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**Original Article**

# Expression of Recombinant Human Amelogenin in Iranian Lizard *Leishmania* and Its Biological Function Assay

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**Abstract**

**Background:** Amelogenins are the major components of enamel matrix proteins. Enamel matrix derivatives (EMD) can be used in periodontal diseases to regenerate periodontal tissues. The main aim of this study was to evaluate expression of full-length functional recombinant human amelogenin (rhAm) in Iranian lizard *Leishmania* (I.L.L.) as an alternative eukaryotic expression system.

**Methods:** Human cDNA encoding a 175-amino acid amelogenin expression cassette was sub cloned into a pLEXSY vector. The construct was transferred into *Leishmania* cells by electroporation. The protein production was surveyed in the transcription and the translation levels. The expressed protein was purified and some of its biological properties were investigated in comparison to EMD and negative control.

**Results:** Expression of rhAm was confirmed by RT-PCR and western blot test in *Leishmania* cells. Purified rhAm significantly inhibited the formation of tartrate-resistant acid phosphatase positive ($TRAP^+$) multinuclear cells in calcitriol stimulated mouse marrow cultures. Moreover, it significantly promoted proliferation and DNA synthesis in L929 mouse fibroblast cells.

**Conclusion:** Functional rhAm was successfully expressed in I.L.L. Easy handling and post translation modification were the main advantages of this expression system. It is suggested to investigate molecular properties of this rhAm in the future.

**Keywords:** Amelogenin, Eukaryotic expression system, Osteoclastogenesis, Lizard, *Leishmania*, Recombinant protein, Iran

## Introduction

Amelogenins play an important role in the mineralization and organization of enamel structure (1).These proteins constitute 90% of the enamel extracellular proteins (2). The human amelogenin gene has been localized to both X and Y-chromosomes with 90% of the transcripts expressed from the X (AMELX) (3, 4). AMELEX contains 7 exons, which undergo alternative mRNA splicing. The most abundant isoform of the native lacks the internal region encoded by exon 4, related cDNA (Gen-Bank Accession No. M86932) encodes for a 175 amino acid protein (5-7). Periodontal disease is a chronic and progressive destruction of the periodontal tissues (8).

Previous studies revealed that amelogenins have a role in the regeneration of all the periodontal tissues: cementum, periodontal ligament, and bone (9-12). Amelogenin inhibits osteoclastogenesis via down regulation of RANKL, M-CSF and fibronectin expression in osteoblasts (13). It also promotes proliferation and migration of fibroblast cells(14). Emdogain® is an acidic extract of extracellular enamel matrix that can be used in the treatment of periodontal disease in human (15). Due to heterogeneity of EMD and protein purification difficulties, many researches were done to reach a large quantity of isoform recombinant amelogenin in the last twenty years (12, 16). The amelogenin molecule is phosphorylated and has many folding in its structure, providing a eukaryotic expression system for reaching an activated recombinant human amelogenin (rhAm).

*Leishmania tarentolae* with an expression vector, pLEXSY, has been used as a eukaryotic protein expression system previously (17, 18). *Leishmania* sp. protozoa are from the family of Trypanosomatidae. Regulation of protein expression in these species occurs mainly on the level of RNA and may be influenced by the structure of the intergenic regions. They have high growth rates and easy handling like *E. coli* and yeast expression systems (19, 20).The post translation modification in these cells is very similar to mammalian cells in comparison to *E. coli* and yeast expression systems (21).

We designed this study to express functional rhAm in Iranian lizard *Leishmania* (I.L.L.) (22) as a novel expression system to obtain functional rhAm.

## Materials and Methods

### Plasmid construction and gene cloning

The human amelogenin gene sequence (Gen Bank Accession Number: M86932 variant: 2), encoding mature amelogenin protein (175 amino acid), was synthesized into pGH vector and added *SalI* and *NheI* restriction sites at up and downstream relatively. The synthesized gene was amplified by M13 primers (Table 1) and digested by using *SalI* and *NheI* restriction enzymes then sub cloned into pLEXSY-hyg2 plasmid that encoded 6His tag gene (EGE-232, Jena Bioscience, Germany). Recombinant pLEXSY-hyg-2-hAm was transformed into *E. coli* XL1-Blue (stratagene, UK). Transformed colony was selected via colony PCR by using P1442 and A264 primers (Table 1) which is flanking the multiple cloning site of pLEXSY-hyg2 plasmid. Final confirmation performed by sequencing the PCR product by using hAm primers (Table 1). The study was approved by the Ethical Committee of the university.

