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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Cleft palate reconstruction by platelet-rich-plasma and stem cell injection: Histological evidences

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Cleft palate (CP) is a common congenital defect. It causes serious problem to cleft-affected children. The gold standard of care is autogenous bone grafting which may cause additional problems together with long and extensive medical interventions. Tissue engineering is a promising solution for a widespread range of defects and disorders. It is reasonable to utilize this novel technology for CP management. Stem cells and growth factors play essential role in tissue engineering, so we evaluate effectiveness of adipose tissue and cord blood stem cells along with platelet rich plasma (PRP) on CP reconstruction. Human umbilical cord blood mononuclear cells (hUCB-MNCs) and human adipose-derived stem cells (hADSCs) were collected and incubated with Bromodeoxyuridine (BrdU) for labeling. The same was done to osteogenically differentiated hADSCs. Palatal bone defects were surgically made in rats. Afterwards, the labeled cells were mixed either with PRP or Aminoplasmal and injected to the defect borders. Immunohistochemistry and morphometry analysis were performed 4 weeks later. Data showed a significant difference in cleft size between cell-injected and control groups while the cleft site was filled with connective tissue rather than osseous tissue. Moreover, immunohistochemistry findings proved the presence of labeled cells in the surrounding tissue. These cells were detectable both in osseous and connective tissues. This study revealed the feasibility of stem cell and PRP application according to CP reconstruction. Hence, further investigation toward tissue engineering in CP may eliminate bone harvesting and its negative consequences.

Key words: Cleft palate, platelet-rich-plasma, cell therapy, stem cells, tissue engineering.

INTRODUCTION

During weeks 8 to 12 of human intrauterine life, palate is formed (palatogenesis). This structure separates oral and nasal cavities from each other. Palatogenesis is a highly coordinated process, depending on anatomical specific

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and precisely timed molecular signals for normal development (Chai and Maxson, 2006). Palatal shelves are two protrusions from lateral walls of the primary oral cavity which fuse in the midline to form the majority of palate named secondary palate. Secondary palate is continuous with the primary palate anteriorly and the soft palate posteriorly (Bush and Jiang, 2012). The interruption in fusion of these segments may cause cleft palate (CP) which is a frequent congenital defect in human kind (Farina et al., 2002). Birth prevalence ranges from 1/500 to 1/2,000 in different races (Marazita et al., 2004).

Cleft palate reconstruction procedure should tackle normal speaking and feeding abilities. At the same time, normal facial appearance should be considered. In this way, one of the major difficulties is bony reconstruction. Therefore, autogenous bone grafts is the gold standard of care of CP now. The most common bone donor sites are tibia, mandible, ilium, cranium and ribs (Batra et al., 2004). Bone harvesting procedure yields limited amount of bone which may not sufficient for grafting and cause failure (Panetta et al., 2008). In addition, bone harvesting procedure is followed by several complications mostly related to the donor site; morbidity is the major problem (Batra et al., 2004). The reported success rate was 41 to 73% after primary grafting; unfortunately most of patients need additional operations (Nguyen et al., 2009). On the other hand, grafting surgery may cause some unwanted consequences such as facial growth disturbance result (De La Pedraja et al., 2000) and mid-face retrusion (Waite and Waite, 1996). Postoperative oronasal fistula development in grafted area still remains a significant challenge of the palatal reconstruction surgery. There is an incidence rate ranging from 11 to 23% (Amaratunga, 1988; Cohen et al., 1991; Emory et al., 1997; Landheer et al., 2010). Allogeneic and synthetic materials are another available choice, but they have some disadvantages as well including risk of infection, immunologic issues, structural integrity and contouring abnormalities (Bostrom and Mikos, 1997). Hence, researches are going on to improve the way cleft palate will be managed in the future.

Tissue engineering opens a new sight of view towards many types of defects and disorders management. Cells, growth factors and scaffolds are essential parts of tissue engineering (Logeart-Avramoglou et al., 2005). An optimal cell source is characterized by availability in large quantities, no immune rejection, no graft versus host disease, no tumorigenicity, predictable differentiation potential for stem cells, and integration into the tissues (Logeart-Avramoglou et al., 2005). Mesenchymal stem cells (MSCs) are appropriate choice for tissue engineering (Diao et al., 2009). Different sources of MSCs are available all over the body including umbilical cord blood (UCB-MSCs), Wharton's jelly (UC-MSCs), bone marrow (BM-MSCs) (Diao et al., 2009) and adipose tissue (ADSCs) (Conejero et al., 2006; Tobita et al., 2008).

The use of MSCs in cleft recostruction is still rare in clinic. However, autologous and allogeneic BM-MSCs along with hydroxyapatite/tricalcium phosphate scaffold were applied in alveolar cleft models. There was no immunological response to neither autologous nor allogeneic BM-MSCs and it showed both cells had the capacity to regenerate bone within craniofacial defects (Kok et al., 2003). Another study combined autologous BM-MSCs with sponge collagen protein and investigated restoration of the alveolar cleft in dog model by these materials. Results showed this method can be used clinically to treat alveolar cleft (Ou et al., 2007). Likewise, desirable results came from another study which used BM-MSCs seeded into different kinds of scaffolds for alveolar bone repairment in dog model (Mylonas et al., 2007).

Adipose tissue and umbilical cord blood have become extremely attractive options for cell-based therapy to avoid pain and stigma associated with the bone marrow harvesting procedure, and also to achieve greater number of stem cells (Mizuno et al., 2012; Seghatoleslam et al., 2012). Rat fat-derived stem cells seeded onto poly-L-lactic acid scaffold also used to repair rat palatal bone defect models, the feasibility of using these cells for healing such defects was demonstrated (Conejero et al., 2006). Moreover, engineered bone from canine ADSCs and autogenous bone graft were compared on a survey; autogenous bone graft was statistically more effective than engineered bone (Pourebrahim et al., 2013).

To improve stem cells' ability to form osteoblasts many studies treat the cells with the osteogenic growth factor BMP2 (Chin et al., 2005; Kang et al., 2011). But they fail to determine levels of osteoinductive effects of BMP2 (Zuk, 2008). In addition, adverse events are reported along with BPM2 application (Woo, 2012). As such, further studies need to find the best growth factors source for regenerative medicine. Platelet-rich-plasma (PRP) is a readily available source of growth factors.

Platelet-rich-plasma is blood derivative defined as high concentration of platelets in a small volume of plasma (Marx, 2001). Platelet's alpha granules contain molecules as platelet-derived growth factor, transforming growth factor β, epidermal growth factor, fibroblast growth factor, insulin-like growth factor I, vascular endothelial growth factor and others. These factors stimulate local inflammation, granulation tissue formation, cell proliferation, cell differentiation, neovascularization and extracellular matrix production which are essential for tissue regeneration (Jamilian et al., 2007; Méndez et al., 2006; Ravari et al., 2011). The rational beyond using PRP in tissue engineering is due to release bioactive factors, which promote angiogenesis and also the extracellular matrix synthesized (Ravari et al., 2011; Shirvan et al., 2013a; Shirvan et al., 2013b). There is a gap in researches on CP reconstruction using stem cells in combination with PRP. However, MSCs have been combined with platelet-rich plasma to heal an alveolar cleft in a 9-yr-old girl, recently (hibi et al., 2006).

The objective of this study is to evaluate usefulness of

hADSCs and hUCB-MNCs along with PRP in cleft palate reconstruction, as an alternative method for bone harvesting.

MATERIALS AND METHODS

Cell isolation and culture

Human adipose-derived stem cells (hADSCs) and human umbilical cord blood mononuclear cells (hUCB-MNCs) are used in this study. All procedures were approved by Mashhad University of Medical Sciences ethics committee, informed consent was obtained from all donors.

The human adipose tissues were obtained by liposuction from the abdominal subcutaneous fat of healthy and non-addicted 30-40 years old women. In order to hADSCs isolation, the adipose tissue samples were washed with phosphate-buffered saline (PBS), and digested by 0.01% type-1 collagenase in PBS (1.5 h at 37°C). Thereafter, hADSCs were sedimented at 600 g for 15 min at 25°C, and cultured in αMEM (Gibco, Invitrogen Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Sigma, St. Louis, MO) (Taha and Hedayati, 2010). Non-adherent cells were removed 24 h later; afterward, cultural medium was changed every third day. Cells were passaged at 70% confluency using tripsin-EDTA (Gibco, Invitrogen Carlsbad, CA). The cells from third passage were harvested, their viability estimated by means of a Neubauer hemocytometer plate and Trypan blue dye exclusion method. These cells were used for characterization by flow cytometer, *in vivo* experiments and osteogeinc differentiation (Rada et al., 2012). For *in vivo* experiments, hADSCs and osteoblastic differentiated cells were labeled with 3 µg/ml bromodeoxyuridine (BrdU) for 48 h.

Umbilical cord blood was collected from umbilical cord after childbirth. Mothers were healthy, non-addicted, and ranging in age from 20 to 40. Standard blood collection bags containing citrate phosphate dextrose adenine (CPDA) were used for this purpose. Cell isolation was done by density gradient technique using Ficoll-Paque solution (Sigma-Aldrich, Inc., St. Louis, MOUSA), and centrifuge at 800 g for 20 min at 25°C. Mononuclear cells (MNCs) layer, the buffy coat, was carefully collected and washed twice with PBS. Then, the cells were re-suspended in fetal bovine serum (10%) supplemented RPMI medium (both from Gibco, Invitrogen Carlsbad, CA) plus 3 µg/ml BrdU, which labels DNA, and incubated in CO_2 incubator at 37°C. After 24 h the cells were harvested and re-suspended in UCB serum for *in vivo* experiment (Seghatoleslam et al., 2012). The hUCB-MNCs viability was assessed and flow cytometric characterization was done as well.

Cell characterization

With the aim of hUCB-MNCs characterization, the expression of hematopoietic stem cells surface markers (CD45 and CD34) were evaluated (Seghatoleslam et al., 2012). Mouse anti-human-CD45-FITC and mouse anti-human-CD34-PE specific monoclonal antibodies (AbDSerotec, Inc., Endeavour House, Kidlington, Oxford, UK) were used.

