Int J Clin Exp Pathol 2016;9(8):8162-8171
www.ijcep.com /ISSN:1936-2625/IJCEP0033003

Original Article

Effects of weightlessness on expression of TLR4/CD14 and chemotactic factors in gingival tissue of rhesus macaques

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Received May 30, 2016; Accepted June 25, 2016; Epub August 1, 2016; Published August 15, 2016

Abstract: Background and objective: Healthy stomatognathic system is a guarantee for nutrient intake of astronauts during long-term spaceflight, but few researches have been carried out to investigate the influence of weightlessness on oral health. This study was aimed to explore the effects of simulated weightlessness on the histology and immune status of gingival tissues of rhesus macaques using a 6w ground-based weightlessness simulation model. Methods: An internationally recognized ground-based microgravity simulation model (-10° Head-down tilted bed rest, HDBR) on rhesus macaques was employed. Fifteen healthy male rhesus macaques weighing 4 to 8 kg were divided into three groups: control group (Group A), 6w -10° HDBR group (Group B) and 6w -10° HDBR followed by 4w recovery group (Group C), according to stratified random grouping design. After the macaques were sacrificed with humanistic care, oral gingival tissues were sampled immediately. The pathological changes of gingival tissues were investigated by hematoxylin and eosin (HE) staining. Histopathochemical method and real-time PCR were performed for the determination of protein and mRNA expression of TLR-4, CD14, MCP-1, MIP-1α and CCL20. Results: Inflammatory cells infiltration was found in the free marginal gingiva of all of the three groups, but it was more severe in group B than in the other two groups. In addition, the cornified envelope of gum in group B was also thinner than that in group A and group C. The expression of TLR-4, CD14, MCP-1, MIP-1α and CCL20 was all slightly reduced by 6w -10° HDBR at both protein and mRNA levels, but only the CD14 IHC scores showed statistically significant difference between Group A and Group B ($P < 0.05$). We also found that all of the reduced expression of TLR-4, CD14, MCP-1, MIP-1α and CCL20 were restored by 4w recovery, however, only MCP-1 IHC scores showed significant difference between Group B and Group C ($P < 0.05$). Conclusion: -10° HDBR for 6 weeks could promote the inflammatory cells infiltration by weakening the local immunity of oral gingiva. This study fills a gap in space medical researches and provides some experimental basis for oral healthcare of astronauts during their spaceflight.

Keywords: Simulated weightlessness, immune response, gingival, rhesus macaque, TLR-4, CD14, MCP-1, MIP-1α, CCL20

Introduction

According to the third national oral health epidemiological investigation, periodontal disease is the primary oral health problems boring the adult population in China, which has a prevalence rate higher than 80% [1]. Such a high prevalence rate attracts our attentions to the oral health of astronauts on spaceflight, especially on their oral healthcare of long-term spaceflight.

With the development of manned spacecraft engineering, the stay of the astronauts on the space station will gradually extend. Thus, the health of astronauts on the space station draws more and more attentions from the people. A body of studies has shown that exposure to the spaceflight and microgravity conditions have profound effects on all of the human physiological systems [2], including the cardiovascular system [3, 4], cardiopulmonary function [2], musculoskeletal system [5, 6]. However, the

effects of weightlessness on oral health, especially on oral immunity that is pivotal for oral health [7, 8], remain to be clarified.

Head-down tilted bed rest (HDBR) is an internationally recognized ground-based microgravity simulation model for studying the effects of microgravity on human systemic functions, especially on the loss of minerals in bone [9, 10]. The similarity of major physiological functions between human and monkey makes non-human primates a suitable model especially for the experiments in weightlessness [11]. The rhesus monkey has been proposed as an ideal model for the effects of space flight on immunity [12]. Thus, in this study, we used rhesus macaques as the subjects to explore the effects of weightlessness on the oral immunity, especially on the gingival tissue due to its sensitivity to the whole oral immune status. This experimental model has been widely used [3, 11, 13].

Our previous studies showed that 30d -6° HDBR can induce inflammation in free and attached gingival tissues [14]. Then we expected to investigate whether the HDBR-induced inflammation was mediated by Toll-like receptor - 4 (TLR4)/CD14 axis or chemotactic factors, such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and CC chemokine ligand 20 (CCL20). Thus, we used rhesus macaques ground-based weightlessness simulation model to investigate the *in situ* effect of weightlessness on the gingival tissues of rhesus macaques by exploring the histopathological changes of the gingival tissues and the changes in the expression of TLR-4/CD14, MCP-1, MIP-1α and CCL20, which play crucial roles in oral immunity, so as to investigate the changes of oral immunity under weightlessness condition.