**Table 1**: Primers sequence used in this study

| Name | Sequence (5'to 3') |
|---|---|
| M13 F | GTAAAACGACGGCCAGTG |
| M13R | GGAAACAGCTATGACCATG |
| hAm F | GTCGACATGGGGACCTGGATT |
| hAm R | GCTAGCATCCACTTCCTCCCG |
| P 1442 | CCGACTGCAACAAGGTGTAG |
| A 264 | CATCTATAGAGAAGTACACGTAAAAG |
| F 3001 | GATCTGGTTGATTCTGCCAGTAG |

### Cultivation of Iranian Lizard Leishmania (I.L.L.)

The I.L.L. cells were cultivated at 26ºC in LB broth medium (Himedia, India) containing 100U/ml penicillin, 100mg/mlstreptomycin (GIBCO, USA) under shaking condition (110 rpm) and passaged by diluting suspension5-10 fold in fresh media every 3-5 days (23).

### Transfection of I.L.L. cells

$10^8$ cells of I.L.L. (22) were washed by electroporation buffer (eppendorf, Germany) and suspended in 1 ml electroporation buffer. The recombinant pLEXSY-hyg2-hAm linearized by *SwaI* (Ferments, Lithuania). Then 50µl of linearized DNA containing 5µgr DNA was added to 450 µl of cell suspension containing $10^8$cell/mlin a 4 mm cuvette. The I.L.L. cells were transfected by using two pulse 2000V (interval: 10S) in a Multiporator (eppendorf, Germany) (24). Stable transfectants were selected on LB broth media containing 100 µg/ml hygromycin (sigma). Integration of the expression cassette into the small subunit rRNA (ssu)

locus of I.L.L. was confirmed by diagnostic PCR reaction by using F3001 forward primer (located on ssu locus of I.L.L. genome) and hAm reverse primer (Table 1).

### Expression of rhAm in the I.L.L. cells

Expression of rhAm in the I.L.L. cells was evaluated by RT-PCR, SDS-PAGE and western blot tests. Total RNA was extracted from the cells by using RNeasy mini kit (QIAGEN). Gene cDNA was synthesized by random hexamers and amplified by PCR with hAm specific primers (Table 1).To determine rhAm protein in cells, transfected promastigotes were analyzed on 12% SDS-PAGE gel. For detection secretory rhAm, supernatant of cultures was filtered (0.2 µm). Then all proteins in supernatant were precipitated by ice-cold trichloroacetic acid 50%. The pellet was prepared and analyzedon 12% SDS-PAGE gel (25). The same procedure was carried out by I.L.L. wild type of *Leishmania* and compared with transformed one.

For Western blot analysis, protein bands were blotted on the nitrocellulose membrane then it was blocked by 3% skimmed milk in TBS buffer (20 mM Tris and 150 mM NaCl, pH 7.6) for 1 hour. Then membrane was washed and incubated with 1/1000 diluted rabbit polyclonal antibody to hAm (Abcam, UK) for 2 hours at room temperature. The washing process repeated again and incubated with 1/2000 diluted alkaline phosphatase conjugated goat polyclonal anti-rabbit antibody (Abcam, UK) for an additional 2 hours at room temperature. Finally, alkaline phosphatase substrate (NBT-BCIP) was added to membrane presoaked to alkaline phosphates buffer. Emdogain® (Straumann, USA) was used as positive control for western blotting test.

### Purification of rhAm by Affinity Chromatography

The rhAm fused to his-tag was purified from cell lysate by using Ni-NTA His•Bind® resins (Novagen, Germany) under nature condition.The purified protein was concentrated and desalted by using Amicon Ultra-15 units with a cut-off of 10 kDa (Millipore, Germany).

