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Hypergravity influences mouse erythrocyte membrane lipid composition and antioxidant potential

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ABSTRACT

To support safe human space exploration, it is important to understand how different effectors, including gravitational forces, influence living organisms. Indeed, altered levels of gravity affect the physiological function of multiple cells, tissues, and organs in living organisms. Previous studies suggested that microgravity modifies plasma membrane permeability and cellular metabolism in erythrocyte, modifying cholesterol and phospholipid levels. However, to support human safe space exploration, it is also relevant to understand the effects of hypergravity. Therefore, the aim of this study was to investigate *in vivo* the impact of hypergravity on lipid phenotype and oxidative stress in mouse erythrocytes.

Animals were housed in the Italian Space Agency's Mice Drawer System (MDS-ASI), a facility designed to house rodents on the International Space Station (ISS) and adapted by Thales Alenia Space to the Large Diameter Centrifuge (LDC-ESA), to expose mice to a $3\times g$ environment for 14 days. After exposure, a tissue-sharing protocol allowed us to purify and analyze erythrocytes.

Our results show that the exposure of mice to altered gravity induced the reduction of unsaturation degree in erythrocyte membranes correlated to a lower stearoyl-CoA desaturase (SCD-1) activity. Moreover, the hypergravity induced both a decline in antioxidant defences, indicated by the significant decrease in total glutathione, and a grow of the inflammatory status, supported by an increase in the AA/EPA ratio.

1. Introduction

As more investigations and research have been conducted over the years, our knowledge of the detrimental consequences induced by extraterrestrial environment exposure on human health has gradually grown. It is now known that astronauts exposed to microgravity suffer from reduced neurological function, declining bone density, atrophied muscles, and significantly impaired immune system [1]. Indeed, the human body undergoes profound changes in response to the alteration of gravity; the most visible early physiological change is the redistribution of body fluids from the lower to the upper part of the body, resulting from the elimination of the gravitational load experienced on Earth. These changes lead to visible alterations in the astronaut's body, such as the so-called "puffy face," due to facial edema, and reduced

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volume of the legs, which gives the characteristic "chicken legs" appearance. Plasma volume is reduced by 10–15 % as intravascular fluids move into the extracellular space because of increased capillary permeability. The redistribution of the blood towards the head causes altered responses of the baroreceptor, nervous, and endocrine systems [2]. Furthermore, space radiation and many other aspects of life in a space environment must be considered, including confinement, isolation, bacterial and viral infections [3,4].

Among the various hematological complications that the human organism undergoes in a space environment, there is a particular type of anemia characterized by loss of erythrocyte mass, increased hemoglobin concentration, reduced plasma volume, increased blood concentration, and decreased levels of erythropoietin [5]. Erythrocytes perceive physical stimuli using specific mechanosensitive structures and the membrane lipid bilayer plays a fundamental role in maintaining the normal physiological functions of the erythrocytes. In these cells, prolonged exposure to the space environment leads to an inhibition of energy production and an increase in oxidative processes. These changes might lead to a modification of the plasma membrane composition, an increase in oxidative stress (in particular an enhancement of lipid peroxidation), and an increase in membrane rigidity. Moreover, in space the altered size and shape of erythrocytes might promote hemolysis that appears to underlie space anemia.

Therefore, to support safe human space exploration, it is important to understand how the different effectors, including gravitational forces, influence organisms. Previous experiments performed by exposing mice to long space flights on the International Space Station (ISS) have demonstrated the effects of microgravity on several tissues including erythrocytes [6–10]. However, a clearer and more complete understanding of the physiological response to gravity requires data from experiments carried out under opposite gravitational conditions. For these reasons, studying the effects of a hypergravity environment on animals is a fundamental step to deepen our knowledge of the physiological response to adverse gravity conditions. Moreover, the application of hypergravity also complies with the gravitational continuum paradigm, making it a possible countermeasure to harmful effects of microgravity [11].