As stated by others, hADSCs was positive to several CD markers including CD9, CD29, CD49, CD54, CD105, CD166, CD44, CD71, CD10, CD13, CD73, CD90, CD59, CD146 and CD55 (Taha and Hedayati, 2010). Here we assessed CD29 and CD90 cell surface markers expression rate by mouse anti-human-CD29-FITC and mouse anti-human CD90-PE (AbDSerotec, Inc., Endeavour House, Kidlington, Oxford, UK).

Briefly, samples were incubated with antibody for 20 min at 25°C in the dark, washed with PBS and run through a BD FACS machine (BD Biosciences, San Jose, CA) which was tuned with respect signal amplification for fluorescein isothiocyanate [FITC (FL1)] and phycoerythin [PE (FL2)]. The isotype control antibodies were used for confirmation. Data analysis was performed by WinMDI 2.9 software.

Osteogenic differentiation

At the third passage of hADSCs osteogenic differentiation induced by a media contained 100 nM dexamethasone, 5 µM ascorbic acid, 0.5 mM β-glycerophosphate, and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). Differentiation occurs after day 14, and a week after that almost all cells will show osteoblast properties (Desai et al., 2013).

Confirmation of osteogenic differentiation

Osteogenic media was removed after 21 days, and cells were washed with PBS and fixed with par formaldehyde 4%. Afterward, the fixative was eliminated, alizarin red solution was added, and cells were incubated at 25°C for 20 min, washed with excess distilled water. Finally, the cells were scanned by a microscope (Olympus, BX51, Japan) attached to a digital camera (Canon, IXUS 950 IS).

Preparation of activated hPRP

A 450 ml blood sample was obtained from blood bank of Mashhad, Iran. The two steps centrifugation method was carry out to remove erythrocyte fraction, and then platelets were sedimented. Subsequently, platelets were re-suspended in appropriate volume of plasma. In order for platelet activation, several freezing and thawing cycles were done; then hPRP was stored at -70°C until use (Mishra et al., 2009).

Surgical procedure

Sixty four 2-months-old female Wistar rats, weighting about 200-250 g, were housed at a constant temperature of 24.5°C and fed normally. All institutional and national guidelines for the care and use of laboratory animals were followed. The rats were anesthetized with Ketamin (80 mg/kg) and Xylasine (5 mg/kg) intraperitoneally. A critical size bony defect was made in the midline of palate, posterior to the upper incisors and anterior to palatal rugae, using a surgical trephine driven by a low-speed dental engine (Takano-Yamamoto et al., 1993). Animals were divided into eight groups (n=8) on the basis of the injected materials to the defect borders: 1) Group N, normal saline; 2) Group P, PRP without cells; 3) Group A, aminoplasmal without cells; 4) Group AP, undifferentiated hADSCs with PRP; 5) Group OP, osteogenically differentiated hADSCs with PRP; 6) Group AA, undifferentiated hADSCs with aminoplasmal; 7) Group OA, osteogenically differentiated hADSCs with aminoplasmal; 8) Group UP, UCB-MCs with PRP. Each rat received just one injection immediately after surgery. The volume of each injection was 300 µl contained 4×10^6 cells. The recovery after operations was uneventful and animals continued to feed normally.

Computerized morphometric measurement

Animals were sacrificed 4 weeks after injection and palates harvested. Images were taken from samples using a digital camera (Canon, SX260 HS), a ruler was used as scale bar in the images

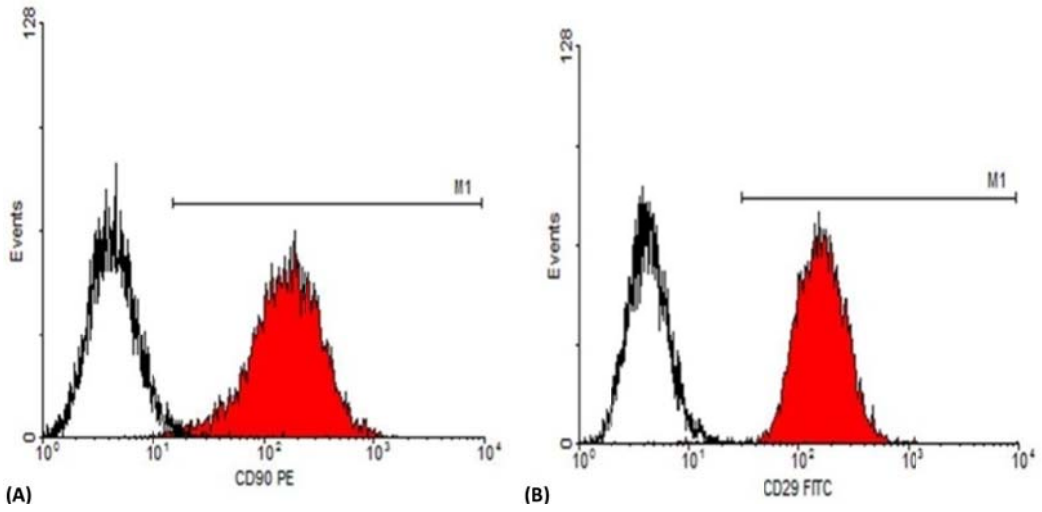

Figure 1. Histogram of hADSCs after the third passage for CD90 PE conjugated stained cells (A) and CD29 FITC conjugated stained cells (B). The first surge shows negative controls.

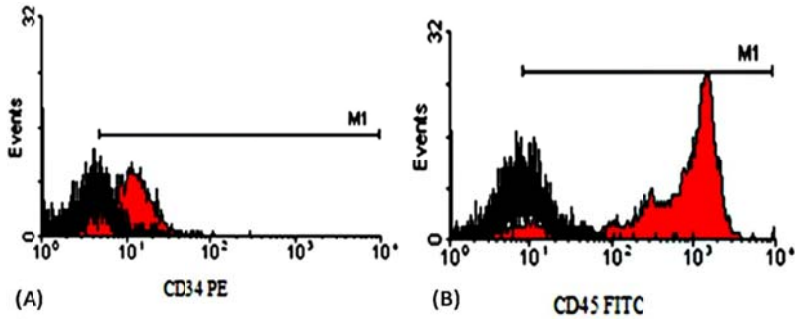

Figure 2. Histogram of hUCB-MNCs after isolation for CD34 PE conjugated stained cells (A) and CD45 FITC conjugated stained cells (B). The first surge shows negative controls.

(Figure 4). The clefts area was measured by freely available AnalyzingDigitalImages software (University of California, Berkeley) adjusted by each picture scale bar (more information presented in the software manual). The cleft size was reported in millimeters squared.

Immunohistochemistry

Palates were fixed with 10% formalin for 4 days, decalcified with 40% acid formic in PBS for 3-4 weeks, dehydrated, and embedded in paraffin. The paraffin blocks were cut in to 7 µm thickness. The sections were deparaffinized, rehydrated and used for immune-histochemistry staining. Heat mediated antigen retrieval was used to unmask antigens. After that, permeabilization was carried out with 1% BSA and 1% tritoin X, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 15 min at room temperature, then non-specific antigen blocking was preformed with PBS containing 1% goat serum. Afterward, sections were incubated with Anti-BrdU or Anti-human osteopntine as primary antibodies and anti-mouse IgG peroxidase conjugated as secondary antibody. Finally, diaminobenzidin (DAB) chromagen was applied to visualize antigens. Counterstaining was done with hematoxylin, cover slipped and scanned by BX51 microscope (100X) attached to a digital camera (Canon, IXUS 950 IS). Cells labeled with BrdU were counted using freely available Image J 1.47v software.

Statistical analysis

Statistical analysis was performed by SPSS 11.5 software (SPSS, Inc., Chicago, Ill.) and one way ANOVA used to compare groups followed by Post Hoc Tukey test.

RESULTS

Flow cytometry analysis

The results obtained from flow cytometry analysis of the hADSCs after the third passage is shown in Figure 1 which confirmed the expression of both CD29 and CD90. The mean percentage of expression was 95.25 and 99.61%, for CD29 and CD90, respectively.

As can be seen in Figure 2, the flow cytometry analysis of the hUCB-MNCs indicates that CD45 and CD34 cell

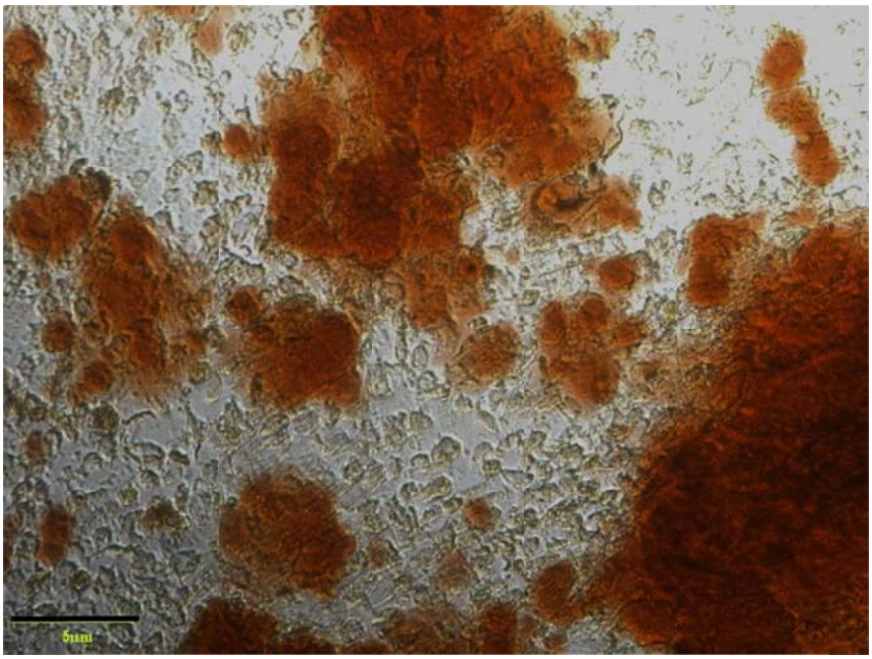

Figure 3. Osteogenic differentiation performed by alizarin red staining. Calcium depositions stain red.

