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Brief title: Yaegashi, RAP altered Hepatic Gene Expression

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None of the authors have conflict of interest.

ABSTRACT

Background: Atrial fibrillation (AF) activates the coagulation system, leading to hypercoagulation of the blood. The liver is a major source of prothrombotic molecules.

Objectives: This study aimed to clarify whether cardiohepatic interactions are involved in AF-related hypercoagulation.

Methods: We compared gene expression profiles of the human liver tissue between AF patients and controls. An AF model was created by rapid atrial pacing (RAP) at a frequency of 1200 bpm in anesthetized 10-week-old Sprague-Dawley rats. Livers, atria, and peripheral blood cells were collected and analyzed after 12 h of RAP.

Results: DNA microarray analysis revealed marked changes in the gene expression profile of the human liver of AF patients. The extrinsic prothrombin activation pathway showed the most prominent change among 354 BioCarta pathways. Twelve hours of RAP also markedly altered the gene expression profile of rat liver. RAP markedly augmented the hepatic mRNA expressions of fibrinogen chains, prothrombin, coagulation factor X, and antithrombin III. The augmented fibrinogen production by RAP was accompanied by increased IL-6 mRNA expression in peripheral blood cells,

enhanced MCP-1 expression in the liver, infiltrated CD11b positive mononuclear cells into the liver, and enhanced STAT3 phosphorylation in hepatocytes nuclei. STAT3 phosphorylation and increased fibrinogen and coagulation factor X production by RAP were suppressed by pretreatment with IL-6 neutralizing antibody.

Conclusion: Rapid atrial excitation mimicking paroxysmal AF remotely altered the hepatic gene expression of prothrombotic molecules. Increased fibrinogen expression in the liver by RAP was mediated by activation of the IL-6/STAT3 signaling pathway in the peripheral blood and liver.

Keywords: Atrial fibrillation; Fibrinogen; IL-6/STAT3 signaling pathway; Liver; Monocyte/macrophage

Introduction

Atrial fibrillation (AF), the most common type of sustained arrhythmia in adults, is associated with a five-fold increase in ischemic stroke incidences.^{1,2} Most strokes in AF patients are believed to be cardioembolic caused by the embolism of left atrial thrombi.³ As shown by the well-known Virchow's triad, thrombus formation in the left atrium (LA) can result from decreased blood flow, increased endocardial dysfunction in the LA, and enhanced blood coagulability.^{4,5} Spontaneous echo contrast formation in the LA is promoted by decreased blood flow velocity and is strongly associated with left atrial appendage thrombus and cardioembolic events.⁶ Previous studies have also determined that AF-associated endocardial dysfunction is characterized by an increase in von Willebrand factor, nitric oxide (NO), and plasminogen activator inhibitor-1 expression and a decrease in NO synthase expression in the LA.^{7,8} Even short-term rapid atrial pacing (RAP) for 8 h induces a marked decrease in tissue factor pathway inhibitor and thrombomodulin expression in the atrial endocardium of rats.⁹ However, the mechanism of AF-induced hypercoagulation of the blood, connected with the third element of Virchow's triad, is poorly understood. Because the liver is an essential

organ synthesizing many coagulation factors and other prothrombic molecules, we hypothesized that a cardiohepatic interaction is involved in AF. Here we examined whether short-term rapid atrial excitation affects gene expression remotely in the liver.

Method

Expanded methods can be found in the Online Supplemental Material.

Rapid Atrial Pacing Model

This study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health using Sprague-Dawley rats, 8–12 weeks of age. It was approved by the Institutional Animal Use and Care Committee of Kanazawa University. An atrial tachycardia rat model was prepared as previously reported.^{9,10} Right atrial stimulation at a frequency of 1200 bpm was performed with 2-ms rectangular pulses using a programmable stimulator. Sham-surgery rats underwent an identical procedure without electrical stimulation. The details are described in Online Supplementary Methods.

Human liver tissue

We screened 465 consecutive patients with non-alcoholic steatohepatitis who underwent liver biopsy from 2003 to 2013 and identified 3 patients with AF without structural heart disease. Three patients who were in sinus rhythm and matched for age, sex, and hepatic histopathological stage (Brunt's pathologic criteria) served as a control group (Supplemental Table 1). There was no statistical difference between the two groups in left atrial diameter. Informed consent was obtained from all patients before liver biopsy. The experimental protocol was approved by the Human Genome, Gene Analysis Research Ethics Committee of Kanazawa University (Approval No. 2014-002), and the study was conducted in accordance with the Declaration of Helsinki.