### Osteoclast differentiation Assay

Osteoclasts were generated from mice bone marrow nucleated cells. 6-week-old Swiss-Webster male mice were sacrificed by cervical dislocation. The procedures were reviewed and approved by the Shahid Beheshti University of Medical Sciences Ethics Committee. These cells were collected from the femurs and tibias of mice, as described by Holliday (26). The marrow cells were suspended in α-MEM that contained 10% FBS and $10^{-8}$ M active metabolite of vitamin $D_3$, 1, 25-dihydroxycholecalciferol [1,25 $(OH)_2D_3$]. Cell suspension was plated in 24-well plate at a density of 500 000 nucleated cells/$cm^2$. The cultures were maintained at 37 ºC in a humidified atmosphere of 98% air and 5% $CO_2$. After 6 days, the media was removed and adherent cells were fixed with 2% paraformaldehyde. Tartrate-resistant acid phosphatase (TRAP) staining was performed according to the kit instruction (sigma, USA). $TRAP^+$ mononuclear cells (less than three nuclei) and $TRAP^+$ multinucleated cells (three or more nuclei) were counted under a 40X microscope from 3 wells in 10 random areas per well in each group (27).

### Proliferation Assay

L929 mouse fibroblast cell line was obtained from National Cell Bank of Iran (Pasteur Institute of Iran). The L929 cell line was sub-cultured and then cell suspension was prepared in DMEM media. The cells were seeded in 96-well cell culture plate at a density $5\times10^3$ per well. After 24 h, it was exchanged with fresh serum free media. EMD and rhAm were added to related groups at the 1µg/ml and 10µg/ml concentrations but not into control group. The cultures were maintained at 37 ºC in a humidified atmosphere of 98% air and 5% $CO_2$for 4 days. Then 20 µl of MTT solution (0.5mg/ml) (Sigma, USA) was added per well and incubated for 4h to form formazan crystals in viable cells. The crystals were dissolved in acidic isopropanol and absorbance was measured at 570nm with reference filter 620 (16).

### DNA synthesis

DNA synthesis was measured by BrdU colorimetric kit (Roch, Germany). The L929 cell culture was

prepared and exposed as the same as MTT assay. The cells were incubated for 2 hours with BrdU and then BrdU incorporation was measured in accordance with the manufacturer's instruction BrdU ELISA kit (16).

### *Statistical analysis*

All experiments were performed at least three times. Descriptive statistics (means and standard deviations) were calculated. Shapiro-Wilk and Levene tests were used to check normality and homogeneity of variance assumptions respectively. One way ANOVA was used for comparison among groups. Pairwise comparisons between groups of interest were performed using Benjamini-Hochberg post hoc test. The $P<0.05$ was considered statistically significant.

## Results

### *Plasmid Construction and gene Cloning*

The hAm gene (531 bp) was sub cloned into *SalI* and *NheI* sites of pLEXSY-hyg 2 (Fig. 1).

The Fig. 2 shows PCR product of recombinant pLEXSY-hyg 2-hAm plasmid (830 bp fragment). Sequencing results confirmed the presence of hAm gene in recombinant construct in an accuracy position.

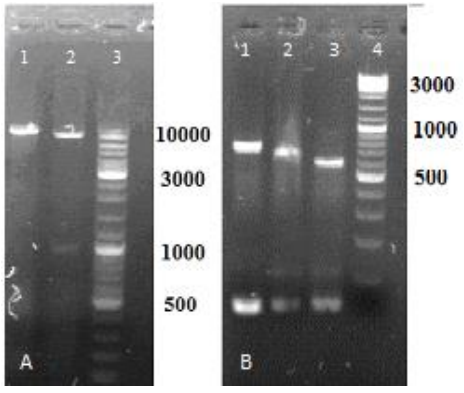


**Fig.1**: Digestion by NheI and SalI restriction enzymes, (A) pLexy-hyg2; lane1, digestion by NheI; lane2, digestion of stuffer fragment by NheI and SalI(1000bp); lane3, DNA size marker100-100000 bp (Fermentas, Lithuania). (B) PCR product digestion; lane1 PCR product 717bp.; lane2 digestion by NheI(618 bp); lane3 digesestion by NheI and SalI 531bp. fragment; lane4, DNA size marker 100-3000 bp (Fermentas, Lithuania)

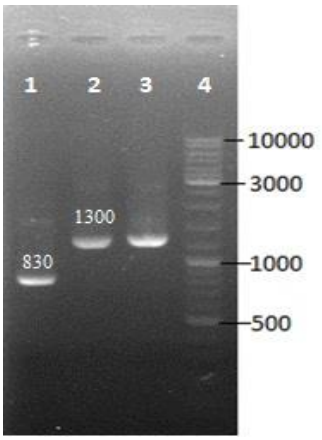


**Fig. 2:** Colony PCR by using P1442 and A264 primers flanking the multiple cloning site of pLEXSY-hyg2 plasmid was performed.; lane 1, desired colony (830 bp fragment)lane 2,3 colony with re-ligation of the stuffer segment of vector with the 1300 bp fragment and lane4, DNA size marker

