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Yours sincerely,

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Title: Effects of positive acceleration (+Gz stress) on liver enzymes, energy metabolism, and liver histology in rats.

Running Title: **Repeated +Gz stress transiently impairs liver function in rats.**

Abstract:

Background: Although clinical data have shown that liver dysfunction occurs in pilots, the precise cause has not been well defined. The present study aimed to investigate rat liver function changes in response to repeated +Gz exposure.

Methods: Ninety male Wistar rats were randomly divided into a blank control group (Group BC, n = 30), a +6 Gz/5 min stress group (Group 6GS, n = 30), and a +10 Gz/5min stress group (Group 10GS, n = 30). The 6GS and 10GS groups were exposed to +6 Gz or +10 Gz, respectively, in an animal centrifuge. The onset rate of +Gz was 0.5 G/s. The sustained time at peak +Gz was 5 min for each exposure, for 5 exposures, and 5-min intervals between exposures for a total exposure and non-exposure time of 50 min. We assessed liver injury by measuring the portal venous flow volume, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), liver tissue malondialdehyde (MDA), Na^+-K^+-ATPase, and changes in liver histology. These parameters were recorded at 0 h, 6 h, and 24 h after repeated +Gz exposure.

Results: The portal venous flow rate was higher in rats from the 6GS group than the 10GS group 0

h after exposure. The 6GS group had significantly lower ALT, AST, and MDA values than the 10GS group 0 h and 6 h post exposure. The Na^+-K^+-ATPase activity in the 6GS group was significantly higher than in the 10GS group 0 h and 6 h post exposure. Hepatocyte injury, determined pathologically, was significantly lower in the 6GS group than in the 10GS group. Conclusions: Repeated +Gz exposure transiently causes hepatocyte injury, affects liver metabolism, and morphological structure.

Key words: Positive acceleration (+Gz); Liver function; Animal models; Liver metabolism; Ischemia-reperfusion injury

Introduction

Exposure to high sustained +Gz (head-to-foot inertial load) is known to have harmful effects on the human body during aviation [1]. With rapid developments in aviation and aerospace technologies, pilots are required to undertake sustained high G-acceleration stress. The characteristics of modern high performance aircraft flight in particular involve high acceleration (**>** 9Gz) that occurs repeatedly and is sustained for 15–45 s, and may exceed the physiological tolerance of human beings, even resulting in pilot incapacitation and subsequent loss of life [2, 3]. Repeated high-acceleration force exposure may result in cumulative adverse stress responses in the body [4]. Accordingly, safe flying is an issue that is attracting broad attention [5].

The vascular beds that ensure hepatic circulation include two distinct blood inflow systems, the portal vein, contributing 70%-80%, and the hepatic artery, contributing 20%-30% to the total hepatic blood flow [6]. Fighter pilots are frequently exposed to high Gz acceleration with the vector oriented in the foot-head direction. Under these conditions, blood and fluids are redistributed in the body and flow along the direction of the inertial force to the lower body [7]. There are some clinical reports in which liver dysfunction has been observed in pilots [8]. However, the reason for these abnormalities remains unclear. An important question to address is whether or not changes in the blood flow direction after +Gz exposure impairs liver function. Moreover, the manner in which portal venous hemodynamics change after repeated +Gz exposure,

and whether or not oxidative stress parameters increase the duration of these changes, are additional questions awaiting answers. To answer these questions, further studies on liver damage and the mechanisms of liver damage induced by high +Gz exposure are needed to provide evidence for effective preventative measures. In recent years, studies on the effects of high +Gz on the heart, brain, and lung have been addressed in considerable numbers of human and animal studies [9-11]. With the above in mind, the aim of the current study was to investigate the effects of high +Gz exposure on liver blood flow, function, and histology.

Materials and Methods

Animals

Male Wistar rats, weighing 200–250 g, were obtained from the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China), maintained under temperature and light-controlled conditions with a 12 h light/dark cycle, and fed standard rodent chow and water *ad libitum*. To avoid fear and stress, the rats were allowed to acclimatize to the rearing environment for 7 days. They were deprived of food for 12 h before the beginning of each experiment. All animal experiments and procedures performed in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of China PLA Air Force General Hospital.

