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ORIGINAL RESEARCH ARTICLE

# Head-out immersion in hot water does not increase serum CXCL1 in healthy men

***Yuta Sakurai[1], Yasunori Umemoto[2], Takashi Kawasaki[2], Daisuke Kojima[2], Tokio Kinoshita[2], Mami Yamashiro[2], Motohiko Banno[1], Hideki Arakawa[3], Fumihiro Tajima[1,2*]***

*[1] Research Center of Sports Medicine and Balneology, Nachikatsuura Balneologic Town Hospital, Tenma-Nachikatuurachou, Higashimuro gun, Wakayama, Japan*
*[2] Department of Rehabilitation Medicine, Wakayama Medical University, Kimiidera, Wakayama, Wakayama, Japan*
*[3] Department of Rehabilitation Medicine, School of Medicine, Yokohama City University, Kanazawa-ku, Yokohama, Kanagawa, Japan*

## ABSTRACT

Exercise-induced production of interleukin (IL)-6 results in the expression of chemokine CXC-motif ligand 1 (CXCL1) in mice. Recent studies described the increase in serum IL-6 levels during immersion of subjects in hot water. The present study investigated the effects of a 20-min head-out immersion in 42 °C water (hot-HOI) on serum concentrations of CXCL1 in eight healthy men. Venous bloods were taken at rest, immediately after hot-HOI, as well as 1, 2, 3, and 4 h after hot-HOI for measurements of serum concentrations of CXCL1, IL-6, tumor necrosis factor (TNF)-α, and high-sensitivity C-reactive protein (hsCRP), while assessing counts of blood cells (CBC) and monitoring core temperature ($T_{core}$). $T_{core}$ and serum IL-6 increased during hot-HOI and remained high until 4 h after hot-HOI. However, serum CXCL1, TNF-α, hsCRP, and CBC remained constant throughout the experiment. In conclusion, the results from our study demonstrated that 20-min hot-HOI increased serum IL-6, but not CXCL1 in healthy men.

***Keywords**: IL-6; TNF-α; hsCRP; hyperthermia; myokine*

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***CORRESPONDING AUTHOR**
Fumihiro Tajima, Department of Rehabilitation Medicine, Wakayama Medical University, 811-1 Kimiidera, Wakayama, Wakayama 641-8509, Japan; fumi@wakayama-med.ac.jp

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## Introduction

During systemic inflammatory state such as sepsis, serum levels of tumor necrosis factor (TNF)-α markedly increase just after an increase in serum interleukin (IL)-6. In contrast, the marked increase in serum level of IL-6 is not preceded by a rise in TNF-α during exercise[1]. Keller *et al.* suggested that the releasing of IL-6 from contracting skeletal-muscle fibers is mainly caused by *IL-6* gene transcription[2]. Therefore, IL-6 is considered a myokine[3,4]. Estimates from recent proteomic studies predict that the number of myokines would be more than 600 and belonging to different families[5].

What is the mechanism of exercise muscle-induced production of IL-6? While the exact stimulus is still being investigated, evidence suggests that local heating can act as a stimulus to express *IL-6* mRNA in skeletal muscle of mouse, *i.e.* the skeletal muscle acts as a "heat stress sensor"[6,7]. In addition, other studies showed that hot water immersion significantly increases IL-6 in humans, and concluded that skeletal muscles secrete IL-6 in response to exercise and heat exposure[8].

Human chemokine CXC-motif ligand 1 (CXCL1) is a 6.4-kDa polypeptide, classified as a chemokine that plays a role in recruiting leukocyte in inflammatory and immune reactions[9]. However, Nedachi *et al.* demonstrated that CXCL1 also works when released from contracting skeletal muscles[10]. Mice, in which the tibialis cranialis muscle over-expresses CXCL1, have lower visceral and subcutaneous fat mass, and the CXCL1-dependent decrease in adipose tissue mass coincides with improvements in glucose tolerance and insulin sensitivity in the whole

body[11]. Other studies have shown that hepatic expression of CXCL1 mediates the liver-protective potential of IL-6, and that CXCL1 is involved in wound healing, acts as a protectant against multiple sclerosis, and has potential neuroprotective effects[12-14].

