglycaemic agent cleared for use in the USA but prohibited in Europe because of its potential adverse side effects. However, studies on rosiglitazone are still on-going, and several have shown its action in promoting the phosphorylation of eNOS Ser-1177 and stimulating NOS in cultured endothelial cells via an AMP-activated protein kinase-dependent (AMPK) mechanism.[14,15] In addition, rosiglitazone may play a protective role in ischaemic brain or hepatic ischaemia/reperfusion injury, which may be partially mediated by alterations in the NO pathway, specifically eNOS and inducible nitric oxide synthase (iNOS).[16–18] Based on the role of eNOS/NO in the pathophysiological processes of DCS and the effect of rosiglitazone on eNOS/NO, the present study investigated its preventive effect on liver injury following rapid decompression and the possible underlying mechanisms with the use of specific markers.

## Materials and methods

### ANIMALS

Male ICR mice (6–9 weeks of age) were provided by the Experimental Animal Centre of Nantong University. All procedures were performed according to the rules of Jiangsu Province Animal Care Ethics Committee and approved by the Animal Care and Use Committee of Nantong University (Approval ID: SYXK (SU) 2007–0021). Mice were housed in a common cage and maintained on a regular day (06:00–18:00)/night (12 h) cycle with free access to food

**Table 1**
Number of mice used for each component of the study; Ros5 – Rosiglitazone 5 mg·kg$^{-1}$; Ros10 – 10 mg·kg$^{-1}$; $n$ = 160

| Test | Control | Vehicle | Ros5 | Ros10 |
|---|---|---|---|---|
| Total (deaths) | 40 (0) | 50 (10) | 20 (4) | 50 (7) |
| Liver function | 10 | 8 | 8 | 9 |
| Histology | 4 | 4 | – | 4 |
| Blood counts | 10 | 8 | 8 | 9 |
| Total NO | 10 | 8 | – | 9 |
| sE-selectin | – | 6 | – | 6 |
| Western-blot | 6 | 6 | – | 6 |

and water. The temperature was maintained at 22 ± 1$^{O}$C. The changes of mice in behaviour, posture and appearance were monitored daily.

Mice were exposed to compressed mixed gas to induce DCS after rapid decompression. The mice were randomly divided into four groups: a control group ($n$ = 40), vehicle group ($n$ = 50) and two rosiglitazone (5 mg·kg$^{-1}$, Ros5 and 10 mg·kg$^{-1}$, Ros10) groups ($n$ = 20 and 50, respectively).[16,18] Mice in the vehicle and rosiglitazone groups were subjected to hyperbaric exposure.

Rosiglitazone was administered intraperitoneally at 45 min before hyperbaric exposure, while the animals in both the vehicle group and the control group were administered with an equal volume of saline (0.1 ml/20 g body weight). The numbers of animals used for each assay are listed in Table 1.

## HYPERBARIC EXPOSURE

Ten mice at a time were placed in a 100-litre tank (Wuhu Diving Equipment Factory, Anhui, China) and were free to move in the cage during each hyperbaric exposure. One hundred and twenty mice in total were exposed to hyperbaric mixed gas. In our pre-experiments, male ICR mice exposed to 911 kPa (absolute) nitrox did not show significant signs of anaesthesia according to electroencephalographic and motor function tests. Compressed air was not used to avoid the potential toxic effect of a high partial pressure of oxygen (21 kPa × 9 = 189 kPa) on the mice, and hyperbaric nitrox was likely to make any DCS symptoms more apparent, thus we chose nitrox for the study.

The chamber pressure was increased at a rate of 16.7 kPa·sec$^{-1}$ up to 203 kPa (atmosphere absolute) using compressed air and then to 911 kPa with pure $N_2$ (Nantong Tianyuan Gas Co., Ltd., Jiangsu, China) at the same rate to give a nitrox mix of approximately 95/5 $N_2/O_2$. The chamber pressure was maintained at 911 kPa for 45 min. The concentration of $O_2$ and $CO_2$ were monitored continuously. Oxygen was supplemented manually while exhaled $CO_2$ was absorbed using soda lime. Rapid decompression was performed at a rate of 100 kPa·s$^{-1}$ to the surface to induce DCS injury.

