



Effects of hypoxic preconditioning combined with altitude training on CD55, CD59 and the immune function of swimmers

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Background: Hypoxic preconditioning refers to a certain intensity and time of hypoxic exposure before hypoxic stress, which makes the body produce endogenous protection to enhance the body's tolerance to subsequent more severe hypoxia. However, there are few studies on the effects of hypoxic preconditioning combined with altitude training on the immune system of athletes.

Methods: Nine swimmers from Shanghai underwent 3-week hypoxic preconditioning [living high-training low (HiLo)] combined with 3-week altitude training. CD55 and CD59 expression in red blood cells (RBCs), CD55 and CD59 expression in white blood cells (WBCs), RBC count, WBC count, T lymphocyte CD3, CD4, CD8 expression, and immunoglobulins IgG, IgM, and IgA were measured 4 times: before the start of hypoxic preconditioning, in the first week of hypoxic preconditioning, at the end of hypoxic preconditioning (i.e., before the start of altitude training), and at the end of altitude training.

Results: CD55 and CD59 expression in RBCs significantly increased in the first week of hypoxic preconditioning ($P < 0.05$), returned to baseline levels at the end of preconditioning, and significantly increased again during altitude training ($P < 0.05$). CD55 and CD59 expression in WBCs decreased significantly during hypoxic preconditioning ($P < 0.05$) and increased significantly during altitude training ($P < 0.05$). CD3 expression first decreased and then increased in the hypoxic preconditioning phase, then decreased again in the altitude training phase. However, there was no significant difference in each phase. CD4/CD8 expression after altitude training was significantly lower than that before altitude training ($P < 0.05$), but was not significantly different from that before the start of hypoxic preconditioning. IgG, IgM, and IgA did not fluctuate significantly throughout the experimental phase.

Conclusions: After hypoxic preconditioning combined with altitude training, the expression of CD55 and CD59 on the surface of RBCs and WBCs increased significantly, and T lymphocyte CD4/CD8 expression also increased. These results suggest an improvement in the complement regulation system and RBC immune function. Hypoxic preconditioning can therefore improve immunity and enhance the physical function of athletes during altitude training.

Keywords: Hypoxic preconditioning; altitude training; swimmers; immune function

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Introduction

Altitude training is an important training method to improve ability in physical endurance sports (1). It can dilate vessels, thicken blood vessels and blood vessel walls, and increase blood volume substantially, thus improving the cardiovascular system of athletes. Altitude training enhances the capacity to tolerate lactic acid by increasing maximal oxygen uptake and hemoglobin concentration. It has been shown that altitude training 3–4 per week can improve swimming ability in elite swimmers, and the improvement can be maintained for a considerable period of time after the end of altitude training (2). Altitude exposure is a type of physiological stress, and training at high altitude will exacerbate this stress. A high altitude environment can alter redox balance, and can induce oxidative stress. Oxidative damage to cellular DNA and proteins causes changes in cellular function (3). Acute and chronic hypoxic exposure at high altitude also leads to an impairment of the homeostatic regulation of Th1/Th2 immune balance (4). Accumulated reactive oxygen species (ROS) induced by altitude training can lead to oxidative stress damage, including apoptosis, lipid peroxidation, and DNA damage, and the occurrence of various diseases is precisely due to the long-term accumulation of peroxidation damage (5). During altitude training, the body receives dual stimulation by hypoxia and training, and the combination of the two stimuli increases and deepens the degree of oxidative stress damage. The exercise-induced immunosuppression and oxidative stress damage caused by altitude training can also induce a variety of diseases, which pose a significant threat to the health of athletes (6). Accelerating the adaptation process of athletes to altitude training so as to quickly improve the training effect is key to the success of altitude training. Some experts propose that hypoxic preconditioning before altitude training can decrease hypoxic stress, thereby reducing its impact on immune function and reducing infection.

