Acceleration of wound healing with use of secretory leukocyte protease inhibitor could be seen by osteopontin expression in *Rattus norvegicus* post tooth extraction

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Acceleration of wound healing with use of secretory leukocyte protease inhibitor could be seen by osteopontin expression in *Rattus norvegicus* post tooth extraction

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Abstract. Tooth extraction can be debilitating due to prolonged inflammation delaying wound healing. Secretory leukocyte protease inhibitor (SLPI) is a non-glycosylated proteomic material that inhibits protease enzymes and has anti-inflammatory properties accelerating wound healing. Increased osteoclast activity is marked by increased osteopontin expression (OPN) during bone resorption. OPN is a phosphorylated glycoprotein secreted by several immune cells. This study aims to determine OPN's expression as an osteoclast activity's overview in wound healing acceleration, following SLPI administration. Thirty-six *Rattus norvegicus* incisors were extracted and randomly divided into 6 groups by treatment type and duration of observation. SLPI was administered in Group 1 and 2 (12 heads respectively) on the socket of rat's teeth, followed by tight stitches. Group 2 additionally received bone graft. Control group K (12 heads) exclusively received tooth socket stitching. Two observations, each on 6 rats, were performed at 7 and 14 days. Subsequently, biopsies were performed and immunohisto-chemically stained with osteopontin antibodies. One-way Anova and Kruskal–Wallis tests showed statistically significant differences ($p<0.05$) between the groups. Results suggest that SLPI administration decreases osteoclast activity in alveolar bone resorption by decreasing the number of active osteoclasts and increasing the number of inactive ones in a Rattus model. Osteopontin expression can be used in overview of osteoclast's activity.

1. Introduction

It has been shown that dental conditions that require tooth extraction are due to abnormalities of the dental support and structure including caries, malposition, supernumerary and idiopathic resorption

[1]. The trauma arising from tooth extraction can affect the wound healing process' duration, especially in the extraction of the third molar.

Prolonged wound healing may cause pain, bleeding, swelling, infection, and impaired mastication [2]. Currently, biomaterial' research is aiming at accelerating wound healing post tooth extraction by inhibiting the inflammatory response, which in turn prevents excessive tissue damage including that of the bone. Studies have shown that proteomic substances, such as secretory leukocyte protease inhibitors (SLPI), are involved in several physiological processes, including sequentially remodeling periodontal ligaments and innate immunity [3]. SLPI is a non-glycosylated proteomic ingredient weighing 11.7 kD. It has the property of inhibiting protease enzymes, i.e., elastase and cathepsin G. It is produced by mucosal cells such as the epithelium, macrophages, and neutrophils. It has been shown that SLPI also has anti-inflammatory properties [4].

Inflammation can be triggered by lipopolicharida (LPS), a bacteria-produced endotoxin around the new wound. LPS will trigger the release of proinflammatory agents, such as nuclear factor kappa B (NFκB), tumor necrosis factor alpha (TNFα), and cytokines such as IL-1, IL-6, and IL-8. As a result, this process will increase the MMPs and osteoclasts. The inflammatory process leads to tissue damage, including bone resorption. Of note, SLPI as a protease inhibitor and anti-inflammatory agent can overcome resorption. This protein has been widely studied in the field of lung, arthritis, and immunology but not in dentistry [5].

Increased osteoclast activity during bone resorption can be seen with an increased osteopontin (OPN) expression [6]. Specifically, OPN is a phosphorylated glycoprotein secreted by active macrophages, leukocytes, and active T lymphocytes. It is located in the extracellular fluid, cell cytoplasm, inflammatory area, and mineralized tissue extra cellular matrix. OPNs include cytokines that mediate cell-cell or cell-matrix interactions. Studies have shown that OPN present in bone facilitates the adhesion between osteoclasts and bone matrix suggesting that it can be used to look at osteoclast activity [7].

In the present study, we aimed at determining the expression of OPN to evaluate osteoclast activity on accelerated wound healing following the addition of SLPI on rat sockets (*Rattus norvegicus*).