Materials and methods

Animals and grouping

Fifteen healthy male rhesus macaques, aged 4 to 8 years and weighing 4 to 8 kg, were from Beijing Institute of Xie'erxin Biology Resource (Beijing, China). All rhesus macaques were solely fed in stainless steel mesh cages at the Laboratory Animal Center of China Astronaut Research and Training Center, with a standard primate diet and had free access to water. Animals were housed in rooms with controlled room temperature (22 ± 2°C) and humidity (55 ± 5%), with 12-h artificial day/night light cycle. All procedures were performed in accordance with the guidelines for the use of experimental animals established by the National Institutes of Health (NIH, USA) and approved by the Institutional Animal Care and Use Committee of China Astronaut Research and Training Center (ACC-IACUC-2014-001).

Animals were divided into three groups: control group without any treatment (Group A, n=5), group treated with head-down tilt for 6 weeks (Group B, n=5) and group treated with head-down tilt for 6 weeks and then recovered for 4 weeks (Group C, n=5), according to stratified random grouping design.

Treatment and sampling

Simulated weightlessness was realized by -10° HDBR. Briefly, macaques in Group B and Group C were fixed on special devices and kept in -10° head-down-tilt for 6 weeks. Before HDBR, macaques received appropriate domestication to ensure they could adapt the restrictive living environment of 6w HDBR. Macaques in Group A were still bred in the cages for 6 weeks with free movement. Macaques in Group C subjected to free movement in the cages for another 4 weeks after 6w head-down tilting.

After the corresponding treatment, macaques in each group were sacrificed under deep anesthesia with hydration ketamine. All macaques received humanistic care before they were sacrificed. Then tissues were sampled immediately from the attached gingiva at buccal side of lower teeth of each macaque. Each sample was divided into two parts: one was fixed in 10% neutral buffered formalin for histopathological or immunohistochemical examinations and the other stored in liquid nitrogen for real time PCR.

Pathological examination

After 24 h fixation with neutral buffered formalin, the gingival mucosa of each macaque were conventionally dehydrated and embedded in paraffin. Then 5 μm-thickness sections were prepared from paraffin-embedded blocks and

Table 1. Specific gene primers used in this study

Gene	Forward	Reverse	Product size (bp)
TLR-4	5'-TTTAGACCTGTCCCTGAACCC-3'	5'-CCAGAACCAAACGATGGACTT-3'	161
MCP-1	5'-AGGCTGGCGAGCTATAGAAGA-3'	5'-AGGCTTCGGAGTTTGGATTT-3'	159
CD14	5'-CAACTTCTCCGAACCTCATCC-3'	5'-AGCCTTGATCGTGTCAGCATA-3'	157
CCL20	5'-TACAGACCGTATCCTTCATCCTAA -3'	5'- CGACGTACAATAAGTTTCACCCA -3'	156
GAPDH	5'-GAGCCAAAAGGGTCATCATCT-3'	5'-TGAGTCCTTCCACGATACCAA-3'	180

subjected to hematoxylin and eosin (HE) staining for morphological evaluation.

Immunohistochemical detection (IHC)

Slides with 5 μm-thickness of paraffin-embedded gingival mucosa tissues of rhesus macaques were treated conventionally to be hydrated and then incubated with 3% hydrogen peroxide (H_2O_2) at room temperature for 20 min to eliminate the endogenous peroxidase. In order to fully expose the antigen sites, slides were immersed in 0.01 mol/L citrate buffer (pH 6.0, Beijing ZSGB Bio-tech. Co., China) and heated in a pressure-cooker for 2 min at the highest pressure and then cooled naturally at room temperature for 30 min. After blocked with 5% BSA in PBS for 30 min at room temperature, slides were respectively incubated with mouse monoclonal antibody to TLR4 (diluted 1:100, ab8376, Abcam, San Francisco, USA), rabbit polyclonal to MCP1 (diluted 1:300, ab73680, Abcam, San Francisco, USA), mouse monoclonal antibody to CD14 (diluted 1:300, NB100-77758, Novus Biological, Littleton, CO, USA), goat polyclonal antibody to CCL20 (diluted 1:1000, AF360, R&D, MN, USA) and rabbit polyclonal to MIP-1α (diluted 1:1000, ab32609, Abcam, San Francisco, USA) at 4°C overnight, followed by sequential incubation with poly pelper reagent and polyer peroxidase-anti-mouse/rabbit or anti-goat IgG (PV-9000/PV-9003 Polymer Detection System, Beijing ZSGB Bio-tech. Co., China) at room temperature for 20 min, respectively. After stained with diaminobenzidine (Beijing ZSGB Bio-tech. Co., China) and counterstained with hematoxylin, slides were observed under a Leica microscope (Leica Microsystems, Wetzlar, Germany). For the negative control, the specific primary antibody was omitted and replaced by phosphate-buffered saline. One human Lymph node tissue served as positive control. Positive and negative controls were performed with each batch of slides.