This study was part of the larger "Tissue sharing program" on mice exposed to hypergravity. Our study specifically focused on how changes in gravity can affect body homeostasis, through the crosstalk of different organs and tissues in a living organism, particularly the erythrocyte metabolism, and survival. The participation of our laboratory in this program, involving several international scientific research groups to maximize the obtainable experimental data, allowed us to study the impact of hypergravity on the membrane lipid composition and antioxidant defence of mouse erythrocytes. The experiments were carried out using the Italian Space Agency's Mouse Drawer System (MDS-ASI) facility, the instrument previously used to expose mice to microgravity on the International Space Station (ISS) [12]. In the present study, we report the *in vivo* effects produced in mice exposed for 14 days to hypergravity (3×*g* environment) on membrane fatty acid profile of the erythrocytes and the activity of some antioxidant enzymes.

2. Material and methods

2.1. Exposure of mice to hypergravity

Six C57BLJ10 mice were exposed to 3×*g* environment (3G group). To do this, the mice were housed in the MDS-ASI, a facility designed to house rodents on the ISS and adapted by Thales Alenia Space to the Large Diameter Centrifuge (LDC-ESA) [13]. The MDS-ASI is an autonomous, multifunctional animal enclosure that allows experimentation in several areas of biomedical interest. The module can accommodate up to six mice, each individually housed. The adaptability of the MDS-ASI to different modules and the stable and controlled environmental conditions of the animal housing system allowed to carry out a study that will not only be reproducible in future investigations but will also provide data comparable to those obtained from the past permanence of mice in a microgravity environment.

In 2019, a first experimental phase, lasting 14 days, was carried out to verify the technical conditions of the study, the welfare of the animals, and the correct functioning of the hardware placed on the LDC-ESA (Fig. 1A); this test was performed at ESA/ESTEC in the Netherlands. The LDC-ESA is part of the Life and Physical Sciences Instrumentation and Life Support Laboratory (LIS) at ESTEC (Netherlands) and enables the study of cells, small animals, and plants in hypergravity condition in the range of 1 to 20×*g* (Fig. 1B). The LDC-ESA is made up of 4 arms with a diameter of 8 m; each arm can support 2 gondolas which can bear a maximum load of 80 kg per gondola. The hypergravity condition inside the gondolas is due to the centripetal forces generated by the rotation. The LDC-ESA is flexible in terms of experiment scenarios, duration, and possible equipment to be use.

To maximize the obtainable experimental data, several international scientific groups were involved in a tissue sharing program as in the previous MDS-ASI microgravity mission to the ISS. Six (1×*g*) ground animals, housed in MDS-like cages were used as controls (GC group).

Due to the Covid-19 situation that postponed the longer exposure experiment to 2023, we decided to analyze samples from 14-day exposure to hypergravity, and present the data in this paper. To this aim, after hypergravity exposure, we collected the blood from 3G and GC mouse groups to purify erythrocytes and analyze the membrane fatty acid phenotype, the activity of different antioxidant enzymes and total glutathione levels.

2.2. Blood collection and fractionation

Blood collection from 5 mice in 3G group (since 1 mouse had to be removed from the study) and 6 mice in GC group was performed by retroorbital sinus puncture. The blood of each animal was divided into 2 microtubes containing EDTA, one for blood cell count and the other for erythrocyte separation from plasma.

After gentle stirring, the tubes were centrifuged at 850×*g* for 15 min. After centrifugation, the plasma was immediately separated and frozen on dry ice. The erythrocyte pellet was then subdivided into microcentrifuge tubes and immediately frozen on dry ice.

A 15 μl aliquot of fresh erythrocytes was used to prepare glutathione extract. For this purpose, cells were homogenized on ice in 10 % metaphosphoric acid, centrifuged at 13,000 rpm for 10 min, and the supernatant was frozen on dry ice for shipment to Italy.

Aliquots of erythrocytes and extracted glutathione samples were shipped to our laboratory in Milan on dry ice and stored at −80 °C for subsequent analysis.

2.3. Lipid analysis

To study the fatty acid composition of cell membrane, and to subsequently measure the activity of antioxidant enzymes, erythrocyte plasma membranes were purified from the hemolysates, then stored at −80 °C, as previously described [14].

