Expression of RANKL by dental cells during eruption of mice teeth

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ABSTRACT

Background : In order for a tooth to erupt, two obvious requirements are needed. First, there has to be alveolar bone resorption of the bone overlying the crown of the tooth such that an eruption pathway is formed. Second, resorption of bony crypt and apposition of new one, third, there has to be a biological process that will result in the tooth moving through this eruption pathway.The amniotic sac contains a considerable quantity of stem cells. These amniotic stem cells are multipotent and able to differentiate into various tissues, which may be useful for human application. Receptor activator of nuclear factor kappa B ligand (RANKL) is concentrated on bone biology, more specifically bone metabolism. RANKL plays a vital role in osteoclastogenesis for bone resorption. This study aimed to evaluate the expression of RANKL marker by dental cells during eruption of the teeth.

Materials and Methods: : forty eight albino Swiss mice of one day old age injected with isolated amniotic stem cells in the anterior region of maxilla (incisors area) other 16 mice injected with saline represents control. Sacrifice 4 mice for each period (4, 7, 10, and 13) day old age. The result were studied histologically and immunohistochemistry.

Results: The present results localized and identified RANKL marker in 3 areas of developing tooth of the studied groups includes overlying, surrounding and apical bone. Positive RANKL with high significant value expressed by osteoclast of overlying bone in Amnion group followed by Control at day 4. In surrounding bone positive expression of RANKL illustrated to be highest in Control followed by Amniotic fluid at day 10.Apical bone shows positive expression of RANKL in amniotic fluid group and it records to be the highest value in comparison to studied groups at day 10.

Conclusion Expression marker RANKL illustrates that amniotic fluid group has a high expression of RANKL in osteoclast surrounding and apical bone areas while control expressed RANKL in osteoclast of overlying bone. The present results opened clinical hopes in dental tissue engineering by application of autologous amniotic fluid and chorion cells.

Key words: RANKL, tooth eruption. (J Bagh Coll Dentistry 2013; 25(1):76-81).

INTRODUCTION

The amniotic sac is the sac in which the fetus develops in amniotes. It is a tough but thin transparent pair of membranes, which hold a developing embryo (and later fetus) until shortly before birth, the inner membrane is the amnion, contains the amniotic fluid and the fetus and the outer membrane is the chorion that contains the amnion and is part of the placenta [(1)] .The amniotic sac is filled with amniotic fluid (clear, pale straw-colored fluid, which gives osmotic and physical protection to the embryo during the remainder of its fetal existence). Amniotic fluid is a good source of stem cells, its intermediate between embryonic stem cells and adult stem cells; they are multipotent stem cells of mesenchymal origin. Amniotic stem cells are able to differentiate into various tissue types such as skin, cartilage, cardiac tissue, nerves, muscle, and bone, and may have potential future medical applications[(2)] . Tooth eruption is a localized process in the jaws which exhibits precise timing and bilateral symmetry. Develop within the jaws and their eruption is a complex infancy process during which they move through bone to their functional positions within the oral cavity [(3)].

RANKL is a member of the tumor necrosis factor (TNF) cytokine family which is a ligand for osteoprotegerin and functions as a key factor for osteoclast differentiation and activation[(4).] RANKL also has a function in the immune system, where it is expressed by T helper cells and is thought to be involved in dendritic cell maturation. T cell activation was reported to induce expression of this gene and lead to an increase of osteoclastogenesis and bone loss [(5)]. RANKL/RANK signaling regulates the formation of multinucleated osteoclasts from their precursors as well as their activation and survival in normal bone remodeling and in a variety of pathologic conditions [(6).]

MATERIALS AND METHODS

Seventy nine Albino Swiss female mice were used in the present study. Those mice were divided into 3 main groups:

1. Experimental group: consisted of 16 mice of one day old of age injected with isolated amniotic stem cells in the anterior region of maxilla (incisors area). Sacrifice 4 mice for each period (4, 7, 10, and 13) day old age. Those 16 mice injected with **amnionic** cells, 4mice for each scarifying periods.
2. Control group: consists of 16 mice of one day old age, injected with normal saline in the anterior incisors region of maxilla. Sacrifice 4 mice for each period (4, 7, 10, and 13) day.

