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Research Article

Simulated microgravity affects carrageenan-induced inflammation process in rats

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Abstract

Weightlessness significantly impacts physiological systems. In the current study, we investigate the effects of 7 days exposure of rats to simulated microgravity (using a modified rat-modeled random positioning machine, working with four experimental animals simultaneously) on local carrageenan-induced inflammation and serum levels of liver enzymes, metabolites (glucose, urea, creatinine), and metabolic hormones (thyroid-stimulating hormone – TSH, aldosterone, cortisol). Male Wistar rats (n=12, m=200 ± 20 g) were evenly divided into RPM (experimental) and RPM-K (control) groups. The RPM rats showed a notable mass decrease compared to the controls. A significant increase in the carrageenan-induced inflammatory response was reached on the 24th hour in the RPM group compared to the RPM-K. Simulated microgravity resulted in lower serum glucose, creatinine, cortisol, and elevated urea levels. In conclusion, 7 days of exposure to random positioning machine-simulated microgravity promotes a pro-inflammatory state, potentially affecting insulin sensitivity, glucose utilization, and muscle catabolism.

Keywords

inflammation, microgravity, random positioning machine, rat

Introduction

Exposure to microgravity affects many physiological functions, including the immune system. Altered immune mechanisms can lead to health-threatening events. Understanding, predicting and counteracting possible negative consequences due to lack of gravity will play a key role in future extended space missions.

Studies based on simulated microgravity have shown some correlations in an inflammatory response in hindlimb unloading (HU) experiments: Interleukin (IL)-6 mRNA levels increased in the gastrocnemius of HU animals (a 12% loss of gastrocnemius mass). The IL-6 upregulation is most likely related to inflammation associated with the atrophic process (Cavey et al. 2017). Atrophic myotubes are known to produce IL-6. NF-κB signaling pathways are activated early in the skeletal muscles of HU rats. In vitro studies in hepatocytes have shown that small amounts of IL -6 cause a significant increase in hepcidin mRNA levels (Memoli et al. 2010).

Microgravity in space conditions attenuated the secretion of cytokines that promote angiogenesis and inflammation. Ligands such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion mol-

ecule-1 (VCAM-1) are affected by microgravity. Furthermore, overexpression of ICAM-1 was found in rats' small intestines after 48 hours of exposure to a random positioning machine (RPM) (Peana et al. 2008). ICAM-1 and VCAM-1, are related to the activity of β2- and α4-integrins. Such changes are responsible for leukocyte recruitment and transmigration during inflammation (Li et al. 2018).

Long-term experiments in mice demonstrated that simulated microgravity (SMG) alters cytokine expression levels in both hippocampus and plasma (Guttmann-Rubinstein 2019). Macrophage function depends on the distinction between spectra of M0 macrophages, pro-inflammatory M1 and alternative pro-healing M2 phenotypes (Ludtka et al. 2021). Clinorotation studies examining the expression of inflammatory and angiogenic genes and proteins have shown that microgravity decreases tumor necrosis factor (TNF)-α expression. Increases in the expression of IL -12 and vascular endothelial growth factor (VEGF) were found in M0, M1, and M2. IL -10 was significantly increased in M1 and M2 macrophages (Ludtka et al. 2021). These observations suggest the hypothesis that phenotypic cytokine expression is related to specific gravity-sensitive signal transduction pathways.

Furthermore, microgravity affects the digestive system and its immune function. Detection of the signaling pathways leading to gastric mucosal changes has been shown to modulate IL -1 activity, leading to positive regulation of the inflammatory response, as well as positive regulation of the neuro-inflammatory response. The results suggest that "SMG" upregulates inflammation-related genes and signaling pathways. The latter may play a central role in the microgravity response (Chai et al. 2021). Secretion of IL -1β was inhibited in microgravity (Licato and Grimm 1999), whereas IL -6 was increased in mice during a 91-day spaceflight (Sandona et al. 2012).

SMG affects carrageenan-induced and Prostaglandin E2 (PGE2)-induced edema in rats (Peana et al. 2002; Peana et al. 2004). In both of their research, the teams have found that short-term (48 hours) SMG has an anti-edematous response in rats' paws. Overall, the available studies on the impact of gravitational stress on cytokine production suggest that microgravity can cause multiple perturbations in secretion patterns at sites of inflammation.

