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The Interaction of Heat and Lipopolysaccharide on the Expression Levels of Receptor Activator of NF-κB Ligand and Osteoprotegerin in Human Periodontal Ligament Cells

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Abstract

Aim: To investigate the expression levels of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in human periodontal ligament fibroblasts when stimulated with heat in infective conditions. Methods: Periodontal ligament fibroblasts were subjected to various temperature increases for 5 min with or without 10 ng/mL lipopolysaccharide (LPS) and then maintained at 37˚C for 6 h. After that, the expression levels of RANKL and OPG were investigated using real-time RT-PCR and ELISA. As a positive or negative control, the cells were cultured at 37˚C with or without 10 ng/mL LPS. Data were analyzed using one-way ANOVA at a significant level of p = 0.05. Results: The mRNA expression levels of RANKL and OPG were both down-regulated when the cells were heated in infective conditions. The release of sRANKL was increased at low temperatures in such infection; while at high temperatures heat treatment down-regulated the release of sRANKL induced by LPS. The relative RANKL/OPG expression ratios were increased at low temperatures in infective conditions. Conclusions: The interactions between heat and LPS would affect the balance between RANKL and OPG in periodontal ligament fibroblasts when they were heated in infective conditions. Such infection may be the reason why bone resorption occurs in the local area after warm vertical compaction.

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Keywords

Heat, Lipopolysaccharide, OPG, Periodontal Ligament Fibroblasts, RANKL

1. Introduction

There are many irregular structures in root canal system, which are difficult to clean and obturate using traditional instruments. The remaining microbes and their by-products in these structures may cause persistent infection and bone lesion in the local area. In order to deal with these complicated structures, ultrasonic endodontic devices and warm vertical compaction techniques were introduced into root canal treatment. However, heat produced by the devices may be a risk factor to the periodontal tissues. It has been reported that heat might induce inflammation, bone resorption or bony ankylosis around the tooth root in the animal models [1]-[4].

Receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) are the key cytokines to regulate osteoclastogenesis and bone resorption [5]. It was suggested that the balanced expression of RANKL and OPG would affect the bone remodeling process around tooth root [6]-[8]. In our previous study, we demonstrated that heat stress at 47˚C would up-regulate the relative RANKL/OPG expression ratio in periodontal ligament fibroblasts (PDLs), which might take responsibility for bone resorption around root surface [9]. However, the temperature rise on the outer surface of teeth during routine warm vertical compaction was often lower than 10˚C [10]-[15]. Hence, its possibility of inducing bone resorption was relatively low. The reason why inflammation and bone lesion occurred after routine warm vertical compaction was unknown.

As is well known, it is difficult to eradicate microorganisms in root canal system. Lipopolysaccharide (LPS), the major virulent factor of Gram-negative bacteria in canals, played a role in the RANKL-induced local inflammation and bone resorption [16]. Hence, endodontic treatments were always performed in a slight infective condition, in which PDLs kept contact with LPS. Since our previous study only investigated the effect of heat stress on the RANKL and OPG in non-infective situations, it was hypothesized that bone lesions induced after routine warm vertical compaction were mainly due to the effect of LPS-induced RANKL expression.

Hence, the study aims to investigate the effect of heat stress on the expression of RANKL and OPG in PDLs in infective situations. The null hypothesis is that LPS exerts no effect on the expression of RANKL and OPG induced by heat.

2. Materials and Methods

2.1. Cell Culture

The study was approved by the ethics committee of West China College of Stomatology and informed consent was obtained from each patient. PDLs were obtained from the middle third of the root surfaces of healthy human premolars, which were extracted under local anaesthesia for orthodontic treatment. The primary PDLs were maintained in Dulbecco's modified eagle's medium (DMEM, Hyclone, Beijing, China) containing 20% foetal bovine serum (FBS, Hyclone, Beijing, China) and antibiotic solution (100 U/mL penicillin and 100 ug/mL streptomycin) under 5% CO_2 in air at 37˚C. After confluence, the cells were detached with 0.25% typsin (Difco laboratories, Detroit, MI, USA) and subcultured in DMEM containing 10% FBS and antibiotic solution. The cultures were used between the fourth and the tenth passage.

