Cerebral morphology in adult mice following Long-term gravity increase

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Özet

Gravitenin akselerasyonu ve rotasyonunda yada parabolik uçuş sırasında artması organizma içerisinde strese neden olur. Bununla beraber, organizma içerisindeki bazı biyolojik değişiklikler artmış graviteye karşı cevap olarak gelişir. Uzun süreli gravite değişikliklerin ve rotasyonun etkilerini belirlemek amacı için, C57BL6 F1 fare beyin dokusunda hipergravite ortamındaki yapısal değişiklikler incelendi. Farelere bir veya iki gravite ortamında uzun-süreli sentrifüj uygulandı ve tedavi edilmeyen grup kontrol grubu olarak kabul edildi. Sentrifüjden 4 hafta sonra, fareler sakrifiye edildi ve beyinleri ascending aortadan %10 formalin solüsyonu ile perfüze edildi. Beyinler çıkarıldıktan sonra, parafine gömüldüler ve seri koronal kesitler ve sistemik üniform rastgele beyin kesitleri analiz edildi. Buna ilaveten, farklı gravite şartlarının nörodejeneratif etkilerini incelemek amacı için glial fibrillary asidik proteinin (GFAP) beyindeki immunohistokimyasal dağılımı incelendi. Sonuçlarımız göstermiştir ki, uzun süreli hipergravite beyin volumünde değişiklik yapmamaktadır ve tüm gruplarda beyinin hücresel morfolojisi normaldir ve dejeneratif değişiklikler gözlenmemiştir. Farelerin beyin morfolojilerinin uygulanan bu şartlarda hipergravite ile etkilenmediği gözlenmiştir.

Anahtar Kelimeler: C57BL6 F1 fare, uzun-süreli sentrifüj, Cavlieri'nin volüm ölçümü, GFAP

Anahtar kelimeler:

Abstract

Increases in gravitational forces that result from acceleration and rotation or parabolic flight can create significant stress for living organisms. Indeed, some biological changes in living organisms have specifically arisen to combat the effects of increased gravitational forces. To determine the potential effects of rotation and long-term gravitational changes, we have investigated the structural changes in C57BL6 F1 mice cerebral tissue under hypergravity conditions. Mice were subjected to long-term centrifugation under one or two gravities and compared with a non-treated control group. After 4 weeks of centrifugation, the mice were sacrificed and their brains were perfused through the ascending aorta with 10% formaldehyde. After removal of the brains, they were embeded in paraffin embedding and the cutting of serial coronal sections and systematic uniform random cerebral sections were analysed and The sterologic cortex and medulla volume estimations were performed. In addition, the immunohistochemical distribution of glial fibrillary acidic protein (GFAP) in cerebrum was determined to reveal any neurodegenerative effects of these different gravity conditions. Our results demonstrate that there were no long-term hypergravitational effects upon the cerebral volume, and that the cellular morphology of the cerebrum in all of the groups remained normal, and hence free from any degenerative changes. Under given conditions mice cerebral morphology has not been effected by hypergavity.

Keywords: cerebrum , C57BL6 F1 mice, long-term centrifugation, Cavlieri's volume estimation, GFAP

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Introductıon

Gravity is one of the main factors that affects creatures all over the world, and terrestrial animals are continuously under its effects. Gravity has a role as an environmental factor, determining the processes of intrauterine and postnatal development, starting from fertilization and extending to every stage of life (1, 2). Knowledge of the effects of gravity on the living organism has also become more important since the beginning of human space travel, and with the possibility of the colonization of space (3). There has been a great deal of research into the effects of microgravity and hypergravity as an altered environmental factor (1 - 12). Hypergravity occurs on a planet that has a greater mass than the Earth, and can also be experienced during parabolic flight. Therefore, this is an important factor for pilots who fly aircraft for long periods. Jet pilots in particular are affected by hypergravity during parabolic flight (6 - 8, 10, 12). The most important effect of hypergravity that has been detected is a loss in total body weight (1, 4, 5). Other systems in the body are also affected by hypergravity (13, 14, 15, 16, 17). In addition, hypergravity can cause venous return inhibition and cerebral ischaemia (6-8, 10, 12, 18) and chemical, genetic and structural disturbances in the brain (19, 20, 21, 22, 23).