Lipids were extracted from purified membranes using chloroform/methanol solution at different concentrations and analyzed according to Ref. [14]. As internal standard (TG C17:0; Sigma-Aldrich) was added to all samples to allow quantification of the analyte; the internal standard is added before the lipid extraction to evaluate the yield of the reaction and the recovery of the analyte under study. The fatty acid methylesters of erythrocyte membranes were obtained after derivatization with sodium methoxide in methanol 3.33 % w/v and were evaluated by Gas Chromatography-Flame Ionization Detector (GC-FID). Activity of stearoyl-CoA desaturase-1 (SCD-1) was estimated as product-to-precursor fatty acid ratio (oleic acid/stearic acid 18:1n-9/18:0).

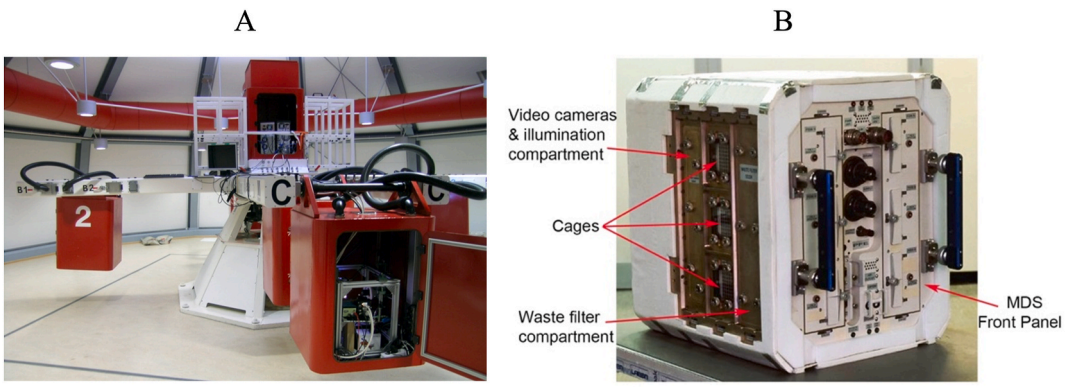

Fig. 1. Instruments for hypergravity experiments
A) the Large Diameter Centrifuge or LDC (Credit ESA): a component of the Life and Physical Sciences Instrumentation and Life Support Laboratory (LIS) useful to study the effects of hypergravity in the range of 1 to 20×*g* in cells, small animals, and plants.
B) the Mice Drawer System (Credit ASI): an autonomous and multifunctional housing system that accommodates animals in stable and controlled environmental conditions.

2.4. Antioxidant defence analysis

The activity of the antioxidant enzymes (catalase, glutathione reductase, glutathione peroxidase, and superoxide dismutase) was measured in the hemolysates according to methods described in Ref. [15]. The hemoglobin concentration in each sample was measured to normalize the data.

Total glutathione levels were determined using a spectrophotometric assay and following the increase in absorbance at 412 nm [15].

2.5. Statistical analysis

Statistical analyses were carried out using the statistical software GraphPad Prism v. 8.0. Student's t-test was performed to compare mice exposed to hypergravity conditions (3G) with control mice (GC). The difference among data was considered statistically significant with a P value < 0.05. The data are expressed as mean ± standard deviation (SD).

3. Results

To study the alterations in the total composition of membrane fatty acids due to exposure to hypergravity condition, we analyzed erythrocyte membranes by GC-FID; the fatty acids composition, which plays a fundamental role in ensuring the correct functionality, deformability and fluidity characteristics of the erythrocyte membrane, is showed in Table 1. The 3×*g* condition for 14 days (3G mice) induced an appreciable reduction of the oleic acid (C18:1) compared to control mice (GC) (from 13.57 ± 0.82 % to 12.56 ± 0.87 %, p = 0.084) correlated to a statistically significant decrease in the estimated activity of the stearoyl-CoA desaturase-1 (SCD-1) (Fig. 2A), indicating that the increased level of gravity gives rise to a modulatory effect on plasma membrane unsaturation and fluidity. Moreover, it is noteworthy that 3G mice, compared to GC mice, showed a statistically significant increase in the ratio between arachidonic (AA, C20:4 n-6) and eicosapentaenoic (EPA, C20:5 n-3) acids, a well-known critical indicator of a potential inflammatory status in mice (Fig. 2B).