Microarray Experiments, Quantitative Real-Time Polymerase Chain Reactions, Western Blot and Histological Analyses

We performed microarray experiments using RNA from the liver tissues of humans and rats. Quantitative real-time polymerase chain reaction (RT-PCR) was performed using RNA from the liver, LA, spleen tissues and peripheral blood cells of rats. Western blot, immunohistochemical

staining and immunofluorescent staining were performed using liver tissues of rats. The detailed methods are shown in Online Supplementary Methods.

Administration of IL-6 Neutralizing Antibody

IL-6 neutralizing antibody (5 μ g/400 kg BW) was intraperitoneally administered 1 day before and immediately subsequent to RAP onset.

Statistical Analysis

Continuous variables are presented as mean $\pm$ SEM for each group unless stated otherwise and were analyzed by ANOVA with Bonferroni post hoc testing for multiple comparisons and unpaired t-test for two-group comparisons. Categorical variables were compared using the chi-square test. Significance was set at $P < .05$.

Results

Gene Expression Profiles of the Human Liver in AF

DNA microarray revealed marked changes in the gene expression profile of the human liver of AF patients. Of 54,675 human genes in the array, 4322 filtered genes demonstrated clear clusters for AF or control (Supplemental

Figure 1). Table 1 displays significantly overrepresented BioCarta pathways in the liver of AF patients. The expression of genes related to coagulation, immunity, apoptosis, and hypoxia was significantly altered in AF. Notably, the extrinsic prothrombin activation pathway, including fibrinogen, showed the most prominent change among 354 BioCarta Pathways (Table 1).

Characteristics of Experimental Rats

At the time of euthanasia, ECG confirmed an atrial paced rhythm in each experimental animal. Arterial blood pressure was not different between 12-h RAP and sham groups (Supplemental Figure 2A). Atrial pacing of 1200 bpm brought the ventricular response from 2:1 to 3:1. Accordingly, the mean ventricular rates of the RAP model demonstrated a slightly higher trend but no statistical difference than the sham model (Supplemental Figure 2B). Additionally, there was no statistical difference between the groups in liver weight/body weight, arterial blood-gas parameters, or serum liver enzymes (Table 2).

Gene Expression Profiles in Rat Liver after 12 h of RAP

DNA microarray analysis revealed marked changes in the gene expression profile of rat liver after 12 h of RAP. Hierarchical clustering with 3737 filtered genes demonstrated clear clusters for the RAP or sham groups (Figure 1).

Quantitative RT-PCR showed that mRNA expression of fibrinogen α , β , and γ chains significantly increased in the liver after 12 h of RAP (2.01 fold, $P = .0007$; 1.34 fold, $P = .0066$; and 1.90 fold, $P = .0032$, respectively; $n = 5$ (Figure 2A). Western blot analysis confirmed significantly increased fibrinogen immunoreactive proteins in the liver after 12 h of RAP (2.84 fold; $P = .0125$; $n = 5$) (Figure 2B). Additionally, 12 h of RAP enhanced the mRNA expression of prothrombin, coagulation factor X, and ATIII in the liver (2.36 fold, $P = .0240$; 1.27 fold, $P = .0206$; and 2.02 fold, $P = .0263$, respectively; $n = 5$) (Figure 2C). Western blot analysis also showed significantly increased prothrombin, coagulation factor X, and ATIII immunoreactive proteins in the liver after 12 h of RAP (2.48 fold, $P = .0098$; 1.93 fold, $P = .0451$; 1.35 fold, $P = .0348$, respectively; $n = 5$) (Figure 2D). The serum levels of fibrinogen, prothrombin, coagulation factor X and ATIII did not change significantly up to 12 h of RAP

(Supplement Table 2).

Upstream Regulators of Fibrinogen Expression

To elucidate the mechanism of enhanced hepatic fibrinogen production with RAP, we quantified fibrinogen gene expression regulators in the LA, spleen and peripheral blood cells. IL-6 and TNF- α mRNA expression was upregulated in peripheral blood cells after 12 h of RAP (1.67 fold, $P = .0444$; 1.82 fold, $P = .0288$, respectively; $n = 5$) (Figure 3A) but not in the LA (Figure 3B) and the spleen (Supplemental Figure 3). IL-1 β mRNA expression was unchanged in the LA, spleen and peripheral blood cells after 12-h RAP.