The cells were seeded at 2×10^5/ml into 35 mm culture plates and incubated at 37˚C. When they reached 80% confluence, they were maintained in DMEM without FBS or antibiotic solution for 24 h. Before stimulation, the culture media were heated in the water tank with experimental temperatures (39˚C, 43˚C, 47˚C, 50˚C) respectively. The cells were divided into three groups. In Group A, the cells were maintained in the pre-heated culture media in an incubator set at the appropriate experimental temperature for 5 min. In Group B, the cells were treated with 10 ng/mL LPS for 24 h first, and then suffered from heat for 5 min as Group A. In Group C, the cells were stimulated with heat and 10 ng/mL LPS for 5 min simultaneously. As a positive or negative control, the cells were cultured at 37˚C with or without 10 ng/mL LPS. LPS comes from *Escherichia coli* (*E. coli*) O111:B4 (Invivogen, Carlsbad, CA, USA).

2.2. RT-PCR Assay and Real-Time PCR Analysis

After stimulation, the PDLs in Group A were incubated at 37˚C for 6 h, while those in Group B and C were cultured at 37˚C with 10 ng/mL LPS for 6 h. Then total RNA from cells were prepared with Trizol reagent (Invitrogen, Carlsbad CA, USA) and reverse-transcribed with SYBR® PrimeScript™ RT-PCR Kit (Perfect Real Time, Takara, Dalian, China) according to the manufacturer's instructions.

Real-time PCR assays were performed with SYBR® PrimeScript™ RT-PCR Kit (Perfect Real Time, Takara, Dalian, China) using ABI 7300 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The primers were synthesized by Takara and the sequences are listed in **Table 1**. The standard PCR conditions were 10 s at 95˚C, followed by 40 cycles at 95˚C for 5 s, 60˚C for 31 s and 72˚C for 30 s. The mRNA expression levels of RANKL and OPG were indicated as the relative expression normalized by glyceraldehydes phosphate dehydrogenase (GAPDH).

2.3. ELISA Assay

After stimulation, the PDLs in Group A were incubated at 37˚C for 6 h, while those in Group B and C were cultured at 37˚C with 10 ng/mL LPS for 6 h. Then the supernatant was collected with centrifugation and used for ELISA assay. The assay was performed according to the manufacturer's instructions. And the ELISA kits for RANKL and OPG were bought from R&D Systems (Minneapolis, MN, USA).

2.4. Statistical Analysis

The relative RANKL/OPG expression ratios were calculated. Values were presented as the mean ± SD. Differences between the experimental groups and the control group were analyzed by one-way ANOVA followed by Tukey post hoc test; and data were considered significant at $p < 0.05$.

3. Results

3.1. Effect of Heat on the mRNA Expression Levels of RANKL and OPG in PDLs in Infective Conditions

Both heat and LPS treatment suppressed the mRNA expression of RANKL and OPG in PDLs in Group B (**Figure 1(a)**) and Group C (**Figure 1(b)**), compared to the negative control group ($P < 0.05$).

3.2. Effect of Heat on the Release of Soluble RANKL (sRANKL) in PDLs in Infective Conditions

Compared with negative control group, heat up-regulated the release of sRANKL at low temperatures, while down-regulated it at high temperatures in infective conditions. There were significant increases in sRANKL at 39˚C in Group B and 43˚C in Group C ($P < 0.05$, **Figure 2(a)**). When the experimental temperature rose further (43˚C, 47˚C in Group B and 47˚C in Group C), the release of sRANKL was decreased gradually but with no significant differences ($P > 0.05$, **Figure 2(a)**). Additionally, sRANKL was also increased at 50˚C when the cells were pretreated with LPS ($P < 0.05$, **Figure 2(a)**).