Experimental groups and animal exposure to acceleration

In this experiment, we chose +6 and +10 Gz as the acceleration test parameters, as reported previously [12, 13]. Ninety rats were randomly divided into three groups: blank control group (Group BC, n = 30), +6 Gz/5 min stress group (Group 6GS, n = 30), and +10 Gz/5 min stress group (Group 10GS, n = 30). The animal centrifuge used was 2 m in radius and was capable of generating a wide range of gravities, from +1 Gz to +15 Gz, with an onset rate of 0.1–6 Gz/s [14]. Each rat was placed into a 15 cm × 5 cm × 3 cm plexiglass box that was clamped to the centrifuge arm, with the rat's head facing the axle centre of the centrifuge for +Gz orientation. In the 6GS and 10GS groups, rats were repeatedly exposed to +6 Gz or +10 Gz stress (each time for 5 min, onset rate of approximately 1 G/s, 5 times with 0 Gz, at 5-min intervals), respectively. The rats in the blank control group were placed in the centrifuge arm and were subjected to the same process as the test groups, but were not exposed to acceleration. To reduce errors, all experiments were performed between 8:00 AM and 11:00 AM in the same quiet and warm environment (room temperature, 18–5 °C). For sample collection, the combined exposure and interval time was 50 min. At 0, 6 h, or 24 h after the last centrifuge run, rats from each group were harvested and blood samples were collected from the inferior vena cava to measure liver enzyme levels (n = 10 per measurement point). Each liver lobe was removed, weighed, and cut into two pieces through the middle with a pair of scissors. One slice of liver tissue was immediately frozen in liquid nitrogen and stored at -80 ℃ to be later used to determine malondialdehyde (MDA) and Na+-K+-ATPase levels. The other piece was fixed with 4% formaldehyde and embedded in paraffin blocks for

routine sectioning and staining with hematoxylin and eosin. At the end of the observation period, all the rats were ultimately killed using chloral hydrate.

Determination of the portal blood flow

Using a color Doppler ultrasound, we evaluated blood vessel diameter and the blood flow velocity in the portal vein. The blood flow volume of the portal vein was calculated with the flow equation: $Q = \pi \times (D/2)2 \times Vmean \times 60$ (Q: flow volume per minute, Vmean: mean blood flow velocity, D: vessel diameter).

Liver function tests

To quantify the extent of liver damage, activity changes in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a serum analyzer (Cobas-Mira Plus; Roche Manheim, Germany).

MDA level measurements

MDA is involved in the metabolism of lipid peroxidation products. The MDA content was measured using a standardized MDA assay kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China), in accordance with the manufacturer's instructions. A peach red color is generated during the condensation reaction between thiobarbituric acid (TBA) and MDA. The results are expressed as nmol/mg protein.

Measurement of Na+-K+-ATPase activity levels

Hepatic Na+-K+-ATPase activity was measured using an ATPase assay kit (Nanjing Jiancheng

Biotechnology Institute, Nanjing, China). Liver tissue samples were homogenized and treated according to the manufacturer's instructions. The measurement of the Na+-K+-ATPase activity is based on the quantification of inorganic phosphate that is formed by adenosine triphosphate decomposition [15]. Na+-K+-ATPase activity was expressed as μmolPi/mg protein/h.

Morphological assessment

Liver specimens were deparaffinized for morphological assessment and stained with hematoxylin and eosin. The histologic damage severity after repeated +Gz exposure was graded using Suzuki's criteria [16]. Liver tissue slices were microscopically examined in a blinded method by an experienced histologist.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 statistical software (SPSS, Chicago, IL, USA). Experimental results were expressed as mean ± standard deviation (SD). Differences were regarded as statistically significant at $P < 0.05$.

Results

Portal venous flow after repeated +Gz exposures

The normal portal venous flow in Wistar rats was 11.468 ± 0.237 mL/min. After repeated +Gz exposure in the 6GS and the 10GS groups, the velocity and flow signals in the portal vein were significantly reduced compared to the BC group ($P < 0.01$ at 0 h post exposure; Fig. 1).

Meanwhile, we found that the portal vein diameter did not change significantly. However, rats in the 6GS group had a much higher portal venous flow volume than those in the 10GS group ($P < 0.01$ at 0 h post exposure). All rats exhibited normal portal venous flow 6 h after repeated +Gz exposure. To summarize, as the G force increased, the portal venous blood flow decreased significantly, but transiently.

Liver function after repeated +Gz exposure

To assess liver cell damage in rats, plasma ALT and AST levels were determined 0, 6, and 24 h after repeated +Gz exposure. ALT and AST values in the BC group were 46.6 ±4.7 IU/L and 110.5 ±7.6 IU/L, respectively.

After repeated +Gz exposure, ALT and AST values in the 6GS and the 10GS groups were higher than in the BC group ($P < 0.01$ at 0 and 6 h time-points after exposure). However, rats in the 6GS group exhibited lower ALT and AST levels than those in the 10GS group, 0 and 6 h post exposure ($P < 0.01$, 6GS group *versus* 10GS group). All rats displayed normal ALT and AST levels 24 h after exposure (Fig. 2). These results demonstrate that the degree of damage to the liver function positively correlated with the increase in G-value, but the abnormalities were transient.