Hypoxic preconditioning refers to a certain intensity and time of hypoxic exposure before hypoxic stress, which makes the body produce endogenous protection to enhance the body's tolerance to subsequent more severe hypoxia (7). Studies in animal models have shown that hypoxic preconditioning can reduce hypoxic damage and play a neuroprotective role in neonatal rats (8), and can significantly increase the exercise capacity of the rats in a hypoxic environment after hypoxic preconditioning (9). A clinical trial demonstrated that hypoxic preconditioning mobilizes endogenous protective mechanisms in the human

body and facilitates an active adaptive response to oxidative stress, inflammatory responses, apoptosis, and excitotoxicity caused by ischemia and hypoxia (10). Furthermore, competitive sports studies have found that 2-week hypoxic preconditioning combined with 4-week altitude training can improve the oxygen carrying capacity of blood, and has a positive effect on the physical function status of swimmers (11). Additionally, 10-day hypoxic preconditioning combined with 8-week sub-altitude training was shown to improve the oxygen carrying capacity of blood and physical function status of rowers (12). However, there are few studies on the effects of hypoxic preconditioning combined with altitude training on the immune system of athletes.

The complement system is activated when pathogens enter the blood circulation. The system is not only an innate immune response of the host against pathogen invasion, but is also a fundamental part of adaptive and humoral immunity (13). Complement regulatory proteins can regulate the progression of the complement cascade at all levels to protect autologous cells (14). Decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) are 2 of the 4 major membrane-binding complement regulatory proteins, and are widely expressed in all circulating cells and most human tissues. CD55 accelerates the degradation of invertase C3 and C5 and prevents the formation of new enzymes. CD59 interferes with the formation of the membrane attack complex (MAC) (15). CD55 and CD59, in addition to being complement inhibitors, are involved in the signaling of specific cellular receptors of the immune system or as ligands for these receptors. They participate in the activation and proliferation of lymphocytes and macrophages, and play important roles in regulating antigen presenting cells and regulating the secretion of cytokines and proinflammatory molecules (16).

This study investigated the changes in CD55 and CD59 expression on the surface of blood cells, as well as immune function in swimmers during hypoxic preconditioning combined with altitude training. The purpose was to further improve the current understanding of the effects of hypoxic preconditioning combined with altitude training on the complement regulation system and the immune function of athletes, so as to provide a reference and scientific basis for this mode of training. The results of this study are of great significance for better understanding the physical function status of athletes, the promotion of fatigue recovery, and monitoring immune levels during altitude training.

We present the following article in accordance with

the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/apm-20-2379>).

Methods

Study subjects

This study included 9 male swimmers from Shanghai. The mean age was 16.9 ± 3.5 years, mean height was 176.6 ± 6.5 cm, mean weight was 74 ± 5.3 kg, and the mean training year was 5.8 ± 2.2 . All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Training arrangements

Living high-training low (HiLo) was performed for 3 weeks in the hypoxic laboratory of the Shanghai Research Institute of Sports Science. The initial simulated altitude was 1,500 m and increased to 2,200 m 3 days later until the end of the hypoxic preconditioning. The participants slept at the hypoxic laboratory from 19:00 pm to 6:00 am, 6 days a week, and underwent normal training in normoxic conditions during the day.

On the second day after the hypoxic preconditioning, they flew to Kunming (2,200 m above sea level) for 3-week altitude training. The training content and training intensity was consistent with the hypoxic preconditioning phase, and was led by the same coach.

Fasting venous blood was collected at 6:00 am for testing 4 times: before the start of hypoxic preconditioning, in the first week of hypoxic preconditioning, at the end of hypoxic preconditioning (i.e., before the start of altitude training), and at the end of altitude training.

Flow cytometry to detect the expression of CD55 and CD59 on the surface of red blood cells (RBCs) and white blood cells (WBCs)

The blood samples were collected into EDTA tubes. Sample preparation was performed within 24 hours after blood collection. The number of RBCs and WBCs expressing CD55 and CD59 was determined by direct fluorescence-labeled flow cytometry. Mouse anti-human CD55-FITC monoclonal antibody (10 μ L, Beckman Coulter, America) and mouse anti-human CD59-PE monoclonal antibody (10 μ L, Beckman Coulter, America) were added into two numbered tubes, respectively. Then, 0.2 μ L EDTA blood

sample was added to 50 μ L PBS solution, mixed thoroughly, and placed at room temperature in the dark for 30 min. PBS solution was then added, centrifuged at 1,000 rpm for 5 min, then the supernatant was aspirated. Finally, 300 μ L PBS solution was added and mixed for detection. CellQuest software (AMS Integrated, America) was used for data collection and analysis. Scatter diagrams with forward scatter and side scatter were used to distinguish blood cell populations. At least 10,000 cells were tested in each sample to analyze the relative expression of CD55 and CD59 on the surface of RBCs and WBCs.