Infiltration of Monocytes/Macrophages with Activation of the IL-6/STAT3 Signaling Pathway in the Liver

MCP-1 mRNA expression was upregulated in the liver after 12 h of RAP (2.60 fold; $P = .0015$; $n = 5$) but not in the LA (Figure 4A) and the spleen (Supplemental Figure 3). Western blot analysis showed that RAP significantly increased MCP-1 immunoreactive proteins in the liver (1.68 fold; $P = .0462$; $n = 5$) (Figure 4B). Immunohistochemical analysis

identified many CD11b-positive cells in the liver after 12 h of RAP (Figure 4C). Western blot analysis confirmed significantly increased CD11b immunoreactive protein in the liver of RAP rats compared with sham (1.27 fold, $P = .0048$, $n = 5$, Figure 4D). The immunofluorescence double-labeling experiments showed the coexpression of IL-6 and CD11b (Figure 4E), suggesting infiltration of activated monocytes/macrophages into the liver. The RAP-induced increase in monocytes expressing IL-6 was accompanied by enhanced STAT3 phosphorylation (2.14 fold; $P = .0023$) (Figure 4D) in the liver. Phosphorylated STAT3 protein was prominent in the nuclei of hepatocytes with RAP (Figure 4C).

Effects of IL-6 Neutralizing Antibody on RAP-induced STAT3

Phosphorylation and Fibrinogen Production

To further study the role of IL-6 in RAP-induced fibrinogen production in the liver, we administered IL-6 neutralizing antibody to the rats. IL-6 neutralizing antibody mildly attenuated the RAP-induced increases in mRNA expression of MCP-1 (Figure 5A), MCP-1 immunoreactive proteins (Figure 5B) and CD11b (Figure 5C) in the liver, but the differences were not statistically significant. STAT3 phosphorylation promoted by RAP was

significantly suppressed by pretreatment with IL-6 neutralizing antibody (0.53 fold vs 12-h RAP; $P = .0012$; $n = 5$) (Figure 5D). Accordingly, IL-6 neutralizing antibody significantly attenuated the RAP-induced increases in mRNA expression of fibrinogen α and γ chains (0.43 fold vs 12-h RAP, $P < .0001$; 0.61 fold vs 12-h RAP, $P = .0087$, respectively; $n = 5$) (Figure 6A) and fibrinogen immunoreactive proteins (0.54 fold vs 12-h RAP; $P < .0001$; $n = 5$) (Figure 6B). IL-6 neutralizing antibody also significantly prevented the RAP-induced increases in mRNA expression of coagulation factor X (0.65 fold vs 12-h RAP, $P = .0001$; $n = 5$) (Figure 6C) and coagulation factor X immunoreactive proteins (0.47 fold vs 12-h RAP; $P = .0146$; $n = 5$) (Figure 6D). On the other hands, mRNA/protein expression of prothrombin and ATIII were not suppressed by pretreatment with IL-6 neutralizing antibody (Figure 6C and 6D).

Discussion

The major findings of this study are as follows: 1) AF markedly affected the gene expression profile of the human liver. The extrinsic prothrombin activation pathway showed the most prominent change among the BioCarta pathways. 2) Twelve hours of RAP mimicking AF markedly altered the

gene expression profile of the rat liver. Hepatic mRNA expression of prothrombic molecules, including fibrinogen chains, prothrombin, coagulation factor X, and ATIII, was augmented by short-term RAP. 3) The augmented fibrinogen production with RAP was accompanied by increased IL-6 mRNA expression in peripheral blood cells, enhanced MCP-1 expression in the liver, infiltrated CD11b positive mononuclear cells into the liver, and enhanced STAT3 phosphorylation in the nuclei of hepatocytes. 4) STAT3 phosphorylation and increased fibrinogen production in the liver by RAP were suppressed by pretreatment with IL-6 neutralizing antibody.

Thrombus formation in AF is known to result from a decrease in blood flow,⁶ impairment of the atrial endocardial function,⁷⁻⁹ and hypercoagulability of the blood, as shown in Virchow's triad.^{4,5} Accordingly, studies have reported hypercoagulability of blood with increased serum TAT in AF patients.^{11,12} Notably, serum fibrinogen and D-dimer levels are elevated in patients just after 12 h following the AF onset.^{13,14} However, the mechanism underlying enhanced blood coagulability associated with AF has not been fully elucidated till date.