The overexpression of IL-6 in murine muscles is followed by marked increase in serum level of CXCL1 and mRNA expression of *CXCL1* in the liver[15]. In an experimental study involving wild-type mice, 1 h of swimming exercise induced increases in serum IL-6 immediately after exercise, and in serum CXCL1 2 h after exercise, whereas the same exercise did not modulate liver *CXCL1* mRNA expression in IL-6 knockout mice[15]. The results suggest a tight muscle–liver crosstalk during exercise, in which exercise-induced IL-6 induces a production of CXCL1 in the liver[15].

Based on the above findings, we hypothesized that heat stress-induced IL-6 increases CXCL1 expression in humans. To test our hypothesis, we measured in the present study serum CXCL1 levels before, during and after 20-min head-out immersion in 42 °C water (hot-HOI) in a group of normal adults.

## Materials and methods

### Subjects

Eight healthy males voluntarily participated in the present study. **Table 1** lists the characteristics of these subjects. None of the subjects were on any medications at the time of the study.

**Table 1.** Characteristics of the eight participating subjects

| Variable | Healthy males (mean ± SD) |
|---|---|
| Age (years) | 25.9 ± 3.5 |
| Height (cm) | 173.3 ± 4.0 |
| Weight (kg) | 70.1 ± 12.0 |
| BMI (kg/m$^2$) | 23.3 ± 3.6 |

### Experimental protocol

The subjects were informed to refrain from strenuous physical activity and alcohol on the day before the study and to refrain from taking any fluids or foods from 2200 h the night before the study, except for tap water, until the completion of the study. They reported to the laboratory at 0800 h on the experiment day. Each subject wore a swimming trunk at the time of the study. A copper-constantan thermocouple probe was inserted into the esophagus and its tip placed at the atrium level to monitor body core temperature ($T_{core}$) throughout the study. A heparinized indwelling catheter was placed into the right antecubital vein. Before the onset of the study, the subjects sat in a room outside of the water tank for 1 h as the control period. After confirmation of stable $T_{core}$, measurements were started during the 10-min rest in the sitting position outside the tank. Then, the subject walked into and sat in the hot (42 °C) water tank, immersing the entire body, except the head, into the water. Measurements were taken after 20 min, then the subject left the tank to sit nearby for 4 h and measurements were repeated during this period. Thus, the entire measurement was of 270 min duration. Venous blood samples were collected just before hot-HOI (pre-HOI), 30 min (at end of HOI), 90 min (1 h post-HOI), 150 min (2 h post-HOI), 210 min (3 h post-HOI), and 270 min (last measurement) from the onset of the study. Blood samples (6 mL each) were withdrawn through the intravascular catheter and were used for the measurements of IL-6, CXCL1, TNF-α, high sensitivity C-reactive protein (hsCRP), hematocrit, and counts of blood cells.

### Analysis of blood samples

Total blood cell counts were assessed by using a cell counter. Hematocrit was determined by centrifugation. Other venous blood samples were drawn into pre-chilled serum venipuncture tubes. The tubes were centrifuged at 3500 rpm for 10 min at 4 °C, and then the serum was separated and stored at −80 °C until analysis. IL-6 was measured by enzyme immunoassay (ELISA) for IL-6 (R&D Systems, Minneapolis, USA) with assay sensitivity of 0.039 pg/mL and intra- and inter-assay coefficients of variability (average CV of different concentrations) of 7.4% and 7.8%, respectively. CXCL1 was assayed using ELISA for CXCL1 (R&D Systems) with assay sensitivity of <10 pg/mL and intra- and inter-assay coefficients of variability of 2.9% and 5.2%, respectively. TNF-α was analyzed using ELISA for TNF-α (R&D Systems) with assay sensitivity of 0.106 pg/mL and intra- and inter-assay coefficients of variability of 5.4% and 8.3%, respectively. IL-6, CXCL1, and TNF-α immunoassays were performed in duplicate by investigators blinded to the study design.

### Statistical analysis

All results were represented as mean ± SEM except when noted otherwise. The differences between two parameters were compared with one-way analysis of variance, followed by multiple

comparisons at various time points. When the *F*-value was significant ($p < 0.05$), the time period and the study condition comparisons were made using Fisher's LSD test.

## Ethics statement

The study protocol was approved by the Ethics Review Committee of Wakayama Medical University and conformed to the Declaration of Helsinki. A signed informed consent was obtained from each subject after a complete explanation of the purpose and risks of the present study.