## LIVER FUNCTION

At 30 min after decompression under isoflurane anesthesia, about 1 ml blood plasma was collected via the retro-orbital venous plexus from 35 rats ($n$ = 10 in control group, $n$ = 8 both in vehicle and Ros5 group, $n$ = 9 in Ros10 group, Table 1). 500 µl were put into a disposable vacuum tube without addition of anticoagulant and centrifuged at 1,000 g for 10 min. The plasma levels of alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) were detected with a biochemical analyzer (AU2700, Olympus, Japan).

## BLOOD CELL COUNTS

500 µl of blood was put into a vacutainer containing $K_2$EDTA (BD Franklin Lakes, NJ, USA). Blood cell counts were performed using an automatic analyzer (XT-1800i, SYSMEX, Japan). White blood cells including lymphocytes and neutrophils, red blood cells, haemoglobin and platelets were measured. The measurements were not self-controlled (before/after pressurisation) as the volume of blood necessary to do so would have been too great to ensure the normal physiological state of mice and thus might have influenced the subsequent results.

## HAEMATOXYLIN AND EOSIN STAINING

At 30 min after decompression, 12 mice ($n$ = 4 of the control, vehicle and Ros10 groups, Table 1) were anaesthetized intraperitoneally with ketamine (70 mg·kg$^{-1}$) and xylazine (10 mg·kg$^{-1}$) and then perfused with 50 mL of normal saline through the left ventricle followed by a 50 mL 4% paraformaldehyde solution. The hepatic tissue was removed quickly and post-fixed for 24 h in 4% paraformaldehyde solution. After paraffin embedding, coronal sections were cut with a thickness of 5 µm on a paraffin microtome (RM2245, Leica, Bensheim, Germany). Sections were then deparaffinized, rehydrated, stained with haematoxylin and eosin, mounted with neutral balata and covered with coverslips. Finally, the sections were examined under a microscope (DM 4000B, Leica, Germany).

## TISSUE PROTEIN EXTRACTION

At 30 min after the end of decompression, livers ($n$ = 16 in the control group, $n$ = 14 in the vehicle group and $n$ = 15 in the Ros10 group, Table 1) were quickly removed from mice killed by decapitation under anaesthesia then homogenized in Radio Immunoprecipitation Assay Lysis Buffer (Beyotime, Jiangsu, China), centrifuged at 14,000 g at 4$^{O}$C for 30 min; the supernatant was then collected and the total protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

## TOTAL NO CONCENTRATION MEASUREMENT

Total NO concentration in the hepatic tissues of the mouse

was measured using a NO assay kit (nitrate reductase method, Jiancheng Bioengineering Institute, Nanjing, China). The livers of mice ($n$ = 10 in control group, $n$ = 8 in vehicle and Ros5 groups and $n$ = 9 in the Ros10 group, Table 1) were harvested at 30 min after decompression as described above and homogenized 1:9 (w:v) in 0.9% saline. The homogenates were then centrifuged at 2,500 rpm for 10 min at 4°C, and the supernatants were taken for NO assay according to the manufacturer's instructions and total protein determination.

SOLUBLE E-SELECTIN DETECTION

About 200 µl blood was obtained from the tip of the tail before compression and at 30 min after decompression ($n$ = 6 vehicle and Ros10 groups, Table 1). The samples were coagulated at 4°C, and the soluble E-selectin (sE-selectin) concentration in the supernatant was subsequently detected using an ELISA kit (Cloud-Clone Corp., Houston, USA). The concentration of soluble E-selectin in serum collected before compression was defined as the baseline level.