In Group B, as the experimental temperature rose, the release of sRANKL was increased first, and then decreased, compared with the positive control. There were statistical increases at 39˚C and decreases at 43˚C and 47˚C, respectively ($P < 0.05$, **Figure 2(a)**). Though the cells in Group C were stimulated with heat and LPS

Table 1. The primer sequences for RANKL, OPG and GAPDH.

Gene	Forward	Reverse	Size (bp)	Tm (˚C)
GAPDH	GTCTTCACCACCATGGAGAAG	GTTGTCATGGATGACCTTGGC	210	58.5
RANKL	CGTTGGATCACAGCACATCAG	GTACCAAGAGGACAGACTCAC	163	58.5
OPG	CACTACTACACAGACAGCTGG	ACTCTATCTCAAGGTAGCGCC	148	58.5

All the primers were designed by ourselves.

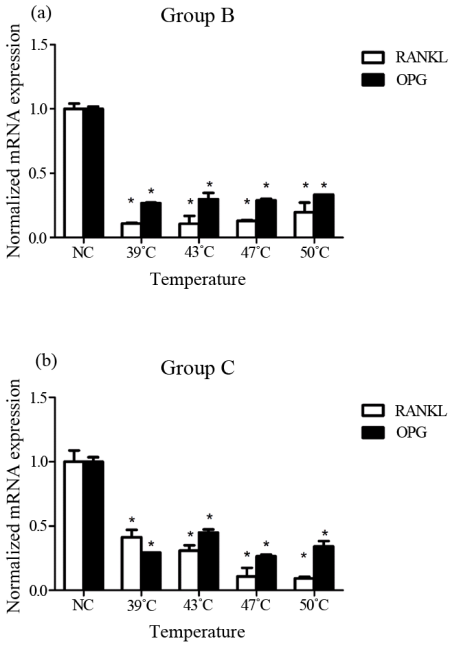

Figure 1. Effect of heat on the gene expression of RANKL and OPG in PDLs in infective conditions (*, $P < 0.05$, compared with negative control (NC) group). (a) The cells in Group B were treated with 10 ng/mL LPS for 24 h first, and then suffered from heat for 5 min; (b) The cells in Group C were stimulated with heat and 10 ng/mL LPS for 5 min simultaneously.

simultaneously, different from Group B, the similar trend existed in sRANKL but with no differences. Heat up-regulated the protein release at 43˚C, while down-regulated it at 47˚C and 50˚C, separately in Group C ($P > 0.05$, **Figure 2(a)**).

Comparing the protein release at the same temperatures between the cells stimulated with and without LPS, it was showed in **Figure 2(a)** that the treatment of LPS interacted with heat stress distinctively at different temperatures. At 37˚C and 50˚C, treatment of LPS induced the release of sRANKL statistically, while at 47˚C it down-regulated sRANKL ($P < 0.05$, **Figure 2(a)**). Besides, the action mode of LPS also played a role. The pretreatment of LPS before heat stress down-regulated sRANKL at 43˚C; however, the simultaneous treatment of heat and LPS induced it at the same temperature (43˚C, $P < 0.05$, **Figure 2(a)**).

3.3. Effect of Heat on the Release of OPG in PDLs in Infective Conditions

Compared with the negative control group, heat stress at 47˚C induced the secretion of OPG when the cells were pretreated with LPS ($P < 0.05$, **Figure 2(b)**). However, there were no significant differences when the cells were treated with heat and LPS simultaneously ($P > 0.05$, **Figure 2(b)**).

Heat stress induced the release of OPG significantly at 47˚C and 50˚C separately in Group B, compared with the positive control group ($P < 0.05$, **Figure 2(b)**). Nevertheless, there were no significant differences between experimental groups in Group C and the positive control.

Comparing the release of OPG at the same temperatures between the cells stimulated with and without LPS, it was showed in **Figure 2(b)** that the treatment of LPS down-regulated OPG at low temperatures (39˚C in Group B and 39˚C, 43˚C in Group C, $P < 0.05$, **Figure 2(b)**). However, when the temperatures rose, there were no significant differences (47˚C and 50˚C, $P > 0.05$, **Figure 2(b)**).