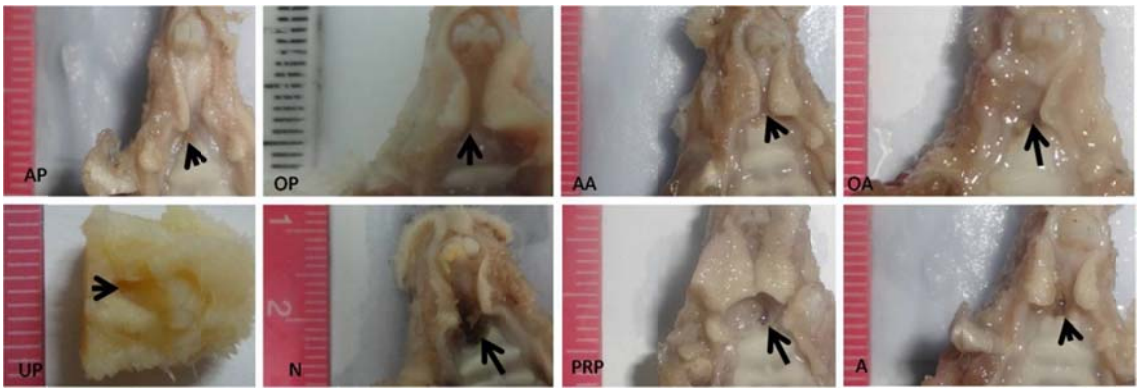

Figure 4. Palates 4 weeks after surgery. (group N, normal saline; group P, PRP without cells; group A, aminoplasmal without cells; group UP, PRP with hUCB-MNCs; group AP, PRP with undifferentiated hADSCs; group AA, aminoplasmal with undifferentiated hADSCs; group OA, aminoplasmal with osteogenically differentiated hADSCs; and group OP, PRP with osteogenically differentiated hADSCs) (Arrows show defect site).

surface markers were expressed among gaited cells. The mean percentage of expression was 89.43% for CD45 and 20.99% for CD34.

Osteogenic differentiation

While osteogenic differentiation takes place cells start to deposit calcium ions in the extra cellular matrix. These nodular calcium depositions are detectable by specific stains like Alizarin red. This is illustrated in Figure 3 that calcium depositions were stained red while cells remain unstained by Alizarin red staining.

Morphometric analysis

Results show the effectiveness of SCs and PRP injection for palate defects reconstruction (Figure 4). This results is considered as significant at the $P<0.05$ level. Image analysis demonstrates withdrawal of defect edges and bone resorption in control group (N) which had wide

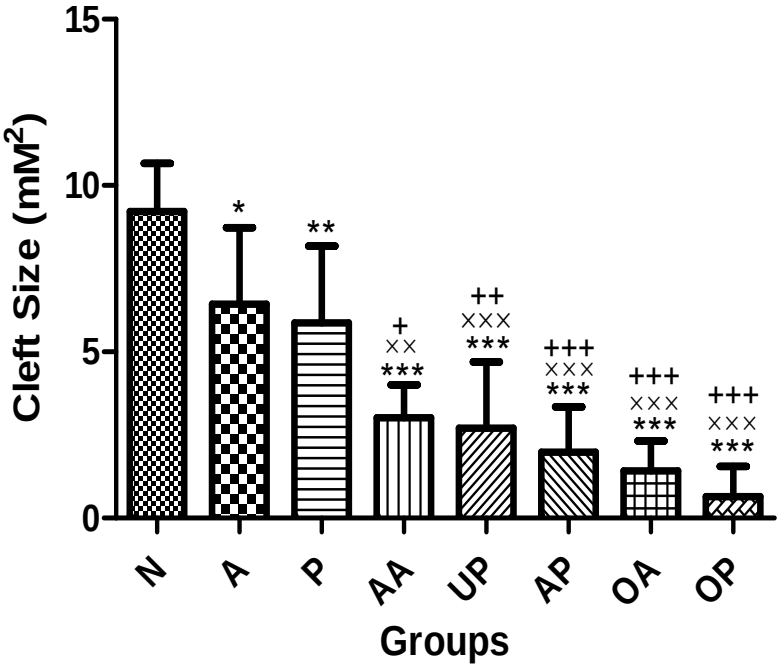

Figure 5. Statistical analysis of cleft size among different groups. Arrows show the defect site. (group N, normal saline; group P, PRP without cells; group A, aminoplasmal without cells; group UP, PRP with hUCB-MNCs; group AP, PRP with undifferentiated hADSCs; group AA, aminoplasmal with undifferentiated hADSCs; group OA, aminoplasmal with osteogenically differentiated hADSCs; and group OP, PRP with osteogenically differentiated hADSCs) (*: P value as compared to N; ×: P value as compared to A; +: P value as compared to P; *: P<0.05; **: P<0.01; ***: P<0.001).

defects. Conversely, all other groups had significant smaller defects in comparison with N. From the data in Figure 4, it is apparent that cell administration regardless the source and differentiation level had significant effect on cleft closure (P<0.001). We did not find any significant difference between PRP and aminoplasmal as carrier, but both were significantly different as compared to N (P<0.01 and P<0.05 for P and A, respectively). It is also shown in Figures 4 and 5 that all experimental groups had statistically significant effect to reduce defect size in comparison with P and A groups (P values are mentioned on Figure 5) (all data in each group passed normality test before ANOVA analysis). Our study did not show any significant difference between differentiated and undifferentiated cells (Figure 5).

Immunohistochemistry

In cell treated groups, BrdU+ cells were detectable at cleft site 4 weeks after injection (Figure 6). Microscopic scanning of stained sections revealed that the defects were filled with connective tissue rather than bony tissue, but the labeled cells were also seen in surrounding bone. The cell counts are summarized on Figure 7. There were significant differences between hADSCs treated groups and hUCB-MNCs (P<0.01), but there were no differences among other groups.

Immuno-staining for human osteopontin confirmed the expression of this protein and its presence in extra cellular matrix on all cell treated groups (Figure 8).

DISCUSSION

The current study showed that stem cells along with PRP could have a positive effect on cleft palate reconstruction. Surprisingly, stem cell application along with aminoplasmal had almost same effect. Contrary to expectations, this study did not observe statistically significant difference between two stem cell types applied in the survey. Another important finding is that osteogenic differentiation seems to have no effect on palate reconstruction procedure.

As mentioned previously, there are several disadvantages related to surgical treatment of cleft palate. In order to address these challenges, tissue engineering had been proposed as an alternative therapy

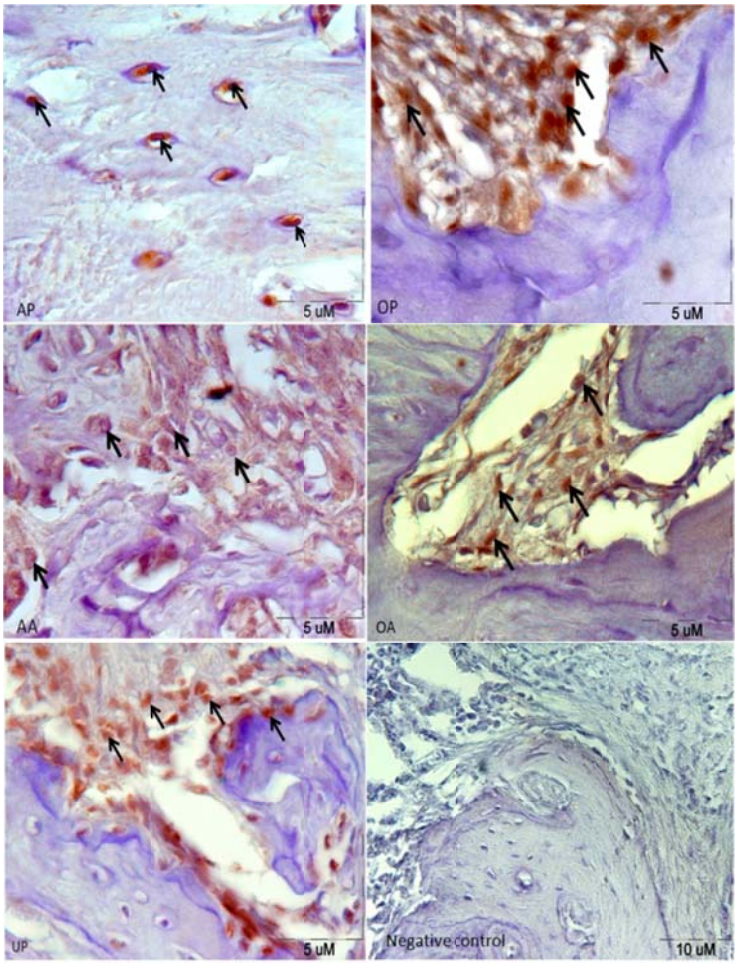

Figure 6. Immunohistochemistry staining against BrdU. Arrows show the stained nucleuses (Group UP, PRP with hUCB-MNCs; Group AP, PRP with undifferentiated hADSCs; Group AA, aminoplasmal with undifferentiated hADSCs; Group OA, aminoplasmal with osteogenically differentiated hADSCs; and Group OP, PRP with osteogenically differentiated hADSCs).

(Conejero et al., 2006; Pourebrahim et al., 2013). However, there are a limited number of experimental studies in this field. Besides, the majority of studies have tried to enhance the standard treatment outcomes by PRP application rather than establish a new method. Therefore, in attempt to use tissue engineering for cleft palate reconstruction, we applied ADSCs and also UCB-MNCs along with PRP.