WESTERN BLOT ANALYSIS

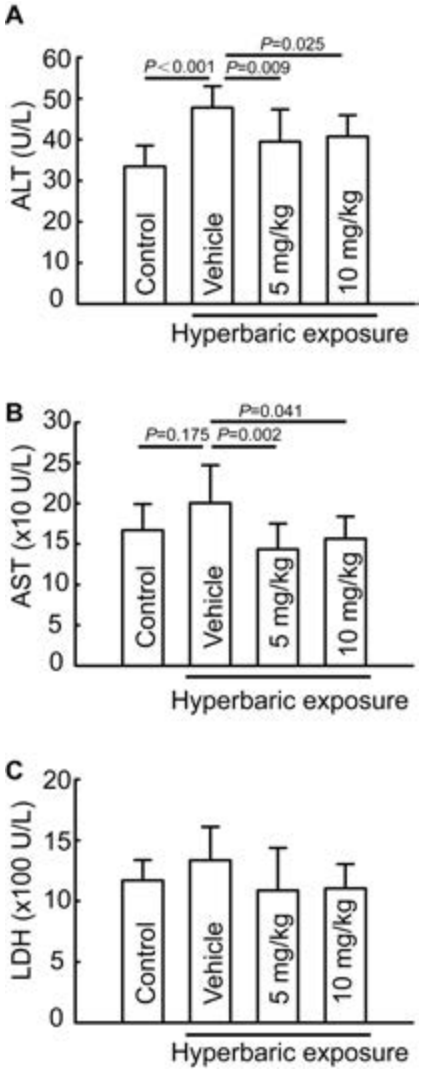
Eighteen protein samples ($n$ = 6 in control, vehicle and Ros10 groups, Table 1) mixed with loading buffer were electrophoresed using 10% sodium lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrically transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated at 4°C overnight in tris buffered saline (TBS) containing 5% defatted milk and detected with the following primary antibodies: eNOS (C–20) (1:200, sc–654, Santa Cruz, Dallas, USA), phosphorylated eNOS (p-eNOS) at Ser1177 (1:500, sc-12972, Santa Cruz), β-actin (1:10,000, Sigma-Aldrich, St. Louis, USA), iNOS (1:1000, #13120, CST, Denver, USA), and E-selectin (H-300) (1:500, SC-14011, Santa Cruz). After several washes in TBS, the samples were incubated for 2 h at room temperature with secondary IRDye 800 CW goat anti-mouse or rabbit (1:10000, Li-COR, Nebraska, USA). Immunoreactivities were captured using a fluorescence scanner (Odyssey Lix, LI-COR) and quantified using the software Image-pro Plus 5.1. Data were obtained from at least three independent preparations.

STATISTICAL ANALYSIS

Normal distribution was tested for with a Shapiro-Wilk Test using SPSS 17.0 software. Data were presented as the mean ± SD, one-way analysis of variance (ANOVA) was used for multiple comparisons (LSD) with variables that were normally distributed. AST levels and the lymphocyte counts were not distributed normally, so these were transformed into ranks and analyzed with univariate analysis of variance. The significance level was established at $P < 0.05$.

**Figure 1**

Effect of rosiglitazone 5 mg·kg$^{-1}$ (Ros5) and 10 mg·kg$^{-1}$ (Ros10) pre-treatment on liver injury in DCS mice; plasma ALT, AST and LDH (mean +/- SD) at 30 min after rapid decompression from 911 kPa ($n$ = 10 in control group, $n$ = 8 in vehicle and Ros5 groups, $n$ = 9 in Ros10 group)




**Results**

Twenty-one of the 120 rats (18%) subjected to pressurisation died, leaving 139 for the various analyses (Table 1). There were no significant differences in mortality between the vehicle and rosiglitazone groups.

LIVER INJURY (FIGURE 1)

The levels of ALT, AST and LDH in the vehicle group were increased to different extents compared to the control group. ($P < 0.001$ for ALT, Figure 1A–C). Both doses of rosiglitazone significantly inhibited these elevations (Ros5, $P = 0.009$; Ros10, $P = 0.025$).