Interpretation of IHC results

TLR4 and CD14 protein expressions are mainly located in cellular membrane and cytoplasm, while MCP-1, MIP-1α and CCL20 are mainly expressed in cytoplasm, characterized by brown granules. According to the cell staining degree and the proportion of positive cells, slides were scoring as follows: 0, 1, 2 and 3 for staining intensity of no staining, yellow staining, tan staining and brown staining, respectively; while according to the proportion of positive cells, 0, 1, 2, 3 and 4 for positive rate < 10%, 10%-25%, 26%-50%, 51%-75% and > 75%, respectively. Five high visions (400×) of each case were selected for the comprehensive score by two experienced pathologists in double-blinded conditions. The total score of each slide was semi-quantified by the formula: total score = intensity score × positive proportion score.

RNA extraction and real-time PCR

The fresh gingival mucosa tissues of each macaque kept in liquid nitrogen were used for total RNA extraction which was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Then, 2 μg of total RNA was reversely transcribed to the first-strand cDNA using AMV First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Foster City, CA). For each sample, 1 μg of cDNA was subjected to real-time PCR amplification with a SG Fast qPCR Master Mix kit (BBI, Shanghai, China). The specific primer pairs were shown in **Table 1**. The real-time PCR reactions were run on a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) under the following conditions: a holding step at 95°C for 3 min, and 40 cycles of 95°C for 7 s, 57°C for 10 s, and 72°C for 15 s. The data were processed using LightCycler® 480 Software System and the relative expressions of target genes were calculated by $2^{-\Delta\Delta Ct}$ meth-

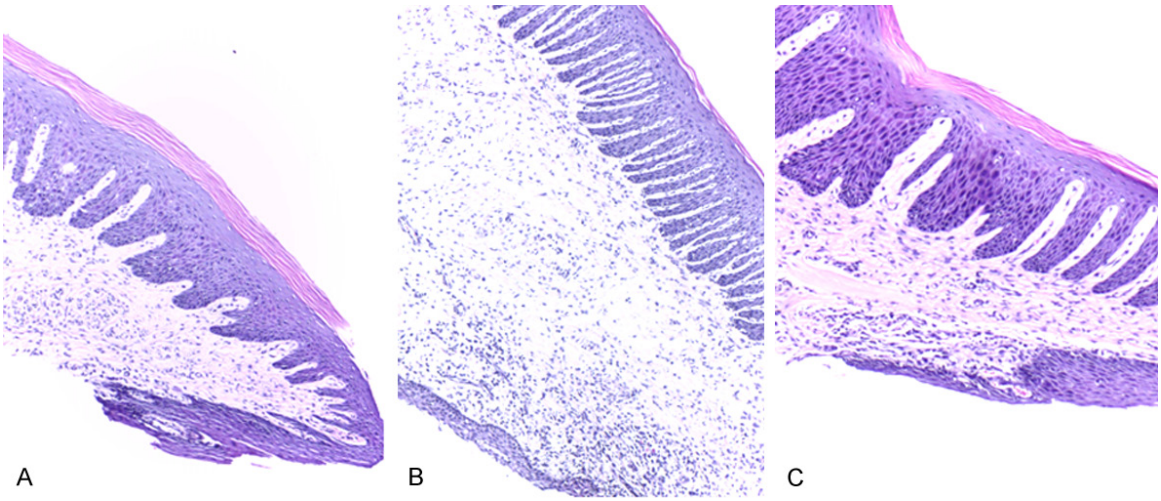

Figure 1. Histological changes in gingival tissues of rhesus macaques determined by HE staining (Original magnification, × 100). The gingival tissues were harvested from Rhesus monkeys in each group, fixed with neutral buffered formalin and paraffin-embedded. Then the 5 μm sections were stained with hematoxylin and eosin. A. Representative of control group; B. Representative of weightlessness group; C. Representative of recovery group.