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3. Pregnant mice group: consists of 15 pregnant mice: 5 out of 15 were used to collect their autologous amniotic fluid at (13 day of gestation period), and stored to be used to their neonatal embryo. While other 10 pregnant mice were scarified to obtain amnionic and chorionic cells from their placenta at (17 day of gestation period).

Collection of amniotic fluid

Amniotic fluid was collected from each 5 pregnant mice at 13 day of gestation period (separately) , by using needle aspiration technique, cleaned their skin and wiped with alcohol, then aspirate the fluid using insulin syringe and preserved the amniotic fluid in sterile tube at -80°C until it used.

Isolation amniotic stem cells from the placenta

Samples were obtained from 10 pregnant mice at 17 day gestation period to isolate Chorion and Amnion, after sacrifice the pregnant mice by over dose anesthesia, the embryos inside amniotic membrane with their placenta will excluded immediately. Then isolate the embryo from the placenta, and carrying the following procedures:

1. The placenta was cleaned from blood clot with a sterile phosphate-buffered saline solution.
2. Removing of amniotic membrane from embryos and put in flask.
3. Take a pair of sterile scissors and carefully cut the outside epithelial layer off. The more cut the more stem cells get. The amnion layer is mechanically peeled off the Chorion.
4. Washing the amnion in Phosphate buffered saline solution (PBS) in several times (8-10X) to remove blood.
5. Mince the tissue thoroughly with a pair of another sterile scissors.
6. To release amniotic epithelial cells, incubate the minced amnion membrane with Trypsin (0.05%) for 10 minutes at 37°C.
7. Treating the remaining tissue in another tube of trypsin (0.05%) for 20 minutes at 37 °.
8. Pooling the cells from the digests.
9. Fuge the filtered cell suspension for 8 minutes at 1200 RPM.
10. Washing the cell pellet with PBS and fuge again.
11. Counting the cells with a hemocytometer and it is advisable to determine the viability of the cells by exclusion of trypan blue dye,
12. Resuspending the pellet in freezing medium by pipetting gently.
13. In order to freeze the cells gradually and safe, place the ampoules in -60°C or less and leave them there for 16-24 hours[7]. **(**All operation was done under sterile condition, using a laminar flow

Monoclonal antibodies RANKL and their Detection kit

Monoclonal antibody (Mouse anti- Mouse) US Biological RANKL (OPGL, ODF,Receptor Activator of Nuclear Factor Kappa Ligand) Immunohistochemistry with Detection Kit, HRP, Mouse Tissue, BioAssay™, US Biological, IHC detection kit,HRP, Mouse Primaries (Catalog No.17506-06)

RESULTS

- Histological and immunohistological tests for detection the expression of CD34 marker were performed on both experimental and control groups for all periods.

Microscopic evaluation of resorption area of overlying tooth germ of mouse **4** days old treated with **Amnion** shows multiple osteoclast cells stained strong positive with DAB of the marker RANKL near resorbed bone. Figure (1).

Area of resorbed bone overlying tooth germ of mouse **4** days treated with **Chorion** illustrates expression of RANKL on osteoclast cell .Figure (2)

Figure (3) shows multinuclear giant cells in resorption bone area of tooth mouse **7** days old treated with **Chorion.** Multiple osteoclast brown color indicating positive histochemical reaction with RANKL marker. The bone in the proximal area of the tooth shows RANKL expression by strong positive stain in figure (4).

View of positive DAB stain for osteoclast indicated expression of RANKL on apical resorbed area of tooth and proximal area of the tooth of mouse 7 days old treated with **Amnion**. Figures (5 and 6).

Figure(7) shows positive RANKL expression in apical resorbed bone area of tooth mouse 10 days old treated with **Chorion**. High power view of immunohistological stain for resorbed bone shows positive RANKL expression. Figure (8)

Apical area of tooth for mouse 10 days old treated with Amnion shows positive RANKL expressed on osteoclast in resorbed bone Figure (9).