**Figure 2**

Haematoxylin and eosin staining of liver sections at 30 min after decompression from 911 kPa; the arrowheads refer to a disordered lobular structure; scale bar 100 µm

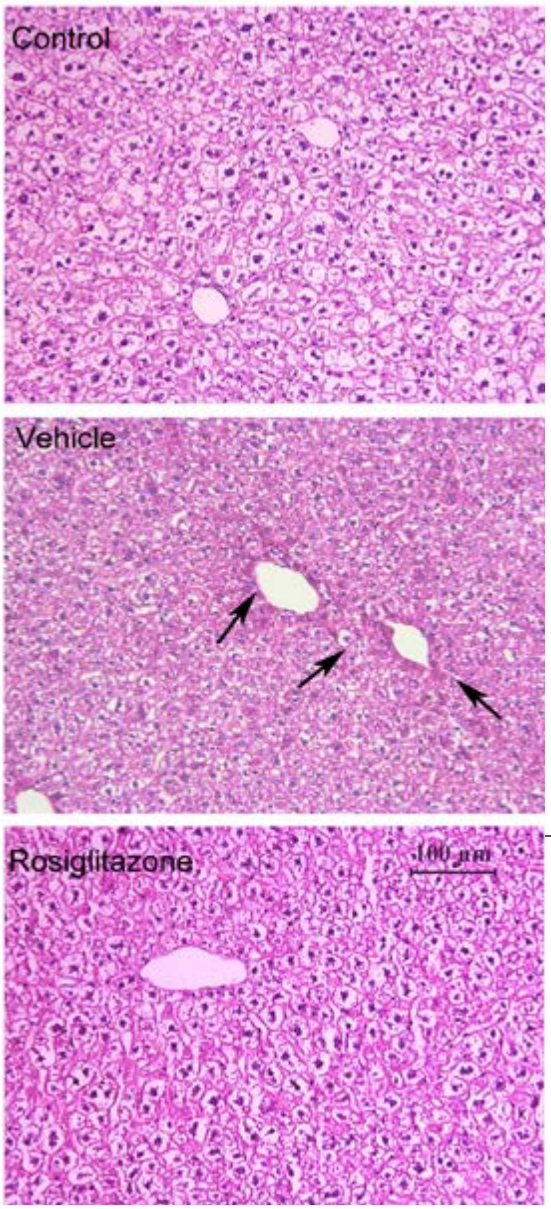
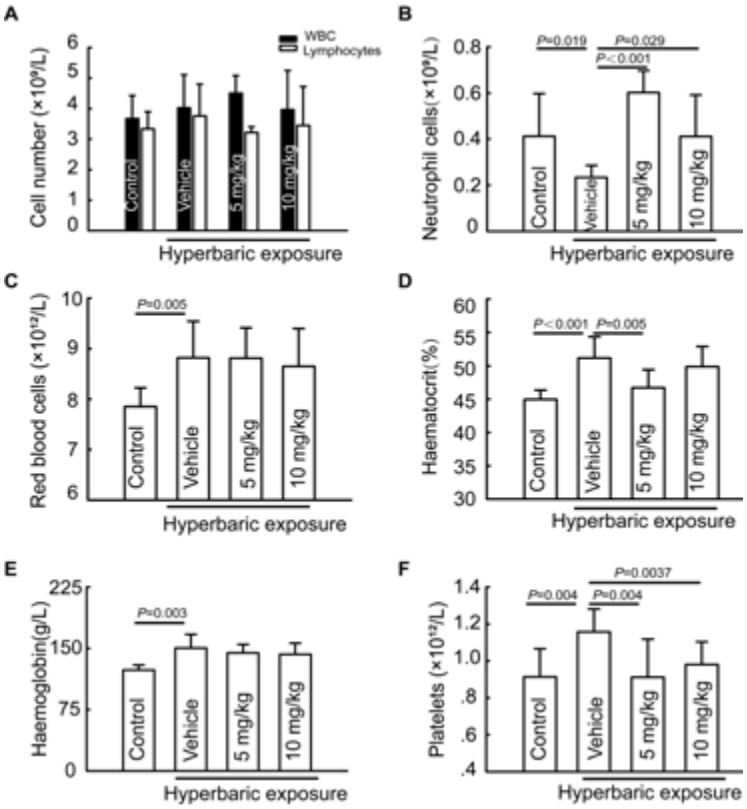



