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Axolotl cells and tissues enhances cutaneous wound healing in mice

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ABSTRACT

Adult mammalian skin wound repair is defective due to loss of the regulation in balancing the complete epithelial regeneration and excessive connective tissue production, and this repair process commonly results in scar tissue formation. However, unlike mammals, adult salamanders repair the wounds by regeneration compared to scarring. To elucidate the healing capability of a salamander, Axolotl, in a different species, here we addressed this question by treating the wounds in mice with Axolotl cells or tissues. Excisional lesions were created on each mouse, and animals in different groups treated by; a-) Axolotl blastema tissue, b-) Axolotl tail tissue, c-) Axolotl blastema cells, d-) Axolotl tail cells, e-) Serum physiologic, e-) Madecassol; respectively. 10 days after the treatments, wound healing success was compared by considering the wound closure rate, histopathological analysis, vascularization and gene expression profiling of cytokines. The results reveal that Axolotl cells or tissues delivered animals demonstrate an improved wound repair capacity. A better reepithelization, granule tissue formation, vascularization and even presence of hair follicles are observed in animals treated with Axolotl samples. Gene expression profiling data discloses the lower levels of pro-inflammatory cytokines in these animals which may indicate the immune-modulating role of Axolotl samples in wound healing.

1. Introduction

The wound is pathological destruction of cells, tissues and/or organs morphologically and functionally. Since the wound may give rise to several pathological disorders, wound healing is an imperative clinical approach in worldwide. An ideally healing is characterized by recovering tissues to normal function, morphological structure and appearance (Lazarus et al., 1994). In mammalians, wounds generally heal with fibrosis and at the end of the healing process the non-functional scar tissue is constituted. However, according to the previous studies, we know that second-trimester fetal skin is capable of performing the perfect scarless-healing (Walraven et al., 2016; Yates et al., 2012). Alterations in immunological profile, collagen formation of skin and the amount of various growth factors and cytokines affect the scar forming potential of tissues (Canesso et al., 2014; Metcalfe and Ferguson, 2007; Walraven et al., 2016).

In spite of the having various cellular periods during the wound healing process; it is possible to divide it into four phases: hemostasis, inflammation,

proliferation and tissue remodeling. After an injury, the first physiological response of organism is wound closure via regulation of coagulation and hemostasis. This reaction provides a safeguarded environment for vascular system and internal organs around the wound site. The clot formation occurs immediately in order to prevent losing blood and also to preserve organism from contamination with external environment. Furthermore, with coagulation, a matrix like a scaffold structure is formed around the wound area to facilitate the infiltration of invaded cells in inflammatory phase (Velnar et al., 2009; Xue and Jackson, 2015). Neutrophils and macrophages are predominant cells which migrate towards the wound zone. By leukocytes infiltration wound area is completely cleaned from pathogens. Moreover, cytokine production by leukocytes assists healing (Mast and Schultz, 1996) and in response to these secretions the proliferative phase starts (Li et al., 2007) however increased and elongated cytokine signaling impairs with wound healing.

In proliferation phase the wounded area has to be rebuilt by formation of new granulation tissue. Fibroblasts and endothelial cells are major contributors of this phase in order to generate proliferation events. These cells accompany re-epithelialization and fibroblasts differentiate into myofibroblasts to provide tensile strength across the wound. Fibroblasts produce basement membrane components such as type 3 collagen. Furthermore, they secrete growth factors such as TGF-β, VEGF and PDGF which contribute to healing process (Kawasumi et al., 2013; Molloy et al., 2003; Pakyari et al., 2013). VEGF is responsible for angiogenesis, forming new blood vessels, and it is one of the significant markers of this process. Since new tissues and cells require sufficient blood vessels for transporting the nutrients and waste products, the level and velocity of angiogenesis have a great impact on healing. After proliferation, during the last phase, tissue remodeling, type 3 collagen is converted into stronger type, type 1 collagen, the connective tissue components are reorganized and repositioned (Lorenz et al., 1993; Yates et al., 2012). The accomplishment of proliferation and remodeling phases determine the fate of repair as either scar tissue forming or scar-free healing.