In the current study, we aimed to investigate the effect of 7 days' exposure to SMG (using recently presented rat-modeled RPM, working with four experimental subjects simultaneously) on the local inflammatory response and whether RPM-SMG stress could increase the serum levels of liver enzymes, metabolites (glucose, urea, and creatinine), and hormones (thyroid-stimulating hormone-TSH, aldosterone, and cortisol) involved in the maintenance of metabolic processes, muscle function, regulation of water and electrolyte balance, and stress response.

Materials and methods

Animals and housing

Twelve male Wistar rats (m = 200 ± 20 g body weight, bred in the vivarium of the Medical University of Plovdiv) were used. The animals were randomly divided into two groups (n=6) named RPM and RPM -K. All animals were acclimatized in the experimental rooms for seven days before the start of the experiment (08:00–20:00 light-dark cycle, temperature 22 ± 1 °C and humidity 55 ± 5%; food and water access were ad libitum). Then, the RPM group was exposed to SMG for 20 h/day for seven consecutive days (from 13:00 h to 9:00 h the next day with only food intake during the experiment). We used a modified RPM with four experimental rat cylinders as previously described (Yotov et al. 2022). A 3D rotational mode (velocity of the inner frame – 0.1575 [rad/s] and the outer – 0.2615 [rad/s]) was used to mimic microgravity effects.

At the same time, the RPM-K group was subjected to the same conditions as the RPM but without rotation by the machine. An identical setup with four cylinders was used for the RPM-K group. For the remaining 4 hours, both groups were returned to their cages and given access to food and water. During the seven days of the experiment, the mass of all animals was measured each day before the start of the session (12:50 h).

Paw-induced inflammation

After the last SMG exposure on the 7-th day, inflammation was induced by a single intraplantar injection of 1% aqueous carrageenan solution (Sigma-Aldrich, Germany). The anti-inflammatory effect was measured by assessing the percent inhibition of hind-paw edema at 2nd, 3rd, 4th and 24th hour. A digital water plethysmometer (Ugo Basile, Italy) was used to measure hind-paw volume. The percentage of edema inhibition was calculated using the following equation:

$$\text{edema inhibition } [\%] = \frac{PV_t - PV_0}{PV_0} x\ 100,$$

PV_0 is the initial paw volume, PV_t is the paw volume at the 2nd, 3rd, 4th and 24th hour following the carrageenan injection.

Blood sample collection and analyses

At the end of the SMG period, blood samples were collected for serum metabolites (glucose, urea, creatinine), enzymes (alanine aminotransferase- ALT, aspartate aminotransferase- AST, amylase, cholinesterase), and hormones (TSH, cortisol, aldosterone). The rats were fixed on their backs in a probe cylinder to restrict their movement. Then a hot compress was placed on the tails and they were fixed outside the cylinder with tape. Pyrogen, endotoxin-free collection tubes were used. The obtained blood samples were centrifuged for 10 minutes. Serum was carefully separated,

aliquoted and frozen at -70 °C. Analyses were performed in a commercial laboratory (external to the university), using Cobas Pro (Roche Diagnostics GmbH, Germany).

Statistics

Statistical analyses were performed with IBM SPSS software (ver.19.0). All data were expressed as mean ± SEM. Data were analyzed by Independent samples T-test for comparison of variables between two groups. Statistical significance was considered at $p < 0.05$.

Results

Effect of simulated microgravity on animal mass change

The mass change during the experiment is presented in Fig. 1. The changes are given in percent compared to day 1 of the experiment. All animals' weight decreased significantly on days 2, 3 and 4 compared to their baseline. The most notable change was in the RPM group (-25.3 ± 3.7%) since day 1.

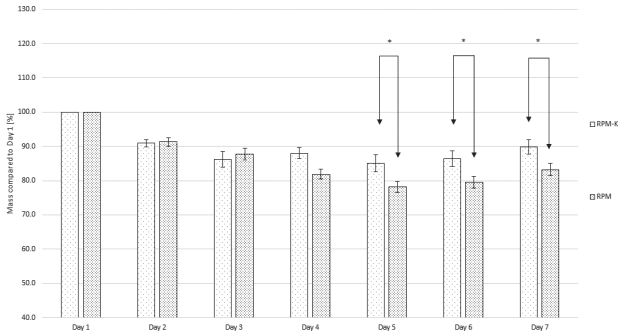

Figure 1. Percentage mass changes through the experiment duration (mean ± SEM), *- p<0.05.