## Results

$T_{core}$ significantly increased immediately after HOI, and was still higher at 1 h post-HOI, 2 h post-HOI, and 3 h post-HOI, but recovered to pre-HOI on the last measurement (**Figure 1**). **Table 2** lists the blood cell counts at the six different time points. Hemoglobin, hematocrit, erythrocyte count, and monocyte count remained constant throughout the study. Serum IL-6 concentrations were significantly higher at 1 h post-HOI, 2 h post-HOI, and 3 h post-HOI, and recovered to pre-HOI level in the last measurement (**Figure 2A**). Serum concentrations of CXCL1 (**Figure 2B**), TNF-α (**Figure 3A**), and hsCRP (**Figure 3B**) remained unchanged throughout the experiment.

## Discussion

This is the first study that examines the serum level of CXCL1 to hot-HOI in young and healthy men. The major findings of this study are the followings: 1) hot-HOI resulted in increased levels of serum IL-6; 2) no changes in serum CXCL1 levels were noted; and 3) the levels of $T_{core}$ and IL-6 returned to baseline at 4 h after hot-HOI. Thus, hot-HOI activated the releasing of IL-6 but did not stimulate the releasing of CXCL1. The increase in serum IL-6 was not induced by dehydration during hot-HOI because hematocrit and erythrocyte count did not change after hot-HOI, compared with the baseline.

It is well known that expressions of mRNA for *IL-6* and other proinflammatory cytokines, such as *TNF-α* and *IL-β*, are mainly regulated by the toll-like receptor (TLR) signaling cascade, which results in nuclear translocation and activation of

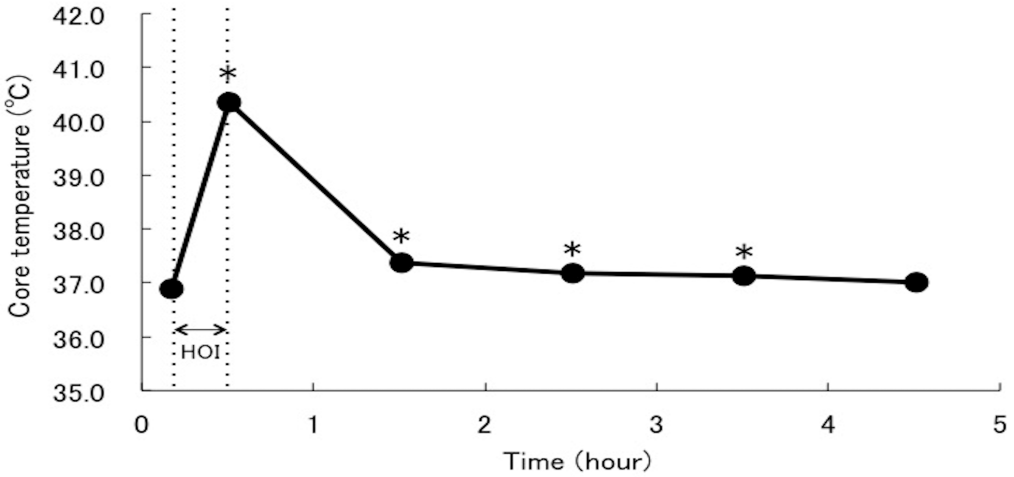


**Figure 1.** Core temperatures before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM. *$p < 0.05$, compared with before immersion.

**Table 2.** Hemoglobin, hematocrit and blood cell count values measured during the study

| | Pre-HOI | Immediately after immersion | After immersion | | | |
|---|---|---|---|---|---|---|
| | | | 1 h post-HOI | 2 h post-HOI | 3 h post-HOI | 4 h post-HOI |
| **Hemoglobin concentration (g/dL)** | 15.0±0.3 | 15.4±0.3 | 15.4±0.3 | 15.0±0.5 | 15.5±0.3 | 15.5±0.2 |
| **Hematocrit (%)** | 44.2±0.8 | 45.6±0.9 | 45.7±0.8 | 44.4±1.3 | 45.9±0.9 | 45.5±0.6 |
| **Erythrocyte count (×$10^5$/μL)** | 49.2±1.1 | 50.7±1.2 | 50.9±1.2 | 49.4±1.6 | 51.2±1.2 | 50.6±0.9 |
| **Monocyte count ($10^2$/μL)** | 1.9±0.4 | 1.4±0.2 | 1.8±0.3 | 1.9±0.3 | 2.1±0.2 | 1.9±0.2 |