Among the vertebrates amphibians particularly urodeles possess an exceptional regeneration capacity. Adult salamanders can regenerate internal organs, central nervous system and extremities throughout their lives (reviewed in (Brockes and Kumar, 2008; Carlson, 2007; Tanaka and Reddien, 2011)). Their astonishing regeneration capacity provides scar-free wound healing after an injury (Godwin et al., 2013; Godwin and Rosenthal, 2014). The link between the scar-free healing and regeneration is still under examination and there are accumulating data on molecular pathways which take roles in both processes. Axolotl limb wounds are repaired by fast re-epithelialization, basement membrane regeneration and reshaping and deposition of dermal components (Seifert et al., 2012). After creating a full-thickness excisional skin wound, re-epithelialization occurs rapidly and keratinocytes cover approximately 1.5 mm-diameter wound in 8h. Although the basal lamina is not overhauled completely in 45 days, epidermal layer return to normal appearance in this time period. At 90th day post-injury, the skin heals perfectly and identifying the injured/non-injured part of skin is not possible (Levesque et al., 2010). A pilot study on Axolotl cutaneous wound healing by following mammalian excisional wound model exhibits the accomplishment of accurate repair without the formation of fibrotic scarring tissue (Seifert et al., 2012). To exclude the effect of aquatic or terrestrial life conditions on scar-free wound repair, metamorphosed Axolotls were investigated for wound healing capacity and it has been postulated that terrestrial metamorphosed Axolotls are also able to perform perfect wound repair (Seifert et al., 2012). Considering this astonishing capability, we reasoned that Axolotl cells and tissues may facilitate the mammalian wound repair.

In this study we explored the effect of Axolotl samples on mammalian wound healing after an experimentally created full thickness excision. We examined wound closure ratio, histopathological results, angiogenesis rate and gene expression profile for cytokines. Axolotl cells or tissues delivered animals demonstrate an advanced wound repair capacity in comparison to control groups; madecassol treated animals as a positive control and serum physiological treated ones as a negative control. Histological analysis and immune-staining results indicate the success in tissue renewal and restoration for experimental groups compared to the control groups. Gene expression profile of cytokines reveals immune-modulating activity of Axolotl samples in mice to enhance the wound repair. Our data suggest that highly regenerative animals hold a great promise to utilize in mammalian wound repair.

2. Material and methods

Ethical statement and animal care:

All experiments were performed in accordance with the government approval guidelines of laboratory animals' care and use. Animal protocols were approved by Istanbul Medipol University ethics committee (authorization number 38828770-E.2305). All mice (n=36, 6 groups consisted of 6 animals each) aged 4-5 months and weighing between 25-30 g were selected and grouped randomly. Animals were caged with free access ad libitum to standard pellet food and water, and following conditions: at room temperature (at around 24°C), with 12h dark: 12h light cycle.

Axolotls used in experiments were purchased

from the Ambystoma Genetic Stock Center (AGSC) and breed in İstanbul Medipol University animal care facility. Animals were lodged in individual aquaria (1 animal/1 aquarium) at ~20 °C in amphibian Holtfreter's solution.

Surgery for the excision wound model and obtaining Axolotl samples:
Ketamine/Xylazine is used for mice general anesthesia in 100 mg/kg and 10 mg/kg body weight doses, respectively. Back of anesthetized animals (n=36) were shaved to remove the hairs and the skin was washed with povidone-iodine solution. By using a 2.5 mm diameter punch biopsy tool, 4 full-thickness excisional skin wounds were made. Each wound was 2.0 mm away from each other and 5.0 mm away from the middle line. Then these mice were randomly grouped to form 6 groups of 6 animals each.

To obtain Axolotl blastemal tissues and cells, tail part of 12 animals were amputated from the mid position of the tail following anesthetize in 0.01% benzocaine (Sigma, St. Louis, MO). Amputated animals were put back to aquaria to allow the regeneration for 1 week. Day 7 blastemas of animals were removed and 6 of them were kept in DMEM medium for 1-2 hours till used in xenograft. Each blastema tissue was cut into four equal pieces to transfer the wound area. The remained 6 blastema tissues were homogenized to have blastema cells by following a protocol described elsewhere (Satoh et al., 2007). For the tail tissue graft and cell treatment, tails of 12 animals were cut from the same position as did for the blastema samples. 1 cm of tail samples were obtained from each animal and half of them was used to obtain the skin cells. The other 6 samples were cut into four equal pieces to use in transplantation.