To evaluate the impact of hypergravity on the potential level of oxidative stress, we analyzed the endogenous antioxidant activity of scavenging enzymes and the amount of total glutathione in erythrocytes (Fig. 3). The enzymatic activity of catalase, glutathione reductase, superoxide dismutase and glutathione peroxidase did not undergo statistically significant changes. On the contrary, a statistically significant decrease was detected in 3G mice compared to controls in the content of total glutathione, which is an important antioxidant in living organisms and is used to neutralize highly reactive compounds, such as hydrogen peroxide and lipid hydroperoxides, while the ratio between glutathione peroxidase activity and total glutathione is significantly increased (Fig. 3).

Table 1
Fatty acid distribution in erythrocyte membranes from control mice (GC) and mice exposed for 14 days to hypergravity (3G).

% Fatty acid	GC	3G
C16:0	32.44 ± 2.93	32.35 ± 2.58
C16:1	1.573 ± 0.415	1.640 ± 0.408
C18:0	15.46 ± 1.45	16.90 ± 1.88
C18:1	13.57 ± 0.82	12.56 ± 0.87
C18.2 n-6	13.78 ± 0.54	13.99 ± 0.37
C18:3 n-6	0.774 ± 0.637	0.248 ± 0.254
C18:3 n-3	1.712 ± 0.884	1.160 ± 0.658
C20:3 n-6	1.442 ± 0.193	1.335 ± 0.275
C20:4 n-6	12.60 ± 2.45	13.21 ± 1.44
C20:5 n-3	0.725 ± 0.166	0.631 ± 0.111
C22:5 n-3	1.004 ± 0.184	1.144 ± 0.247
C22:6 n-3	4.919 ± 1.130	4.829 ± 0.987
SFA	47.90 ± 3.79	49.25 ± 2.44
MUFA	15.14 ± 1.13	14.20 ± 1.05
PUFA	36.96 ± 4.45	36.55 ± 2.82
n-6 PUFA	28.60 ± 2.90	28.79 ± 1.54
n-3 PUFA	8.359 ± 1.702	7.764 ± 1.430

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n-6 PUFA, omega-6 polyunsaturated fatty acids; n-3 PUFA, omega-3 polyunsaturated fatty acids.

4. Discussion

Gravitational force and oxidative stress have played a fundamental role in the development and evolution of life on our planet [16]. The alteration of the levels of gravity, exposure to radiation, and the effects caused by reactive oxygen species can lead to the onset of changes in the skeletal, muscular, immune, and cardiovascular systems with consequences such as space anemia, alteration of cellular metabolism, carcinogenesis and alteration in the control of inflammatory processes [17, 18].

The cell membrane composition plays a key role in determining the erythrocyte resistance to mechanical stress and is known to be influenced by external events such as hypothermia, hypoxia, and variations in gravitational force [19–22]. The plasma membrane consists of a lipid bilayer composed of phospholipids, cholesterol, and glycolipids. Modifications of the ratio and/or percentage distribution of the fatty acids present in glycolipids and phospholipids might change the membrane's