**Figure 3**

Effect of rosiglitazone pre-treatment on blood cell counts 30 min after decompression from 911 kPa (mean +/- SD); A–F: white blood cells, lymphocyte, neutrophil and red blood cell and platelet counts, haematocrit and haemoglobin concentration ($n$ = 10 in control group, $n$ = 8 in vehicle and Ros5 groups, $n$ = 9 in Ros10 group)




A clear lobular structure and organized hepatic cell cords were observed in control mice, whereas disorganized hepatocytes and a disordered lobular structure were observed in the vehicle group (Figure 2). However, normal hepatic architecture was preserved in the Ros10 group.

## BLOOD CELL COUNTS (FIGURE 3)

Red blood cell counts and haematocrit (Figure 3C and D), haemoglobin (Figure 3E) and platelets (Figure 3F) were significantly increased after decompression in the vehicle group. The increase in haematocrit was prevented in the Ros5 group compared to the vehicle group ($P$ = 0.005), and in both rosiglitazone groups the levels of platelets were significantly reduced (Ros5 $P$ = 0.004 and Ros10 $P$ = 0.037, Figure 3). Additionally, the neutrophil counts were significantly decreased after rapid decompression ($P$ = 0.019, Figure 3B), and this reduction was prevented by both rosiglitazone groups (Ros5 $P$ < 0.001 and Ros10; $P$ = 0.0029, Figure 3B).

## HEPATIC TOTAL NO PRODUCTION AND iNOS EXPRESSION (FIGURE 4)

Total NO production and iNOS expression in hepatic tissue were significantly elevated in the vehicle group after decompression ($P$ < 0.001 and $P$ = 0.016, respectively *vs.* the control group), however, these elevations were inhibited by rosiglitazone pre-treatment (Ros5 $P$ < 0.001 and Ros10 $P$ = 0.048, respectively *vs.* the vehicle group, Figure 4).

## SERUM SE-SELECTION CONCENTRATION AND HEPATIC E-SELECTIN EXPRESSION (FIGURE 5)

Following rapid decompression, the concentration of sE-selectin in the serum and E-selectin expression in hepatic tissue in the vehicle group were remarkably elevated ($P$ = 0.017 and $P$ = 0.011, respectively). Pre-treatment

**Figure 4**

Effect of rosiglitazone pre-treatment on total NO production and iNOS expression in the liver (mean +/- SD) at 30 min after decompression from 911 kPa; A: total NO concentration; ($n$ = 10 in control group, $n$ = 8 in vehicle group, $n$ = 9 in Ros10 group); B: examples of iNOS expression; C: iNOS levels ($n$ = 6 in each group)

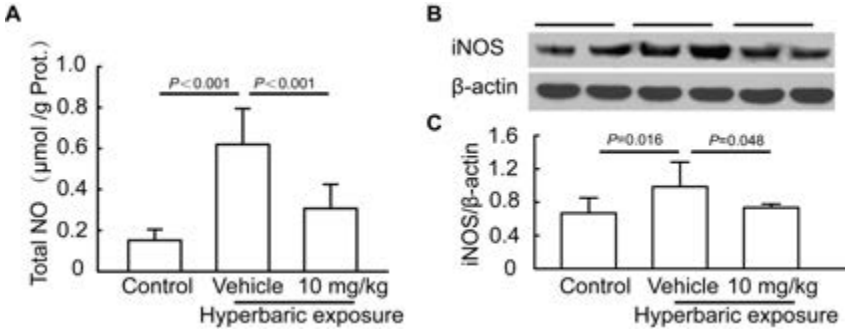



**Figure 5**

Effect of rosiglitazone pre-treatment on the levels of serum sE-selectin concentration and E-selectin expression in hepatic tissue (mean +/- SD) at 30 min after rapid decompression from 911 kPa; A – change in sE-selectin ($n$ = 12 in the control group, and $n$ = 6 in the vehicle and rosiglitazone groups); B – examples of E-selectin expression (Western blot); C – mean values of E-selectin in the liver