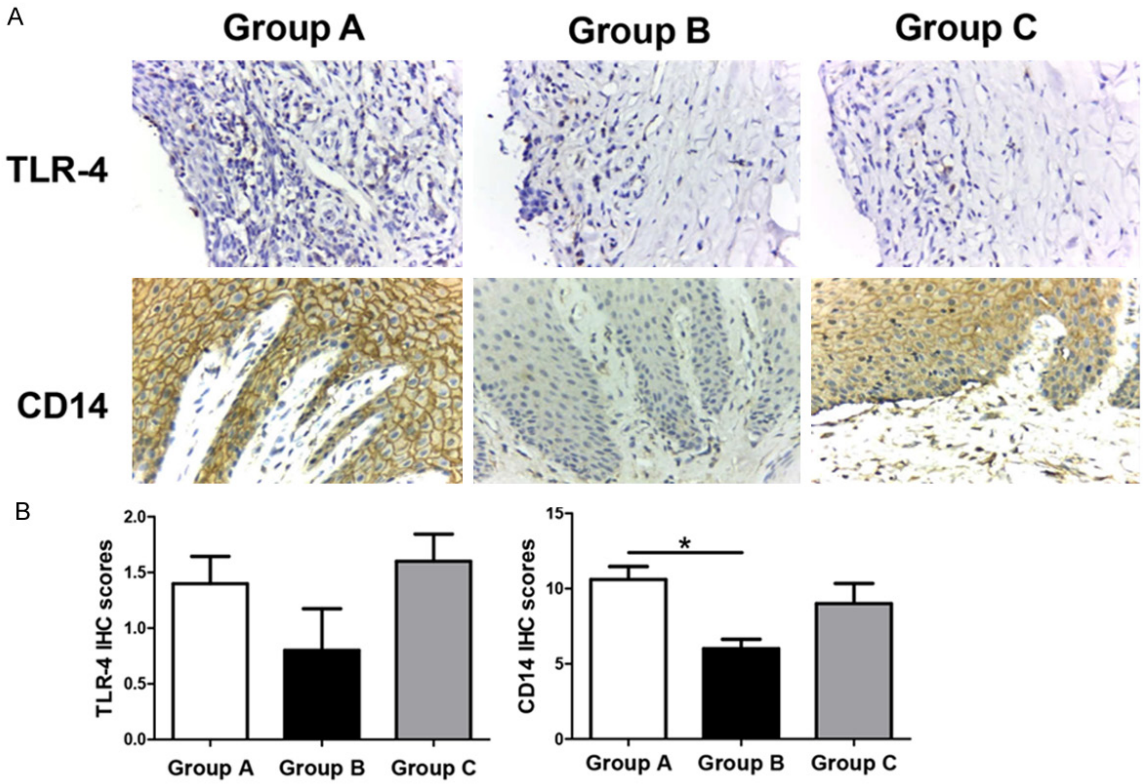

Figure 2. Expression of TLR-4 and CD14 in the gingival tissues of rhesus macaques determined by immunohistochemistry (Original magnification, × 400). The gingival tissues were harvested from Rhesus monkeys in each group, fixed with neutral buffered formalin and paraffin-embedded. The 5 μm sections were subjected to IHC assay with the indicated antibodies. A. Representatives of the expression of TLR-4 and CD14 in each group; B. Statistical analysis of the IHC scores, data are shown as means ± S.E. (n = 5). *, $P < 0.05$ vs. Group A. Group A, control; Group B, weightlessness; Group C, weightlessness followed by recovery.