The liver is an essential organ that synthesizes many bioactive proteins,

including acute-phase inflammatory proteins (e.g., C-reactive protein) and coagulation factors. Animal models of acute kidney injury have shown that acute impairment of renal function affects hepatic inflammatory responses;^{15,16} thus, renal-hepatic crosstalk is likely present in acute kidney disease. Acute coronary syndrome is accompanied by systemic manifestations such as proinflammatory responses and coagulation system activation;¹⁷ therefore, cardiohepatic interaction is implicated in acute coronary syndrome. In a previous study by our group, marked changes in hepatic gene expression were observed in the mouse model of myocardial infarction and ischemia/reperfusion.¹⁸ However, AF-related changes in hepatic gene expression have not been addressed. To the best of our knowledge, our data provides the first evidence that AF remotely and markedly alters liver gene expression profiles, primarily those involved in the extrinsic coagulation pathway.

The IL-6-dependent STAT3 phosphorylation has recently been shown to be involved in regulating acute phase proteins, including fibrinogen.¹⁹ IL-6 is mainly secreted by mononuclear cells such as monocytes and macrophages. Based on previous reports demonstrating that AF causes atrial endocardial dysfunction⁷⁻⁹, we initially hypothesized that the LA may

be involved in RAP-induced IL-6 production. However, in our RAP model, IL-6 mRNA expression was increased in peripheral blood cells but not in the LA. In fact, in human paroxysmal (and persistent) non-valvular hypertensive AF patients, IL-6 and TNF- α mRNA in monocytes were upregulated in accordance with our results.²⁰ Acute-onset AF activates platelets within minutes to initiate platelet-mononuclear cell interaction.²¹ This phenomenon may underlie peripheral mononuclear cell production of IL-6 and TNF- α .

In the animal model of subacute myocardial infarction, mononuclear cells were recruited in the peripheral blood, and the highest number of exited mononuclear cells was found in the liver, followed by the draining lymph nodes and the spleen.²² Mononuclear cell migration into the liver is mainly facilitated by profound secretion of MCP-1 by non-parenchymal liver cells such as Kupffer cells, liver stellate cells, and damaged hepatocytes.²³⁻²⁵ Our data demonstrate RAP-enhanced MCP-1 expression, CD11b positive mononuclear cells in the liver, and STAT3 phosphorylation in the nuclei of hepatocytes. Moreover, the RAP-induced STAT3 phosphorylation and increased fibrinogen production were suppressed by pretreatment with IL-6 neutralizing antibody. These findings

suggest that the activated peripheral blood monocytes infiltrate the liver and secrete IL-6 to promote fibrinogen production via STAT3 phosphorylation in hepatocytes. On the other hand, in LA and spleen, the increases of MCP-1 and IL-6 expression were not observed. These findings might imply that activated mononuclear cells affect predominantly to the liver in the present animal model.

Coagulation Factor X plays a central role in the coagulation cascade. Factor X is synthesized in the liver, and its promoter activity is significantly increased by expression of GATA-4.²⁶ A previous study reported that IL-6 stimulation increased the binding of GATA-4 to the HAMP promoter.²⁷ Our data demonstrated that RAP-induced coagulation factor X production was suppressed by IL-6 neutralizing antibody, suggesting the involvement of IL-6 in the production of factor X, as well as fibrinogen in AF.

Interestingly, several studies have indicated that serum IL-6 levels are associated with AF occurrence.²⁸⁻³⁰ Additionally, in a large cohort of anticoagulated permanent/paroxysmal AF patients, serum IL-6 level was an independent predictor of cardiovascular events and death, and the addition of IL-6 resulted in improved prediction performance of the

CHA₂DS₂-VASc clinical risk stratification schemes.³¹ These observations support the idea that IL-6 is involved in the pathogenesis of AF and AF-related thromboembolism.

Study Limitations

In the analysis of human liver, we only included small number of patients with liver disease due to ethical reason. Although the patients' characteristics including histological fibrosis stage of the liver were not different between AF and control groups, the interpretation of the transcriptomics might be confounded by the presence of steatohepatitis and other comorbidities.

