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Effect of acupuncture stimulation on salivary human beta-defensin 2 after a single strenuous exercise in young male subjects

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Abstract

[Objective] The aim of this study was to examine the effect of acupuncture stimulation on mucosal immune function after a single period of intense exercise by measuring salivary human beta-defensin 2 (hBD2).

[Methods] Seven healthy young men (age, 23.4 ± 0.6 years) randomly participated in two experimental sessions: exercise–rest (ex–rest) and exercise–acupuncture (ex–acp) experiments, with a crossover design. Subjects exercised on a cycle ergometer for 60 min at 75% of their maximal oxygen uptake. In the ex–acp experiment, acupuncture stimulation was applied to meridian points (LU6, LI4, ST36, and ST6) for 30 min after the exercise session. In the ex–rest experiment, subjects rested without acupuncture. Saliva samples were collected before exercise, immediately after the exercise, and 1, 2, 3, and 24 h after exercise. Samples were evaluated for hBD2 concentrations using enzyme-linked immunosorbent assay. Salivary total protein (TP) was measured using Pierce 660nm Protein Assay. Finally, the salivary hBD2 level was shown as an hBD2-to-TP ratio (hBD2/TP).

[Results] The salivary flow rate significantly increased after 2 h in the ex–acp subjects ($P < 0.05$), whereas there was no significant change in the ex–rest experiment. The hBD2 concentration did not significantly change in the ex–rest and ex–acp experiments. The hBD2/TP did not significantly change in ex–acp experiment. In contrast, the hBD2/TP of the ex-rest experiment significantly decreased immediately, 1, and 2 h after exercise ($P < 0.05$).

[Conclusion] Acupuncture stimulation attenuated the decrease in hBD2 level induced by strenuous exercise. These results suggest that acupuncture treatment might contribute to improving the impairment of mucosal immune function mediated by hBD2 following intensive exercise.

Key words: *Acupuncture, oral immune function, saliva, anti-microbial peptide, strenuous exercise*

I. Introduction

Acupuncture is part of oriental traditional medicine and has been used by many populations to treat a variety of symptoms for a long time. It has been used for the treatment of chronic pain such as low back pain, osteoarthritis, and tendinitis in recent years[1)]. Therefore, many athletes have had their orthopedic diseases treated by acupuncture for maintaining and enhancing their physical performances[2,3)]. However, acupuncture treatment has had reported efficacy not only in orthopedic disorders but also in medical diseases such as upper respiratory tract infections (URTIs) and asthma[4,5)].

Athletes regularly undertake intensive training to improve their physical performances. However, repetitive intensive exercise can lead to disorders such as infections, overtraining syndrome, and chronic fatigue[6)]. URTI, particularly constitute the most frequent infection in highly trained athletes[7)]. Reports of URTIs in exercising populations tend to favor a higher incidence in endurance athletes than that in sedentary or moderately exercising subjects in various sports[8)]. In fact, most athletes experience the morbidity of URTI despite major competitive conditions[9)]. Therefore, it is necessary to establish an effective program for the prevention of URTIs in athletes.

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Most pathogens causing URTIs invade mucosal tissue such as the oral cavity, respiratory tract, and nose. Antimicrobial peptides (AMPs) have broad-spectrum antimicrobial activity against bacteria, viruses, and fungi by preventing the attachment of infectious agents to mucosal surfaces. Human beta-defensin2 (hBD2) is an epidermal AMP and plays a crucial role in protecting the host from pathogenic invasion through the inhibition of viral entry and disruption of viral envelope[10,11]. Low level of salivary hBD2 is reported to be associated with a high incidence of URTI[12]. Therefore, salivary hBD2 contributes to preventing URTIs.

There is little information on the response of salivary hBD2 to exercise. Previous work in our laboratory indicated that salivary hBD2 levels decreased after an acute bout of intensive exercise in sedentary subjects[13] and that continuous intensive training decreased the resting salivary hBD2 in athletes (Hanaoka et al., submitted). Furthermore, Usui et al.[12] reported that the resting salivary hBD2 was significantly lower in elite marathon runners than in sedentary subjects as evaluated in a cross-sectional study.