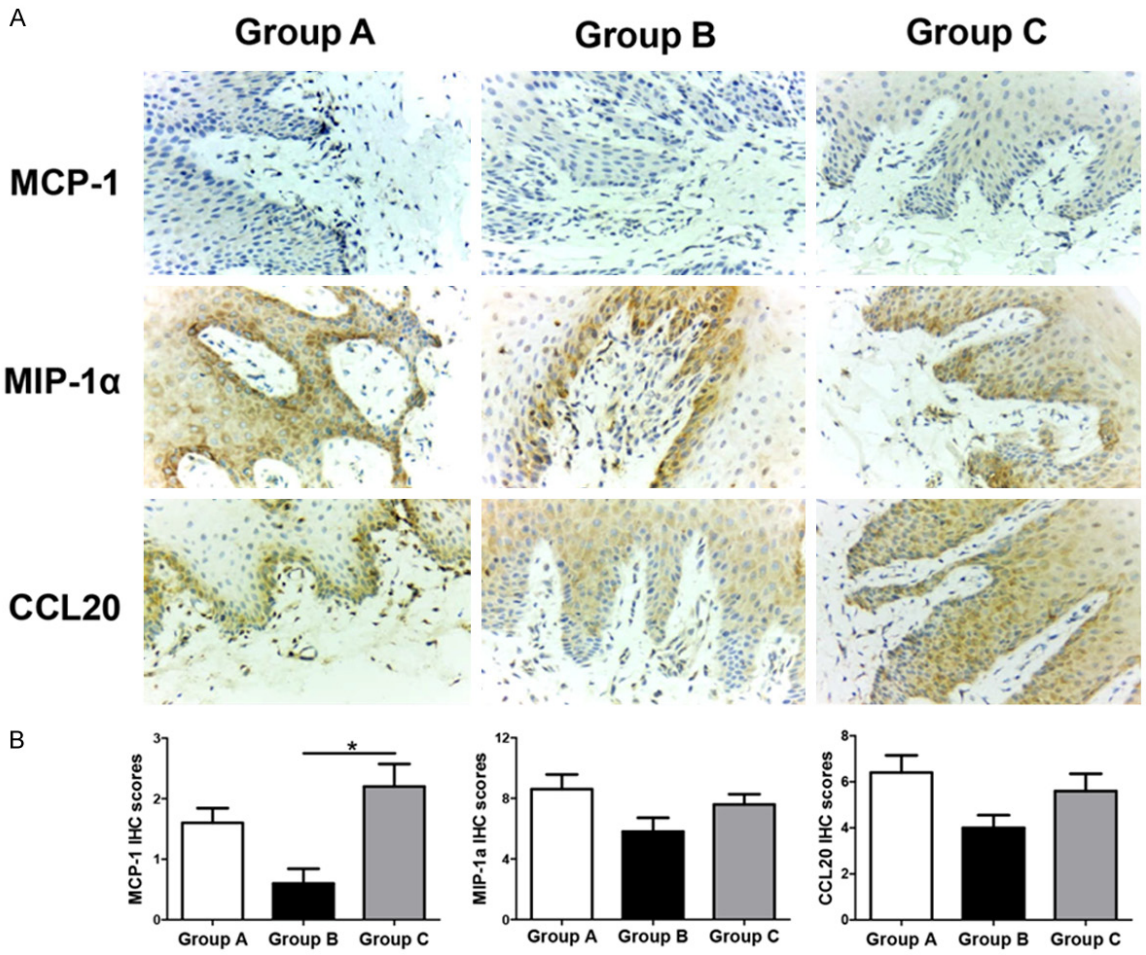

Figure 3. Expression of chemotactic factors MCP-1, MIP-1α and CCL20 in the oral gingival tissues of rhesus macaques determined by immunohistochemistry (Original magnification, × 400). The gingival tissues were harvested from Rhesus monkeys in each group, fixed with neutral buffered formalin and paraffin-embedded. The 5 μm sections were subjected to IHC assay with the indicated antibodies. A. Representatives of the expression of MCP-1, MIP-1α and CCL20 in each group; B. Statistical analysis of the IHC scores, data are shown as means ± S.E. (n = 5). *, $P < 0.05$ vs. Group A. Group A, control; Group B, weightlessness; Group C, weightlessness followed by recovery.

od. PCR reaction of each sample was run in triplicate.

Statistical analysis

Statistical analysis was performed using SPSS (version 18.0, SPSS, Chicago, IL, USA). The quantitative data were expressed as mean ± standard error (SE) if in accordance with normal distribution or they were shown as median (range). Intergroup differences were compared using one-way analysis of variance. In case of homogeneity of variance, LSD test was performed. In case of heterogeneity of variance, Dunnett's T3 test was employed. If the data were in abnormal distribution, the differences were compared by rank sum test. Differences with $P < 0.05$ were considered statistically significant.