In the rat with short-term RAP, the expressions of IL-6, TNF- α and IL-1 β was enhanced in the liver but not in the LA and spleen. However, it is not rationale to think that the liver is the only organ affected by AF, because RAP augmented IL-6 mRNA expression in the peripheral mononuclear cells, suggesting the systemic inflammatory effects of AF. In fact, the recent study reported that the number of monocytes in the spleen was reduced and that of macrophages infiltrating the LA was increased on 2 days in the pressure overload-induced AF model.³²

In this study, the mRNA/protein levels of fibrinogen, prothrombin, coagulation factor X and ATIII in the liver were increased after 12-h RAP, however, they were unchanged in the peripheral blood, possibly due to the short duration (12 h) of RAP. In the rats suffering from turpentine-induced inflammation, the peak of fibrinogen level was observed at 24 h in the liver and 40 h in plasma.³³ The duration of RAP in this study was up to 12 h due to difficulty of maintaining the hemodynamics of rats for more than 12 h under the anesthesia.

Although we determined that the IL-6/STAT3 signaling pathway is involved in RAP-induced hepatic fibrinogen production, we could not exclude the existence of other mediators, such as hemodynamic stress and/or autonomic nerve activity. Liver congestion may be involved in the changes in hepatic gene expression after 12 h of RAP. However, in this study, there was no statistical difference in arterial blood pressure, heart rate, liver weight/body weight, arterial blood gas parameters, or serum liver enzymes between the 12-h RAP and sham groups.

Conclusion

Short-term rapid atrial excitation mimicking paroxysmal AF markedly altered the hepatic gene expression profile and promoted the production of prothrombotic molecules in the rat liver. Augmented hepatic fibrinogen expression by RAP was partially mediated by the activation of the IL-6/STAT3 signaling pathway in the peripheral blood and liver.

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Figure legends

Figure 1.

Gene expression profile in rat liver. Hierarchical clustering analysis and heat map depicting 3737 filtered genes (fold change ≥ 1.2) in rat liver for the 12-h rapid atrial pacing (RAP) and sham groups. (n = 4)

Figure 2.

The effect of rapid atrial pacing (RAP) on expressions of coagulation factors in the rat liver. The effect of RAP on mRNA expression of fibrinogen chains (A); protein expression of fibrinogen (B); mRNA expression of prothrombin, coagulation factor X, and antithrombin III (ATIII) (C) and protein expression of prothrombin, coagulation factor X, and ATIII (D) in the liver. Both n = 5. * $P < .05$; ** $P < .01$; *** $P < .001$ vs sham. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

Figure 3.

Upstream regulators of fibrinogen expression. The effect of rapid atrial pacing (RAP) on mRNA expression of interleukin (IL)-6, tumor necrosis factor (TNF)- α , and IL-1 β in the left atrium (LA) (A) and peripheral blood cells (PBCs) (B). Both n = 5. * $P < .05$ vs sham. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

Figure 4.

Infiltration of monocytes/macrophages with the activation of the interleukin (IL)-6/ signal transducer and activator of transcription (STAT) 3 signaling pathway in the liver. A: The effect of rapid atrial pacing (RAP) on mRNA expression of monocyte chemoattractant protein (MCP)-1 in the liver and left atrium (LA). Both $n = 5$. $*P < .05$ vs sham. B: Western blot analysis of liver tissue for MCP-1. Both $n = 5$. $*P < .05$ vs sham. C: Immunohistochemical analysis for IL-6, cluster of differentiation (CD) 11b, and phospho-STAT3 (pSTAT3) in the liver. After 12 h of RAP, immunostaining indicative of IL-6 and CD11b was abundant in the liver (brown color). Also, pSTAT3 protein was prominent in the nuclei of hepatocytes after RAP (brown color). D: Western blot analysis of liver tissue for CD11b and STAT3 phosphorylation. Both $n = 5$. $*P < .05$ vs sham. $**P < .01$ vs sham. E: The immunofluorescence double-labeling experiments revealed coexpression of IL-6 (green) and CD11b (red) in the liver. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

Figure 5.

The effects of interleukin (IL) -6 neutralizing antibody (NA) on rapid atrial pacing (RAP) -induced monocytes/macrophages infiltration and

signal transducer and activator of transcription (STAT) 3 phosphorylation.