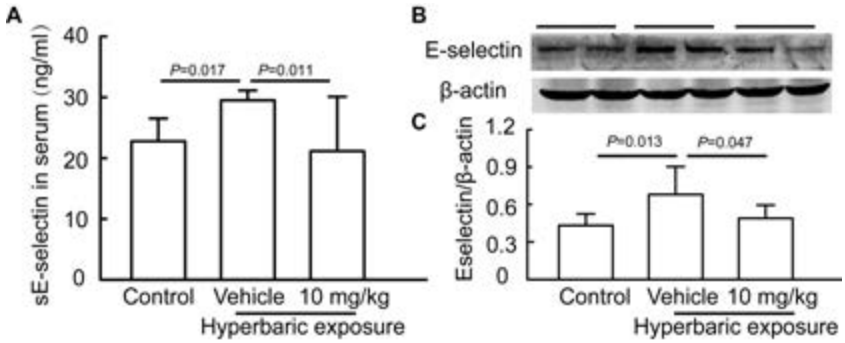
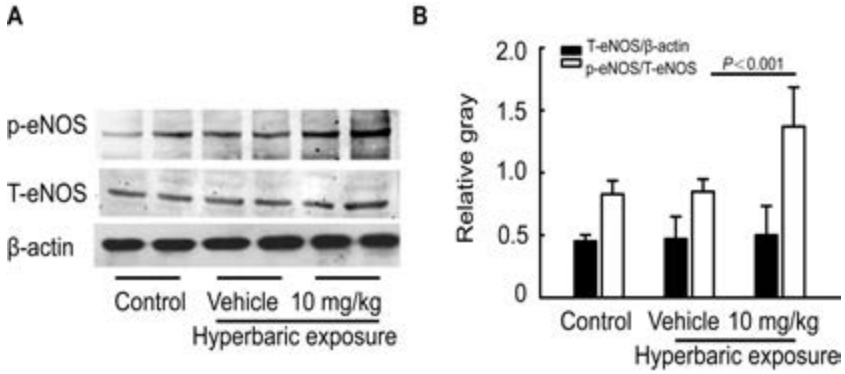



**Figure 6**

Effect of rosiglitazone pre-treatment on the phosphorylation of eNOS in the liver (Western blot analysis) at 30 min after rapid decompression from 911 kPa; A: examples of T-eNOS, p-eNOS and -actin expression; B: T-eNOS and p-eNOS level relative to β – β-actin (mean +/- SD, $n$ = 6 in each group)




with rosiglitazone prevented these elevations (Ros5 $P$ = 0.013 and Ros10 $P$ = 0.047, Figure 5).

PHOSPHORYLATION OF eNOS (FIGURE 6)

The expression of T-eNOS remained unchanged after decompression and in the presence of rosiglitazone. Moreover, rapid decompression did not change the level of p-eNOS; however, pre-treatment with rosiglitazone (Ros10) elevated the expression level of p-eNOS and the ratio of p-eNOS / T-eNOS in the hepatic tissue ($P$ < 0.001, Figure 6).

## Discussion

The main finding in this study was that pre-treatment with rosiglitazone reduced liver injury in mice following rapid decompression, as assessed by morphological hepatic lobule alterations, the ALT and AST levels and blood cell counts. In addition, we found that rosiglitazone pre-treatment significantly decreased sE-selectin level in the serum and promoted the phosphorylation of eNOS in the liver of mice after rapid decompression. These results suggest that the reduction in liver injury by rosiglitazone pre-treatment may be mediated via stimulation of eNOS and the resultant elevation of endothelial function. Moreover, the decreases in iNOS expression, total NO production in the liver and of neutrophil counts in blood suggest that rosiglitazone also may have an anti-inflammatory action.