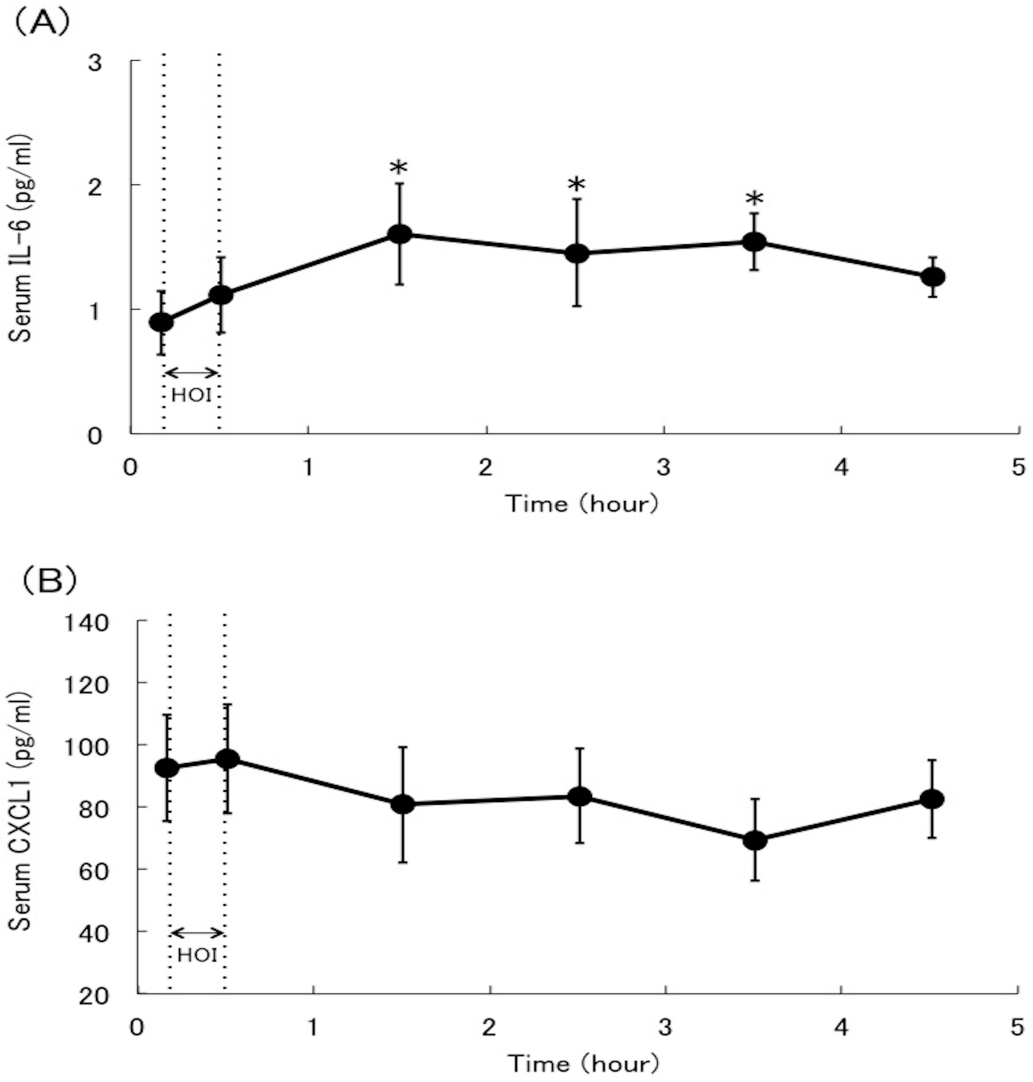


**Figure 2.** Serum IL-6 (**A**) and CXCL1 (**B**) levels measured before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM. *$p < 0.05$, compared with before immersion.

NF-κB during systemic inflammatory state such as sepsis. In contrast, exercise-induced increase in serum IL-6 appears in advance of a rise in TNF-α[1]. The stable levels of TNF-α, hsCRP, and monocyte count throughout the present measurement indicate the lack of inflammatory response during hot-HOI. Therefore, the increase in serum IL-6 is unlikely to be induced through TLR receptor signaling cascade. On the other hand, the simultaneous recovery of $T_{core}$ and IL-6 strongly suggests that $T_{core}$ is a strong activator for releasing IL-6.