Glial fibrillary acidic protein (GFAP) is the principal component of 8-9-nm intermediate filaments in mature astrocytes of the central nervous system (CNS). Over a decade ago, the value of GFAP was recognized as a prototype antigen in nervous tissue identification and as a standard marker for fundamental and applied research at an interdisciplinary level (24). As a member of the cytoskeletal protein family, GFAP is thought to be important in modulating astrocyte motility and shape, through providing structural stability to astrocytic processes. Following an injury in the CNS of higher vertebrates arising as a result of trauma, disease, genetic disorders or chemical insult, the astrocytes become reactive and respond in a specific manner, undergoing astrogliosis. Astrogliosis is characterised by the rapid synthesis of GFAP and it has been seen as an increase in the protein content or by immunostaining with a GFAP antibody (24).

Cavalieri's volume estimation technique is a very old and worthwhile tool that remains as effective as the Archimedian fluid replacement volume estimation technique (25, 26). Increases in either subcompartments of the brain or in the total volume of the brain that result from possible astrogliosis can be shown by Cavalieri's volume estimation. Therefore, we have investigated the effects of long-term hypergravity conditions on cerebral cell morphology by determining the distribution of GFAP and by using the Cavalieri's volume estimator to reveal potential volume changes in mice cerebrums that could originate from astrogliosis.

Materials and methods

Eighteen adult male mice were divided into three groups of six for this study. The mice were kept in cages that were attached to a radius of a centrifugation device (Fig. 1). One group of the mice was subjected to centrifugation at one gravity (1G) and another group at 2G (hypergravity) in the animal centrifuge device for 4 weeks, and the third group was kept separate as a control group. Centrifugation was performed 6 hours/day and 6 mice were treated at once. After the 4 weeks of centrifugation, the mice were deeply anaesthetized with chloroform and perfused through the ascending aorta with 10% formaldehyde. Their brains were removed and further post-fixed with 10% formalin for 24 h. The samples were washed and soaked in a graded series of ethanol and were then embedded in paraffin. Systematic randomised cerebral sections of 5 μm-thickness taken from the blocks and prepared for both histochemical and immunohistochemical staining. Hematoxylin-eosin dye was used for sterological volume estimations. The volumes of the both the cerebral cortex and medulla were estimated stereologically using Cavalieri`s methods. An Olympus BX 41 microscope with an LCD camera (Samsung SAC-410PA South Korea) and a 17-inch computer monitor were used. A transparent grid of 2.25 cm^2 squares was used for point counting at 40x magnification. On average, 150-200 points were counted per sample on 12 to 15 sections to arrive at a coefficient of error (CE) between 5 - 10% (26). In order to evaluate volumetric differences, student’s t-test was used (26).

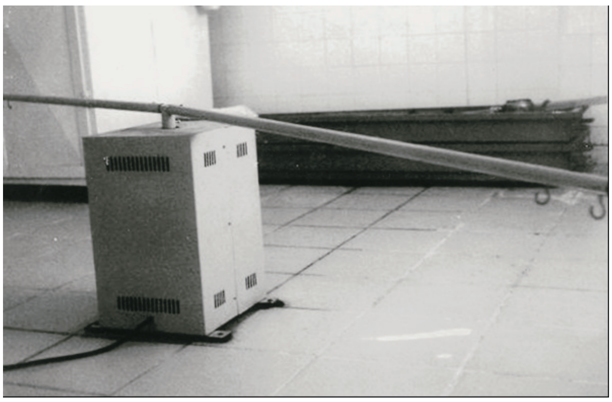

Figure 1A. Rotation device.

The distribution of GFAP was determined using immunohistochemical techniques. The slides were first incubated at 60°C overnight and then immersed in xylene for 30 min. After washing with serial concentrations of ethanol, the sections were washed with distilled water and phosphate buffered saline (PBS) for 10 min. They were then immersed in 2% trypsin in Tris buffer at 37 °C for 15 min, before a final wash with PBS. The sections were drawn with a Dako pen (Dako S-2002) and incubated in 3% hydrogen peroxidase for 15 min, to inhibit endogenous peroxidase activity. They were then washed with PBS and stained with an anti-GFAP antibody (1/100, Neomarkers RB-087-A) for 18 h. They were then washed with PBS three times for 5 min each, followed by an incubation with biotinylated IgG before streptavidin peroxidase was administered (Universal Dako LSAB2 kit). The incubation steps were interspersed with three washing steps. After washing with PBS three times for 5 min following the secondary antibody, the sections were washed with Dako DAB Substrate system for 5 min to reveal the immunoreactivity, and then washed with Mayer's hematoxylin. Finally, the sections were covered with mounting medium and observed under an Olympus BX 40 light microscope. Coloured pictures were taken with 100 ASA Fuji colour film. The control samples were processed in an identical manner, but in the absence of the primary antibody. The ethics committee at Celal Bayar University, Faculty of Medicine approved the study protocol.