RBC count and WBC count

A total of 1 mL EDTA blood sample and the Sysmex blood-cell counter were used for routine blood tests.

Detection of immune system indexes CD3%, CD4%, and CD8%

CD3%, CD4%, and CD8% were measured by Flow cytometry. Anticoagulant blood (50 μ L) was put into a clean tube, then 3 μ L mouse anti-human CD4+ monoclonal antibody labeled with RPE fluorescein and CD8+ monoclonal antibody labeled with FITC fluorescein were added and gently mixed. Anticoagulant blood (50 μ L) was put into another clean tube, and 3 μ L mouse anti-human CD4+ and CD8+ were added then gently mixed. After the two labeled tubes were incubated at room temperature in the dark for 15 min, 100 μ L hemolysin was added and gently mixed to destroy the RBCs. Then, 1 mL double-distilled water was added and placed at room temperature in the dark for 5 min. After centrifugation (1,200 rpm, 5 min), the supernatant was aspirated and RBC debris were removed. After centrifuging again (1,200 rpm, 5 min), the supernatant was collected. Finally, 0.5 mL PBS solution was added for detection. CellQuest software was used for detection and data analysis.

Detection of immunoglobulins IgG, IgM, and IgA

A total of 1 mL serum was used to detect immunoglobulins IgG, IgM, and IgA with the Hitachi 7100 automatic biochemical analyzer.

Statistical analysis

All data were analyzed using SPSS 19.0. After testing

Table 1 Changes in decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) expression in red blood cells (RBCs) after hypoxic preconditioning combined with altitude training (mean fluorescence intensity)

Characteristics	Before the start of hypoxic preconditioning	Hypoxic preconditioning week 1	At the end of hypoxic preconditioning (before the start of altitude training)	At the end of altitude training
RBC count ($10^{12}/L$)	5.01±0.23	5.13±0.31	5.25±0.34	5.67±0.27 ^{ab}
CD55 in RBCs	8.86±1.80	15.39±3.31 ^a	7.84±1.19 ^b	13.78±7.56 ^c
Change rate		73.70%	-6.84%	55.53%
CD59 in RBCs	23.86±4.19	46.94±9.49 ^a	20.31±5.54 ^b	28.56±8.25 ^{bc}
Change rate		96.73%	-14.87%	19.70%

^a, P<0.05 vs. before the start of hypoxic preconditioning; ^b, P<0.05 vs. hypoxic preconditioning week 1; ^c, P<0.05 vs. at the end of hypoxic preconditioning (before the start of altitude training).

normality, the indexes met the normal distribution. Repeated measures analysis of variance was used. All indexes all were expressed as mean ± SD, and P<0.05 indicated a significant difference.

Results

Effect of hypoxic preconditioning combined with altitude training on CD55 and CD59 expression in RBCs

As shown in *Table 1*, CD55 expression in RBCs increased substantially after the start of hypoxic preconditioning and increased by 73.70% after 1-week of preconditioning, which was significantly higher than before the start of hypoxic preconditioning (P<0.05). Furthermore, CD55 expression decreased to baseline levels at the end of preconditioning, which was not significantly different from levels before the start of preconditioning. CD55 expression increased significantly during the subsequent altitude training, and was significantly higher than before altitude training (P<0.05). The changes in CD59 expression were similar to that of CD55. CD59 first increased then decreased in the preconditioning phase, and then increased again during the altitude training phase with significant differences compared to before altitude training. RBC count during the hypoxic preconditioning phase continued to increase, but there was no significant difference overall. RBC count during altitude training was significantly higher compared to before the start of preconditioning and in the first week of preconditioning (P<0.05).