2. Methods

This research is an experimental laboratory post-test-only control group design. Thirty-six Rattus norvegicus incisors were removed from the left mandible and randomly divided into 6 groups. Samples were subsequently subdivided by treatment type and duration of observation. Socket of rat teeth of Group 1 (12 head) were given SLPI and stitched tightly. Sockets of rat teeth of Group 2 (12 heads) were given a mixture of SLPI and bone graft and then stitched tightly. In the control group K (12 heads), the tooth sockets were exclusively stitched tightly. Two observations, each on 6 rats, were performed at 7 and 14 days. Subsequently, biopsies were performed and immunohistochemically stained with osteopontin antibodies.

OPN expression data was obtained by counting the number of osteoclast cells. The latter had a brown cytoplasmic color following immunohistochemistry staining. The extra cellular matrices appear yellowish and adhere to the bone tissue. Cells were counted manually on a five-field inspection using a scanning microscope (dot slide OlyVIA program, Olympus) at 400X magnification.

3. Results

Results of OPN expression in osteoclast cells are as shown in Table 1 and Figure 1:

Table 1. Mean OPN expression in osteoclast cells on days 7 and 14.

	Control (K)		SLPI (P1)		SLPI + BG (P2)	
	day-7	day-14	day-7	day-14	day-7	day-14
Active osteoclast	200[b]	432[b]	82[a,b]	245[a,b]	230[a,b]	883[a,b]

Table 1. *Continue*

Non-active osteoclast	503[b]	368[b]	931[a,b]	782[a,b]	1568[a,b]	638[a,b]

[a]There is a significant difference vs. the control group.
[b]There is a meaningful difference between days 7 and 14.