Results

In this study, we initially determined the cerebral cortex, medulla and total cerebral volumes under control conditions and after the long-term 1G and 2G conditions, as described in the methods. For the calculation of the volume estimates, the total number of points per sample and the average slab thickness were multiplied by the area per point (corrected for magnification) (Fig. 2A). The mean volumes in the control group were: cortex, 3.86 mm^3; medulla, 1.95 mm^3; total cerebrum, 5.81 mm^3. Following the statistical analysis, the mean volumes in the 1G group (cortex, 3.81mm^3; medulla, 2.03 mm^3; total cerebrum, 5.84 mm^3; CE = 0.04) and the 2G group (cortex, 3.80mm^3; medulla, 2.07 mm^3; total cerebrum, 5.87 mm^3; CE = 0.05) showed no significant differences across each of the volume measurements between the three groups (cortex, p= 0.75; medulla, p=0.08; total cerebrum, p=0.75) (Table) (Figure 1B).

Table Details of the cerebral volume measurements and the statistical analysis of the three groups studied.

	Cortex volume ($mm^3 \pm$ SD)	Medulla volume ($mm^3 \pm$ SD)	Total volume ($mm^3 \pm$ SD)	Statistical difference to control (P)
Control (n=6)	3.86 ± 0.07	1.95 ± 0.02	5.81 ± 0.13	-
1G (n=6)	3.81 ± 0.10	2.03 ± 0.04	5.84 ± 0.09	0.08
2G (n=6)	3.80 ± 0.09	2.07 ± 0.03	5.87 ± 0.15	0.75

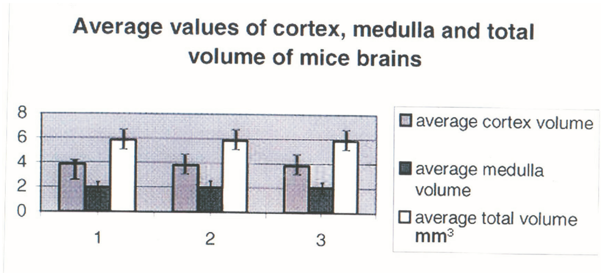

Figure 1B.The graphic of the cerebral volume estimations.

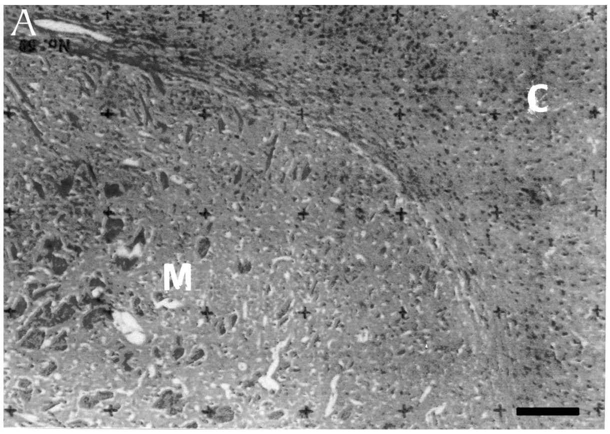

Figure 2A.Application of Cavalieri's technique Bar 40µm..

There was no histopathological changes among the histological sections of the groups stained with Hematoxylin – Eosin (Figure 2 B,C,D).

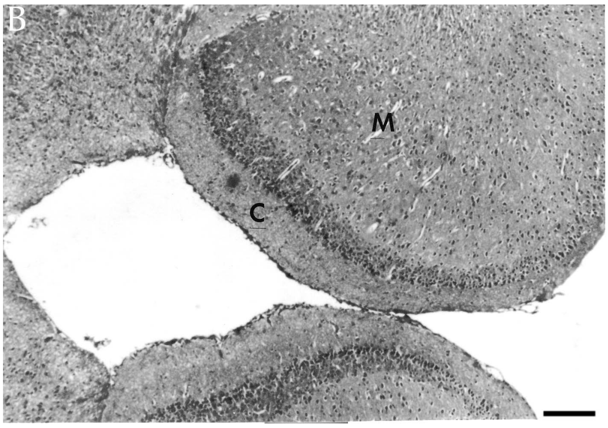

Figure 2B. Hematoxylin-eosin staining of a control mouse cerebrum (magnification, x40) Bar 20μm.

Figure 2C. Hematoxylin-eosin staining of a 1G mouse cerebrum (magnification, x40) Bar 20μm..