Acupuncture treatment is reported to have an effect on URTI symptoms[8], however, there are few reports examining the efficacy of acupuncture with respect to immune function. Secretory immunoglobulin A (SIgA) is one of the proteins, which have crucial role of host defense against URTI. Thus, decreased SIgA levels following intensive exercise are linked to elevated susceptibility to URTI[14,15]. Continuous acupuncture stimulation has been reported to counteract intense exercise-induced decrease in SIgA and, moreover, had positive effects on physical condition in athletes during competitions[3]. In addition, acupuncture stimulation attenuated SIgA decrease following an acute bout of intensive exercise[16]. However, there has been no study demonstrating the effects of acupuncture on the salivary hBD2 response to acute exercise. Demonstration of the availability of acupuncture treatment on salivary hBD2 might contribute to the establishment of an effective conditioning program in terms of improvement of mucosal immune function and prevention of URTIs.

The purpose of this study was to examine the effect of acupuncture stimulation on salivary hBD2 after acute high-intensity exercise. We hypothesized that acupuncture treatment can abate the decrease in salivary hBD2 induced by intensive exercise.

Ⅱ. Materials and Methods

1. Subjects

Seven healthy sedentary young men (age, 23.4 ± 0.6 years) participated in this study (Table 1). All subjects provided written informed consent prior to study participation. None of the subjects smoked, exercise habit or was taking any type of medication. All subjects did not conduct intensive exercise during the study period. They took part in two experimental sessions: (1) control (exercise–rest: ex–rest) experiment and (2) acupuncture treatment (exercise–acupuncture: ex–acp) experiment, with a crossover design. There was a time period of a week or more between two experiments. The study de-

Table 1 characteristics of subjects.

	ex-rest	ex-acp
n	7	
Age (yr)	23.3 ± 1.8	
Height (cm)	172.6 ± 2.6	
Body mass (kg)	66.1 ± 5.2	
Fat (%)	17.4 ± 2.5	
Body Mass Index (kg/m^2)	22.2 ± 1.2	
VO_{2max} (ml/kg/min)	44.5 ± 5.0	
Maximal Borg scale during exercise	18.4 ± 1.1	17.7 ± 0.6
Maximal Heart Rate during exercise (bpm)	176.4 ± 6.7	171.0 ± 10.2

Subjects took part in two experimental sessions: (1) control (exercise–rest: ex–rest) experiment and (2) acupuncture treatment (exercise–acupuncture: ex–acp) experiment, with a crossover design.
All values are described as mean ± SD.

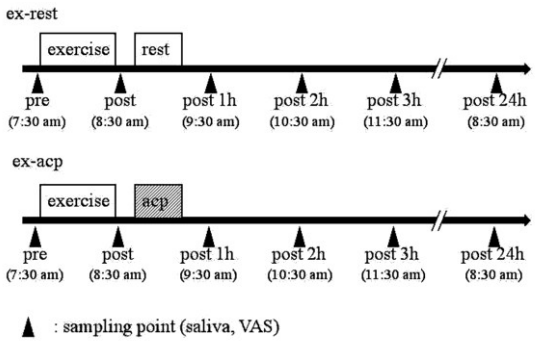

Fig. 1 Experimental design of the study

* Saliva collect method: Saliva production was stimulated by chewing a sterilized cotton swab for 2 min at a frequency of 1 chew per second.
Subjects took part in two experimental sessions: (1) control (exercise–rest: ex–rest) experiment and (2) acupuncture treatment (exercise–acupuncture: ex–acp) experiment, with a crossover design.
In both experiments, subjects performed an exercise on a bicycle, equipped with an ergometer, for 60 minutes at 75% VO_{2max}. After the exercise, subjects in ex-acp experiment were administered acupuncture stimulation for 30 minutes, while subjects in ex-rest experiment rested in recumbent position.

sign is presented diagrammatically in Fig. 1. Subjects were required to abstain from alcohol, caffeine, and strenuous activity to remain in the trial. This study, which conforms to the principles outlined in the Declaration of Helsinki, was approved by the Ethics Committees of the University of Tsukuba (The authorization number: 24-57).