On day 5, a significant difference ($p = 0.026$) was observed between the weight of animals from RPM (78.14 ± 1.67%) and RPM -K (85.06 ± 2.44%). Similarly, a remarkable change was observed between the results of both groups on day 6 ($p = 0.027$) and day 7 ($p = 0.045$). After day 5 of the experiment, all animals began to gain weight. However, the rats from both groups ended the experiment with a lower weight than their baseline.

Effects of simulated microgravity on carrageenan-induced inflammation

The animals subjected to SMG showed an insignificant decrease in paw swelling in the 2nd hour compared with the RPM-K group. On the contrary, in the 3rd and 4th hour, the same rats demonstrated a slight increase in paw volume compared to the animals not treated with SMG. Significance was reached at the 24th hour ($p = 0.031$), when the rats from the RPM group showed an increased hind-paw compared to the animals from the other group (Fig. 2).

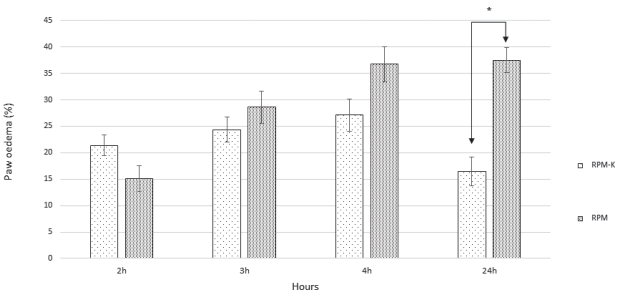

Figure 2. Effect of simulated microgravity on carrageenan-induced inflammation, *- p<0.05.

Effect of simulated microgravity on serum enzyme levels

The levels of liver enzymes (AST and ALT) in the serum of animals subjected to SMG did not show a significant change in comparison between the two groups. In RPM rats the concentration of amylase was decreased when compared to the RPM-K animals. However, significance was not reached. The same tendency was detected for plasma cholinesterase (Table 1).

Table 1. Effect of simulated microgravity on serum enzyme levels.

Enzyme levels	RPM-K	RPM
AST [U/L]	141.6 ± 0.27	140.7 ± 9.79
ALT [U/L]	38.7 ± 0.11	36.9 ± 1.9
Amylase [U/L]	1836.3 ± 0.47	1670.3 ± 66.54
Cholinesterase [U/L]	185.3 ± 0.47	166.75 ± 14.58

Effect of simulated microgravity on serum metabolite levels

Creatinine and glucose levels in the serum of the animals subjected to microgravity were significantly decreased ($p < 0.001$) compared to the control group. On the contrary, the level of urea was notably increased ($p < 0.001$) compared to the RPM-K groups. The urea/creatinine ratio was also significantly increased in the RPM animals in comparison to the control ones ($p < 0.001$) (Table 2).

Table 2. Effect of simulated microgravity on serum metabolite levels.

Metabolite levels	RPM-K	RPM
Creatinine [μmol/L]	45.5 ± 0.28	36.25 ± 0.75***
Glucose [mmol/L]	12.10 ± 0.004	6.96 ± 0.4***
Urea [mmol/L]	4.55 ± 0.28	6.67 ± 0.16***
Urea/Creatinine Ratio	100 ± 1.0	184 ± 2.5***

***p < 0.001 when compared to the control group.

Effect of simulated microgravity on serum hormone levels

When evaluating the hormones, we did not detect any serious changes in the level of TSH between the two groups. The aldosterone serum level was insignificantly decreased in the RPM animals. In the same animals we detected significantly ($p < 0.001$) lower level of cortisol in comparison to the control group (Table 3).

Table 3. Effect of simulated microgravity on serum levels of some biochemical markers.

Hormone levels	RPM-K	RPM
TSH [μUI/L]	0.0035 ± 0.00029	0.0038 ± 0.00025
Aldosterone [ng/dL]	11.15 ± 0.28	8.02 ± 1.56
Cortisol [nmol/L]	36.25 ± 0.28	12.87 ± 0.27***

***$p < 0.001$ when compared to the control group.

Discussion

Findings of the current investigation demonstrated that 7 days' exposure to SMG using RPM (in 20:4 hours modality) produced alterations in the body weight of the animals. The registered differences between RPM and RPM-K groups can be explained by the activation of central neuronal mechanisms independent of stress-induced hypophagia (Harris et al. 2002). However, the mobility restriction and the applied food diet should have also affected the observed changes.