Tissue MDA levels after repeated +Gz exposure

Oxidative stress in rat hepatocytes was assessed by measuring the level of liver tissue MDA. MDA is a marker of lipid peroxidation that is generated by free oxygen radical insults on the cell membrane. Liver tissue samples were harvested 0, 6, and 24 h after repeated +Gz exposure. MDA

concentration in both the 6GS and the 10GS groups had increased 0 h and 6 h after exposure. The MDA concentration in the 6GS group was lower than in the 10GS group 0 h (2.89 ± 0.24 *versus* 3.32 ± 0.25 nmol/mg protein, $P < 0.01$) and 6 h (2.64 ± 0.18 *versus* 3.18 ± 0.19; $P < 0.01$) post-exposure (Fig. 3). Notably, the 6GS and 10GS group MDA concentrations did not recover to normal levels 24 h after exposure. Based on these data, it was concluded that repeated +Gz exposure may induce lipid peroxidation in the rat liver.

Evaluation of Na+-K+-ATPase activity

Na+-K+-ATPase is a cell membrane enzyme that is highly susceptible to lipid membrane peroxidation and free radical reactions [17]. Loss of its activity is a signal of indirect membrane damage. Na+-K+-ATPase activity decreased significantly after exposure in both the 6GS and the 10GS groups, compared to the BC group. The 10GS group had lower Na+-K+-ATPase activity than the 6GS group 0 h (0.73 ± 0.05 μmolPi/mg protein/h *versus* 0.85 ± 0.04 μmolPi/mg protein/h, $P < 0.01$) and 6 h (0.78 ± 0.05 μmolPi/mg protein/h *versus* 0.87 ± 0.03 μmolPi/mg protein/h, $P < 0.01$) post exposure. There was no significant difference between the 10GS and the 6GS groups 24 h after exposure (Fig. 4).

Histopathological observations in the liver after repeated +Gz exposure

The hepatic pathological injury after repeated +Gz exposure was assessed and scored according to Suzuki's criteria [16]. The structure of the hepatic lobules and liver antrum were clear, and cellular edema was not obvious in the BC group (Fig. 5A; Suzuki's score = 2.12 ± 0.35). At the 0

h time-point after exposure, the hepatic sinus cord-like structure was maintained in the 6GS group (Fig. 5B; Suzuki's score = 3.21 ± 0.13), whereas it was less well maintained in the 10GS group, which presented with hepatocyte edema (Fig. 5C; Suzuki's score = 4.63 ± 0.25). At the 6 h time-point post exposure, hepatocyte edema had been significantly relieved in the 10GS group (Fig. 5E; Suzuki's score = 3.53 ± 0.31; $P < 0.01$). There was no significant score difference between the 0 and 6 h time-points after exposure in the 6GS group (Fig. 5B and D; Suzuki's score = 3.21 ± 0.13 *versus* 3.24 ± 0.28; $P < 0.01$). The hepatic histology profiles in both the 6GS and the 10GS groups were nearly normal 24 h after exposure (Fig. 5F; Suzuki's score = 2.14 ± 0.33. Fig. 5G; Suzuki's score = 2.13 ± 0.36).

Discussion

In this study, the effects of high +Gz acceleration on rat liver function was investigated. To this end, we devised an animal model of short-term repeated +Gz exposure. We chose Wistar rats as the experimental subjects because the human Glisson's capsule is similar to that of rats and the model is simple and easy to control. Hepatic energy metabolism and an optimal intracellular environment rely on an adequate blood supply. Hepatocytes are very sensitive to ischemia and/or hypoxia in liver tissue [18, 19]. Therefore, factors related to ischemia and/or hypoxia will definitely influence their metabolism [20]. Direct action and stress response caused by +Gz exposure can result in significant hemodynamic changes between the upper and the lower body, in

important organs, and on the body surface, which is similar to hepatic ischemia-reperfusion (I/R). Indeed, repeated +Gz exposure may cause hepatic I/R. Ischemia is defined as inadequate blood supply to an organ or part of an organ as a result of obstructed blood flow [21]. Ischemia results in a sharp fall in cellular ATP concentration, loss of the ATP-dependent Na+/K+ pump, cellular edema, and increased cytosolic calcium concentration, all of which lead to cell damage [22]. Our findings were consistent with those reported effects, with some significant differences between the acceleration exposed and the control rats observed.

Color Doppler Ultrasound is a well-established method for assessing hemodynamic changes in liver circulation that occur under various physiological conditions [23, 24]. As described by Kim *et al* [25], exposure to high +Gz acceleration forces acting along the body axis from the head to the feet severely reduces blood supply to the internal organs.