### *Transfection and Selection of Recombinant I.L.L. cells*

Recombinant pLEXSY-hyg2 plasmid was transfected into I.L.L. promastigote by electroporation. They were cultured in the presence of hygromycin (up to100 µg/ml). Resistant transfected promastigotes were surveyed by PCR reaction. The PCR products of transfected promastigotes 1828 bp and non-transfected promastigotes are shown in Fig.3.

### *Analysis of Expression Recombinant hAm*

RT-PCR was performed on transfected and non-transfected I.L.L. promastigotes by using hAm specific primers. The length of desired product should be 537 bp (Fig. 4). Expressed hAm protein was electrophoresed on SDS-PAGE, transferred on nitrocellulose membrane and analyzed by western blot compared to cell culture supernatant. SDS-PAGE gel staining results show that two bands 19kDa and 50kDa in recombinant cell lysate were stronger than the control and there is no obvious difference between case and control groups in cell culture supernatant.

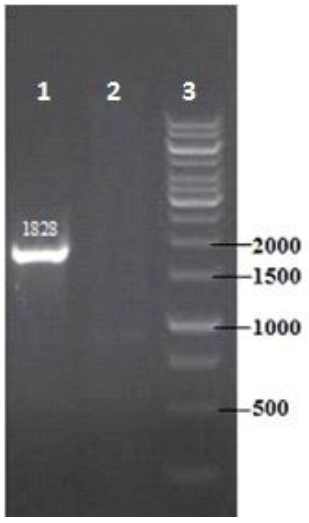


**Fig.3:** Confirmation of genomic integration by PCR. Diagnostic PCR was performed by using forward primer, hybridizing to the ssu sequence (F3001) and reveres primer of hAM within expression cassette. Lan1, transfected I.L.L; lane2 wildtype control, lane 3,DNA size marker

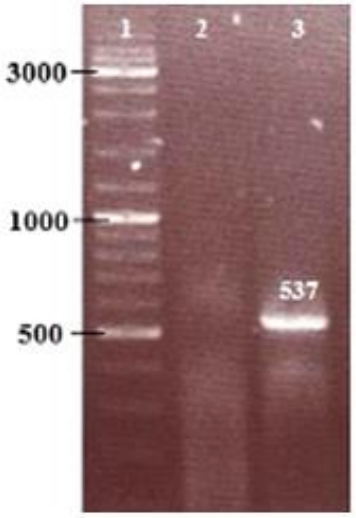


**Fig.4**: RT - PCR analysis of gene expression. PCR was performed by using hAm forward and reverse primers.lane1, DNA size marker; lane2, non-transfected control cDNA; lane2, transfected DNA

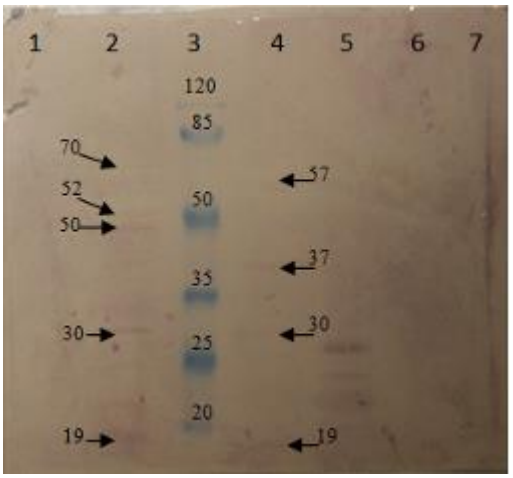


**Fig.5**: Analysis of recombinant hAm expression by using the specific polyclonal antibody to hAmin western blot test. lane1, non-transfected cell lysate; lane2, transfected cell lysate; lane3, protein size marker in kDa.;lane4, purified rhAm from cell lysate by using Ni-NTA His•Bind® resins, lane5, positive control (Emdogaine) for western blot test; lane6, supernatant of cell culture related to transfected I.L.L.; lane7, supernatant of cell culture related to non-transfected I.L.L

Theoretical molecular weight of rhAm include signal peptide and His-tag is about 23 kDa and without them is about 20 kDa. In western blot test 19 kDa, 30 kDa, 50 kDa, 52kDa and 70 kDa isoforms were observed in recombinant cell lysate but not in culture supernatant comparison to control group (Fig. 5).