Furthermore, RPM-SMG has a pro-inflammatory effect on our test animals. These results agree with recent studies which indicate that both spaceflight and SMG in vitro and in vivo contribute to immune dysfunction and triggering of an inflammatory state (Paul et al. 2020). Weightlessness has a significant impact on the endocrine system, bone and muscle metabolism (Strollo 2000), digestive system (Yang et al. 2020) and kidney function (Liakopoulos et al. 2012). We found lower glucose and creatinine levels in rats exposed to SMG whereas urea concentrations were increased. These results could be explained by muscle wasting, inhibition of hepatic gluconeogenesis and/or increased insulin-dependent glucose transport in skeletal muscles.

In the present study, SMG promoted the enhancement of local inflammatory response to sub-plantar carrageenan injection. On the contrary, Peana et al. (2002) found that two days of exposure to SMG in RPM significantly reduced the carrageenan-induced inflammatory edema not only soon after the injection but also at the 3rd and 5th hour. Our results showed that SMG reduces inflammatory response only at the 2nd hour, while subsequent measurements demonstrated an increase in edema with significance at the 24th hour. One possible explanation for this discrepancy is the difference in the duration of exposure to SMG. Another one could be the experimental protocol used. Our experimental design might lead to initial suppression of the activity of inflammatory enzymes followed by their stimulation (possibly enhanced by an adaptive mechanism in the 4 hours of rest). Differences in the initial weight (age of the rats), breading of the animals and the diet during the experiments could also play a role in the dissimilarities observed.

Carrageenan-induced inflammation has two phases. The first phase occurs within 1 hour after injection and is mediated mainly by histamine and serotonin. Stimulation of cyclooxygenase and increased production of prostaglandins are observed during the second phase, which develops approximately 3 hours after induction of inflammation (Perez 2016). Since the results of the current study showed enhancement of inflammatory edema after the 3rd hour of carrageenan injection, we can propose that SMG increases cyclooxygenase activity. Indeed, in vitro studies have shown that SMG can promote prostaglandin synthesis. Cazzaniga et al. (2016) found that RPM rotation upregulates cyclooxygenase 2 (COX-2) in human bone mesenchymal stem cells. On the other hand, animal studies show that SMG inhibits intestinal COX-2 activity (Peana et al. 2008) and decreases tissue prostaglandin levels in rats with PGE2-induced hind paw edema (Peana et al. 2004). Further studies on the influence of SMG on cyclooxygenase activity in the settings of carrageenan-induced inflammation are needed to clarify the role of prostaglandins in the observed effect.

Numerous other mechanisms are also responsible for the pro-inflammatory effects of microgravity. In human umbilical vein endothelial cells, two-dimensional clinorotation induces endoplasmic reticulum stress, which stimulates inducible nitric oxide synthetase (iNOS), leading to activation of the NF-kβ pathway and endothelial inflammation (Jiang et al. 2020). The release of nitric oxide plays a role in both the early and late phases of carrageenan-induced edema (Omote et al. 2001). Therefore, we can assume that this is another explanation for the observed pro-inflammatory effect of SMG. Microgravity also increases the expression of adhesion molecules such as E-selectin, VCAM-1 and the cytokine monocyte chemoattractant protein-1 in the common carotid artery in HU (Liu et al. 2014). One limitation of the current study is that the inflammatory and adhesion molecules levels have not been measured in the exudate of the inflamed hind paw.