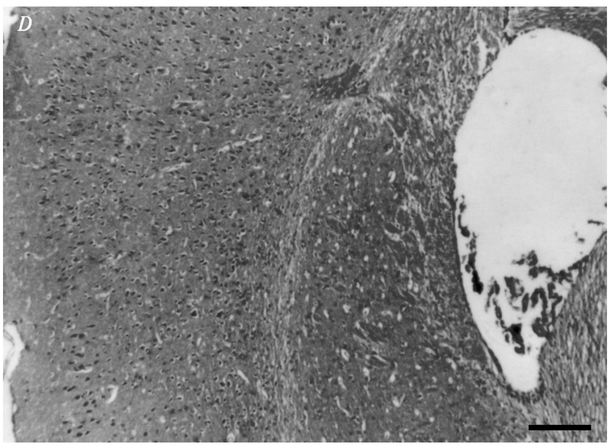

Figure 2D. Hematoxylin-eosin staining of a 2G mouse cerebrum (magnification, x40) Bar 20μm.

As illustrated in Figures 3 A (control), B (1G) and C (2G), 5 μm serial sections, following an examination of the randomly selected, stained the cellular morphology of the cerebrum in the three groups was seen to be normal, without any ischaemic areas being detected. Furthermore, there were no visible systematic differences between the cell layers. In addition, while the indirect immunoreactivity assay revealed the distribution of GFAP in the astrocytes, no differences in the GFAP distribution of immunoreactivity were seen between these three groups.

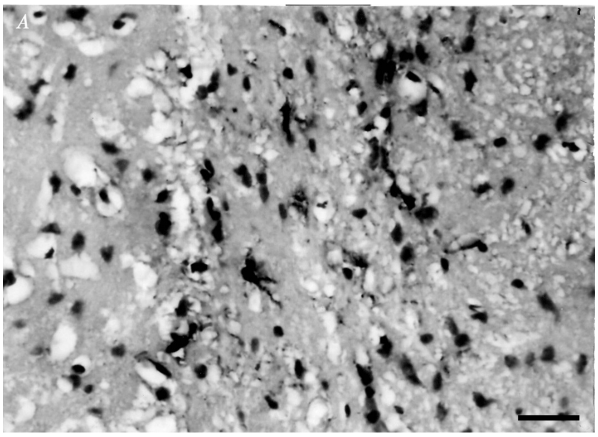

Figure 3A. Immunohistochemical distribution of GFAP in the control (magnification, x40) Bar 20μm.

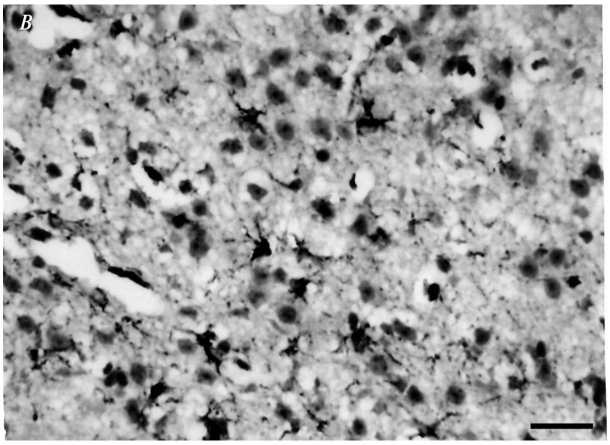

Figure 3B. Immunohistochemical distribution of GFAP in 1G (magnification, x40) Bar 20μm.

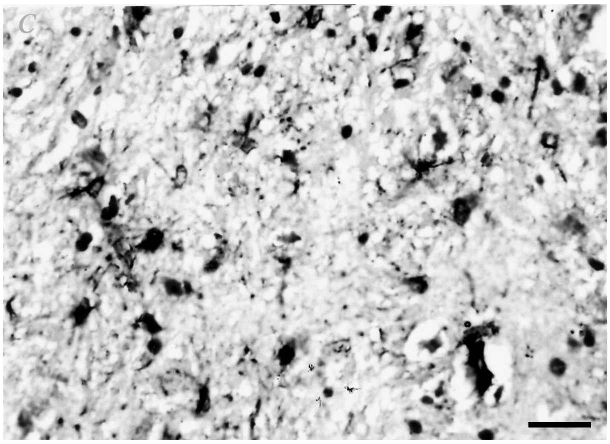

Figure 3C. Immunohistochemical distribution of GFAP in 2G (magnification, x40) Bar 20μm.