### *Purification of rhAM*

Purification of rhAm from cell lysate was performed by using Ni-NTA His•Bind® resins. Western blot test showed three bands in 19 kDa, 30 kDa, 37 kDa and a weak band in 57 kDa (Fig. 5).

### Osteoclast differentiation assay

Osteoclast precursors in mouse bone marrow can be induced to form multinucleated osteoclast-like cells in the presenceof1, 25(OH) $_{2D3}$ (28). Formation of TRAP$^+$ multinuclear cells was inhibited significantly in presence of rhAm in all doses [10ng/ml, 100ng/ml and 500ng/ml ($P$=0.0001)] in comparison to control group. Similarly EMD was inhibited multinuclear formation in comparison to control group [10 ng/ml ($P$= 0.039), 100 ng/ml ($P$=0.009) and 500ng/ml ($P$=0.0001)]. Counting of TRAP$^+$ mononuclear was increased significantly in presence of rhAm in all doses in comparison to control group [10ng/ml ($P$=0.006), 100ng/ml ($P$=0.0001) and 500ng/ml ($P$=0.0001)]. Also number of the TRAP$^+$ mononuclear was increased significantly in the presence of EMD in all doses in comparison to control group. [10ng/ml ($P$= 0.023), 100ng/ml ($P$=0.014) and 500ng/ml ($P$=0.0001)] (Fig. 6).

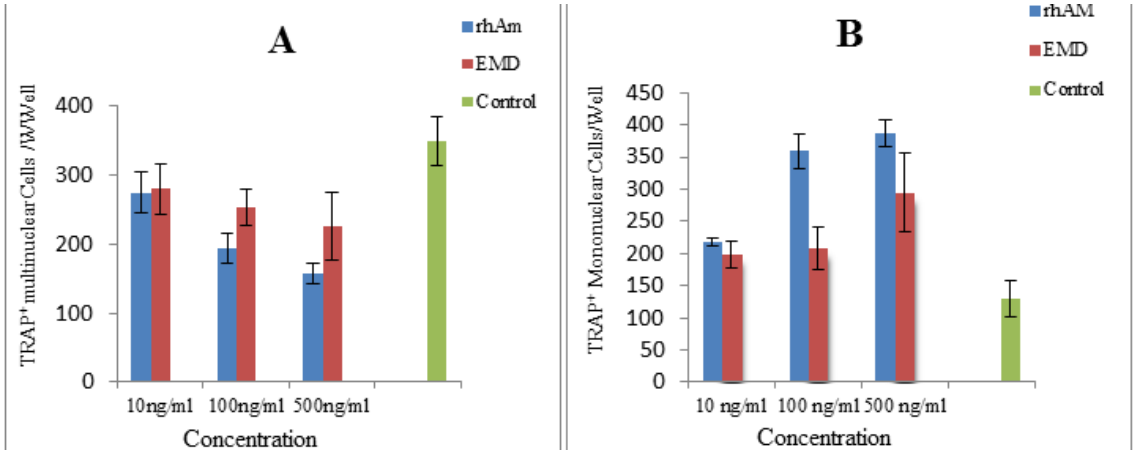


**Fig.6:** Bar chart of counting TRAP+ multinuclear cells, part A, and TRAP+ mononuclear cells, part B, in expose to different doses of rhAm and EMD in comparison to control group. Data are means ± SD of result from three determinations.

A) Formation of the TRAP+ multinuclear was inhibited in all concentration of rhAm and EMD groups in comparison to control group. There was significant difference between rhAm and EMD groups in 500 ng/ml in number of TRAP+ multinuclear ($P$=0.04) but not in10 ng/ml and 100 ng/ml doses. B) There was an increase in the number of TRAP+ mononuclear cells in all groups in comparison to control group. There was no significant difference between rhAm and EMD group in 10 ng/ml dose, but there was in 100 ng/ml ($P$=0.0001) and 500 ng/ml ($P$=0.005) in the number of mononuclear cells. Bars represent standard deviations (SD)

### Proliferation Assay

L929 fibroblast cell line was incubated in serum free DMEM with rhAm or EMD for 4days. MTT assay showed that exposed cells with rhAM were proliferated in a dose dependent manner in comparison to control group (Fig. 7).