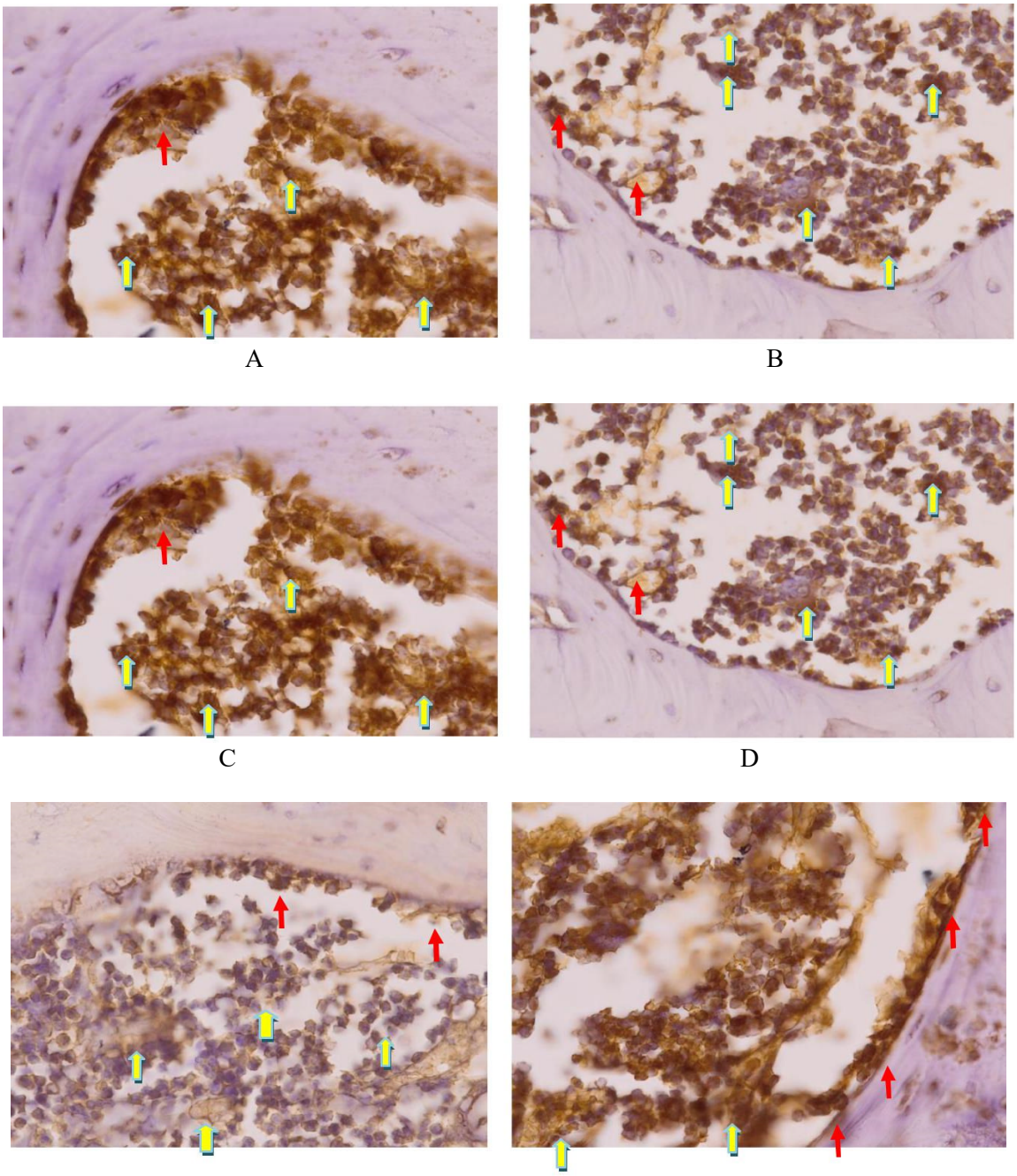

Figure 1. Figure A. An overview of OPN expression in osteoclast cells in the control group on day 7 day (Arrows indicate activated osteoclast cells and inactive osteoclasts). Successive images B, C, D, E, and F represent, respectively, the control group on day 14, SLPI treatment group on day 7, SLPI treatment group on day 14, SLPI + bone graft treatment group on day 7, and SLPI + bone graft treatment group on day 14.

4. Discussion

OPN is one of the most abundant non-collagen proteins in the bone matrix. OPN contains an arginine-glycine-aspartate (RGD) motif, which binds integrin, and allows bone cells to adhere to the mineral matrix. OPN has been known to be involved in rapid bone resorption, as observed in a study of bone loss induced by *in vivo* ovariectomy and *in vitro* bone-parathyroid (PTH) surgery. It is well known that OPN is required for stimulation of osteoclastic bone resorption and suppression of osteoblastic bone formation in mice undergoing removal of OPN genes [8].

SLPI has been used as an anti-inflammatory agent. The potential of SLPI being developed into an inflammation inhibitory due to its protease inhibition is interesting to investigate. It has been described that protease is an enzyme secreted by osteoclasts for bone matrix resorption [9]. Osteoclast inhibition through the second level [10], located in the osteoclast property barrier, can slow down and even prevent bone resorption. The key to bone resorption is the bond between osteoclast and mineral matrix on the bone surface. OPN, a major cell- and hydroxyapatite-binding protein synthesized by osteoblasts, mediates these bonds. Several studies have reported that OPN is expressed in osteoclasts and also present in the bone matrix [11-14].

Immunohistochemical studies have shown that osteoclast cells can be classified in 2 groups based on their osteoclast expression: those attached to the bone expressing a yellowish brown color in the cytoplasm (called active osteoclasts); those not attached to the bone expressing the yellowish brown color in the cytoplasm (called non-active osteoclasts) Asou et al. observed that once the bone resorption process is initiated, osteopontin in bone mineral matrix captures the osteoclasts and the vitronectin receptor on osteoclast plasma membrane, leading to the above-mentioned phenomena [14].

Various observations were performed 7 and 14 days following the procedure, based on the duration of wound healing in rats. In the post-extraction week, blood clot organization was characterized by the presence of fibroblasts and endothelial cells at the center of the socket, followed by neutrophils, macrophages, and osteoclast cells. These latter cells were involved in destroying necrotic cells and bone fragments. On days 10 to 15, osteoid and immature bone begin to form on the edge of the tooth socket [15]. Osteoid formation indicates that the process of bone resorption has begun to decline, so the length of observation was limited to 14 days.