2. Preliminary measurements

The maximal oxygen uptake (VO_{2max}) of the subjects was determined through the use of an incremental bicycle exercise test that was undertaken approximately 1 week before commencing the main trial. Subjects performed a continuous incremental exercise test on a cycle ergometer bicycle (AE310S; Minato Medical Science, Osaka, Japan) until exhaustion and their breath-by-breath oxygen uptake and carbon dioxide production. The protocol consisted of 2 min of unloaded pedaling and subsequent incremental exercise. The workload was increased to 60, 80, and 100 W for 2 min. Subsequently, the workloads increased by 30 W every 3 min until exhaustion. Objective criteria for maximal effort included at least two of the following; (1) increased workload without corresponding increase in VO_2; (2) respiratory exchange quotient equal to or greater than 1.10; or (3) pedal cadence less than 50 rpm despite maximal voluntary effort. The highest O_2 uptake over a 30-s period was defined as VO_{2max}. After at least 1 week of the VO_{2max} test, subjects underwent a submaximal cycle exercise test. In the submaximal cycle exercise test, subjects exercised on a cycle ergometer for 1 h at 75% of their VO_{2max}. This preliminary measurements procedure was tried a similar method of a previous study[17].

3. Saliva collection

Saliva samples were collected at rest before exercise (pre, 7:30 am); immediately after exercise (post, 8:30 am); 1, 2, and 3 h after exercise (post 1 h, 9:30 am; post 2 h, 10:30 am; post 3 h, 11:30 am); and 24 h after exercise (post 24 h, 8:30 am). Subjects came to the laboratory without breakfast (except for water). Subjects were free to drink water during the experiment. They rinsed their mouths thrice for 30 s with 100 ml of mineral water and then rested for at least 5 min. Saliva production was stimulated by chewing a sterilized cotton swab (Salivette; Sarstedt, Germany) for 2 min at a frequency of 1 chew per second. Saliva was separated from the cotton swab by centrifugation at 3,000 rpm for 15 min. Saliva flow rate (ml/2 min), which is volume of secreted saliva during 2 min, was converted to milliliters based on an assumed saliva density of 1 g/ml, as described in previous studies[16]. After the sample volume was measured, samples were frozen at −80°C and stored until analysis.

4. Salivary hBD2 concentration

Salivary hBD2 concentration was measured by enzyme-linked immunosorbent assay (ELISA), using a commercially available ELISA kit for hBD2 (Phoenix Pharmaceuticals Inc., USA).

5. Subjective evaluations of physical fatigue conditions

To assess subjective physical fatigue condition, a visual analogue scale (VAS) ranging from 0 (no fatigue) to 100 mm (extreme fatigue) for self-perceived fatigue was used at the same points as the sample collections.

6. Acupuncture stimulation

After the submaximal cycle exercise test, acupuncture stimulation was performed by an experienced acupuncturist. As described in a previous study[16], the following bilateral acupuncture points were used LU6 (Kongzui, Kousai) in the forearms, LI4 (Hegu, Goukoku) in the hands, ST36 (Zusanli, Ashi no Sanri) in the legs, and ST6 (Jiache, Kyousha) on both sides of the face (Fig. 2). After standard disinfecting of the insertion sites, disposable stainless steel needles (50-mm long, 0.20 mm in diameter; SEIRIN, Shizuoka, Japan) were inserted and manipulated until the subjects felt a sensation from the needles. Electrodes were connected to three points (LU6, LI4, and ST36) and then attached to an electric stimulator (OhmPulser LFP-4800; Zen Iryoki, Fukuoka, Japan). The points were electrically stimulated with a low-frequency current of 2 Hz for 30 min. At the same time, the fourth point (ST6) was manually stimulated for 30 min, every 5 min, until needle sensation was reached.