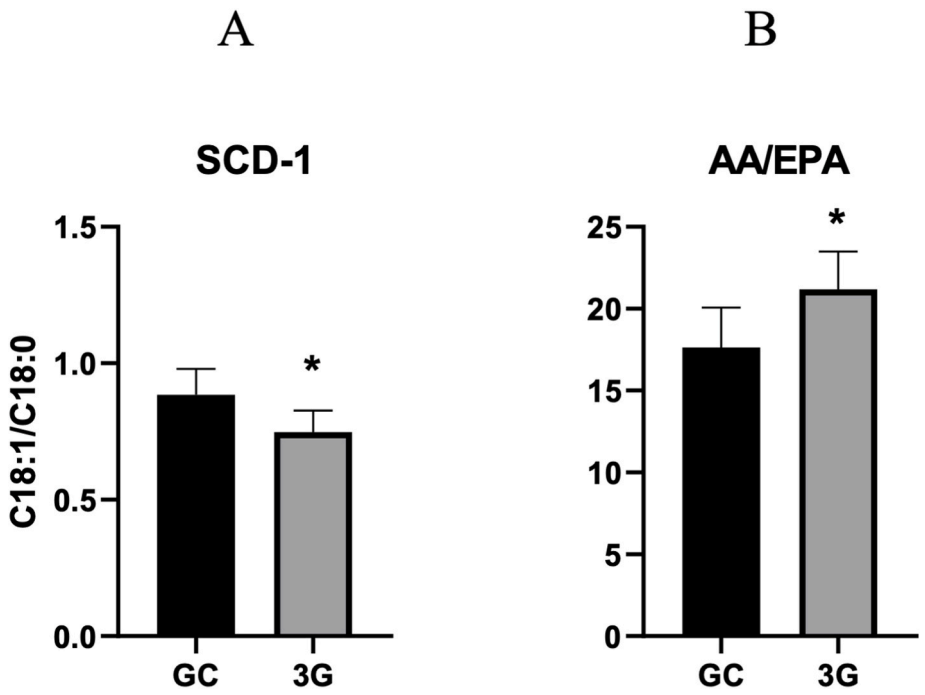

Fig. 2. Effects of hypergravity on unsaturation and inflammation status in erythrocytes from control mice (GC) and mice exposed for 14 days to 3×*g* (3G).
A) The activity of stearoyl-CoA desaturase-1 (SCD-1) is estimated as product-to-precursor fatty acid ratio (oleic acid/stearic acid 18:1n-9/18:0).
B) The AA/EPA, marker of the levels of inflammation, is calculated as arachidonic acid and eicosapentaenoic acid ratio. The data are expressed as mean ± standard deviation, * P-value <0.05, Hypergravity (3G) vs Controls (GC)).