Results

Histological changes in oral gingival tissue of rhesus macaque induced by weightlessness

From the gross observation, no obvious ulceration was found in all of the three groups. However, rhesus monkeys in group B had a certain degree of periodontal inflammation, presenting a bit of swelling in gum. The pathological examinations showed inflammatory cells infiltration could be found in the free marginal gingiva of all of the three groups (**Figure 1**), but it was more severe in group B (**Figure 1B**) than

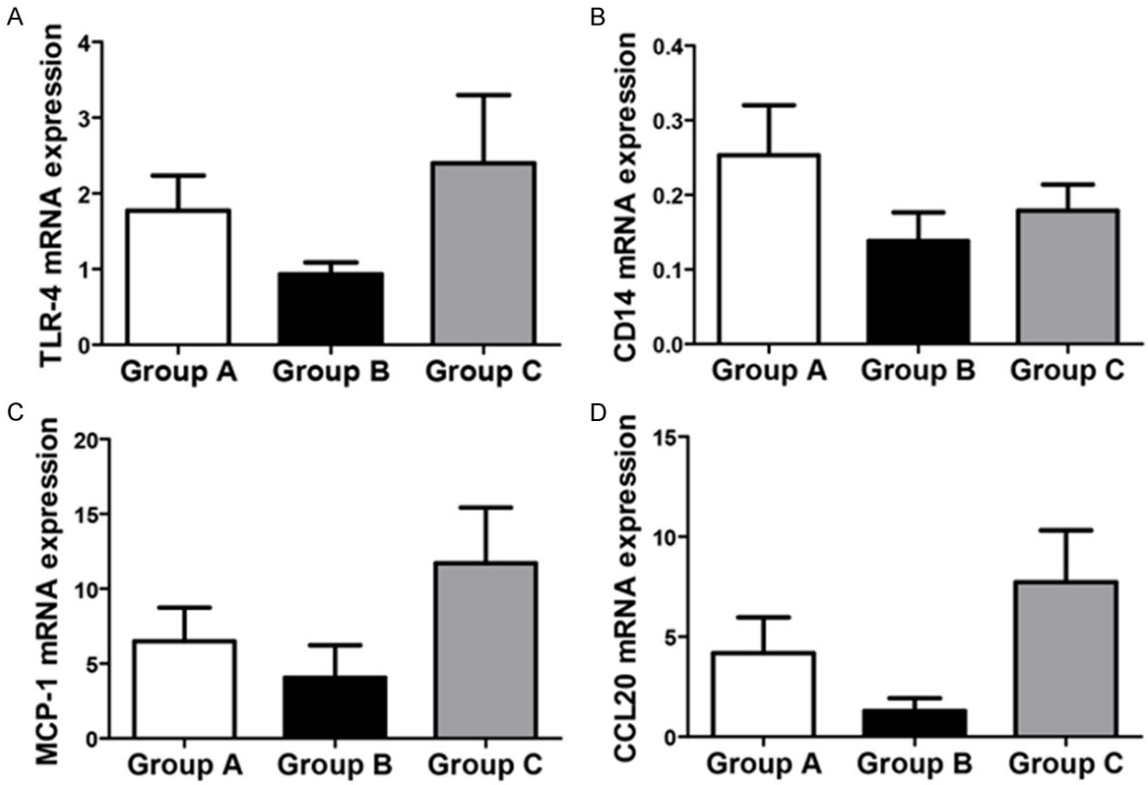

Figure 4. mRNA expression of TLR-4, CD14, MCP-1 and CCL20 in the gingival tissues of rhesus macaques determined by real-time PCR. The gingival tissues were harvested from Rhesus monkeys in each group and stored in Liquid nitrogen. The total RNA was extracted from all of the gingival tissues using Trizol reagent and then subjected to cDNA synthesis and the subsequent real-time PCR. A. The expression of TLR-4 mRNA; B. The expression of CD14 mRNA; C. The expression of MCP-1 mRNA; D. The expression of CCL20 mRNA. The differences were not statistically significant.

in the other two groups (**Figure 1A** and **1C**). The inflammatory cells infiltration could be commonly seen in the free marginal gingiva of macaques. In addition, we also found that the cornified envelope of gum in group B was thinner than that in group A and group B.