Figure (10) illustrates osteoclast cell expressed RANKL marker in resorbed bone of tooth mouse 10 days old **(Control**).

Multiple osteoclast cells in resorbed bone area shows positive RANKL marker with positive mononuclear cells either free in adjacent area or in pay like cavity of apical and proximal resorbed bone of tooth mouse of 10 days old treated with **amniotic fluid** Figures (11, and 12).

- Coincidence of expression marker for **RANKL** in studied groups illustrated that Amniotic fluid shows high expression of RANKL in surrounding and apical areas while overlying area expressed high RANKL marker in Control group

DISCUSSION

The present results localized and identified RANKL marker in 3 areas of developing tooth of the studied groups includes overlying, surrounding and apical bone.

The expression of RANKL with high significant value by osteoclast of surrounding and apical bone at day 10 in amniotic group could be explained as fallow.

1.The mononuclear cells recruited to the dental sac must fuse to form osteoclasts for resorption of alveolar bone for the eruption pathway[8] . Each time correlates with the maximal burst of osteoclastogenesis in each species.

2. A major burst of osteoclastogenesis occurs at day 3 and the molecular regulation of this by the dental sac is critical for eruption. In essence, two molecules known to promote osteoclastogenesis, CSF-1 and RANKL, are required for this major burst [9]. Although RANKL also is expressed in the dental sac at day 3, its gene expression is not unregulated at this time [10]. However, the down-regulation of OPG at day 3 would result in a ratio of RANKL/OPG that would favor osteoclastogenesis. The maximal expression of CSF-1 at this time would also promote osteoclastogenesis, given that CSF-1 upregulates the expression of RANK in the osteoclast precursors to enhance cell-to-cell signaling of RANKL and RANK [11].

3.A minor burst of osteoclastogenesis at day 10 prior to eruption appears to require one or two new genes, as well as an alternation of expression of genes also expressed at day 3 (major burst). Specifically, CSF-1 expression is reduced at day 10 but its function, in part, appears to be replaced by vascular endothelial growth factor (VEGF) which is maximally expressed in the dental sac at days 9–11[12,13].

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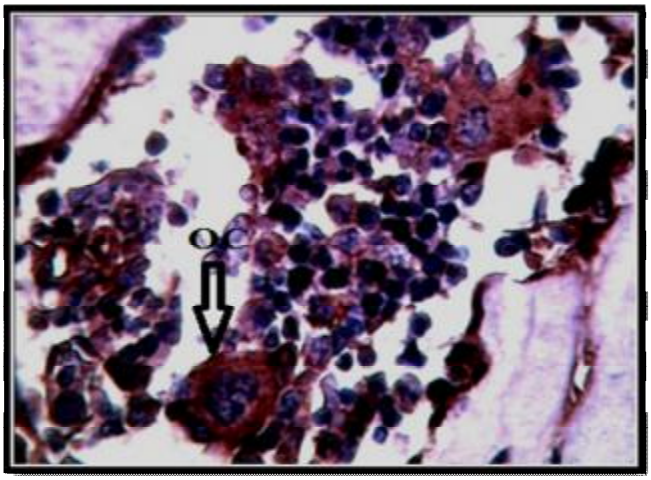

Figure 1: RANKL expressed on osteoclast cell (OC) in bone resorption area of overlying tooth germ of mouse 4 days old treated with Amnion. DAB stain with counter stain hematoxylin, X400.

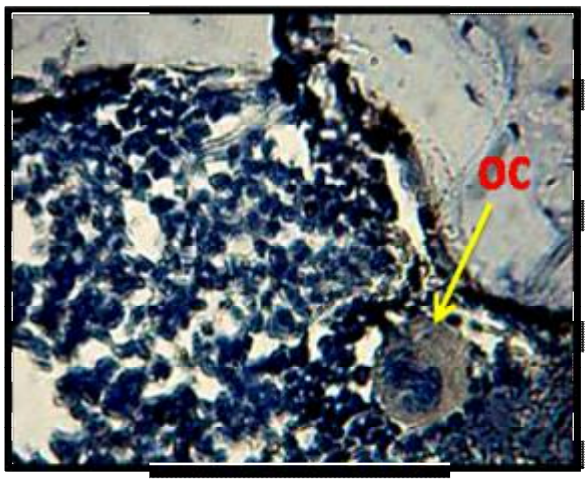

Figure 2: RANKL expressed on osteoclast cell (OC) in resorbed bone overlying tooth germ of mouse 4 days old treated with Chorion. DAB stain with counter stain hematoxylin. X400.