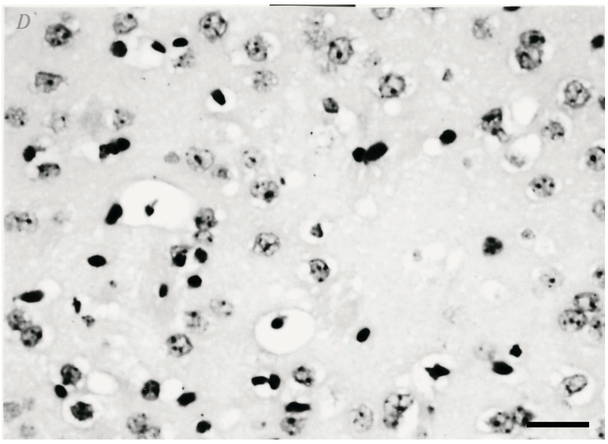

Figure 3D. The control slides for immunohistochemistry (magnification, x40) Bar 20μm.

Discussion

Obvious morphological and physiological changes have been shown to occur in living systems exposed to altered gravity (5, 11). In the CNS, specific changes can take place due to the ischaemic effects of acceleration, centrifugation and parabolic flight. The volumes and cellular morphologies of the cerebrums in the present study were not affected by the rotation or the long-term changes in gravity. Previous studies have demonstrated that hypergravity can cause cerebral ischaemia and changes in cerebral blood flow (6, 7, 8, 10, 11, 12). However, to date, the cerebral volumes have not been estimated under such conditions. When humans live in space stations in the future, centrifugation will need to be used for the generation of artificial gravitational forces in space (3), and therefore it is important to determine the combined effects of both hypergravity generation and rotation. In addition, the main example of hypergravity is in parabolic flight in an aircraft. Therefore, it is especially pilots who suffer from the associated loss of consciousness and central vision (7, 8). When blood flow in the different areas of the brain has been measured, there have been no statistically significant differences seen (6, 7), and spinal cord blood circulation was not seen to be changed (7). However, Tripp et al. observed that the regional cerebral tissue oxygen was decreased by 13% in men compared to 9% in women under hypergravity conditions. Following the end of the hypergravity exposure, both the men and the women showed slow recoveries in their cerebral oxygen saturation values to their pre-baseline levels (10). As the most effective volume estimator in unbiased stereology, Cavalieri's volume estimation tool was used in this study to estimate the cortex, medulla and total cerebral volumes of the samples in each of the three groups. As an index of efficiency of the volume estimations, the coefficient of error calculations were found to be within the expected intervals of between 5 - 10% (27). The Cavalieri's volume estimation findings indicated that no astrogliosis or pathologies related to the long-term centrifugation that could have resulted in volume increases in the cerebrums took place under the hypergravity conditions used in the present study.

GFAP is an intermediate filament protein in astrocytes, and its expression increases in response to injury, neurodegenerative diseases and aging (28, 29). GFAP also appears to be regulated by local changes in neuronal activity. Immunoreactivity and mRNA expression of GFAP both serve as useful neurodegenerative markers, because increased expression of GFAP corresponds to a characteristic cellular hypertrophy that is known as astrogliosis (24). GFAP was chosen as the marker for potential gravity-induced neurodegeneration in our study; however, no significant changes were seen in the GFAP immunoreactivity here. The turnover of GFAP is estimated to span from several hours to several weeks (30), and thus an increase in its expression might be expected to have been revealed. Similarly, Cai et al. (1997) showed that Hsp70 mRNA expression in rat brain can be induced by repeated hypergravity exposures and that this increased Hsp70 mRNA expression may have an important role in self-protection against brain damage induced by hypergravity exposure. In addition, in a study by Santucci et al. (2002), the responses of CD-1 mice exposed to 2G hypergravity showed significant increases in central nerve growth factor (NGF) levels and minor changes in brain-derived neurotrophic factor (BDNF) levels after rotation (19). Pathological changes in the brain have also been related to gravity levels, gravity onset rates, duration of high-gravity exposure, and individual difference factors (31), and they also depend on the condition (32) and body size (33, 34) of the subject. Therefore, the lack of increases in the astrogliosis indicator in the present study with respect to hypergravity-related ischaemia might also result from the relative size of the mice or the gravity levels investigated. Although the result of some of these previous studies have suggested that there can be a decrease in cerebral blood flow (which can cause cerebral ischaemia) under the influence of different hypergravity levels on different animals, in the present study, there was no evidence of an effect of long-term 1G or 2G conditions on either cerebral volume or cellular morphology.

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