As shown in Figure 1 the number of active osteoclasts in the P1 group on day 7 was lower compared to the K and P2 groups. This is consistent with study by Taggart et al. results showing that SLPI administration can suppress the number of active osteoclasts by inhibiting osteoclast cell maturation through the NFκB barrier mechanism [4]. When the SLPI enters the macrophage cells and is localized to the cytoplasm and nucleus, it will compete with NFκB in binding to the DNA through IJB degradation. As a consequence, the NFκB product decreases, and the receptor activator for NFκB (RANK-L) becomes inactive, thus disrupting the maturation of preosteoclast into osteoclast cells. This immature preosteoclast cell cannot be attached to the bone and remains floating around it so as to be deactivated. Therefore, active osteoclasts tend to be reduced in the SLPI group on day 7 (P1O1). This phenomenon is of interest because although this osteoclast is not attached to the bone, it still expresses osteopontin. This mechanism can be explained as follows: OPN is not expressed in monocyte circulation but is dramatically regulated during macrophage differentiation and is one of the major macrophage products [16]. OPN is known to be induced in macrophages by several inflammatory cytokines, including TNF-α, IL-1β, IFN-γ, and IL-6, and other factors including angiotensin-II, oxidized LDL and phorbol-esters, known as OPN inducers in macrophages [17]. Differentiation of macrophages in wound healing involving bone is osteoclasts. Since the osteoclast maturation barrier effect on NFκB by SLPI is likely to cause signaling barriers between OPN in bone extracellular matrix and that in intracellular (iOPN) osteoclasts.

The number of osteoclast cells with non-active OPN expression on day 7 of the SLPI group (P1O1) was doubled when compared to the control group. This phenomenon can be explained by the fact that on day 7 post-extraction of rat teeth, an increase in the number of macrophages in tooth sockets could

be seen (Baskar, 1980) [15]. In the first week there was an increase of neutrophil cell activity as well as macrophages involved in the destruction of necrotic cells, bone fragments or sharp bone fragments. Only 2%–5% of these macrophages will become osteoclasts or preosteoclasts (Steinbeck, 2001) [18]. At this stage, there is still a slight signal to the surface of the bone because the number of macrophages that become preosteoclasts is low.

On day 14, the osteoclast activity via OPN expression in the SLPI group (P1O2) was similar to that of SLPI and control groups on day 7. Specifically, they showed a decrease in the number of active osteoclasts. The decrease was statistically significant ($p<0.05$) compared with that in the control group. This indicates that on day 14, SLPI maintains the capability of suppressing the number of active osteoclasts by inhibiting the maturation of preosteoclasts into osteoclasts.

In the present study, bone graft is added to SLPI as a comparison group. In the SLPI group with bone graft (P2), the number of active osteoclasts on day 7 was slightly increased ($p<0.05$) compared with those in the K and P1 groups. It has been shown that this may be due to the resorbable nature of hydroxyapatite in bone grafts, which tends to increase the number of osteoclasts [19]. The unusual finding of an increase in OPN expression in active osteoclasts, followed by a doubled increase of osteoclast cells were nulled when compared with the control. Such findings are made possible by the role bone graft has as a scaffold in the socket, causing the SLPI to last longer in the socket on day 7 [19]. This makes the immature osteoclasts to be higher in number compared to the control group or the group where the SLPI is given alone. OPN expression followed the same condition or decreased. But this has not been observed on day 14.

Day 14 OPN expression in the active osteoclast cells of the P2 group actually increased sharply compared to groups K and P1. It is noteworthy that SLPI's effect was untenable on day 14 of the P2 group. A possible cause for this finding was the effect of hydroxyapatite in the bone graft used in this study. According to the da Cruz et al. findings hydroxyapatite, resorbed by osteoclast, had peaked in the second week [19]. Osteoclast accumulation occurring in this group may be a possible explanation of such findings.

The effect of a single dose of SLPI (0.18 μg) used in this study represents one of the causes of the unexpected increase in the number of active osteoclasts in this group. According to Taggart et al. the dose used was the maximum amount that can cause RANK inhibition in macrophages [4]. The presence of hydroxyapatite triggers an increase in the number of osteoclasts. Therefore, the increased number of macrophages did not balance the number of SLPI cationic molecules entering the macrophages. This is according to the picture in the P2O2 group showing that inactive osteoclasts decreased when compared to the P1 group.

Based on the results obtained from this study, further research is needed to understand the relationship between osteopontin and SLPI in the mechanism of alveolar bone resorption barrier. A wider application of SLPI in dentistry relies on many unknowns being answered first. To this end further research on SLPI should be performed.

5. Conclusion

The expression of osteopontin on the sockets of the Rattus norvegicus teeth after administration of the SLPI can be used as an overview of osteoclast activity. Osteopontin expression in active osteoclast cells decreased with the administration of the SLPI, as observed on days 7 and 14 of the post-tooth extraction wound of Rattus norvegicus, while in non-active osteoclasts increased with the administration of the SLPI.

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