Nitrogen bubbles that appear in the blood, extracellular space, and intracellular space during decompression can promote neutrophil activation, stimulate the release of inflammatory mediators IL-6, as well as cell adhesion molecules E-selectin, L-selectin and intercellular adhesion molecule-1, thereby triggering inflammatory cascades in tissues.[6,19] In this study, sE-selectin, a marker of endothelial activation and systemic inflammatory response syndrome,[20] was elevated in serum after decompression. In addition, damage to the vascular endothelium by gas bubbles in DCS may provoke diapedesis, which may be the reason for the decrease in neutrophil counts in the present study. Furthermore, stimulation of NO production through up-regulation of iNOS expression, may promote inflammatory responses contributing to tissue injury that is accompanied by increased leukocyte activation and endothelial adherence.

Rosiglitazone pre-treatment significantly prevented the increase in ALT and the decrease in neutrophils as well as inhibiting iNOS expression and total NO production. This suggests a potential inhibitory effect of rosiglitazone on inflammatory responses and consequently, exerting a protective effect during DCS. We believe that this protective effect of rosiglitazone may be partially mediated by inhibiting iNOS activity.[21] Previous studies have also shown that rosiglitazone can attenuate inflammatory responses and exert a protective effect in experimental models of ischaemia and intracerebral haemorrhage.[22] Thus, the present results of rosiglitazone treatment on liver injury induced by rapid decompression are consistent with the literature.

Bubble precursors (gas nuclei) adhering to the endothelium are able to grow into bubbles during decompression.[12] To some extent, the amount and size of bubble formation during decompression is dependent on the basal synthesis of NO, specifically for NO derived from eNOS in the endothelium.[8,12] Thus, altering the properties of the vascular endothelium via exogenous NO administration or mediators of endogenous NO up-regulation might reduce DCS risk and severity, which may be mediated by altering the endothelial surface tension and ultimately interfering with bubble formation.[7,23] In this study, we found an increase in the endothelial activation marker sE-selectin in the serum and E-selectin expression in hepatic tissue following decompression, indicating an activation of the endothelium. These increases were inhibited by rosiglitazone, suggesting that its protective effect may involve actions on endothelial function.

Rosiglitazone has been reported to be able to acutely stimulate NOS in cultured endothelial cells via an AMPK-dependent mechanism. eNOS-mediated NO generation can serve a protective function, thereby preserving endothelial integrity, improving tissue perfusion, and abrogating injury.[7,14] Rosiglitazone promoted the phosphorylation of eNOS in the liver in the present study. In general, damage to endothelium during rapid decompression will increase the microvascular permeability and extravasation of plasma, which will result in haemoconcentration.[24] The haematolical data suggest that some level of haemoconcentration had occurred and that rosiglitazone pre-treatment prevented this. Thus, a potential effect of rosiglitazone may be to alleviate endothelial injury during rapid decompression.[25,26]

As there were no significant differences in the results between the two rosiglitazone groups, we did not measure the effects of 5 $mg \cdot kg^{-1}$ rosiglitazone on indices including E-selectin, total NO level and the level p-eNOS. The present findings suggest a mechanism underlying rosiglitazone-induced protection that involves the phosphorylation of eNOS, that increases endothelial NO production, preserves vascular integrity and function and, as a result, the liver function of mice after rapid decompression.

## Conclusions

The present study demonstrated that pre-treatment with rosiglitazone could protect mice against the liver injury induced by rapid decompression. This preventive action may be mediated, at least in part, by limiting inflammatory processes and preserving endothelial integrity and function. Therefore, targeting eNOS/NO pathways may serve as a strategy to reduce liver injury in DCS.

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