### DNA synthesis

Incubation of L929 fibroblast cell line was performed with rhAm or EMD in a serum free media for 4 days. BrdU test revealed that DNA synthesis was increased in rhAm group in a dose dependent manner in comparison to control group (Fig. 8).

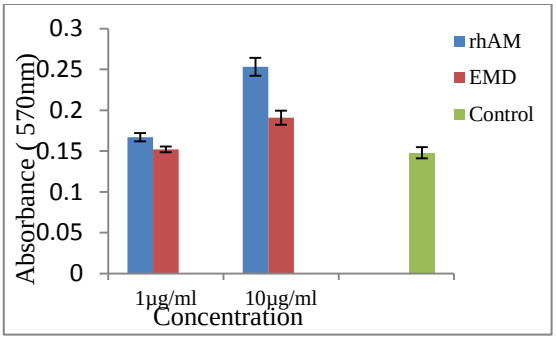


**Fig. 7**: Proliferation assay was performed by using L929 mouse fibroblast cell line and MTT salt. Cell proliferation significantly increased in the presence of

1µg/ml rhAm (*P*=0.015), 10µg/ml rhAm (*P*=0.0001) and10µg/ml EMD (*P*=0.0001) but not in1µg/ml EMD in compare to control group. There was a significant difference between 1µg/ml rhAm treated group and 1µg/ml EMD treated group (*P*=0.04). Also there was a significant difference between 10µg/ml rhAm treated group and 10µg/ml EMD treated group (*P*=0.0001). Bars represent standard deviations (SD)

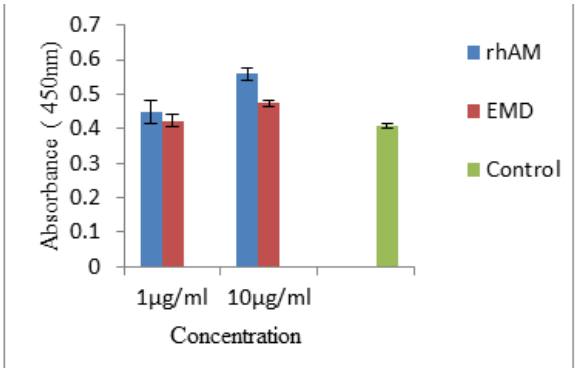


**Fig. 8**: Brdu incorporation in L929 cell line was significantly increased in the presence of 1µg/mlrhAm (*P*=0.041), 10µg/ml rhAm (*P*=0.0001) and10µg/ml EMD (*P*=0.003) but not in1µg/ml EMD in comparison to the control group. There was a significant difference between 10µg/ml rhAm treated group and 10µg/ml EMD treated group (*P*=0.0001) but not in 1µg/ml concentration. Bars represent standard deviation (SD)

## Discussion

Amelogenins are the major components of enamel matrix proteins. These proteins are translated from mRNA splicing variants then undergo post-translational modification and they play an important role in the mineralization and organization of enamel structure (29-31). These highly conserved proteins have tendency to the unique physiological characteristic of self-assemble into spherical mono dispersed aggregates (15).

In this study we transfected I.L.L. cells with the pLEXSY-hyg2-hAM vector included hAm gene (Gen Bank Accession Number: M86932 variant: 2) that encoded a full-length recombinant human amelogenin fused with the his-tag in C-terminal. The western blot test confirmed that two stronger bands (19 and 50 kDa) in SDS-PAGE gel were amelogenin in transfected I.L.L. cells. Also the western blot test revealed additional bands in 30, 52 and 70 kDa. This result is due to the nature of amelogenin proteins to tendency to form aggregation (10, 16, 32). To determine exact molecular weight characterization of produced rhAM, it is suggested to use mass spectrometry. Enamel Matrix Derivative extracted from porcine enamel can be used for restoration three periodontal tissue types in periodontal diseases (33). EMD is composed of heterogenic proteins therefore many researches and efforts were done to reach a large quantity of isoform recombinant amelogenin (10, 16, 32).Expression of rhAm has been reported in different expression systems. For the first time, in 1996, Deutsch et al. prepared a human cDNA, encoding for the 175-amino-acid amelogenin, by RT- PCR and sub-cloned into pGEX-KG expression plasmid for over-expression in *E. coli* (32). The *E. coli* expression system lacked cell machinery to encourage correct folding and post translational modification of proteins. Taylor et al., expressed rhAm in the eukaryotic baculo virus expression system (10). Cheng et al. used yeast, *P. pastoris,* to express rhAm, and then they investigated the biological and molecular characteristics of rhAm (16).