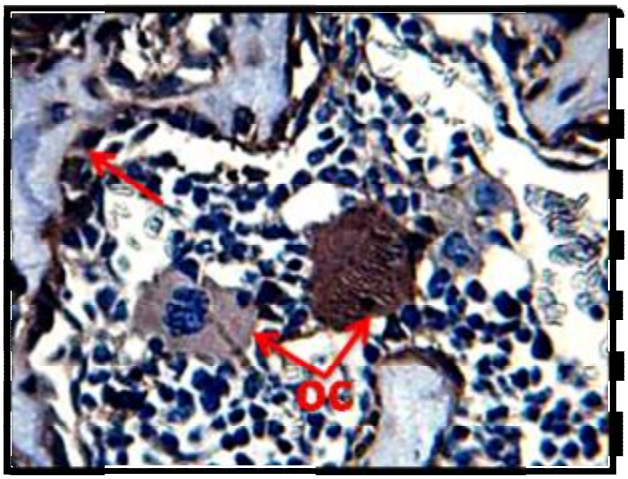

Figure 3: RANKL expressed by osteoclast cell (OC) in resorption bone area of tooth mouse 7 days old treated with Chorion. DAB stain with counter stain hematoxylin, X400

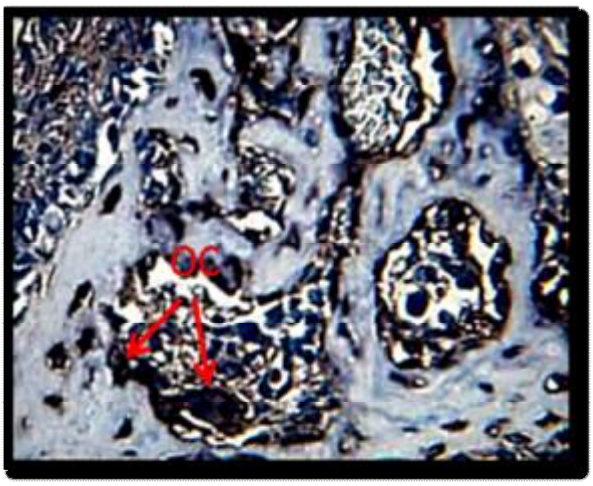

Figure 4: Positive RANKL expression by osteoclast (OC) seen in bone surround the tooth of previous figure (3) DAB stain with counter stain hematoxylin,. X400

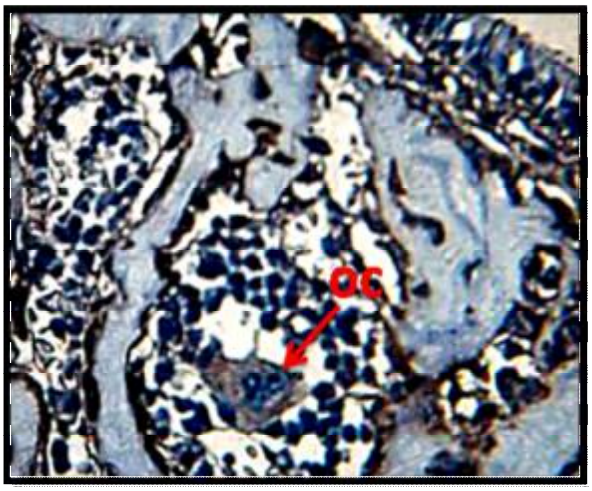

Figure 5: RANKL demonstrated on osteoclast cell (OC) in apical resorbed area of tooth mouse 7 days old treated with Amnion. DAB stain with counter stain hematoxylin, X40.