Platelet-rich-plasma as a storage vehicle of growth factors is applied recently in tissue engineering. The growth factors are platelet-derived growth factor, transforming growth factor-b1 and -b2, platelet-derived epidermal growth factor, platelet-derived angiogenesis factor, insulin growth factor-1 and platelet factor-4 have influence on bone regeneration. In addition, basic fibroblast growth factor, epithelial cell growth factor, interleukin-1 and osteonectin (major protein in mineralization) in the alpha-granules of the platelets (Aghaloo et al., 2002; Marx et al., 1998; Marx, 2004; Tozum et al., 2003). This implies that PRP may influence bone formation through a variety of pathways. Others revealed that PRP stimulated proliferation of bone cells derived from human trabecular bone (Gruber et al., 2002) and rat bone marrow (Oprea et al., 2003). Some studies revealed positive effects of PRP on bone regeneration in combination with bone grafts (Ohva et al., 2005). Additional studies revealed that PRP had a stimulating effect on the initial cell growth and matrix mineralization of rat bone marrow cells (Dolder et al., 2006).

Many studies have been performed on combinations of PRP and mesenchymal stem cells, *in vitro* (Ccho et al., 2011; Dohan et al., 2010; Mishra et al., 2009; Xie et al., 2012). Almost all of these studies' results showed that PRP increased cell proliferation but divergences were found regarding the stem cell differentiation capacity to osteoblasts (Dohan et al., 2010) or chondroblasts

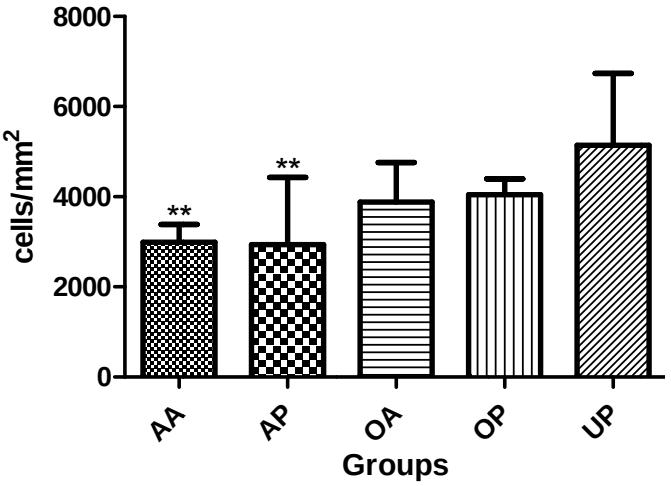

Figure 7. Cell count among groups (Group UP, PRP with hUCB-MNCs; Group AP, PRP with undifferentiated hADSCs; Group AA, aminoplasmal with undifferentiated hADSCs; Group OA, aminoplasmal with osteogenically differentiated hADSCs; and Group OP, PRP with osteogenically differentiated hADSCs)(**: P<0.01).

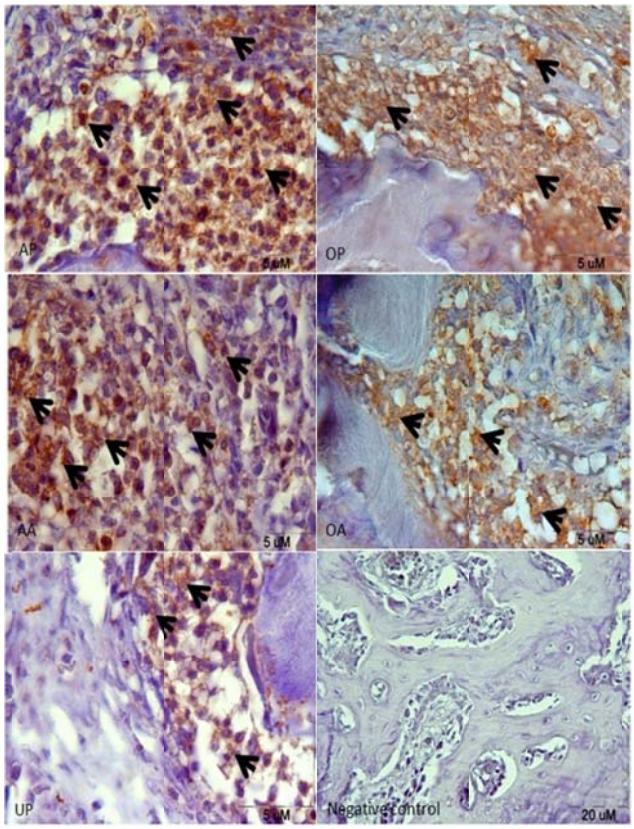

Figure 8. Immunohistochemistry staining against osteopontin. Arrows show stained extra cellular matrix (Group UP, PRP with hUCB-MNCs; Group AP, PRP with undifferentiated hADSCs; Group AA, aminoplasmal with undifferentiated hADSCs; Group OA, aminoplasmal with osteogenically differentiated hADSCs; and Group OP, PRP with osteogenically differentiated hADSCs).

(Drengk et al., 2009; Mishra et al., 2009). This could be due to the differences between PRP preparation methods. In addition, some other surveys showed supportive results toward usage of PRP in wound healing and tissue regeneration due to high concentrations of PDGF and TGF are secreted from platelet α-granules after activation. These growth factors are described as mitogenic and attractant for mesenchymal stem cells, which may mediate regenerative effects (Amable et al., 2013; Eto et al., 2011; Roubelakis et al., 2014).

Judging by the PRP composition and its effects described here, our findings support the use of PRP along with stem cells in CP regeneration. Our study revealed the promising effects of PRP in cleft palate closure. This may be due to elimination of bone resorption and also to aid tissue regeneration. Also, this finding is in agreement with Hibi et al. (2006) which used a mixture of MSCs and PRP for an alveolar cleft osteoplasty in a 9-year-old female patient successfully. Nevertheless, results remain highly controversial due to lack of studies that assessed PRP and stem cell effectiveness on reconstruction of maxillary defects, simultaneously. Despite this, there is limited number of surveys towards clinical application of PRP in maxillary auto-graft surgery which leads to arguable subject (Luaces-Rey et al., 2010; Marukawa et al., 2011).

The rationale behind using stem cells is their ability to differentiate into a wide range of cell types. Considering of tissue engineering principles of stem cells could have the essential role in angiogenesis and osteogenesis. It is obvious that well-established tissue nutrition improves the regeneration (Kanczler and Oreffo, 2008). Adipose tissue is a readily available source of stem cells in body. Adipose derived stem cells have been demonstrated to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes and neurons, depending on the microenvironment, *in vitro* (Gimble et al., 2007). In addition, umbilical cord blood is an abundant source of pluripotent stem cells. The isolated cells from this tissue have been shown to have the capacity to differentiate into different cell lineages (Harris and Rogers, 2007). Furthermore, PRP is utilized in tissue engineering due to its bioactive factors stored in platelet granules. These bioactive factors play important roles in cell proliferation, cell differentiation and angiogenesis (Shirvan et al., 2013a).

This study produced results which corroborate the findings of previous studies in this field (Conejero et al., 2006; De Kok et al., 2003). Conejero et al. (2006) made a surgical defect on rat palate and tried to repair it by osteogenically differentiated ADSCs seeded onto poly-L-lactic acid scaffolds. The samples were harvested 6 or 12 weeks after treatment. Results demonstrated the feasibility of reconstructing bony defects with differentiated ADSCs. Although there was newly formed bone in differentiated cell treated group, but they found connective tissue in undifferentiated cell treated animals (Conejero et al., 2006). Likewise, our data showed connective tissue in all cell treated animals.

Unfortunately, our assessments was done only at 4 weeks following the injections; as we demonstrated the presence of cells and osteopontin in the area it could be possible to find bony material after 6 or more weeks.

Further supporting data comes from other studies which applied other types of stem cells to regenerate alveolar defects including BMSCs, PDLSCs (Kim et al., 2009), autologous and allogeneic BMMSCs (De Kok et al., 2003; Ou et al., 2007).

This experiment did not detect any significant difference between differentiated and undifferentiated cells. This is incontrarywithpreviouslymentionedstudywhichdetermined differentiated cells more effective (Conejero et al., 2006).

While this study presents promising data towards cell therapy for cleft palate closure, it failed in addressing bony tissue defect. However, an earlier study compared tissue regeneration of engineered bone with ADSCs and autogenous bone graft in bilateral maxillary alveolar cleft model. Their results found bone autograft significantly more effective than engineered bone (Pourebrahim et al., 2013).

What is surprising is that aminoplasmal as an injectable mixture of amino acids showed a therapeutic effect. Unfortunately, there was no comparable study in this field. It is remarkable that we choose aminoplasmal as a carrier to omit PRP's association effects, but surprisingly we found it effective itself. So, one of the limitations of this study is the absence of groups with stem cells treatment without PRP or aminoplasmal.

Conclusion

This study proved that the stem cells along with PRP could enhance cleft palate closure in rat models. It seems that the presented method could apply for those cases with small defects to eliminate bone grafting consequences; also PRP and stem cells as adjuvant with autografting may be effective in wide defects. However, caution must be applied towards clinical administration unless more researches are done on this subject. Further work needs to be done to set the exact procedure for clinical use, and also to determine the stem cells effects without PRP or aminoplasmal.

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Conflicts of interest

Authors declare that they have no conflict of interest.

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Full Length Research Paper

Comparison study of oral disc morphology of Saudi *Bufo dhufarensis* and *Rana ridibunda* tadpoles and their oral deformities

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The microanatomy of the oral discs of the tadpoles of *Bufo dhafarensis and Rana ridibunda* were described. Tadpoles of stage 49 and 41, respectively were examined and analyzed using scanning electron microscope. In *Rana* tadpoles, the mouth was ventral and the oral disk opening had the same vertical and horizontal opening size. While in *Bufo* tadpoles, the mouth was anterio-ventral and the oral disc had its horizontal opening larger than the vertical one. The border of oral disc of tadpoles of *B. dhufarensis* was surrounded with 22 marginal papillae and 8 submarginal papillae and a dorsal gap was observed. The oral disc of *Bufo* had less number of marginal papillae where they were short and had broad smooth ends with no apical parts. On the other hand, the border of the *Rana*'s oral disc was surrounded by 50 marginal papillae and 10 submarginal where the dorsal gap was also observed. Both marginal and the submarginal papillae were long, numerous, closely spaced and ending with 3-7 apical parts in each papilla. The labial tooth row formula (LTRF) of the tadpoles of *B. dhufrenensis* was 2/3, while that of the tadpoles of *R. ridibunda pallas* was 2(1)/3(1-2). In *B. dhufarensis*, the labial teeth were keratinized, short ending with 4-7 terminal cusps and arranged as a single row. On the contrary, the labial teeth of *R. ridibunda pallas* were elongated, closely spaced and numerous. These labial teeth were noticed to be either vertically positioned or curved downwards carrying 3-4 terminal cusps in their free ends. Herein, we described Saudi Arabian Amphibian *B. dhufrenensis* and *R. ridibunda pallas* by utilizing scanning electron micrographs. In the present study, we described deformities in the oral disc of *B. dhufrenensis* and *R. ridibunda pallas* tadpoles in their natural conditions.