Simulated weightlessness reduced the expressions of TLR4 and CD14 in oral gingival tissue of rhesus macaques

Due to the inflammatory cells infiltration in gingival tissues of rhesus macaques and the histopathological changes induced by simulated weightlessness, we expected if TLR4/CD14 axis, a crucial pathway in the recognition of microorganisms, could be changed by simulated weightlessness. Thus, we detected the expression and distribution of TLR4 and CD14 in the gingival tissues of rhesus macaques by using IHC method. Results showed that TLR4 mainly expressed in the infiltrated inflammatory cells, and there were no marked difference for TLR4 expression among the three groups (**Figure 2**). CD14 was highly expressed in the base layer of the gingival mucosa of control animals (Group A). After 6w -10° HDBR (Group B), the expressions of CD14 were significantly reduced as compared with that in Group A. However, with the 4w recovery (Group C), the expression of CD14 increased comparable to the level in Group A (**Figure 2**).

Simulated weightlessness had mild effects on the expression of chemotactic factors MCP-1, MIP-1α and CCL20 in oral gingival tissues of rhesus macaques

We also explored if chemotactic factors plays a role in the rise of inflammation cells infiltration. We detected the expression of MCP-1, MIP-1α and CCL20 in the gingival tissues of macaques by using IHC method. MCP-1, MIP-1α and CCL20 were all expressed in the cytoplasm of

mucosal epithelial cells. MCP-1 was mildly expressed in Group A and C, but almost not expressed in Group B. Statistical difference was found between Group B and Group C but not between Group A and Group B or between Group A and Group C. MIP-1α and CCL20 were highly expressed in all of the three groups, although they were decreased slightly in Group B, no statistical difference was found (**Figure 3**).

Simulated weightlessness slightly reduced the mRNA expressions of TLR4, CD14, MCP-1 and CCL20 in oral gingival tissue of rhesus macaques

In order to verify if weightlessness could affect the immune response of gingival mucosa at mRNA level, we detected the mRNA expression of *TLR4, CD14, MCP-1* and *CCL20* by using real-time PCR. Due to lack of an optimal specific primer pair for *MIP-1α*, no determination of *MIP-1α* mRNA was carried out. The results revealed that all of the mRNA expression of *TLR-4, CD14 and MCP-1* were slightly reduced by 6w -10° HDBR, but they would be restored after 4w recovery. Nevertheless, all of the differences showed no statistically significant difference (**Figure 4**).

Discussion

Weightlessness has a variety of effects on the human physiological functions. It has been reported that about half of the astronauts executing Apollo missions had minor bacterial or viral infections within a week after their return, although the effects only lasted for short time [15]. The reactivation of latent herpes viruses has also been observed in crew during flight and within 1 week of return, indicating the downregulation of cellular immunity [16, 17]. In addition, Mehta et al. recorded subclinical activation of Epstein-Barr virus, varicella-zoster virus and cytomegalovirus in 14 of the 17 astronauts who undertook short-duration flights on Space Shuttle [18]. All these evidences urged us to explore the effects of weightlessness on the oral immunity due to the large number of oral flora existing in oral cavity.

We employed a ground-based simulated weightlessness model on rhesus macaques in this study to explore the histopathological change of gingival tissues under 6w -10° HDBR condition and if it can be restored after 4w recovery. The pathological examination revealed that inflammatory cells infiltration could be seen in the gingival tissues of all of the three groups, especially in the HDBR group. Because no oral care had been taken for the macaques during the experiment, all of the macaques had periodontal diseases in different degrees, and the HDBR group was in the most severity, suggesting that 6w -10° HDBR could aggravate the inflammation in gingival tissues, which was consistent with the results from human test [14]. However, after 4w recovery, the inflammatory cells infiltration was obviously alleviated, indicating that the effect of weightlessness on the oral health was reversible.

In the current study, we found that the cornified envelope of rhesus macaque gum was attenuated as compared with that in the control group and the recovery group. Cornified envelope is the outermost layer of gum, formed by cross-linked proteins and lipids, which is the main composition of the epithelial barrier. It possesses high resistance to dissolve and strong flexibility, so as to protect the epithelial cells in the inner layer. Kim W *et al.* demonstrated that spaceflight can promote the number of viable cells, biofilm biomass, and thickness of biofilm by Pseudomonas aeruginosa [19]. Oral microbial community is a typical biofilm, the bacteria in which have some special properties [20, 21]. When bacteria in biofilm-forming state, they have greater resistance to antibiotics. Due to the multiple species characteristics of dental plaque biofilm, oral microbial interactions may influence the whole human community. Thus, we speculated that the thinning cornified envelope may be induced by the accumulation of biofilm biomass.