3.4. Effect of Heat on the Relative RANKL/OPG Expression Ratios in Infective Conditions

According to the release of sRANKL and OPG, their relative expression ratios were calculated. In Group B, heat stress up-regulated the relative RANKL/OPG expression ratios at 39˚C and 50˚C, but down-regulated them at

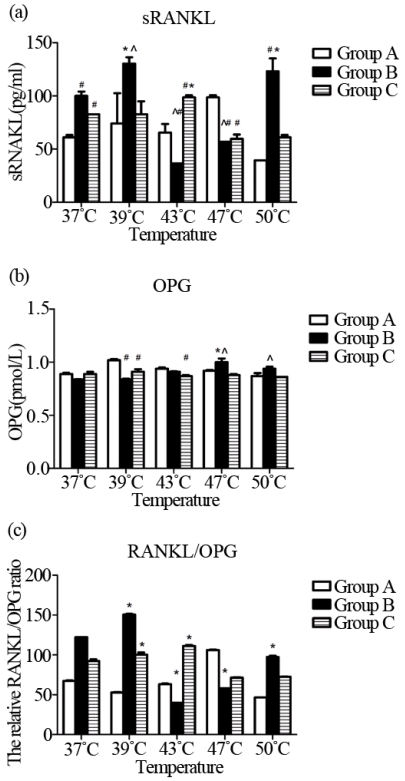

Figure 2. Effect of heat on the release of sRANKL and OPG in PDLs in infective conditions (*, $P < 0.05$,compared with negative control group; ^, $P < 0.05$, compared with positive control group; #, $P < 0.05$, there is significant difference when comparing the release of sRANKL or OPG at the same temperatures between the cells stimulated with and without LPS). (a) Effect of heat on the release of sRANKL in PDLs in infective conditions; (b) Effect of heat on te release of OPG in PDLs in infective conditions; (c) Effect of heat on the relative RANKL/OPG expression ratios in PDLs in infective conditions.

43˚C and 47˚C respectively, compared with the negative control group ($P < 0.05$, **Figure 2(c)**). However, when the PDLs were irritated with heat and LPS simultaneously, the relative expression ratios were only induced at 39˚C and 43˚C ($P < 0.05$, **Figure 2(c)**). It was distinctive from the effect of treatment in Group B.

4. Discussion

As is well known, it is difficult to eradicate the microorganisms in root canal system completely. The remaining bacteria and their by-products would exert an effect on the periodontal tissues at all times. Warm vertical compaction technique will only be performed when the residual bacterial load is reduced to a non-pathogenic number. Hence, the aim of this study is to investigate the effect of heat stress on the mRNA expression levels and protein release of RANKL and OPG in PDLs in such slight infective conditions. It is suggested that LPS is the major virulent factor, which plays a pivotal role in local infection [17]-[19]. Therefore in the present study low concentrations of LPS were used to stimulate PDLs to imitate such slight infective situations.

After the cells were stimulated with heat and LPS, we found that the mRNA expression levels of RANKL and OPG were significantly decreased. It was consistent with our previous study [9], which showed that heat stress down-regulated the gene expression. Although it had been reported that the mRNA expression levels of RANKL and OPG were up-regulated when the PDLs were stimulated with *E. coli* LPS for 48 h, there was no difference when the cells were irritated for 24 h [20]. And Krajewski *et al.* [21] also found that the gene expression of RANKL rose slightly when the cells were treated with LPS isolated from *Porphyromonas gingivalis* for 48 h. It

indicated that short-term treatment of LPS had no influence on the gene expression of RANKL and OPG. In the present study, the cells were irritated with LPS just for a short term (30 h for Group B and 6 h for Group C). Hence, the treatment of LPS may have no influence on the heat-induced gene expression of RANKL and OPG.