Effect of hypoxic preconditioning combined with altitude training on CD55 and CD59 expression in WBCs

As shown in *Table 2*, CD55 expression in WBCs decreased

significantly by 45.54% in the first week of hypoxic preconditioning (P<0.05), and remained at this decreased level at the end of hypoxic preconditioning. CD55 expression in WBCs increased substantially during altitude training, and was significantly higher than in the first week of hypoxic preconditioning and at the end of hypoxic preconditioning (before the start of altitude training) (P<0.05). CD59 expression in WBCs continued to decrease after the start of the hypoxic preconditioning phase, and reached minimum levels at the end of preconditioning, with a significant decrease of 58% compared to before the start of hypoxic preconditioning (P<0.05). CD59 expression also increased after the start of altitude training, and was significantly higher than that at the end of hypoxic preconditioning (before the start of altitude training). WBC count increased and then decreased during the hypoxic preconditioning phase. At the end of preconditioning, it was significantly lower than before preconditioning (P<0.05). WBC count increased again during altitude training, which was significantly higher than before preconditioning and before altitude training (P<0.05).

Effects of hypoxic preconditioning combined with altitude training on the immune system

As shown in *Table 3*, during hypoxic preconditioning, the changing trend of CD3% was not significant. A comparison of each phase with the phase before preconditioning showed that CD3% first decreased and then increased during hypoxic preconditioning, and decreased again at the end of altitude training, but there were no significant differences. During 3-week hypoxic preconditioning, CD4/CD8 showed an increasing trend, and reached the highest point at the end of hypoxic preconditioning, which was

Table 2 Changes in decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) expression in white blood cells (WBCs) after hypoxic preconditioning combined with altitude training (mean fluorescence intensity)

Characteristics	Before the start of hypoxic preconditioning	Hypoxic preconditioning week 1	At the end of hypoxic preconditioning (before the start of altitude training)	At the end of altitude training
WBC count ($10^9/L$)	6.29±1.36	6.76±1.29	5.26±1.54 ^a	7.05±1.61 ^{ac}
CD55 in WBCs	18.84±4.99	10.26±2.52 ^a	10.60±6.64	16.87±4.25 ^{bc}
Change rate	–	–45.54%	–43.73%	–10.46%
CD59 in WBCs	5.50±3.26	2.67±1.41	2.31±0.36 ^a	3.30±0.36 ^c
Change rate	–	–51.45%	–58.00%	–40.00%

^a, P<0.05 vs. before the start of hypoxic preconditioning; ^b, P<0.05 vs. hypoxic preconditioning week 1; ^c, P<0.05 vs. at the end of hypoxic preconditioning (before the start of altitude training).

Table 3 Changes in T lymphocytes and their subsets (CD3%, CD4%, CD8%, and CD4/CD8) and immunoglobulins (IgG, IgM, and IgA) during hypoxic preconditioning combined with altitude training

Characteristics	Before the start of hypoxic preconditioning	Hypoxic preconditioning week 1	At the end of hypoxic preconditioning (before the start of altitude training)	At the end of altitude training
CD3%	65.47±5.18	63.21±4.37	66.46±5.83	65.62±4.89
CD4%	33.47±3.25	32.21±3.19	33.54±3.43	31.26±3.07
CD8%	30.01±4.14	28.34±3.14	28.22±4.05	29.56±3.92
CD4/CD8	1.35±0.34	1.38±0.32	1.45±0.36 ^a	1.21±0.39 ^{bc}
IgG (g/L)	10.69±2.10	11.16±2.16	10.51±1.81	10.61±2.64
IgA (g/L)	1.62±0.45	1.57±0.67	1.55±0.34	1.58±0.52
IgM (g/L)	0.92±0.31	1.13±0.45	0.95±0.46	0.97±0.44

^a, P<0.05 vs. before the start of hypoxic preconditioning; ^c, P<0.05 vs. at the end of hypoxic preconditioning (before the start of altitude training).

significantly different from that before preconditioning (P<0.05). It began to decrease during altitude training and was significantly different from before altitude training (P<0.05).

In the hypoxic preconditioning phase, IgG and IgM increased and then decreased, while IgA continued to decrease, with no significant difference in each phase of preconditioning. In the altitude training phase, all three immunoglobulins increased slightly, but there was no significant difference in each phase of training.