Assigned groups are:
Group 1 (n=6): Axolotl blastema tissue graft; Blastema tissues were grafted to open wounds.
Group 2 (n=6): Axolotl tail tissue graft; Graft of tail tissues to open wounds was performed.
Group 3 (n=6): Axolotl blastema cells treatment; Cell suspension of blastemal tissue (100 μl in serum physiologic) was topically applied to each wound.
Group 4 (n=6): Axolotl tail cells treatment; Cell suspension of tail tissue (100 μl in serum physiologic) was topically applied to each wound.
Group 5 (n=6): Control group (Serum Physiological treatment); 100 μl of serum physiologic was administrated to the each wound.
Group 6 (n=6): Positive control group (Madecassol treatment); Madecassol cream was topically applied to cover the each wound area for the first three days, once a day.

Macroscopic wound healing assessment:
Photographs were taken at day 0 and 10 for each wound with a ruler by using a Canon model digital camera to calculate the wound closure rate. All pictures were taken from the top view of the wounds with an angle of 90 degrees. To calculate the wound surface area and evaluate the picture results, Image J (Image J.2.0 software, NIH) image analyzer was used.

Sample preparation:
Animals were sacrificed on day 10, and skin covering the wound site including the original wound margin was removed for further analysis. Out of 4 wounds, two of them were fixed in 4% formaldehyde to use in histological analysis. The other two wounds were frozen immediately with dry ice to use in cryo-sectioning for immunostaining or to extract RNA for gene expression profiling.

Histological analysis
Samples incubated in 4% formaldehyde for 24h at room temperature were washed under tap water for 24h. As a next step, incubation in ascending alcohol series (70, 90, 96 and 100%) were followed for 1 h each at 60˚C. Samples were kept in toluene for 1h at room temperature and this step is followed by overnight incubation in paraffin at 60˚C. Cooled paraffin embedded samples were cut into 5 μm thin sections using a microtome (Thermo-Microm HM 340E). Paraffin sections were stained either with hematoxylin and eosin (H&E-Bio-Optica Mayer's Hematoxylin an Eosin Y Plus) or Trichrome Stain Kit (Empire genomics, Cat# BPK2916) according to producer's protocol. Sections were covered with 'Mounting medium' and examined by light microscopy (using Axio Zoom V16 microscope) to identify the general histological structures.

Criteria for evaluation of samples based on microscopy are described elsewhere (Galeano et al., 2006). Epidermal and dermal organization, thickness of granulation layer and angiogenesis are the main parameters to compare the differently treated groups.

Immunohistochemical analysis:
Dry ice fixed wounds were embedded in O.C.T. (Optimum Cutting Temperature) and have been cut into 8 μm thin sections using cryostat (Leica). Sections at - 80˚C have been incubated at room temperature for 1 hour to get dry. Afterwards the fixation with ice cold aseton for 15 minutes, the tissues sections have been incubated at room temperature for 1 hour.

Cryo-sectioned tissues were fixed in 4% PFA and washed in PBS and 0.3% TritonX-PBS. Blocking was performed in 0.1% TritonX-PBS and 10% goat serum. Following the blocking, sections were washed in PBS and incubated overnight in VEGF antibody (1:100, rabbit, Abcam, ab46154) at +4°C. After washing in

PBS, sections were incubated 1h in goat-anti rabbit 488 antibody (1:400, Abcam, ab150077) at room temperature. Excess secondary antibody was removed with PBS wash. For counterstaining DAPI (Life Technologies, P36931) was used to label the nucleus. LSM 780 NLO Multiphoton microscope (Zeiss) was used for imaging.

q PCR analysis:

Total RNA was extracted from frozen wound tissue samples using RNeasy Mini kits (Qiagen, Crawley, UK) by following the manufacturer's protocol. cDNA was synthesized from 1 μg of wound RNA using Superscript II reverse transcriptase (ThermoFisher Scientific) and random primer mix (NEB). Quantitative Real-time PCR (qPCR) was applied with specific primer sets (Supplementary Table 1) using the iQ-SYBR Green Supermix (Biorad) following producer's instructions and a qPCR thermal cycler (Biorad). IL-1α, IL-1β, IL-4, IL-10, Interferon gamma (IFNγ) and tumor necrosis factor (TNF)-α expression levels were measured as three replicates. The housekeeping genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used for normalization of the samples.