Key words: Oral disc, tadpoles, *Rana ridibunda*, *Bufo dhufarensis,* labial tooth row formula, deformities.

INTRODUCTION

The marsh frog, *Rana ridibunda* is the only true frog reported from the oasis of Al-Hassa and Al-Qatif regions in Saudi Arabia. This frog has been subjected to several studies to determine the seasonal changes in population

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structure, the breeding behavior, the tadpole development and the individual growth in Saudi climate (Briggs, 1980, 1981; Haas, 2003; Al-Shehri and Al-Saleh, 2005a, b, 2008). Meanwhile, recent research on the amphibian chromosomes of the Arabian Peninsula and *Bufo dhufrenesis* has just started, and few papers have been published (Haas, 2003; Al-Shehri and Al-Saleh 2005a, b, 2008). It is very important to study and protect such creatures because they are under grave threat not only due to general habitat alteration but also to climate change (Fellers et al., 2001). However, more interest has been concentrated on studies of comparative aspects of buccal anatomy related to feeding of Saudi amphibian tadpoles mainly on taxonomy and distribution.

Variations in the size and shape of the oral disc, the papillae at the margins of the oral disc, the shape of the jaws, the numbers of teeth rows and any gaps in those rows are all important features in identifying tadpoles of different species (Duelman and Trueb, 1986). Even among closely related taxa and in many cases, they seem to reflect lineage and habitats (Grandison, 1981; Duelman and Trueb, 1986; Channing, 2001).

Dental formula of a tadpole depicts number and arrangement of tooth rows on its oral disc. The number which is written on the left of "/ "refer to the anterior labium, while that which is written on the right is for posterior labium. Open numbers indicate total number of tooth rows on each labium, number in parenthesis are the number of interrupted rows in order of arrangement on labium. A dental formula of 2(2)/3(1-2) indicates 2 rows on anterior labium where the second one is interrupted with a median gap,while that of the posterior labium has 3 rows where the first and second ones only are interrupted (Channing, 2001).

In *Bufonidae*, both protruded oral disc and keratinized mouth parts were present; the teeth formula was 2/2 or 2/3. In Ranidae, the formula was smaller, 3/3 or more (5/3, 2/4, 3/4 or 6-7/6). In some Hylidae, Pipidae *"Xenopus laevis"* and Rhinophrynidae*"Rhinophrynus dorsalis*" the oral disc were not protruded and the formula was 2/2, 2/3 or 2/4 (Nascimento et al., 2005; Alcalde and Blotto, 2006; Rossa-Feres and Nomura, 2006; Altig, 2007; Vieira et al., 2007; Bekhet, 2012). These keratinized teeth are derived from cells present in the base of the tooth ridge (Gosner, 1959). The function of teeth in frogs is primarily to grasp prey or to position it for swallowing. The oral apparatus of tadpoles of different anuran tadpoles is different and that this difference reflects different feeding habits. The ontogeny of the labial teeth row structure of anuran tadpoles inhabiting temperate regions has been studied by several workers (Bonacci et al., 2008; Tolledo et al., 2009; Erik et al., 2010; Lunaet al., 2012).

Oral deformities may be used as biomonitoring tool, both for detecting contamination and for determining the efficacy of occurrence of deformities in unimpacted and in contaminated wetlands (Cooke, 1981).

Oral deformities have not generated widespread media attention because they are not linked to the declines in amphibian populations, although the pathogens which are believed to cause mortality can also cause deformities in larval mouth parts (Morell, 1999). The developing larvae may remain longer in ponds with longer hydroperiods and thus have a greater incidence of developing oral deformities (Snodgrass et al., 2000). The variability of oral deformities as pigmentation in keratinized cells in tooth rows and jaw sheaths may also be related to either seasonal changes in temperature (Rachowicz, 2002) or infection by *Batrachochytrium dendrobatidis* (Vieira et al., 2013). Deformities in the oral discs have been reported for animals either exposed to the organic pesticide DDT (Osborn et al., 1981) or to coal combustion residues (Rowe et al., 1996, 1998a, b; Peterson et al., 2008). However, Dunson and Travis (1994) stated that the oral deformities have an effect on the feeding ability and growth.

In the current work, the circum oral disc of the Saudi tadpoles was described in details. Here, we complemented these data by taxonomizing the tadpoles of *B. dhufarensis* and *R. ridibunda pallas.* In addition, we investigated oral deformities in the marginal papillae and tooth rows of *B. dhufrenensis* and *R. ridibunda* tadpoles.

MATERIALS AND METHODS

Manipulation

Fertilized eggs from some of the available Saudi Dhufar toad *B. dhufarensis* (Al-Derayya village, Riyadh) and true frog *R. ridibunda* (Al Hassa oasis) were collected from natural ponds with fine mesh hand net .In the laboratory, eggs hatched and tadpoles were reared. After hatching, the larvae were fed on a 20 g meal of boiled spinach daily. The experimental stages were 49 for *Bufo* and 41 for *Rana* were selected according to the normal table of Sedra and Michael (1961), for circumoral studies, since at these stages the tadpoles have typical morphology and the oral disc is fully developed and functional. Both stages are similar in characters and developmental rate of growth.

Experimental design

A minimum of 15 tadpoles for each species were euthanized using 1:10000 MS-222 in distilled water, they were examined using a dissecting microscope to detect the selected stages, then incubated on the surface of moist tissue, in a Petri dish at 25°C.

Scanning electron microscopy

These specimens were fixed in a 2-3% glutaraldehyde solution for 3-4 h at room temperature, followed by three 15 min washes in 0.1 M 50, 70, 80, 95%, three changes at 100%, for 15 min each and a final 5 min wash in acetone 100%. Specimens were critical point dried in CO_2, mounted on aluminum stubs and sputter coated with gold. Structure of oral discs was examined and photographed using a scanning electron microscope attached to acomputer. Terminology used to describe features of the oral cavity follows Wassersug (1997).

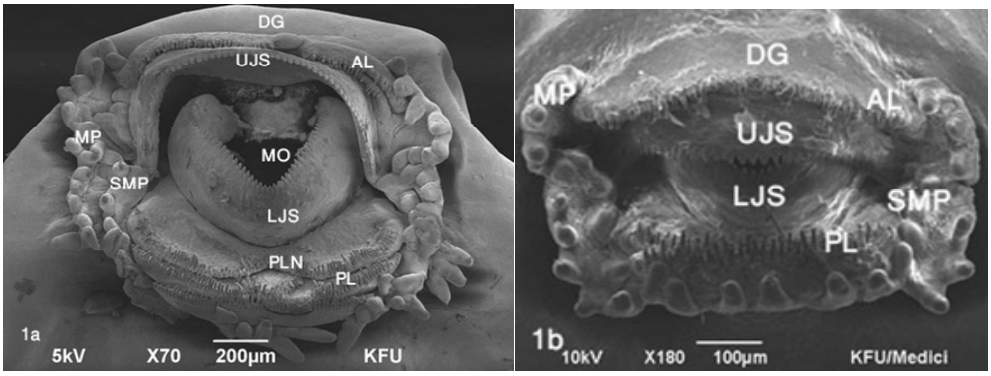

Figure 1. Scanning electron micrographs of the oral disc of: a) *Rana ridibunda* and (b) *Bufo dhufarensis* showing the anterior labium (AL), the dorsal gap (DG), the lower jaw sheath (LJS), the mouth opening (MO), the marginal papillae (MP),the posterior labium (PL), posterior labial notch (PLN), submarginal papillae (SMP) and the upper jaw sheath (UJS).

Table 1. The morphometric measurements (mean ± S.E. (range) of the oral disc components in tadpoles of *B. dhufarensis* and *R. ridibunda* species.

Morphometric measurements	*Rana ridibunda*	*Bufo dhufarensis*
ODW	1.13 ± 0.089 (1.04-1.19)	2.18 ± 0.165 (2.1-2.45)
DGMP	1.05 ± 0.1 (0.98-1.4)	0.50 ± 0.60(0.48-0.52)
LMP	0.23 ± 0.21 (0.12-0.3)	0.11 ± 0.02 (0.09-0.1)
ATRL1	1.55 ± 0.95 (1.3-1.45)	1.01 ± 0.9 (0.9-1.1)
ATRL2	1.65 ± 0.11 (1.0-2.0)	1.25 ± 0.15 (1.16-1.2)
PTRL1	1.45 ± 0.09 (1.42-1.5)	0.95 ± 0.09 (0.84-0.9)
PTRL2	1.30 ± 0.05 (1.3-1.4)	0.80 ± 0.75 (0.7-0.85)
PTRL3	0.85 ± 0.8 (0.65-1.5)	0.45 ± 0.65 (0.35-0.41)

Oral disc width: ODW; dorsal gap of the marginal papillae: DGMP; length of marginal papillae: LMP; anterior tooth row length 1-2: ATRL1-2; posterior tooth row length 1-3: PTRL1-3. Mean ± S.E.(range).

Quantitative data

The measurements taken using a stereomicroscope with measuring device and converted later into mm are: ODW = maximum oral disc width; DGMP = dorsal gap of the marginal papillae. The mouth parts include: MP = number of marginal and SMP = submarginal papillae; LMP = length of the marginal papillae; ATR = number of anterior tooth row; PTR = number of posterior tooth row; ATRL = anterior tooth row length; PTRL = posterior tooth row length.