Levels of ALT and AST can be used as measures of hepatic damage, and were used in this study to assess damage incurred due to repeated +Gz exposure. The 6GS group was associated with less cellular damage than the 10GS analogue; this was reflected by the lower serum ALT and AST levels. The results indicate that the degree of functional liver damage increased gradually with increasing G value. Zhang *et al* [26] reported that repeated +10 Gz stress had some impact on the oxygen radical metabolism in the rat liver. MDA is widely used as an indicator of oxidative stress, which is one of the end products of lipid peroxidation in the liver [27]. The results of our study showed that rats in the 6GS group had less hepatic MDA than those in the 10GS group. After

repeated +10Gz exposure, the oxygen and nutrients supplied to the liver were reduced. After exposure, rats in the 6GS group presented with less oxidative stress-induced damage than rats in the 10GS group, as manifested by the lower MDA levels. In this study, changes in the MDA levels were in accordance with those caused by ischemia and/or hypoxia in rat livers [28], which also points toward ischemia or hypoxia as one of the main causes of high +Gz stress-induced liver injury.

Early research in this field found that positive acceleration affected the physiological index of the liver. Daligcon *et al* [29] reported that hyper-G stress increased levels of circulating catecholamines and glucagon, both effective stimulators of hepatic gluconeogenesis, and that continued hyperglycemia may be due, in part, to the control of the insulin-stimulated uptake by muscle tissues. They also found that hyper-G stress not only increased circulating and blood glucose levels, but also increased the content of liver glycogen. This was attributed to an increased rate of gluconeogenesis and the key role that epinephrine plays during the beginning of centrifugation exposure [30]. Later research reported that hypergravity exposure caused significant injury to the liver [31].

Our study has some limitations. First, we did not measure the blood flow changes in the hepatic artery due to technical limitations. This may be possible through technical advances in the future or the use of larger animal models, and we plan to actively pursue this research avenue in the near future. Additionally, other serum liver parameters such as alkaline phosphatase, gamma-glutamyl

transferase, bilirubin, and serum lactate were not measured. We also plan to assess these parameters in future work. Moreover, physiological differences between rats and human may render the data obtained herein non-transferable to human pilots under similar conditions of exposure.

In summary, the main findings of the study can be summarized as follows: first, short-term repeated exposure to either +6 Gz or +10 Gz temporarily reduced the portal venous flow. Second, ALT and AST levels only slightly increased in response to G exposure and soon reverted back to normal. An increase in G force resulted in additional liver damage. Third, evidence of oxidative damage was found, which may have been due to liver ischemia. Finally, repeated exposure was associated with transient depression of the liver metabolism, as indicated by the decrease in Na+-K+-ATPase activity. Although the rat data may not be directly extended to that of pilots, because of similar conditions of +Gz exposure, this model may be helpful in identifying more potential adverse effects of high +Gz stress on the human liver, and help develop practical effective protective measures. In the future, we will further expand our study to explore the effects of +Gz exposure over longer durations on liver function, with a view to elucidating the underlying pathophysiological mechanisms and proposing feasible protection to decrease adverse +Gz effects. This could be accomplished by applying an understanding of aviation medicine to aeronautical engineering technology development. This would be significant in aviation progression by ensuring flight safety, extending pilot flying-life, promoting good performance of combat aircrafts,

and improving fighting capacity. These factors are essential for the development of a new generation of high-performance fighter aircrafts.

FIGURE LEGENDS

Fig. 1 Comparison of the rat portal venous blood flow 0, 6, and 24 h after repeated +Gz exposure in the blank control (BC) group, +6 Gz/5 min stress (6GS) group, and +10 Gz/5 min stress (10GS) group. **a** Portal vein blood flow, **b** Portal flow velocity.

Fig. 2 Comparison of rat serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels 0, 6, and 24 h after repeated +Gz exposure in the blank control (BC) group, +6 Gz/5 min stress (6GS) group, and +10 Gz/5 min stress (10GS) group. **a** Alanine aminotransferase (ALT), **b** Aspartate aminotransferase (AST).

Fig. 3 Comparison of the liver tissue malondialdehyde (MDA) levels 0, 6, and 24 h after repeated +Gz exposure in the blank control (BC) group, +6 Gz/5 min stress (6GS) group, and +10 Gz/5 min stress (10GS) group.

Fig. 4 Comparison of the rat liver Na^+-K^+-ATPase activity 0, 6, and 24 h after repeated +Gz exposure in the blank control (BC) group, +6 Gz/5 min stress (6GS) group, and +10 Gz/5 min stress (10GS) group.

Fig. 5 Pathological changes in the liver tissue 0, 6, and 24 h after repeated +Gz exposure in the blank control (BC) group, +6 Gz/5 min stress (6GS) group, and +10 Gz/5 min stress (10GS) group.