3. Statistical analysis

All statistical analyses were performed with SPSS 18.0 software program. The closure rate for each treatment is calculated as means ± Standard deviation. The significance of differences between groups was analyzed by one-way ANOVA for the macroscopic assessment of wound healing. Multiple comparisons between groups were carried out by using Dunnett's test. According to Post-hoc tests; $p<0.05$ was considered as statistically significant.

4. Results

Macroscopic wound healing assessment

The crust overlying the open wound surface was observed to be less in the treatment groups (Fig. S1). For all samples, day 0 wounds (Fig. S1a-f) have been repaired at day 10 (Fig. S1a'-f') and regarding the macroscopic view, healing process seems active at different rate among the control and treatment groups (Fig. S1). As shown in figure 1, various treatments have diverse impact on wound closure size.

Wound area was calculated at day 0 and day 10, and the ratio of day 10 to day 0 demonstrates the success of wound closure. Among the Axolotl blastema cell, blastemal tissue and tail cell treated animals no significant difference was observed (Fig. 1). Remained wound size for these groups was significantly less than the wound size of the tail tissue grafted animals (Fig. 1; $p<0.01$). For the Axolotl cell or tissue treated animals a highly significant improvement in wound closure

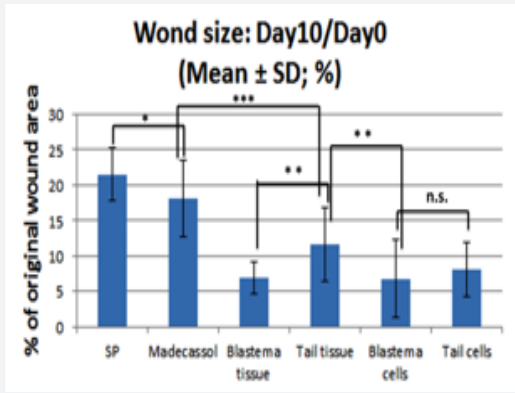

Fig. 1. Temporal changes in wound size

Statistical analysis of wound size change in control and experimental groups. The data presents the means ± standard deviations (SD). Wound closure rate is higher in Axolotl cells or tissue administrated animals than madecassol or SP treated animals.

rate was noticed in comparison to no treatment control (serum physiological administrated) or madecassol treated animals (Fig. 1; $p<0.001$). Madecassol treated animals exhibit significantly better wound closure compared to no treatment control (Fig. 1; $p<0.05$). Relevant statistical data is presented in supplementary data (Supplementary Table 2).

Histological analyses

Wound healing areas were histologically examined to demonstrate histopathological variations among control and treatment groups (Fig 2). Samples were stain with Hematoxylin& Eosin (a-a') and Masson Trichrome (b-b') to demonstrate the granulation tissue and collagen density; respectively.

In Axolotl blastema tissue grafted animals (group 1) complete epithelization of the wound is observed and wound surface is covered with mature epithelial cells (1a-1a'). As a consequence of effective wound healing, wound edges were not distinguishable (1a-1b). Epidermal thickness was significant (1a-1a'), hair follicles were detected (1a', white arrows), dermal papilla was formed (1a'-1b') and epithelial and connective tissue integrity was notable (1a'-1b', stars) for blastema tissue grafted animals.

Axolotl ail tissue grafted animals (group 2) exhibits relatively moderate wound healing (2a-b'). Wound edges were not in contact with the epithelium (white arrow, 2a'). In the granulation tissue, cell density and newly synthesized thin collagen fibers are observed (star, 2b').

As a sign of potent healing, dense vascularization is noted in dermal layer (black circle, 2a'). Around the