RESULTS

Ultrastructure, morphometric and numeric measurments of oral disc

In tadpoles of *Rana*, the oral disc structures were present and located ventrally (Figure 1a). The vertical and horizontal length of the opened oral cavity was almost the same (1.13 ± 0.089 mm). The border of oral disc was surrounded by 50 marginal papillae and 10 submarginal papillae (Table 1). There was a dorsal gap (1.05 ± 0.1) that lacked the marginal papillae, their number was 50 and they were 0.23 ± 0.21 mm in width. Sixteen papilla out of the marginal ones were located antero-laterally (eight papillae on each side). And the remaining 34 papillae were located post-laterally (17 papillae on each side). Meanwhile, 5 submarginal papillae were found on each side of the oral disc. It was also found that the marginal and submarginal papillae were the last structures undergoing atrophy during metamorphosis. Concerning the jaw sheaths, it consisted of a curved edge upper jaw sheath and a V-shaped lower one. The edges of both jaw sheaths were serrated along the length of the jaw sheaths. It was obvious that the upper jaw sheath embraced the lateral ends of the lower jaw sheath.

Furthermore, the labial tooth row formula (LTRF) of the tadpoles of *Rana* was 2(1)/3(1-3), indicating that these tadpoles had two anterior tooth row ridges that bear the keratinized teeth named the “A” rows with a median gap on the row A-1, and three posterior tooth row ridges named

Table 2. The numeric measurements of marginal, submarginal papillae and number of teeth per labial tooth row of specimens examined.

Components of oral disc	Species	
	R. ridibunda	*B. dhufarensis*
MP	50	22
SMP	10	8
ATR1	30	25
ATR2	41	23
PTR1	52	34
PTR2	39	29
PTR3	22	16
LTRF	2(1)/3(1-2)	2(0)/3(0)

Marginal papillae: MP; anterior tooth row 1-2: ATR1-2; posterior tooth row 1-3: PTR1-3; SMP: submarginal papillae.

the "P" rows with median gaps observed in the P1 to P3 (Figures 1a and 4a).The length of ATRL1 was 1.55 ± 0.95 mm and that of the ATR2 was 1.65 ± 0.11 mm. The width of the PTR1, 2 and 3 were 1.45 ± 0.09, 1.30 ± 0.05 and 0.85 ± 0.8, respectively (Table 1). Furthermore, the number of teeth was 30 and 41 in ATR1 and 2, respectively. In addition, the number of teeth was 52, 39 and 22 in PTR1, 2 and 3, respectively (Table 2).

On the other hand, in tadpoles of *Bufo,* the oral disc and their keratinized mouth parts were present and extented anteriolly (Figure 1b). The horizontal length (width) of the oral opening was double in length of that of the vertical one, which was 2.18 ± 0.165 mm (Table 1). The border of the oral disc was surrounded by 22 marginal papillae and 8 submarginal papillae, where their length was 0.11 ± 0.02 mm. A dorsal gap was found with 0.50 ± 0.60 mm width. Eight out of twenty two marginal papillae were located antero-laterally (four on each side), and the remaining 14 papillae were located post-laterally (8 on each side). Meanwhile, the 8 submarginal papillae were equally divided on each side.The upper jaw sheath was straight with slight upward curvature, while the lower one was horizontally straight. Both upper and lower sheaths were serrated. The LTRF of the tadpoles of *Bufo* was 2/3 indicating that the tadpoles possessed two anterior and three posterior tooth rows. Both tooth rows lacked the median gap (Figures 1b and 5a). The width of the ATR1 and 2 were 1.01 ± 0.9 and 1.25 ± 0.15 mm, respectively. And the PTR1, 2 and 3 were 0.95 ± 0.09, 0.80 ± 0.75 and 0.45 ± 0.65 mm, respectively (Table 1). Furthermore, ATR had 25 teeth in the first row and 23 in the second one. While PTR had 34 teeth in the first row, 29 in the second one and 16 in the third row (Table 2).

Ultrastructure of marginal and submarginal papillae

Both the marginal and the submarginal papillae of *Rana* were found to be elongated, numerous and closely spaced, in which they ended with 3-7 apical parts (Figure 2a and b). There is individual variation in the size and spacing among these papillae, these marginal papillae border the oral disc, except for a dorsal gap. Two lateral folds are clearly visible so the oral disc laterally emarginated. In *Bufo*, the marginal and submarginal papillae were less in number and shorter than in *Rana*. They were also found to be widely-spaced from each other and their ends were smooth, broad and lack apical parts. the rosteral (dorsal) gap is small and also the oral disc is emarginated (Figure 3a and b).

Labial teeth

In *Rana ridibunda,* the labial teeth were elongated, closely spaced, numerous in number and arranged in one row for each labium. The teeth were noticed to be either vertically positioned or curved downwards ending with three terminal cusps (Figure 4a and b). On the other hand, the tooth rows in *Bufo* were uniserial carrying keratinized labial teeth. Each tooth consisted of three distinct regions; a distal head with 5-7 terminal cusps, an intermediate region known as the neck and the rest of the tooth body known as the base (Figure 5a and b).

Malformed oral structures

It was observed in some cases, that the *B. dhufarensis* had malformed oral structures represented in the marginal and submarginal papillae on both side of the oral disc. In some cases, malformation was found in the presence of teeth on the marginal papillae forming toothed marginal papillae (TMP) and toothed submarginal papillae (TSM) (Figure 6a, b, 7a and b),these teeth had the same structure as that of the labial teeth. Moreover, in some cases, it was shown that two or three marginal papillae were fused into one single marginal papilla

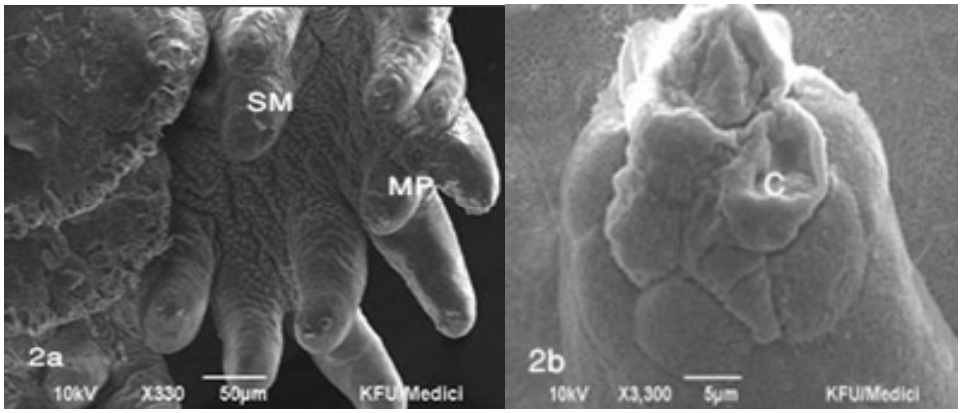

Figure 2. Scanning electron micrographs in *Rana ridibunda* showing a) marginal (MP) and submarginal papillae (SM), b) enlarged end of the marginal papillae with 5 apical parts (C).

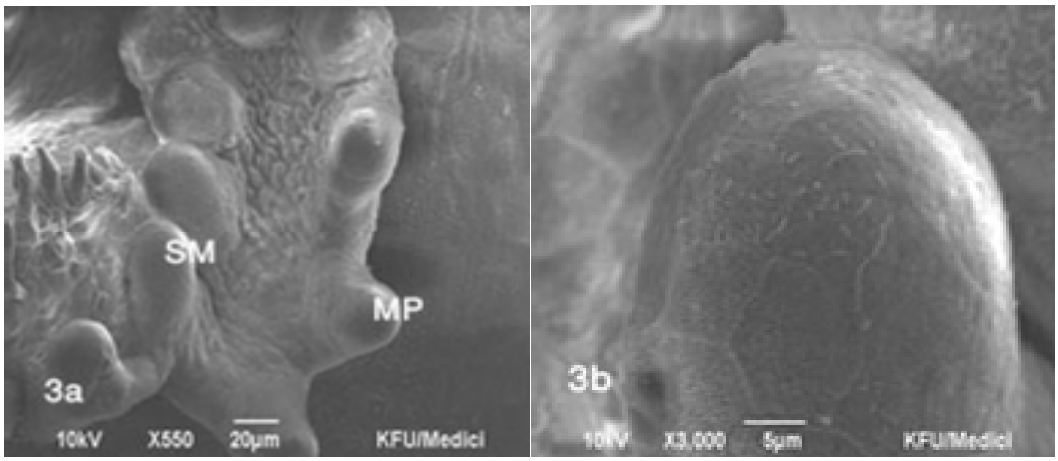

Figure 3. Scanning electron micrographs of *Bufo dhufarensis* showing a) the marginal papillae (MP) and submarginal papillae (SM), b) enlarged marginal papilla of *Bufo* (broad, smooth and lacking of apical parts).

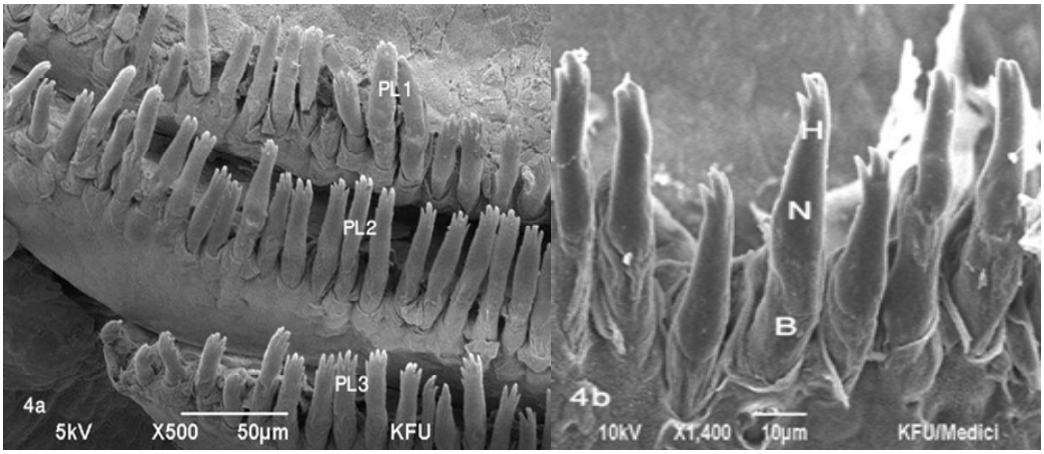

Figure 4. Scanning electron micrographs showing labial teeth in *Rana ridibunda* pallas: a) Vertical positioned posterior labial teeth rows (PL1-3) and their cusps, b) enlarged labial teeth, where the head (H), neck (N) and base (B) were obvious, ending with 3 cusps (C).