One of the new eukaryotic expression systems *Leishmania* tarentolae (Jena Bioscience LEXY host P10) has been used to express many proteins (18, 34).In this study, we used Iranian lizard *Leishmania* (22) for the expression of rhAm for the first time. Some advantages of this system are a) easy handling like *E. coli* and yeast expression systems. b) Cultivation in low cost media like LBc) eukaryotic protein synthesis and post translation modification more likely to mammalian cells than yeast cells. d) High cell density in suspension culture e) human safety d) extremely stable over hundreds of generation (21). To investigate biological function of rhAm in our lab, the effect of this product on ostoclastogenesis and fibroblast proliferation was surveyed.

Mouse bone marrow cultures contain both osteoblasts and osteoclasts. By adding 1,25 $(OH)_2D_3$ to culture media, osteoblasts were stimulated to increase production of RANKL, which stimulates

osteoclasts progenitors to mature into osteoclasts (26). Previously Nishiguchi demonstrated that amelogenin inhibits osteoclastogenesis via down regulation of RANKL,M-CSF and fibronectin expression inosteoblasts (13). In this study, the effect of rhAm on ostoclastogenesis was determined in three doses (10, 100 and 500 ng/ml) compared to similar doses of EMD and control. Multinucleated cell formation was inhibited by different doses of rhAm and EMD. Expectedly, the mononuclear cells' counting was increased in the presence of different doses of rhAm and EMD in comparison to control group. Leucine-rich amelogenin peptide (LRAP) inhibited osteoclast formation in the co-cultures of osteoclast progenitor and cementoblast/periodontal ligament cells of mice. LRAP significantly reducedRANKL expression and the TRAP-positive cells in 10 and 100 ng/ml concentrations (9). Porcine recombinant amelogenin significantly decreased the number of human $TRAP^+$ odontoclastic cells in appropriate cell culture environment (35). Our study supported previous studies and revealed that rhAm has biological activity. On the other hand, EMD promoted migration and proliferation of gingival fibroblasts (36). rhAm (expressed in yeast) and EMD increased proliferation and DNA synthesis in human PDLF (16). Amelogenin stimulate fibroblast signaling, proliferation and migration via integrin interactions (14). In our study, MTT assay and BrdU incorporation assay were performed to investigate proliferation effect of rhAm L929 fibroblast cells. MTT assay showed that exposed cells with rhAM were proliferated in a dose dependent manner in comparison to control group. Also the proliferative effect of rhAm significantly was more than EMD at the same concentration. In this regard, BrdU test revealed that DNA synthesis was increased in the presence of rhAm in a dose dependent manner in comparison to the control group. Our investigation supported previous studies and confirmed some of the biological properties of amelogenin protein. Also this study supported responsibility of amelogenin for therapeutic effect of EMD. Further, it revealed that produced rhAm in I.I.L. has biological activity. In this study, we could not indicate any secreted amelogenin in media supernatant. This may be due to the lower efficiency of the LmSAP (acid phosphatase of *L. mexicana*) signal peptide in recombinant I.L.L. Secreted protein may be enhanced by altering some amino acids codon in signal peptide gene (37).

The final yield of purified protein was about 4 mg/l that was low in comparison to protein expressed in *L. tarentolae* (Jena Bioscience LEXY host P10)(17, 21) but it was compatible to protein expressed in I.L.L. (20, 25). It is suggested to characterize I.L.L. and its proteolytic enzymes.

## Conclusion

We expressed and purified rhAm in a domestic *Leishmania* host as a new eukaryotic expression system for the first time. The biological activity of the rhAM was corroborated. The main advantages of this expression system are its easy handling like *E. coli* and yeast expression systems, post translation modification and cultivation in low cost media such as LB.

## Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

## Acknowledgements

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