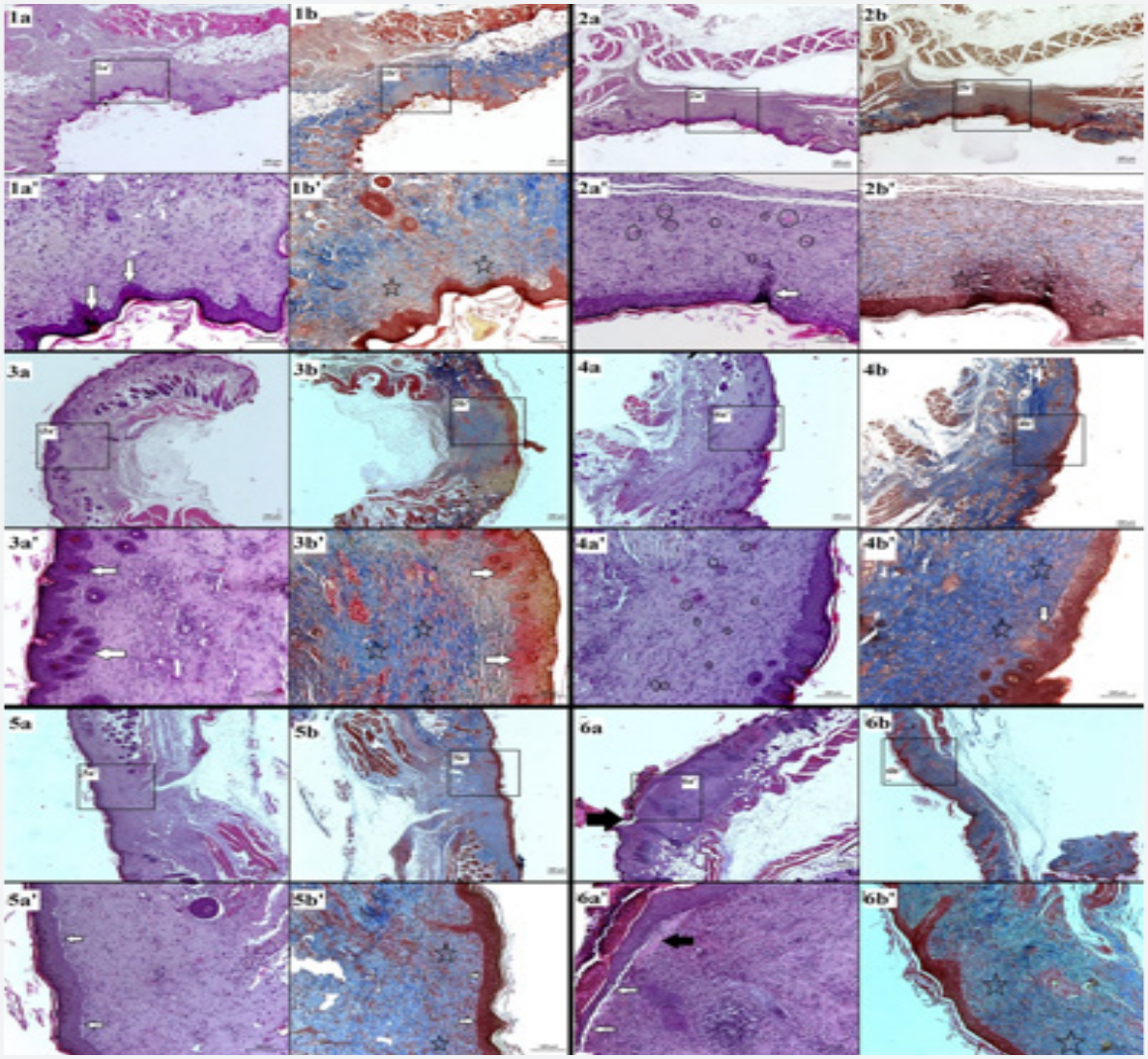

Fig. 2. Photomicrograph of histopathological sections of mice wound tissues.
Sections are stained with Hematoxylin&Eosin **(a-a')** and Masson trichrome; **(b-b')**. The area surrounded with black frame is indicated in high magnification. White arrow; hair follicles **(1a')**, stars; granulation tissue **(1b')**, black circles; angiogenesis **(2a')**, white arrow; wound edges **(2a')**, stars; granulation tissue **(2b')**, white arrow; hair follicles **(3a' and 3b')**, stars; granulation tissue **(3b')**, black circles; angiogenesis **(4a')**, white arrow; hair follicles **(4b')**, white arrow shows the separation of the dermis and epidermis and loss of dermal papilla **(5a' and 5b')**, stars; granulation tissue **(5b')**, white arrow shows the separation of the dermis and epidermis and loss of dermal papilla **(6a')**, black arrow; inflammatory cells, stars; granulation tissue **(6b')**
(a-b; 40X magnification, bar=200 μm and a'-b'; 160X magnification, bar=100 μm)

epidermal layer, inflammatory cells were recognized (2a'). No dermal papilla is noticed and epidermal flatting was accompanied with degenerated epithelial cells (2a'-2b').

Animals treated with Axolotl blastema cells (group 3) display a remarkable healing success in terms of epithelization, granulation tissue and vessel network formation (3a-b'). Wound edges are not distinguishable and observed skin histology at day 10 resembles undamaged skin histology (3a-3a').