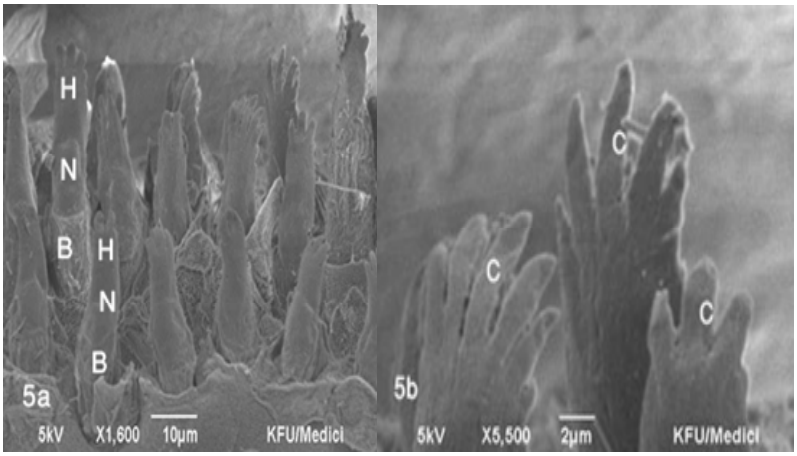

Figure 5. Scanning electron micrographs in *Bufo dhufarensis* showing: a) three rows of labial teeth, where the head (H), neck (N) and base (B) were obvious, b) enlarged labial teeth with 5-7 cusps (C).

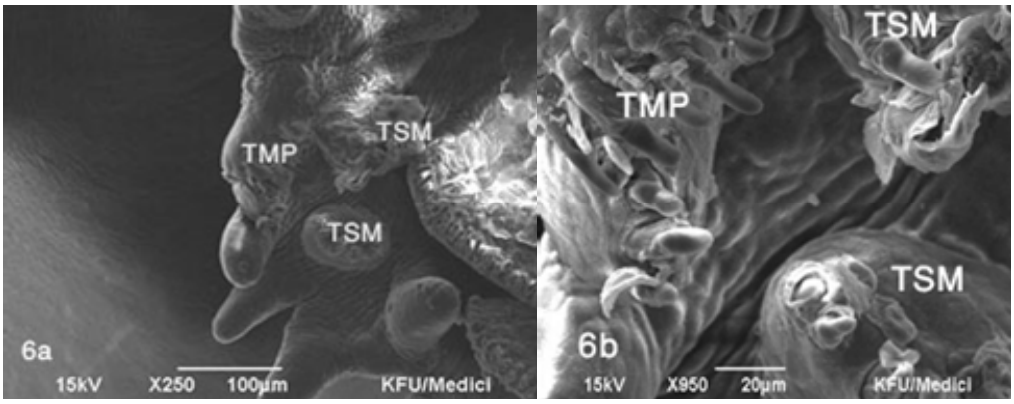

Figure 6. Scanning electron micrographs of *Bufo dhufarensis* showing: a) right side of the mouth with malformed teethed marginal and submarginal papillae, b) enlarged part of teethed submarginal papillae (TSM) note the tooth carrying 3 cuspes.

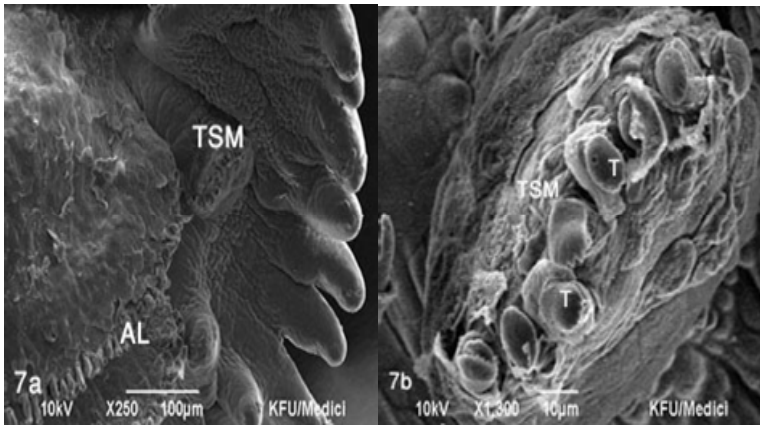

Figure 7. Scanning electron micrographs of *Bufo dhufarensis* showing: a) left side of the mouth with malformed teethed submarginal papillae (TSM); b) magnified part of (TSM).The teeth (T) carrying 5-9 cusps.

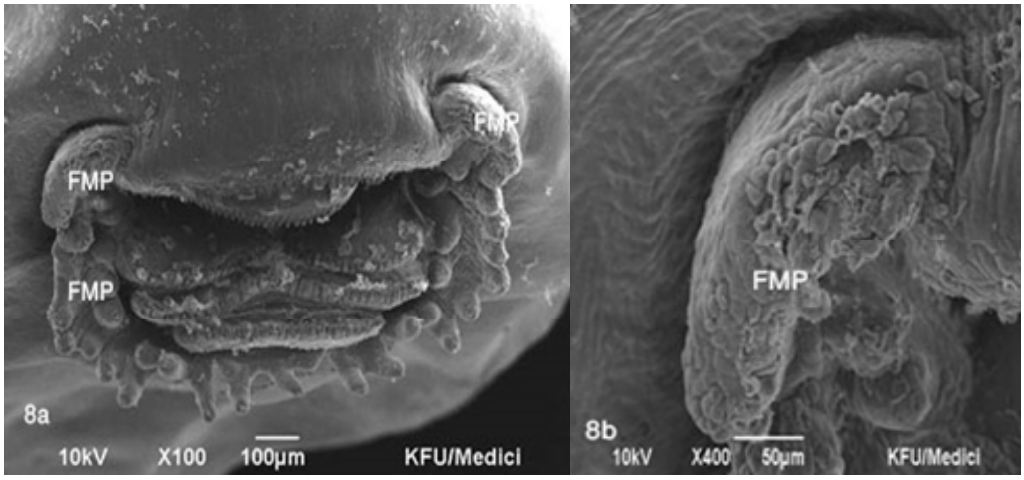

Figure 8. Scanning electron micrographs of *Bufo dhufarensis* showing: a) malformed fused marginal papillae (FMP) on both sides of the mouth; b) magnified part of FMP.

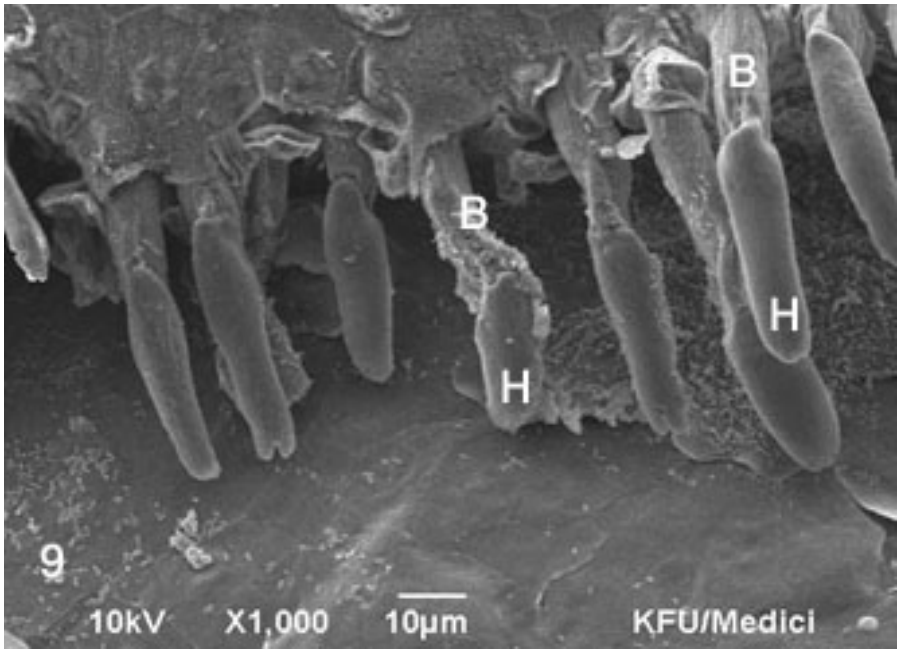

Figure 9. Scanning electron micrographs of *Bufo dhufarensis* showing: a) malformed anterior labial teeth with elongated basal part (B). Note the head (H) ends with 0-2 cusps.

(FMP) at both sides of the oral disc (Figure 8a and b). In other cases, it was noticed that labial teeth appeared with malformation forms such as the appearance of basal part of the teeth and lacked cuspes (Figure 9a) or underdeveloped labial teeth without head and neck or shorted one (Figure 10 a and b).

On the other hand, in some cases, in *Rana* it was shown that the postrior labial teeth changed their position from straight to downwardly curved teeth (Figure 11).