Discussion

The expression of CD55 and CD59 on the RBC surface after hypoxic preconditioning increases significantly, which is a compensatory effect of the body on changes in the environment. A hypoxic environment induces immune cell

activation and affects immune function (17). CD55 and CD59 expression on the RBC surface were significantly increased in swimmers during the early phase of 2-week HiLo training and rowers during the early phase of 3-week HiLo training, indicating that CD55 plays a key role in preventing complement activation and preventing complement attack on RBCs (18,19). In this study, after 1-week of hypoxic preconditioning, CD55 and CD59 expression were higher than before training. Increased CD55 expression is beneficial for the maintenance of RBC stability and prevents the occurrence of cell hemolysis. After 1-week of hypoxic preconditioning, CD55 and CD59 expression in RBCs decreased significantly until the end of hypoxic preconditioning. The decreased expression of CD55 and CD59 proteins on RBC membranes may be due to impaired synthesis of GPI-anchors or abnormal coupling of the membranes and proteins on RBC precursor

membranes (20). Complement system disorder is partially responsible for increased hemolysis and tissue damage (21).

The significant increase in CD55 and CD59 expression in RBCs during altitude training is an adaptive adjustment of the body to re-hypoxic exposure. The adjustment is effective for maintaining the function of RBCs and reducing complement attack on RBCs. Hypoxic preconditioning combined with altitude training can increase the expression of CD55 and CD59, which is beneficial for resisting complement attack.

During 3-week hypoxic preconditioning, RBC count increased slightly, but there was no significant difference, suggesting that the expression of CD55 and CD59 on the RBC surface was not related to RBC count, but only related to the regulation of the complement system. During the following 3-week altitude training, RBC count increased substantially, and was significantly higher than before hypoxic preconditioning. Gao *et al.* (11) found that in a study of swimmers who underwent 2-week hypoxic preconditioning combined with 4-week altitude training, RBC count change did not change significantly during the hypoxic preconditioning phase. This result was consistent with our findings. They also found that RBC count reached the maximum value in the third week of altitude training, which was significantly higher than before hypoxia and in the first week of hypoxia, and decreased in the fourth week. Different times and modes of hypoxic training have different effects on RBCs, which may be related to the different height and intensity of hypoxic training.

The WBC count first increased and then decreased during hypoxic preconditioning, which was consistent with the results of Gao *et al.* (22) who examined changes in WBCs during HiLo training of rowers. The increase in WBC count at the early phase of preconditioning was likely to be a stress phenomenon of the body to the hypoxic environment, while the subsequent decrease in WBC count might be due to the deepening of hypoxic stimulation and the training load, resulting in greater impact on the body. Wang *et al.* (23) found that in a study of boxers who underwent 4-week HiLo, WBC count decreased in the first week, and then increased until the end of HiLo, with no significant difference at each phase. This result is different from our findings. In our study, the WBC count decreased significantly at the end of hypoxic preconditioning, which might be related to different training load and time. Most studies have found that the WBC levels of athletes in different events decreases significantly during sub-altitude training and altitude training (24). In this study,

during altitude training, WBCs significantly increased and immunosuppression was weakened in swimmers, suggesting that after 3-week hypoxic preconditioning, the body adapted well to altitude training, and hypoxic preconditioning improved the immunity of the athletes. At the same time, this study also found that the changes in CD55 and CD59 expression on the WBC surface was not significantly related to WBC count, which is consistent with the results of Zhao *et al.* (25) who demonstrated that the expression of CD55 and CD59 on the WBC surface is related to the complement regulatory system itself and not the WBC count.

The function of CD55 and CD59 is not limited to the innate immune system by regulating the complement system. It has been shown that CD55, CD59, and other complement regulators are able to reduce T cell responses and proliferation (26), which may explain the expression of CD55 and CD59 in WBCs and its subtypes. The expression of CD55 and CD59 varies greatly in different cell lines, while CD59 is more highly expressed on RBCs (27), which is also consistent with the results of this study. In this study, the expression of CD59 in RBCs was significantly higher than in WBCs. It has been reported that the regulation of CD55 and CD59 by exposure to nucleated cells, proinflammatory molecules, and cytokines (e.g., C-reactive protein, TNF- α , IL-1) is an adaptive response to a chronic inflammatory state (28), associated with long-term systemic complex activation (29). However, the biological functions of these molecules are related to the cell membranes which express CD55 and CD59. RBC membrane proteomes, unlike WBCs (as well as other nucleated cells), are rarely altered but remain constant in the activated state of the cells (30). This may be why the expression of CD55 and CD59 on the RBC surface is not the same as that shown in this study.