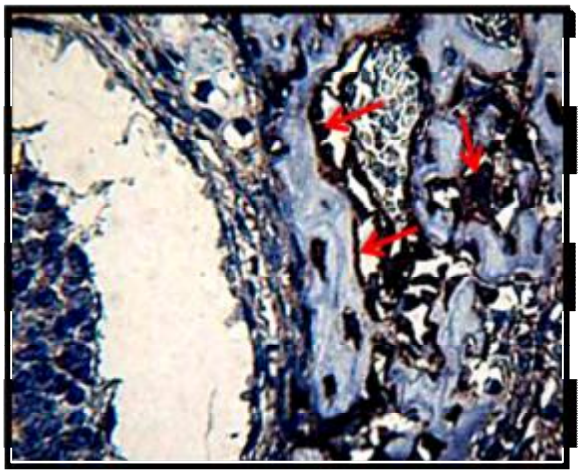

Figure 6: Positive RANKL expression seen in resorbed bone (arrow) of surrounding area in the tooth of previous figure (5). DAB stain with counter stain hematoxylin, X400.

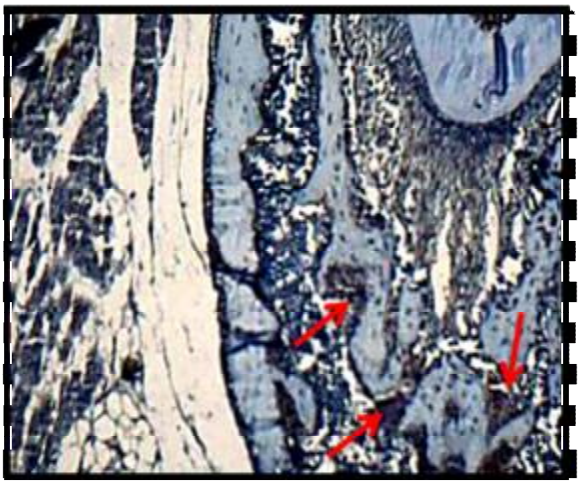

Figure 7: Positive RANKL expression (arrow) seen in apical resorbed bone area of tooth mouse 10 days old treated with Chorion. DAB stain with counter stain hematoxylin, X200.

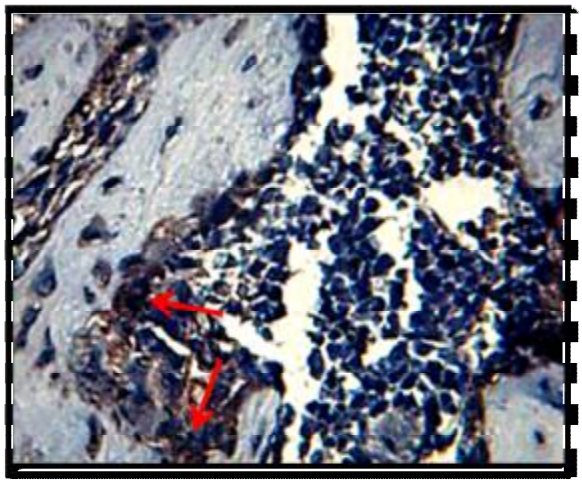

Figure 8: High magnification view of previous figure (7) shows positive RANKL marker by osteoclast cells in resorbed bone (arrow). DAB stain with counter stain hematoxylin, X400.

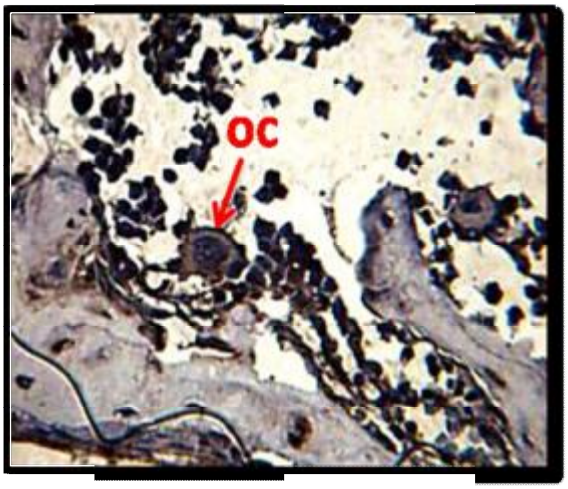

Figure 9: Positive RANKL expressed on osteoclast in resorbed bone (apically) for tooth mouse 10 days old treated with Amnion. DAB stain with counter stain hematoxylin, X400.