DISCUSSION

The oral disc of the examined tadpoles of *R. ridibunda* and *B. dhufarensis* had the same general organization of keratinized jaw sheaths and with keratinized tooth rows,

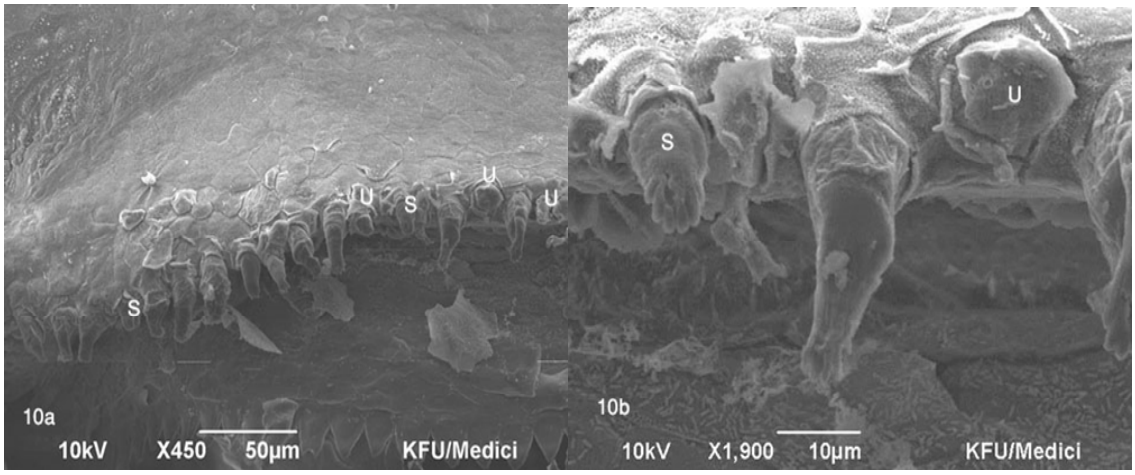

Figure 10. Scanning electron micrographs of anterior labial teeth row in *Bufo dhufarensis* showing: a) malformed short teeth (S) with 7 cuspes or undeveloped labial tooth (U) without cusps; b) Enlarged part of malformed teeth.

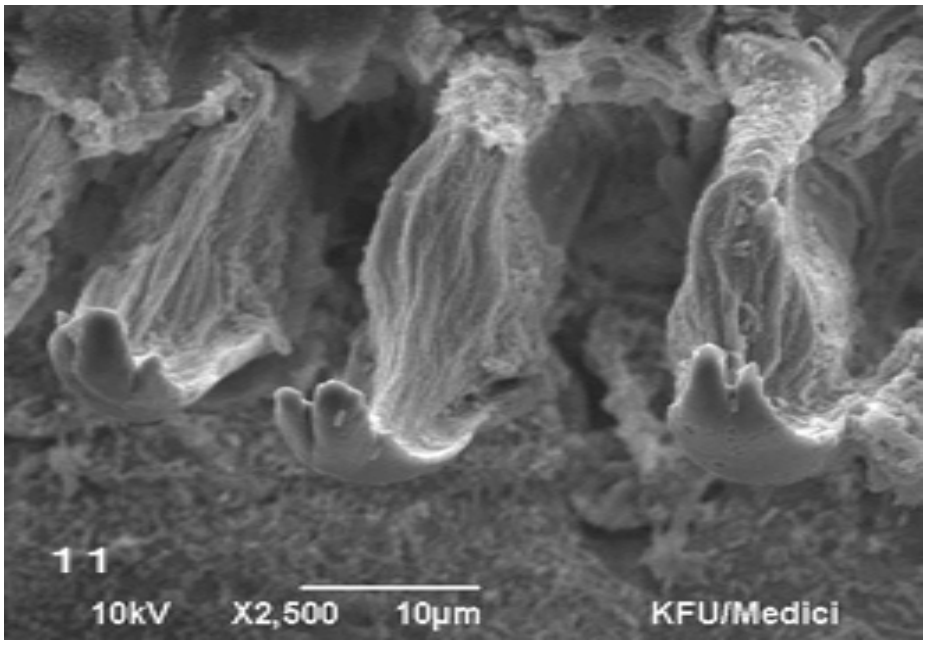

Figure 11. Scanning electron micrographs of posterior labial teeth row in *Rana ridibunda* showing labial teeth changing their position from straight to downwardly curved teeth with 2-3 cusps.

which are the most common feature of the oral discs of most tadpoles (Thibaudeau and Altig, 1988; Altig and Johnston, 1989). However, the oral discs of these two species differed specifically in orientation. It was anterio-ventral in *Rana*, while in *Bufo* it was unique in having anterior disc. The anterio-ventral disposition of oral disc of *Rana* tadpoles indicated their mainly detritus feeding habits and it grazed on algal vegetation and it also filter feeds the planktonic bloom of the pond (Khan and Mofti, 1994a, b).

Nevertheless, some differences in the arrangement and morphology of the mouthparts were observed. First, the arrangement of the upper and lower jaw sheaths varied among the two species. The horizontal width of the oral disc was double its vertical height in *Bufo*, while the width and the vertical height had almost the same size in *Rana.*

The differences among the shape and size of the oral discs could be related to the nature of the food particles ingested by both species. In fact, *Rana* tadpoles feed probably by taking large bites of macrophytes and algae attached on submerged substrates, while *Bufo* tadpoles ingest smaller particles of detritus and algae generated by rasping food (Savage, 2002; Kinne et al., 2004). The present results support their feeding behavior.

The structure, length and arrangement of the marginal and submarginal papillae also varied among the investigated species. The distribution pattern of the marginal papillae of *Rana* was taxonomically and ecologically the most common (Altig and Johnston, 1989) particularly in ranid tadpoles (McDiarmid and Altig, 1999). On the other hand, a dorsal gap among the marginal papillae was found in *Bufo* tadpoles; a result which matches with the configuration that occurred commonly in most bufonids (McDiarmid and Altig, 1999). In fact, Van Dijk (1981) assumed that the presence of a ventral gap in the row of marginal papillae of bufonidae tadpoles could play a role as a "weir-like flow-controlling structure"which acts as a barrier against water flow. The marginal papillae have tactile and chemosensory functions and help to control the water flow conveying food particles towards the mouth (McDiarmid and Altig, 1999), but the functional significance of the differences of this papillary pattern is still not understood. In *B. variegata*, it was found that the marginal papillae surrounded the entire oral disc (Altig and Johnston, 1989). They confirmed that this configuration was found only in some larval types, mainly in stream inhabitants of several families. While in *Rana* tadpoles, the lack of dorsal gap was probably because they are carnivores and live in small ponds. Haas (2003) found that the tadpoles use their complete papillary row as a filter for water flow and for a better adhesion to the irregularities of substrates. The presence of a gap in the row of labial papillae was among the apomorphic characters that defines the ranids, while this character is absent in Bufonidae (Haas, 2003).

Another characteristic was present, which was the presence of multiserial teeth in the LTRF. It was 2(1)/3(1-3) in *Rana* and 2/3in *Bufo*. This finding is in accordance with the observation of McDiarmid and Altig (1999) in Bombinatroids and that of Grillitsch and Grillitsch (1989) and Tubbs et al. (1993) in *Bufo* species. On the contrary, the present observation is in contrast to the previous finding of Bekhet (2012) in *B. regularis* where LTRF was 2(1)/3(2). Our observation supports the uniform morphology of the oral apparatus in the genus *Bufo* as proposed by McDiarmid and Altig (1999). Some authors have noticed that the number of upper labial tooth rows in *R. dalmatina* can vary between 3 and 5 rows (Nikolsky, 1915; BarbadilloEscriva, 1987). In contrast, both Grillitsch and Grillitsch (1989) and Picariello et al. (1996) reported that the LTRF of *R. graeca* and *R. italic* tadpoles were 3(2,3)/4(1). The variation between the present tooth rows in *R. ridibunda* and that of *R. dalmatina* may be related to pond dimensions. It is known that tadpoles in temporary ponds have few labial tooth rows because the progress of development and the metamorphosis are induced before the full development of the oral structures (Vences et al., 2002). They also assumed that the first tooth row on the upper labium is always longer than the lower tooth rows. The lower tooth rows have a similar length in *Bufovariegata*, but in *Rana,* their lengths decrease from the proximal to the distal row (Altig and Johnston, 1989). They suggested that the tooth row lengths are correlated to the microhabitats of the tadpoles. Indeed, the morphologies, which occupy standing water, may have a shorter distal lower tooth row than the proximal row, whereas in species that live in running water, the lower tooth rows are typically long.

In the present work, it was found that the labial teeth had the same pattern in both species, but some differences in their morphology can be recognized. The teeth in *Rana* were long and ended with 3 cusps either straight or curved, whereas that of *Bufo* were wider, short and ended with 5-7 cusps.

Regarding the deformities, extra keratinized structures in the marginal and submarginal papillae in the *Bufo* tadpoles was observed. These results coincided with the same oral deformities that aided in stuffing large pieces of food into oro-pharyngeal passage of the tadpole (Atlig and Johnson 1989; Khan and Mufti 1994a; Hopkins et al., 2000; Drake et al., 2007). In addition, oral anomalies such as eroded jaw sheaths and gaps in tooth rows were also reported in the natural ponds (Altig, 2007). Drake et al. (2007) reported oral deformities (teeth in the marginal papillae, tooth rows and jaw sheaths) of tadpoles from 13 population samples. The frequency of oral deformities can be high in natural population due to the presence of *Batrachochytrium dendrobatidis* infection, which exerts a strong influence on the occurrence and type of oral deformities in tadpoles. The chytrid fungus *B. dendrobatidis* was found to cause oral abnormalities in the species *R. muscosa* (Fellers et al., 2001) and in *Rhinella quechua* (Barrionuevo et al., 2008). *B. dendrobatidis* (chytrid)-induced mouthpart deformitiesin *B. fowleri* and *Hyla chrysoscelis* tadpoles. On the other hand, the incidence of oral deformities increased in the American Bullfrog (*Lithobates catesbeianus*) tadpoles, due to the exposure to coal combustion residues (Rowe et al., 1996, 1998a and b, 2001; Peterson et al., 2008). However, the jaw sheaths had significantly more deformations than labial teeth (Venesky et al., 2010). All or part of the previous reasons could result in the deformities found in the present work. Further environmental studies are required to investigate the present observed malformations.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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