TLR-4/CD14 axis plays crucial role in the innate immune system. TLR-4 is most well-known for recognizing lipopolysaccharide (LPS), a component of Gram-negative bacteria, which also widely exist in oral cavity. CD14 acts as a co-receptor along with the Toll-like receptor TLR-4 and MD-2 for the detection of LPS [22]. Periodontal disease is initiated by the accumulation of a broad variety of bacteria. Based on ligand recognition, TLR-4 interacts with most periodontal pathogens and activates signaling pathways by combining with CD14, resulting in

immune responses against microbial infections. In this study, we found that both the protein and mRNA expressions of TLR-4 and CD14 in the macaque gingival tissues were reduced by -10° HDBR compared with those in control group, but the downregulation could be restored after 4w recovery. Unfortunately, the changes could only show an obvious trend, because only the difference of CD14 IHC score showed statistical significance between the control group and HDBR group. Our pathological results showed that HDBR promoted inflammatory cells infiltration in the gingival tissues, but the expressions of TLR-4 and CD14 in the HDBR group was reduced at both protein and mRNA levels, instead. This is maybe a result of impaired innate immune induced by weightlessness, which has been verified by several studies [23, 24]. Marcu, O *et al.* applied Drosophila (fruit fly), which shares many similarities with human innate immunity at the level of molecular and genetic pathways, to investigate the effect of spaceflight on innate immunity, and found that the phagocytosis of plasmatocytes was attenuated following spaceflight, and in parallel, the constitutive expression of pattern recognition receptors and opsonins that specifically recognize bacteria, antimicrobial peptide (AMP) pathway and immune stress genes were also reduced [23]. Kaur I *et al.* also reported that the astronauts' monocytes exhibited reductions in ability to phagocytose Escherichia coli following 5-11 days of spaceflight [24].

Monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and CC chemokine ligand 20 (CCL20) are all cytokines that belong to the CC chemokine family, which play vital roles in various inflammatory diseases including periodontitis [25-27]. Our results showed that MCP-1 could be slightly decreased following 6w -10° HDBR, but then return to normal after 4w recovery. MCP-1 can recruit monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection [28]. CCL20 (also refers as macrophage inflammatory protein (MIP)-3alpha) and MIP-1α can attract activated T and B lymphocytes and immature dendritic cells in host responses to bacterial infection. However, although MCP-1, MIP-1α and CCL20 were decreased to some extent, the inflammatory cells infiltration was still severe in the HDBR group. This may be another evidence for the loss of function of immune cells induced by HDBR. Recently, one report on Science revealed that IAV-infected monocytes from older humans have intact inflammasome responses but the antiviral resistance is impaired [29], which may partly explain the phenomenon in our study. Stowe RP *et al*. also showed that neutrophils increase in circulating leukocyte subsets but their chemotactic ability decrease significantly after short-term spaceflight [30], indicating that not only the cytokines production of immune cells but also the chemotactic ability of them could be reduced by weightlessness. Thus, we may attribute the aggregation of inflammatory cells in oral gingival tissues of HDBR group to some else mechanisms, such as the redistribution of blood and others, but not the homing environment in the gingival tissue of rhesus macaque.

Our results can only show a change trend of the effects of stimulated weightlessness on the structure of oral gingival tissue and its immune status, because the differences were not very statistically significant between the groups. As we known, rhesus macaques have complicated genetic background, just like human. Thus, 5 rhesus macaques in each group may be a fatal weakness of this study. This may be complemented in the following studies. Another limitation of this study is all of the rhesus macaques having some degree of gingivitis due to lack of oral care for them. But it is perhaps a model more suitable to the real conditions owing to the high prevalence of periodontal disease in China.

In conclusion, our results revealed that 6w HDBR could promote the inflammatory cells infiltration in oral gingival tissues of rhesus macaques. The thinning cornified envelope of macaque gum indicated a reducing protection of the epithelial cells against the oral microbial community. The decreased expression of TLR-4/CD14 axis, MCP-1, MIP-1α and CCL20 induced by HDBR suggested an inhibitory effect of 6w HDBR on the local immunity of oral gum. This study filled a gap in space medical researches and provides some experimental basis for oral healthcare of astronauts during their spaceflight.

Acknowledgements

This work was supported by Natural Science Foundation of China (No. 30870598). We also would like to appreciate all of the staff of the

Laboratory Animal Center of China Astronaut Research and Training Center for their kindly supports during the animal experiment.

Disclosure of conflict of interest

None.

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