Epidermal and dermal structures are well developed and alignment of epithelium and connective tissue is appropriate (3b-3b'). Collagen bundles are well defined in connective tissue (3b', stars). Five different layers were distinguishable from stratum basale to stratum corneum at the epidermis. In dermis, extensive hair follicles are detected and their extension to open surface is recognized is (white arrow, 3a').

Axolotl tail cells administrated animals (group 4) exhibit a successful wound repair (4a-b'). Complete epithelization and a sheet of epithelial cells around

the wound area is noted (4a-4a'). Lack of widespread dermal papilla and instead flatting epidermal layer was noticed in the epidermis (white arrow, 4a). Dense vascular structures in the dermal layer (black circle, 4a') and intense collagen fibers at the subepithelial area is noteworthy (star, 4b').

Control group animals (serum physiologic treated-group 5) demonstrate the lowest level of healing and repair success among the all groups. Reepithelization in samples is noted however observed epidermal layer is flat and no dermal papilla structure is detected (5a-5a'). Collagen bundles were intense at the subepithelial region (star, 5b'). Epidermal layer is separated from dermal layer and intercellular junctional connection is lost (white arrow, 5a'-5b').

Animals treated with madecassol have a thin epidermal layer and a delay in wound healing manifestations compared to Axolotl tissue or cell treated aniamls. In three of six animals, wound edges were not in contact with epithelium and it is represented with black arrow in the figure (6a-6a'). Separation of the dermis from epidermis and loss of intercellular junctional connection is observed (white arrow, 6a'-6b'). Collagen fibers situated parallel to epidermal layer are recognized at the subepithelial area (star, 6b'). Presence of intense inflammatory cells was noticed at dermal layer.

Immunostaining

VEGF staining was performed to monitor the vascularization variations among the groups (Fig. 3). Axolotl blastema tissue (4a-a'), tail tissue (4b-b'), blastemal cells (4c-c') and tail cells (4d-d') administrated animals exhibit a better vascularization level around the wound site than the negative control (4e-e') and madecassol treated (4f-f') animals. In higher magnification (4a'-f') a well-established vessel network is observed in Axolotl treated animals (4a'-d'; white arrows for representation of vessels) whereas the presence of vessels are barely detected for control groups (4e'-f'). No non-specific staining was detected for negative control (Fig. S2).

Expression levels of cytokines in different groups were analyzed by qPCR. Levels of pro-inflammatory cytokines (IL-1α, IL-1β, IFNγ and TNFα) were examined for the samples harvested at day 10. To assess the differences in mRNA expression levels cytokines, GAPDH and β-actin were used as reference