Existing data from spaceflights and SMG in experimental animals show that weightlessness is associated with liver damage. Alterations in liver carbohydrate and lipid metabolism, inflammation, increased apoptosis and altered metabolic capacity for xenobiotics are observed (Vinken 2022). Elevated serum ALT and AST levels are one of the most commonly used markers of liver injury due to increased release from damaged hepatocytes (Sookoian and Pirola 2015). Their concentrations are increased in rats exposed to space flight for 14 days and 18.5 days, respectively (Macho et al. 1982; Merrill et al. 1990). The enlarged activity of ALT has been shown not only in plasma but also in the liver (Macho et al. 1991a). Experimental data on the effect of SMG on liver transaminases are conflicting. Earlier studies using the HU model showed no increase

in serum concentration of AST (Merrill et al. 1992). However, more recent studies showed that long-term tail suspension (14, 28 and 42 days) caused a significant increase in serum levels of AST and ALT, upregulation of miR-223 and inhibition of hepatocyte proliferation (Chen et al. 2017). Du et al. (2015) showed that 8-week SMG in the rat model HU leads to high serum levels of transaminases, changes in liver morphology, alterations in mitochondria and endoplasmic reticulum and increased hepatic levels of proteins involved in the regulation of apoptosis. The above-mentioned data demonstrate that SMG induces liver injury. The results of our study show that RPM-SMG does not cause a significant increase in serum levels of AST and ALT. Probably, this is due to the shorter duration of RPM -SMG exposure and the time course required for a significant elevation of serum transaminases as a marker of liver injury under SMG conditions is much longer than 7 days. Cholinesterase is another enzyme used to evaluate liver function. Its decreased serum level reflects hepatocellular injury as a result of decreased synthesis of this enzyme (Santarpia et al. 2013). The present study showed only an insignificant decrease in cholinesterase level compared to RPM-K. We can assume that it is a more sensitive marker of liver damage under SMG conditions.

Results of spaceflight and ground-based studies in humans (prolonged head-down bed rest and dry immersion experiment) have demonstrated that weightlessness causes insulin resistance with increased plasma glucose and decreased glucose tolerance (Strollo et al. 2022). Previous experiments in animals exposed to space flight showed increased plasma glucose and insulin levels after 7- and 14-day flight (Macho et al. 1991a; Merrill et al. 1992), but this was not observed in rats exposed to hypokinesia by 14-day tail suspension (Macho et al. 1991a). Wang Y et al. (2019) found increased fasting glucose, impaired glucose metabolism in the liver and upregulated transcription levels for the pro-inflammatory cytokine TNF-α in mice exposed to a 4-week HU model. In the last study, significant differences in fasting glucose levels were observed between controls and animals exposed to SMG starting at week 3. Du et al. (2015) found that 2 months of tail suspension leads to low levels of plasma glucose and hepatic glycogen. Other studies showed that HU increased plasma glucose for 7 and 14 days (Bederman et al. 2013). Therefore, studies on the effect of SMG on blood glucose show controversial results. This is likely due to differences in experimental conditions, duration of exposure and numerous other confounding factors. Our results showed that a 7-day exposure to RPM-SMG significantly decreased plasma glucose levels (due to the dietary regime and/or rotation/rest ratio). The duration of exposure likely affects insulin sensitivity and consequently glucose utilization. Experiments with one-day hindlimb exposure show the development of insulin resistance with elevated fasting glucose and insulin levels (O'Keefe et al. 2004a). On the other hand, SMG (7 days HU) enhances the effect of insulin on glucose transport by increasing the intracellular insulin signaling cascade. This has been associated with significantly lower glucose levels in the insulin tolerance test (O'Keefe et al. 2004b).

Weightlessness conditions are associated with loss of muscle mass. Muscle catabolism leads to increased urea production, while muscle wasting reduces creatinine production (Gunst et al. 2019). Bed rest experiments with healthy male volunteers showed that continuous hypokinesia reduces plasma volume, body weight, and muscle mass and causes an increase in plasma and urea with decreased plasma creatinine concentrations (Bilancio et al. 2014). Studies of these metabolites in rats exposed to spaceflight and a tail suspension model showed an increase in both plasma creatinine and urea in landed animals, whereas a decrease in creatinine and an increase in plasma urea were observed in animals exposed to simulated microgravity (Merrill et al. 1992). The results of the present study are consistent with these observations. We found a significant increase in plasma urea and a decrease in plasma creatinine after 7 days of simulated microgravity in RPM. A limitation of this study is that urinary urea and creatinine concentrations and renal function were not examined.