A: mRNA expression of monocyte chemoattractant protein (MCP)-1 in the liver. B: Protein expression of MCP-1 in the liver. C: Western blot analysis of liver tissue for cluster of differentiation (CD) 11b. D: Western blot analysis of STAT3 phosphorylation. Both $n = 5$. $*P < .05$; $**P < .01$ vs sham; $^{\dagger\dagger}P < .01$ vs 12-h RAP group. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

Figure 6.

The effects of interleukin (IL) -6 neutralizing antibody (NA) on rapid atrial pacing (RAP) -induced fibrinogen production and other coagulation molecules. A: mRNA expression of fibrinogen chains. B: Protein expression of fibrinogen. C: mRNA expression of prothrombin, coagulation factor X and antithrombin III (ATIII). D: Protein expression of prothrombin, coagulation factor X and ATIII. Both $n = 5$. $*P < .05$; $**P < .01$; $***P < .001$ vs sham; $^{\dagger}P < .05$; $^{\dagger\dagger}P < .01$; $^{\dagger\dagger\dagger}P < .001$; $^{\dagger\dagger\dagger\dagger}P < .0001$ vs 12-h RAP group. GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

Table 1. Significantly overrepresented BioCarta pathways in the liver of AF patients

Gene and Frequent Pathway	LS permutation p-value
Extrinsic prothrombin activation pathway	0.00001
Overview of telomerase protein component gene hTert	0.00001
Transcriptional regulation	
Complement pathway	0.00016
p53 signaling pathway	0.00020
Hypoxia and p53 in the cardiovascular system	0.00027
CTCF: first multivalent nuclear factor	0.00040
Estrogen-responsive protein Efp controls cell cycle and breast tumors growth	0.00062
Acute myocardial infarction	0.00068
Apoptotic signaling in response to DNA damage	0.00088
Lectin-induced complement pathway	0.00098
Downregulated MTA-3 in ER-negative breast tumors	0.00173
ER-associated degradation (ERAD) pathway	0.00222

IL4 signaling pathway	0.00254
Basic mechanism of action of PPARa, PPARb(d), and PPARg and effects on gene expression	0.00342
Activation of cAMP-dependent protein kinase, PKA	0.00364
Regulation of eIF4e and p70 S6 kinase	0.00373
Signaling pathway from G-protein families	0.00407

Table 2. Characteristics of Experimental Animals

	Sham	4-h RAP	8-h RAP	12-h RAP
	(n = 5)	(n = 5)	(n = 5)	(n = 5)
BW, g	410.2 ±	390.8 ±	390.4 ±	426.4 ±
	17.4	23.5	13.0	31.6
LW/BW, ×10⁻²	3.90 ± 0.06	3.93 ± 0.21	4.21 ± 0.11	3.89 ± 0.25
<i>Arterial blood gas parameters</i>				
pH	7.324 ±	7.389 ±	7.389 ±	7.365 ±
	0.008	0.019	0.015	0.023
pCO₂, mmHg	53.4 ± 2.7	41.8 ± 3.5	45.3 ± 2.1	44.1 ± 6.0
pO₂, mmHg	73.1 ± 4.2	96.9 ± 8.4	84.3 ± 10.6	82.2 ± 7.7
HCO₃, mmol/L	27.0 ± 1.1	24.4 ± 1.0	26.7 ± 0.9	24.4 ± 2.8
BE	0.44 ± 0.8	-0.04 ±	1.74 ± 0.77	-0.84 ±
		0.56		2.37
Hb, g/dL	14.1 ± 0.6	14.4 ± 0.7	14.8 ± 0.6	14.4 ± 1.3
Lactate, mmol/L	2.62 ± 0.22	2.20 ± 0.23	2.18 ± 0.25	2.60 ± 0.30

Serum liver enzymes

AST, U/L	72.2 ± 7.3	71.0 ± 7.4	71.4 ± 4.1	71.2 ± 3.3
ALT, U/L	34.4 ± 5.0	31.6 ± 1.6	35.6 ± 1.8	35.8 ± 2.2
ALP, U/L	497.6 ±	500.6 ±	573.2 ±	489.6 ±
	28.9	27.8	57.1	46.3

Values are presented as mean ± SEM. No statistically significant differences in these parameters were observed between the groups. ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BE = base excess; BW = body weight; Hb = hemoglobin; HCO₃ = bicarbonate; LW = liver weight; pCO₂, = partial pressure of carbon dioxide; pO₂, = partial pressure of oxygen.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6