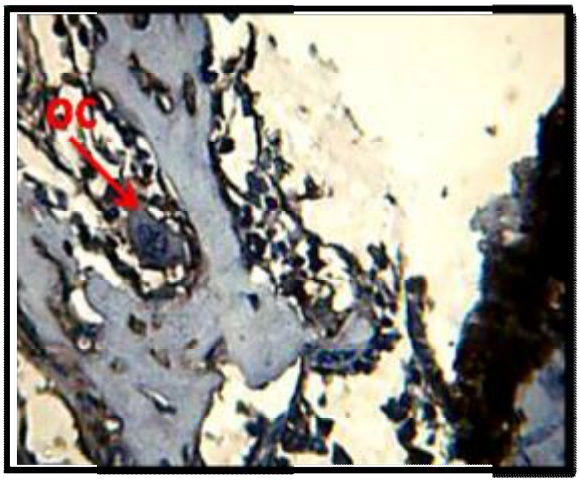

Figure 10: Osteoclast cell expressed RANKL marker in resorbed bone of tooth mouse 10 days old (Control). DAB stain with counter stain hematoxylin, X400.

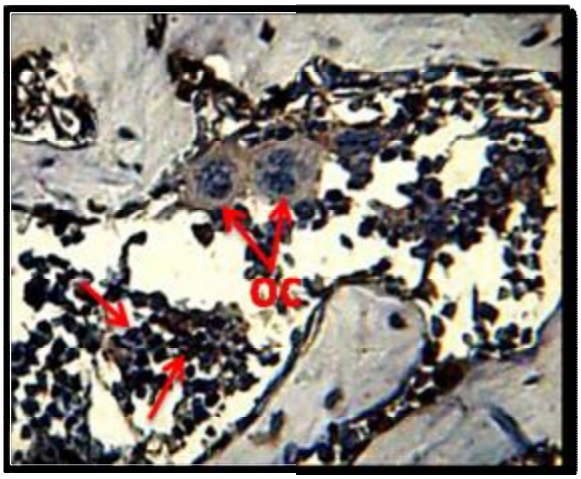

Figure 11: Multiple osteoclast cell (OC) in apical resorbed bone area shows positive RANKL marker with positive mononuclear cells (nearby) (arrow) section related to tooth mouse 10 days old treated with amniotic fluid. DAB stain with counter stain hematoxylin, X400.

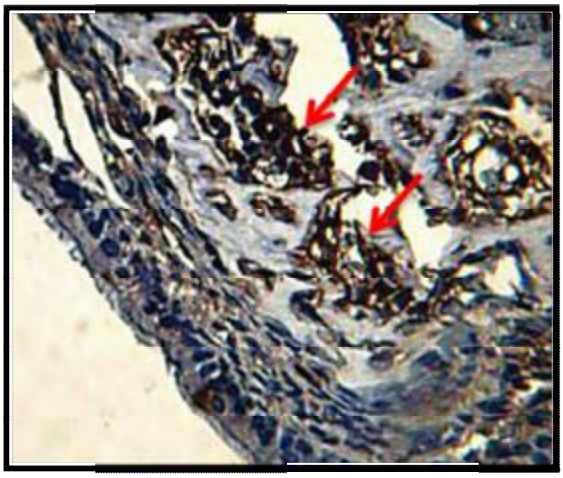

Figure 12: Mononuclear cells (arrow) presented in pay like cavity of proximal resorbed bone of tooth mouse of 10 days old treated with amniotic fluid. DAB stain with counter stain hematoxylin, X400.