Previous studies demonstrated the interactions between $T_{core}$ and IL-6 production during exercise. Rhind *et al.* reported that the decrease in $T_{core}$ during endurance exercise is associated with attenuation of the IL-6 response[16]. Starkie *et al.* reported the augmentation of the circulating IL-6 response when endurance exercise is performed in a hot environment, and concluded that releasing IL-6 from contracting skeletal muscles is a temperature-related phenomenon[17]. Another study described the upregulation of IL-6 in skeletal muscle following exposure to heat, both *in vitro* and *in vivo*[6]. Our results of changes in IL-6 level after hot-HOI strongly suggest that the increase in $T_{core}$ is an independent factor responsible for the increase in serum IL-6. The significant rise in IL-6 level after hot-HOI corresponds to the findings of previous study[8]. Interestingly, hyperthermia is reported to induce the release of IL-6 from skeletal muscle tubules in mice[6]. Considered together, it seems that

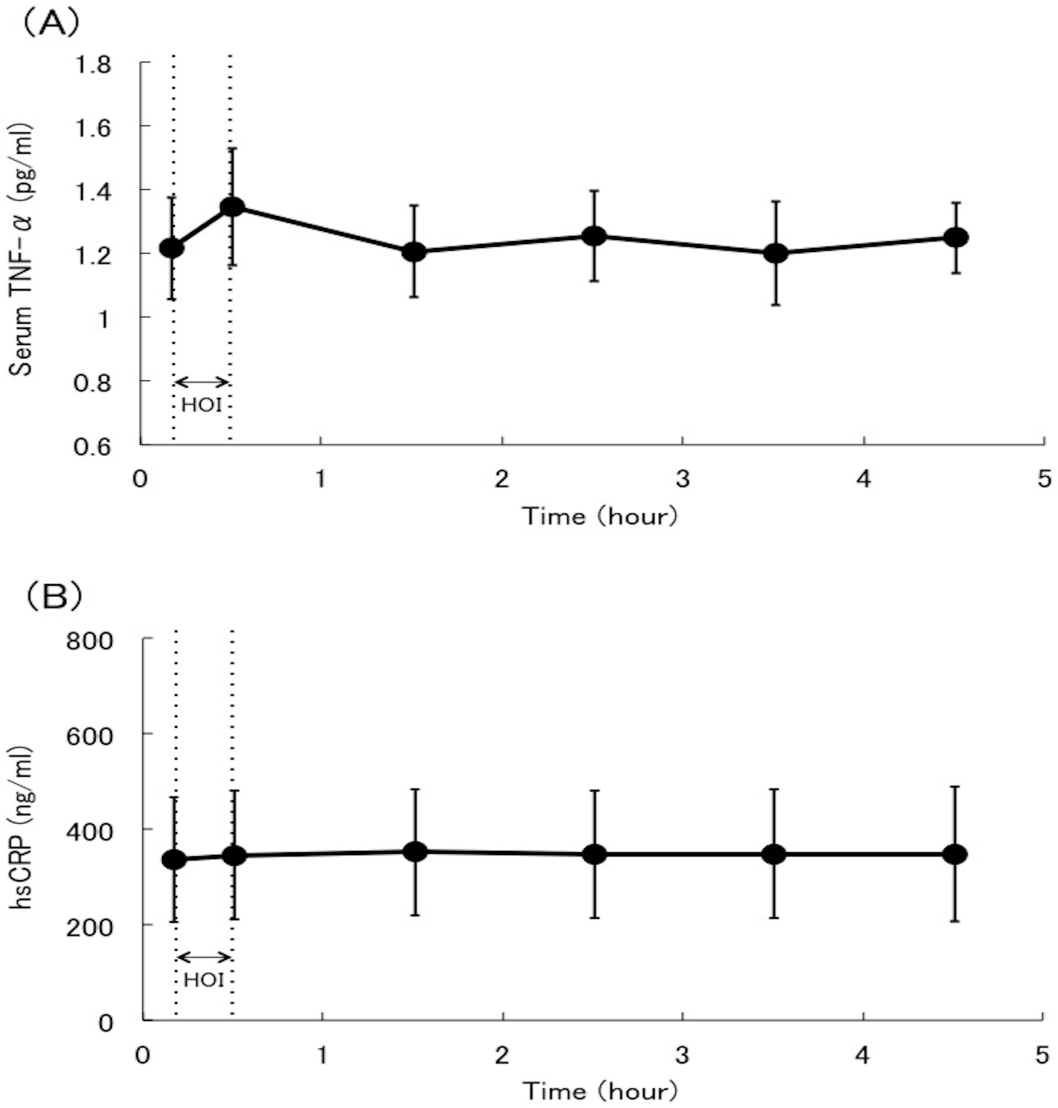


**Figure 3.** Serum TNF-α (**A**) and hsCRP (**B**) levels before and after head-out immersion (HOI) in hot water (42 °C). The left side of the plot represents data before HOI. Time 0 represents the onset of the study. The time between the two vertical dashed lines represents the time spent in hot-HOI. Data are mean ± SEM.

skeletal muscles are one of the organs which produce IL-6 at least after hot-HOI.

Serum CXCL1 protein levels and *CXCL1* mRNA expression levels in muscle and liver increase in swimming mice[15]. Importantly, the induction of CXCL1 expression is highest in the liver; the liver being the main source of releasing CXCL1 after exercise[15]. The same study demonstrated that IL-6 is released from the muscle-regulated liver CXCL1 expression[15]. One hour of swimming exercise would induce a 5-fold increase in serum IL-6 in wild-type mice immediately after exercise, and the increase was still seen at 2 h post-exercise, which was coupled with an increase in serum CXCL1[15]. In another study of mice, 30 min of treadmill running induced a 2.8-fold increase in serum IL-6, together with an increase in serum CXCL1[10]. In the present study, hot-HOI induced a 2-fold increase in serum IL-6 but no change in CXCL1. Therefore, the stability of CXCL1 and increase in IL-6 level suggest that hyperthermia *per se* contributed to the decrease in CXCL1 expression.