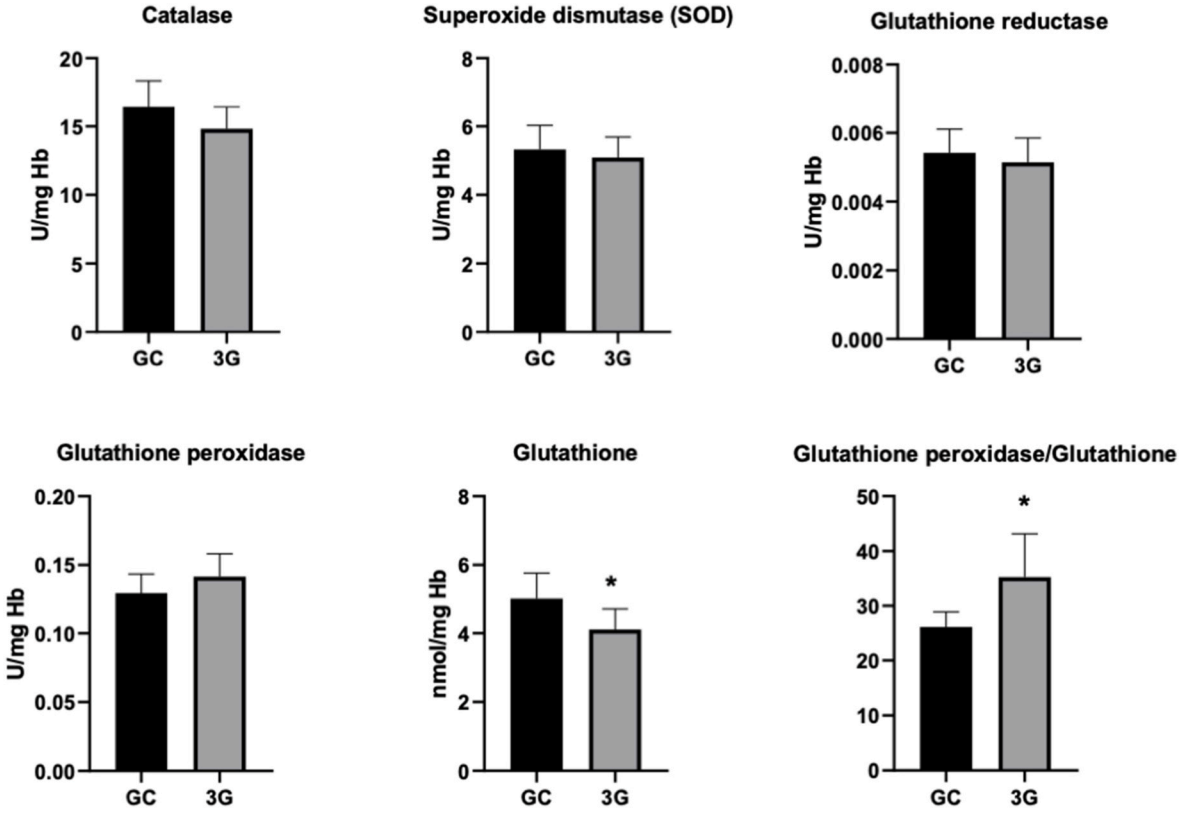

Fig. 3. Analysis of oxidative stress in erythrocytes from control mice (GC) and mice exposed for 14 days to hypergravity (3G).
The activity of antioxidant enzymes (GSH peroxidase, GSH reductase, superoxide dismutase and catalase) has been measured in all samples in triplicate by a spectrophotometric assay and expressed as units (U) per mg of hemoglobin (Hb). Total glutathione is quantified as nmol/mg of Hb. The data are expressed as mean ± standard deviation, * P-value <0.05, Hypergravity (3G) vs Controls (GC)).

permeability and fluidity. Additionally, these changes may increase vulnerability to oxidative stress, which would make membranes more fragile.

Our results show that the exposure to hypergravity for 14 days is sufficient to promote a change in the distribution of fatty acids in erythrocyte membrane, with a decrease of MUFA oleic acid which, although not statistically significant, correlates closely with a significant decline of SCD-1 activity. SCD-1 is a key enzyme in fatty acid metabolism, and altered SCD activity is associated to multiple adverse health outcomes [23,24]. Diet, hormone levels, and environmental conditions are potential factors affecting SCD activity [24–26]. Moreover, as the MUFA produced by SCD-1 are the major substrates for the synthesis of complex lipids, including phospholipids, the decrease of SCD-1 activity might reduce the degree of fluidity and permeability of the membrane, inducing greater sensitivity towards oxidative stress [27,28]. SCD-1 also regulates lipogenesis to provide essential lipid resources for rapid proliferation and cell structure [29,30]. These changes could probably be related to the hepatic and general metabolism of mice. Indeed, we must not forget that a part of the erythrocytes is continuously newly synthesized, and another part is subjected to lipid exchanges with other circulating particles, such as lipoproteins, exosomes, and microvesicles, whose lipids could be influenced by both intrinsic (i.e. hepatic and adipocyte metabolism) and extrinsic (i.e. space environment and nutrition) factors [31,32]. These observations are strongly supported by preliminary data in mice exposed to 3×*g* gravity for 27 days; indeed, the analysis of fatty acid composition in erythrocyte membranes from these mice shows a stronger effect with statistically significant reduction in the percentage of not only oleic acid (C18:1) but also palmitoleic acid (C16:1). Therefore, further investigations into the lipid profile of the liver and plasma of the mice under study will help us to better understand the mechanism that produced these results. Indeed, *in vivo* an increase in oxidative stress and inflammatory response leads to an impairment of liver and kidney function [33], attenuates lipid synthesis, improves mitochondrial FA oxidation, and causes abnormal lipogenesis [30,34,35]. Unfortunately, the clear relationship between oxidative stress and SCD-1 activity still remains poorly understood.

Oxidative stress refers to an imbalance between the processes that lead to the generation of reactive oxygen species and the processes of neutralization of reactive intermediates and repair of damage in biological systems. Alterations of the normal redox state in cells can generate negative effects that damage the fundamental components of structure and correct cellular functioning such as proteins, lipids, and DNA [36]. Reactive species are normally produced in the cell during metabolism, but overproduction or decreased levels of antioxidants can be harmful to cells. Normally, low levels of free radicals play an important role in the living organism's defence, but if their production is not strictly controlled, oxidative stress can be responsible for the onset of chronic and degenerative diseases, accelerating the aging process and causing acute and chronic pathologies [37]. Exposure to space radiation, hypoxia, and microgravity results in increased cellular production of reactive oxygen and nitrogen species [38].