Artificial simulated hypoxic training had no significant effect on T lymphocytes in athletes. Gao *et al.* (31) found that in a study of the immune function of female rowers who underwent 4-week HiLo, CD4 and CD4/CD8 increased significantly after the start of HiLo until the end of training, while CD8 did not change significantly during the whole process. This result is similar to our findings. In this study, CD4/CD8 was significantly increased during 3-week hypoxic preconditioning, suggesting that hypoxic preconditioning in HiLo mode improved the immune function of the athletes. During the subsequent altitude training, CD4/CD8 continued to decrease, indicating that the altitude training model inhibited cellular immunity. At the end of the altitude training, although CD4/CD8 was

significantly lower than before altitude training, it was not significantly different from that before the start of hypoxic preconditioning. This indicates that altitude training after hypoxic preconditioning inhibited T lymphocyte immunity, but did not have a significant effect on immune function, only on immune balance. The reasons behind this are likely related to the increase of RBCs and hemoglobin during hypoxic preconditioning, which improved the oxygen carrying ability of blood and the aerobic capacity of muscles, which in turn improves immunity.

CD3 first decreased and then increased during the hypoxic preconditioning phase, but there was no significant difference, suggesting that the hypoxic preconditioning phase had little effect on CD3. This result is similar to the previous finding that CD3 does not change significantly after 3-week HiLo in rowers. Xu *et al.* (32) found that by comparing four different hypoxic preconditioning modes, the hypoxic preconditioning mode of HiLo had little effect on T lymphocyte immunity. Chang *et al.* (33) found that in a study of 3 sub-altitude training modes on the Chinese Short Track Speed Skating Team, CD3 decreased significantly after the end of 3-week altitude training, indicating that altitude training greatly stimulated CD3. In this study, although CD3 decreased slightly at the end of altitude training after 3-week hypoxic preconditioning, there was no significant difference compared with that before hypoxic preconditioning and before altitude training, indicating that hypoxic preconditioning caused an adaptive immune response.

There are few studies on the effects of altitude training on immunoglobulins. Wang *et al.* (34) found that the levels of the three immunoglobulins in rowers did not change significantly during 8-week sub-altitude training (1,400 m), and IgM showed a significant increase 2 weeks after leaving the altitude environment. Furthermore, Gao *et al.* (35) found that the levels of the 3 immunoglobulins in rowers were basically stable without significant changes during 8-week altitude training (2,800 m). In this study, we found that although the trends of IgG, IgM, and IgA were different during hypoxic preconditioning, the overall changes were not significant. At the end of hypoxic preconditioning, the levels of all three immunoglobulins tended to decrease, indicating that hypoxic preconditioning inhibited cellular immunity. The small increase in the three immunoglobulins during altitude training suggested that the cellular immune response tended to improve. However, considering that the range of change of the immunoglobulins was not large, and there was no significant difference in each phase of hypoxic

preconditioning combined with altitude training, it is possible that hypoxic preconditioning and altitude training had little effect on cellular immunity.

Conclusions

Our study demonstrated that during hypoxic preconditioning, the complement regulation system and RBC immunity were inhibited, and T lymphocyte immunity was out of balance. After hypoxic preconditioning combined with altitude training, the expression of CD55 and CD59 on the surface of RBCs and WBCs increased significantly, and T lymphocyte CD4/CD8 increased. These results suggest an improvement of the complement regulation system and RBC immune function after hypoxic preconditioning. Hypoxic preconditioning can improve immune cell levels, and therefore have a beneficial effect on the physical function of athletes during altitude training.

It is suggested that appropriate hypoxic preconditioning be performed before altitude training in the future, which can improve the body's immune function and reduce the risk of disease during altitude training.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

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