Previous studies described a close relationship between increased expression of *IL-6* mRNA in the skeletal muscle in a hot environment and those of heat shock protein *(HSP)-72* mRNA, and that treatment with KNK437, a heat shock factor (HSF) inhibitor, significantly reduced *IL-6* mRNA expression[6]. The main pathway involved in regulation of releasing IL-6 in various stressed

conditions, including hyperthermia, activates mitogen-activated protein kinases (MAPK), or more specifically, stress-activated protein kinases (SAPK) such as c-jun N-terminal kinase (JNK)[18]. JNK increases a transcription of IL-6 through the downstream activation of c-fos and c-jun, which dimerize and bind to the activator protein-1 (AP-1) regulatory element on the IL-6 promoter. Welc *et al.* examined the heat-induced transcriptional control of IL-6 in C2C12 muscle fibers, and demonstrated that the regulation of releasing IL-6 in hyperthermia is directly controlled by HSF-1 and AP-1 signaling[7]. Nedachi *et al.* have recently succeeded in establishing an advanced *in vitro* muscle exercise model using highly developed C2C12 myotubes that possess electric pulse stimulation (EPS)-evoked vigorous contractile activity[10]. They found marked upregulation of CXCL1 expression in contractile C2C12 myotubes after 24-h EPS. Moreover, they strongly suggested that the NF-κB signaling pathway is directly involved in CXCL1 expression in response to EPS-evoked contractility[19]. However, NF-κB signaling was reported to be downregulated by hyperthermia in mouse myoblast cells[20]. These results of the above studies might explain the lack of change in serum CXCL1 level and the increase in serum IL-6 level after hot-HOI.

The other possible explanation for the lack of change in serum CXCL1 level after hot-HOI would be related to an insufficient expression level of IL-6; even serum IL-6 significantly increased after hot-HOI. Furthermore, there was no contraction of skeletal muscles and/or lower energy expenditure because sitting would be associated with the lack of altering in the serum CXCL1 level[15]. However, it is not clear the reason why serum CXCL1 remained unchanged after hot-HOI.

## Conclusion

The present study demonstrated that 20 min of hot-HOI did not affect CXCL1 expression in young healthy men, but increased serum IL-6 levels, probably through the increase in core body temperature.

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## Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of their article.

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