7. Statistical Analyses

All data were represented as means ± standard deviation (SD). For all analyses, $p < 0.05$ was inferred as statistically significant. All variables were analyzed using Wilcoxon signed-rank test. Comparison at each time (pre, post, post 1 h, post 2 h, post 3 h, and post 24 h) between experiments was applied to t-test. Statistical analysis was performed using a StatView ver. 5.0 (SAS Institute Inc., Japan).

Ⅲ. Results

Saliva flow rate

Table 2 shows the changes of saliva flow rate in the ex–rest and ex–acp experiments. There is no significantly between each point. The saliva flow rate significantly increased at post 2 h in the ex–acp experiment ($P < 0.05$), whereas in the ex–rest experiment, there was no significant change.

Salivary hBD2 concentration

Table 2 shows the changes of salivary hBD2 concentration in the ex–rest and ex–acp experiments. There is no significantly between each point. The salivary hBD2 concentration in the ex-rest significantly decreased immediately post, post 1 h, and post 2 h ($P < 0.05$), otherwise, the ex–acp experiment, the hBD2/TP did not significantly change.

Physical fatigue

Table 3 shows the change of VAS score relating to subjective fatigue in ex–rest and ex–acp experiments. There is no significantly between each point. The VAS score significantly increased for both experiments immediately after the exercise ($P < 0.05$).

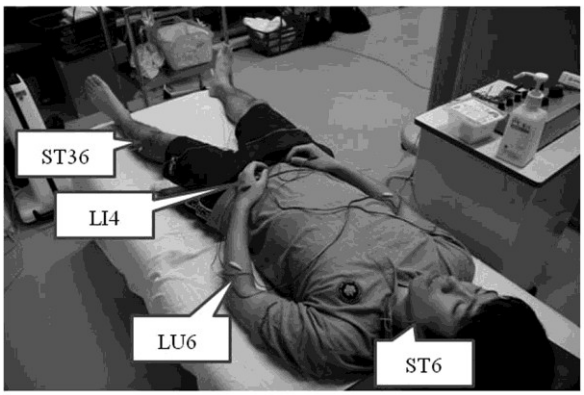

Fig. 2 The situation of an acupuncture stimuli

Acupuncture needles were inserted through the skin to a depth of 5-10mm, and manipulated until the subjects felt a sensation from the needles. Electrodes were connected to three points (left LI4-left LU6, right LI4-right LU6, left ST36-right ST36) and then attached to an electric stimulator. The points were electrically stimulated with a low frequency of 3Hz for 30 min. At the same time, the forth point (ST6) was manually stimulated for 30 minutes until needle sensation was reached every 5 minutes.

Table 2 The change of saliva data in ex-rest and ex-acp experiments

	pre (7:30)	post (8:30)	post 1h (9:30)	post 2h (10:30)	post 3h (11:30)	post 24h (8:30)
Saliva flow rate (ml/2min)						
ex-rest	1.8±1.0	1.9±0.8	2.2±0.7	2.4±0.7	2.4±0.7	2.1±0.8
ex-acp	1.9±0.7	1.9±0.5	2.5±0.6	2.7±0.7*	2.3±0.8	2.2±0.9
hBD2 concentration (pg/ml)						
ex-rest	552.6±585.1	288.3±315.9*	236.9±256.6*	201.2±186.5*	299.6±278.0*	454.1±599.8
ex-acp	606.6±844.1	491.7±654.3	574.7±775.2	384.6±449.7	532.2±606.0	777.4±403.1

Saliva samples were collected at rest before exercise (pre, 7:30 am); immediately after exercise (post, 8:30 am); 1, 2, and 3 h after exercise (post 1 h, 9:30 am; post 2 h, 10:30 am; post 3 h, 11:30 am); and 24 h after exercise (post 24 h, 8:30 am). Saliva flow rate (ml/2 min), which is volume of secreted saliva by chewing a sterilized cotton swab for 2 min, was converted to milliliters based on an assumed saliva density of 1 g/ml, as described in previous studies. Salivary hBD2 concentration was measured by enzyme-linked immunosorbent assay (ELISA).