Microgravity affects the body's fluids and electrolytes control. It is observed that plasma volume is reduced, there is extravasation of fluids and decreased urine flow rate (Norsk 2000). This is associated with changes in the levels of hormones involved in the regulation of sodium and water balance in the body. Since microgravity increases sodium reabsorption (Liakopoulos et al. 2012) we aimed to assess whether this is related to increased aldosterone plasma levels. The results from our study showed that there is no significant difference in plasma levels of this hormone between rats exposed to SMG and the control group. These data are consistence with earlier studies in humans which demonstrated that aldosterone decreases early in the microgravity conditions and remains at preflight levels later during it (Leach et al. 1988). Experiments with rats exposed to hypokinesia demonstrated that aldosterone levels are elevated initially and tend to decrease with long-term exposure (Macho et al. 1992). More recent studies also found elevated aldosterone levels after short-term hindlimb unloading in rats (Sullivan et al. 2004). Neri et al. (2002) found that rats exposed to simulated microgravity have low aldosterone plasma levels and increased adrenomedullin concentrations. The last is a peptide produced by adrenal medulla and vascular endothelial cells which causes vasodilation and inhibits aldosterone production. Therefore, we might speculate that the observed non-significant decrease of plasma aldosterone levels in rats exposed to SMG is due to increased adrenomedullin. Changes in plasma levels of other hormones probably contribute to the adaptation of fluid and electrolyte control in the conditions of microgravity. It was found that plasma anti-diuretic hormone levels are elevated in HU rats for 7 days followed by a decline to the baseline on the 14th day. This could explain reduced diuresis and natriuresis in the early period of spaceflights or short-term exposure to microgravity (Chung et al. 2012).

Activation of the hypothalamus-pituitary-adrenal axis and increased cortisol production are observed in response to stressful events (Pulopulos et al. 2020). Since spaceflight and exposure to microgravity are undeniable stress factors, it can be expected that they lead to an elevation in serum cortisol levels. Earlier studies in humans showed that serum concentrations of this hormone are increased during spaceflight (Leach et al. 1988; Macho et al. 1991a). However, changes in cortisol levels show great individual variations and are dependent on a variety of factors such as the duration of the flight. Data from experiments with animals exposed to spaceflight with different duration (7, 14 and 18,5 days) showed an increase in serum cortisol (Macho et al. 1982; Macho et al. 1991b). Increased activity of cortisol-dependent liver enzymes, such as thyrosine aminotransferase and tryptophan pyrolyse, was also observed (Macho et al. 1991b). On the other hand, Grindeland et al. (1990) found that cortisol levels are lower in-flight animals, but significance is not reached in comparison with controls. Despite the increased cortisol levels in animals subjected to spaceflights, some results showed that in groups exposed to SMG, the concentrations of the hormone are lower (Macho et al. 1982; Merrill et al. 1992). Our results showed that SMG significantly decreased serum cortisol levels. The observed difference between data about animals subjected to spaceflight and those exposed to SMG on the Earth might be due to specific stress associated with spaceflight and simulated weightlessness in the RPM.

Microgravity may lead to mild hypothyroidism (Strollo 2000). Reduced thyroxine (T4) and triiodo-L-thyronine levels, and morphological changes in the thyroid gland of rats exposed to spaceflight have been found (Plakhuta-Plakutina et al. 1990). Studies in astronauts showed increased serum TSH and T4 levels in the post-flight recovery period after short- and long-term space flights (Macho et al. 1991a, b). Lower thyroxine levels were also observed in rats flown for 14 days on the COSMOS 2044 unmanned space vehicle whereas in animals exposed to simulated microgravity, no significant changes in this hormone were found (Merrill et al. 1992). Our results are in agreement with this finding. There were no significant differences in TSH concentration between animals from the RPM-K group and those exposed to simulated microgravity.

Conclusions

In the current research, animals were exposed to SMG for seven days in a modified rat-modeled random positioning machine. The results demonstrated an enhancement of response to carrageenan-induced inflammation. In addition, we found that in animals subjected to experimental conditions of RPM-SMG for a relatively short period of time (7 days) are observed lower serum glucose, creatinine and cortisol concentrations with elevation of urea levels.

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The study is approved with a license №327, accepted with Protocol 26/09.12.2021 by the Animal Health and Welfare Directorate of the Bulgarian food safety agency (BFSA, https://bfsa.egov.bg/wps/portal/bfsa-web-en/home) and it is approved with Protocol №5/17.06.2022 from the Ethical committee of Medical University-Plovdiv. The authors confirm that all performed experiments followed the relevant guidelines and regulations of the Republic of Bulgaria and the European Union.

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