Though the function mode of LPS is different between Group B and Group C, the similar trend existed in the release of sRANKL. The treatment of LPS made PDLs to respond to heat stress at low temperatures. The release of sRANKL were significantly increased at 39˚C in Group B and 43˚C in Group C ($P < 0.05$, **Figure 2(a)**), compared with the negative control. It was different from Group A, in which heat stress had no influence on the sRANKL. Lim *et al.* [22] has suggested that pre-existing inflammation would compromise the heat tolerance in cells. Hence, in such infective situations the release of sRANKL was up-regulated at low temperatures, which was mainly attributed to the effect of LPS. And the temperature difference between Group B and Group C may be due to the different action time.

When the release of sRANKL in Group B was compared to the positive control, we found that heat treatment at special temperatures (43˚C and 47˚C in Group B, $P < 0.05$, **Figure 2(a)**) down-regulated LPS-induced sRANKL release. Cooper *et al*. [23] found that heat treatment ranging from 39.5˚C to 40˚C reduced the production of TNF-α induced by LPS in macrophages. It was also reported that the release of pro-inflammatory cytokines were decreased *in vivo* when mice were injected with LPS and suffered from heat for 15 min further [24]. Since LPS promoted the expression of RANKL via induction of some pro-inflammatory cytokines, such as TNF-α [17] [18] and IL-6 [25], heat treatment at special temperatures may impair LPS-induced sRANKL release through its inhibitory effect on these pro-inflammatory cytokines. This was related to heat shock proteins (HSPs) [26] and heat shock factors (HSFs) [27] induced by heat in PDLs [28].

Besides, we found that the release of sRANKL was increased gradually in Group B when the experimental temperature rose from 43˚C to 50˚C. It indicated that the inhibitory effect of heat on LPS-induced sRANKL release became less obvious. Hence the release of sRANKL was significantly up-regulated at 50˚C. It was consistent with Lin's study [29]. They found that when heat exceeded a proper value, LPS and heat had synergistic effect on the inflammation; large quantities of pro-inflammatory cytokines would be secreted.

In our previous study [9], heat treatment up-regulated the release of OPG at 39˚C. However, LPS was reported to exert an opposite effect [30]. Therefore, the release of OPG in PDLs when they were suffered from heat in infective conditions would be determined by the interactions between heat and LPS. As it was shown in **Figure 2(b)**, the release of OPG in Group B was only increased at 47˚C, compared with the negative control group. It suggested that at low temperatures the effect of LPS and heat strsee may be equivalent on OPG; while at high temperatures the effect of heat stress may become dominant. This can be verified further when the release of OPG in Group B were compared with that in Group A or with the positive control. At low temperatures (39˚C), there was a significant decrease in OPG when the cells were compared between those treated with and without LPS; while at high temperatures OPG was increased gradually under the same infective condition when the temperature rose (47˚C and 50˚C in Group B, $P < 0.05$, **Figure 2(b)**).

According to the changes of RANKL and OPG, their relative expression ratios were calculated. There were significant increases at low temperatures (39˚C in Group B and 39˚C, 43˚C in Group C, $P < 0.05$, **Figure 2(c)**) and decreases at high temperatures (43˚C and 47˚C in Group B). It was absolutely opposite to our previous study, which showed that in non-infective situation the ratio was down-regulated at low temperatures (39˚C and 43˚C) and up-regulated at high temperature (47˚C) [9]. These differences may be due to the complicated interactions between heat stress and LPS treatment. The balanced expression of RANKL and OPG in the local area would be determined by these two treatments. The increases of this ratio induced by heat in the present study, which indicated a possibility of bone resorption at low temperatures, may be attributed to infective condition. Since the temperature rise on the root surface during routine warm canal filling was generally lower than 10˚C [10]-[15], bone resorption which occurs after routine warm vertical compaction may be due to the uncontrolled infection in the local area. It reminds all the dentists that the most important thing in endodontic treatment is still to control infection.

5. Conclusion

These results suggested that our null hypothesis was invalid. The interactions between heat and LPS would affect the balance between RANKL and OPG in PDLs when they were heated in slight infective conditions. Such infection may be the reason that bone resorption occurs in the local area after routine warm vertical compaction.

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