To evaluate the levels of oxidative stress induced by hypergravity in mice, endogenous antioxidant activity and total glutathione levels were measured in their erythrocytes. The results show that mice subjected to the 14-days exposure to hypergravity have a lower content of total glutathione, which is an important protector to oxidative stress, compared to controls. However, no significant differences were found in the activities of several antioxidant enzymes including glutathione peroxidase and glutathione reductase suggesting that the reduction of total glutathione is likely due to insufficient hepatic synthesis or accelerated degradation. Nevertheless, the increase of the ratio between glutathione peroxidase activity and total glutathione suggest that the reduction of glutathione might negatively impact the scavenging activity of peroxidase. To have a complete view of the effects induced by exposure to hypergravity on erythrocytes it will be necessary to correlate them with the data of hepatic metabolism that will be an object of future studies.

Moreover, a growing body of evidence supports the impact of altered gravity on spaceflight-associated immune dysfunction and alterations in inflammation in astronauts both during spaceflight and upon their return to Earth [39]. A valuable important indicator for monitoring inflammatory status and maintaining health is the AA/EPA ratio, with a higher ratio corresponding to higher levels of inflammation. Therefore, the statistically significant higher AA/EPA ratio, we observe in erythrocyte membranes from 3G mice compared to controls, suggest that even 14 days of exposition of animals to hypergravity may induce a proinflammatory status, with a concomitant reduction in the anti-inflammatory capacity. Indeed, the effects of n-3 and n-6-PUFAs on inflammation have been extensively investigated in numerous studies, suggesting that PUFA status might modulate inflammation and metabolic function in the organism. In general, n-3 PUFAs, as EPA, promote the synthesis of lipid mediators that exhibit anti-inflammatory properties and are involved in the resolution of inflammation [40]. On the contrary, n-6 PUFA, as AA, may contribute to inflammation by the eicosanoid production that play a role in proinflammation, pro-platelet aggregation, and immune responses [41]. In addition, several studies suggest that EPA-derived lipid mediators temper the inflammatory response not only by reducing neutrophil infiltration and regulating the cytokine-chemokine axis [42], but also by improving total antioxidant capacity. Indeed, EPA-eicosanoids are able to reduce the production of reactive oxygen species and increase the expression and activity of antioxidant enzymes, such as catalase and glutathione peroxidase, so to mitigate oxidative stress. On the contrary, higher AA levels are related to higher oxidative stress, especially lipid peroxidation [43].

5. Conclusions

In recent decades, space exploration has allowed us to deepen our knowledge in the biological and biomedical fields, introducing new techniques and tools for studies on cellular and physiological behaviour in an extreme environment. Exposing cells or animals to an environment with different gravity and radiation means creating a stressful situation able to affect normal cellular and physiological processes so as to discover new weaknesses, or strengths, in biological systems. Exploiting these findings could reveal new biomarkers, drug targets, nutritional and exercise countermeasures. This is important not only for improving the health and well-being of the astronauts during future space travel, but also for the resulting medical approaches.

The present study provides a deeper understanding of the biochemical responses to hypergravity. The data collected highlight the reduction of MUFA levels in erythrocyte membrane correlated to a lower SCD-1 activity. Furthermore, we report that hypergravity induces both a decline in antioxidant defences, indicated by the significant decrease in the amount of total glutathione, and an increase of the inflammatory status suggested by an increase in the AA/EPA ratio. To extend the physiological and biochemical role of these observations detected in erythrocytes to in toto organism, it will be fundamental to correlate our data with those obtained in different tissues and plasma by the other researchers of the team.

CRediT authorship contribution statement

Angela Maria Rizzo: Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Giampaolo Murgia:** Writing – review & editing, Investigation. **Antonio Lentini:** Writing – review & editing, Investigation. **Stefania Zava:** Writing – review & editing, Investigation. **Francesca Ferranti:** Supervision, Project administration. **Sara Tavella:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Daniela Santucci:** Writing – review & editing. **Jack J.W.A. van Loon:** Writing – review & editing, Funding acquisition, Conceptualization. **Irma Colombo:** Writing – original draft, Methodology, Formal

analysis, Data curation. **Paola Antonia Corsetto:** Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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