Subjects took part in two experimental sessions: (1) control (exercise–rest: ex–rest) experiment and (2) acupuncture treatment (exercise–acupuncture: ex–acp) experiment, with a crossover design. n = 7

All values are described as mean ± SD.

Significant difference: * $P < 0.05$ vs. pre.

Table 3 The changes of subjective fatigue in ex-rest and ex-acp experiments

	pre (7:30)	post (8:30)	post 1h (9:30)	post 2h (10:30)	post 3h (11:30)	post 24h (8:30)
Subjective fatigue (mm)						
ex-rest	31.3±17.0	69.4±22.4*	44.9±12.9	27.3±8.9	27.3±14.1	26.6±10.0
ex-acp	33.3±21.3	66.4±13.9*	42.4±8.4	33.4±8.7	27.1±5.9	30.9±16.8

Subjects took part in two experimental sessions: (1) control (exercise–rest: ex–rest) experiment and (2) acupuncture treatment (exercise–acupuncture: ex–acp) experiment, with a crossover design. n = 7

The visual analogue scale for subjective physical fatigue was used at the same points as the sample collections. This rating was assessed using a 100 mm continuous scale, ranging from 0 (no fatigue) to 100 mm (extreme fatigue).

All values are described as mean ± SD.

Significant difference: * $P < 0.05$ vs. pre.

Ⅳ. Discussion

The present study investigated the effect of acupuncture on mucosal immune function modulated by salivary hBD2 after a single bout of intensive exercise. The results of this study revealed that acupuncture stimulation attenuated the decrease in hBD2 level induced by intensive exercise. Therefore, acupuncture stimulation might contribute to improving impaired mucosal immune function following intensive exercise.

Salivary hBD2 contributes to preventing URTIs through antiviral activities, such as inhibiting viral entry and disrupting viral envelope[10]. In this study, salivary hBD2 significantly decreased after intensive exercise until post 2 h in the ex-rest experiment. Furthermore, a previous study in our laboratory indicated that intensive exercise transiently decreased salivary hBD2[13]. In contrast, Usui et al.[18] reported that intensive exercise transiently increased salivary hBD2. However, new data in our laboratory showed that continuous intensified exercise decreased resting salivary hBD2 secretion (Hanaoka et al., submitted). In the cross-sectional study, resting salivary hBD2 in elite marathon runners was lower than that in sedentary subjects[12]. Accordingly, it is possible that high-intensity exercise might down-regulate oral immune function modulated by salivary hBD2, thereby increasing susceptibility to URTIs. Exercise intensity of using this study 75% VO_{2max} was estimated high intensity by ACSM[19]. Therefore, this exercise intensity corresponded to high intensity. Additionally, many previous studies have been adopted similar exercise intensity to decreased immune function.

Acupuncture has been used to treat URTIs[8]. In addition, acupuncture stimulation was reported to activate cytokines and immune cells, suggesting that it can up-regulate immune function[20]. In this study, salivary hBD2 did not show significant change after exercise in the ex-acupuncture experiment, whereas hBD2 decreased after exercise until post 2 h in the ex–rest experiment. Therefore, acupuncture stimulation might suppress intensive exercise-induced decrease in hBD2 level and, therefore, has the potential for bolstering hBD2 secretion. Considering this result, the benefit of acupuncture for immune function might be important to initiate treatment as early as possible following exercise training. Future research should investigate the development of more effective and specific protocols relating to acupuncture stimulation for bolstering mucosal immune function.

In these results of the study, salivary hBD2 concentration in ex-acp experiment did not change but in ex-rest experiment statistically decreased after the exercise. These results raised the possibility that there is not sufficient volume occurred the depressed of the hBD2 in ex-acp experiment. However, the protocol of this study adopted same subjects with crossover design and they were monitored their VO_2 to maintain their exercise intensity during exercise test in both experiments. Additionally, value of subjective fatigue was similar in both experiments. Although salivary SIgA level did not change immediately after the intensive exercise, it decreased significantly after 30 min and 60 min of exercise[18]. Therefore, hBD2 concentration might possibly decrease at 60 min after the exercise in ex-acp experiment. Future study needs to examine the hBD2 reactivity to exercise and the effect of acupuncture on hBD2 response to exercise. hBD2 plays an important role in the defense against pathogen infection by effective killing of Respiratory Syncytial virus and Influenza virus A such as occurring in URTI. In this study, the experiment using acupuncture stimulation after intensive exercise did not decrease hBD2 concentration.

The molecular mechanisms underlying the down-regulation of hBD2 by intensive exercise have been unclear. In the previous study, many different cytokines regulated the expression of hBD2. Tumor necrosis factor (TNF)-□ and interleukin (IL) -1□ enhance or IL-17 from T-helper (Th) 17 cell moderate induced the expression of hBD2. Although TNF-□ and IL-1□ have traditionally been understood to be the main inducer cytokines of acute phase reactions, the majority of studies have shown that the circulating concentration of these cytokines is either unchanged following exercise load in this study[21]. Possible mechanisms on the expression of hBD2 might be related to dendritic cell (DC) and cytokine responses to exercise. DC stimulate Th17 cell through IL-23 to produce IL-17, thereby inducing the secretion of hBD2 from epithelial cells. Sugama et al.[22] reported that strenuous long-lasting endurance exercise, such as a duathlon race (consisting of 5 km of running, 40 km of cycling, and 5 km of running), decreased IL-17 and IL-23 levels in plasma and urine, respectively. In this study, it is possible that decreases in IL-17 and IL-23 under intensive exercise can induce the down-regulation of hBD2 production. However, these previous studies about regulating hBD2 reported in blood or urine not in tissue. In the future study, there will the research using the animal model to investigate for more detailed mechanism of exercise on hBD2.

The result of this study suggested that acupuncture stimulation can attenuate the decrease in hBD2 after intensive exercise. Acupuncture relieves symptoms by triggering the body to produce more endorphins. Guo et al.[23] suggested that acupuncture can stimulate peripheral nerves through the brain to release endogenous opioids. Moreover, endogenous opioids such as endorphins might regulate the production of cytokines by Langerhans cell, which is one of the dendritic cells in epidermal tissue[24]. It is, therefore, possible that acupuncture might stimulate dendritic cells through up-regulation of endorphins to produce IL-23 and then activated the production of IL-17 from Th17, leading to an induced secretion of hBD2.

However, there is no information on relationship between acupuncture and cytokines, such as IL-17 and IL-23. Future studies must examine the effects of acupuncture on mechanisms of hBD2 secretion through immune cells and cytokines.

The present study has a certain limitation. It is necessary for athletes to have more information on effective methods of acupuncture treatment, which is one of the most effective medical treatments for athletes training for competition. However, there is little information on the effects of acupuncture on immune response to exercise. Therefore, future research needs to determine the combination of acupuncture points and quality of stimulus used in acupuncture for the up-regulation of immune function. Furthermore, this could contribute to the establishment of an effective conditioning program for the prevention of URTIs.

In this study, it was observed the differences response to exercise on hBD2 both experiments, though the exercise conducted under the same condition. In the ex-acp experiment, hBD2 did not decreased immediately after the exercise but there is the possibility that the hBD2 might be decreased at post 1h, post 2h after the exercise. Because hBD2 decreased immediately post, post 1h and post 2h after the exercise in the ex-rest experiment. In future, it is necessary to further test the accuracy of this protocol.

Ⅴ. Conclusions

In conclusion, acupuncture stimulation attenuated the decrease in salivary hBD2 level, induced by intensive exercise. These results suggested that acupuncture stimulation might contribute to improve the impairment of mucosal immune function mediated by hBD2, following intensive exercise. Accordingly, acupuncture may be a useful conditioning method for bolstering immune function and preventing URTIs.

Acknowledgements

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Conflict of interest

The authors have no conflicts of interest to disclose.

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