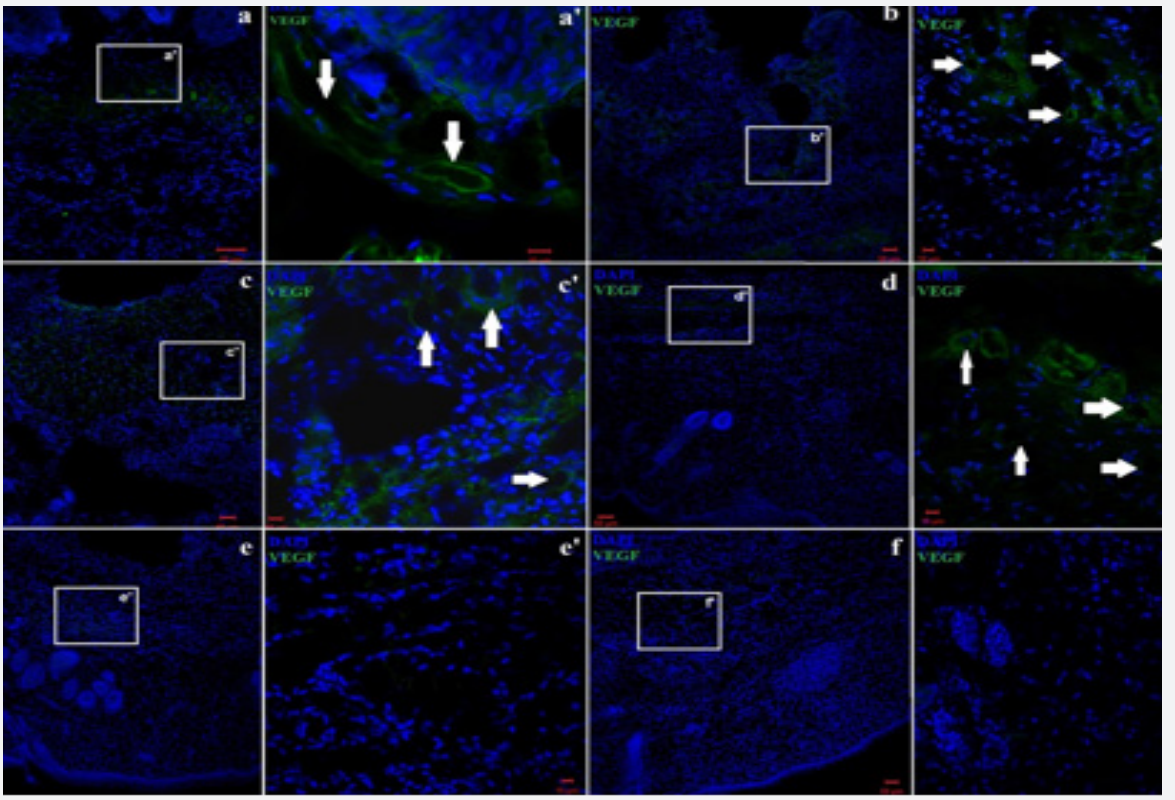

Fig. 3. Angiogenesis levels by VEGF and DAPI staining

White frames in low magnification images (10x magnification, bar=50 μm) are revealed with high magnification (40x magnification, bar=10 μm). A significant increase of VEGF expression in the groups of 1,2,3 and 4 can be recognized (a-d) which is indication of angiogenesis in and around the granulation site. e)SP treated group and f) Madecassol group.

genes and investigated cytokines were normalized to these house-keeping genes. Animals treated with Axolotl cells or tissues display a significant ($p<0.05$) decrease in IL-1α, IL-1β, IFNγ and TNFα levels at the wound site at day 10 (Fig. 4). The striking difference in relative mRNA levels between SP and Axolotl samples treated groups was observed for TNFα expression (Fig. 4). Madecassol treated animals demonstrate significant decrease in IL-1α and IL-1β levels compared to SP treated animals, however no significant difference were observed for IFNγ and TNFα mRNA levels between these two groups (Fig. 4).

We couldn't detect any significant difference in expression levels of anti-inflammatory signals such as IL-4 and IL-10 (data not shown) among the groups.

5. Discussion

Wound healing is a complex process of repair and restoration of damaged tissues. Wound closure and tissue reformation capacity differs greatly among the animals and potential of the successful repair arises as a combination of genetic background and the applied treatment. Molecular mechanisms behind the exceptional regeneration and self-repair capability of Axoltl are not fully explored yet. However, exploitation of this unique feature without the foreknowledge of the exact cascade(s) is still worthy to examine whether it may improve the mammalian cutaneous wound healing. In this study we search the adequacy of Axolotl tissues and cells to heal the experimentally damaged mice skin tissue.

As observed in fig. 1 all animals close the wounds with comparable grades and a visible scar. To evaluate the closure rate for different groups, day 10 and day 0 wound areas are calculated for each wound. Since statistical data rather than macroscopic investigation is more reliable to draw a meaningful conclusion, average closure rate among groups is compared. As shown in fig. 2, Axolotl tissues or cells administration significantly accelerates the wound healing in comparison to medacassol and serum physiologic treated control groups. Better healing profile than prominent medication (madecassol) provides new evidences of self-repair potential of Axolotl to promote restoration of damaged tissues even in another species. No significant macroscopic differences between Axolotl blastema tissue, blastema cells and tail cells administrated animals may indicate negligible variations in wound repair above a certain threshold for wound closure success. Interestingly, wound size at day 10 of tail tissue grafted animals is significantly higher than the other Axolotl tissue or cell treated animals' wound size. This is probably due to less toleration of tail tissue graft by mice. Madecassol is commonly used medicine which acts in increasing antioxidative activity, enhancing collagen synthesis and angiogenesis (Bylka et al., 2013) and therefore it accelerates the wound healing process. Not surprisingly, a real benefit of madecassol treatment is observed when compared with serum physiologic delivered animals.

Although wound closure rate is a good indication of wound repair capability, tissue remodeling and

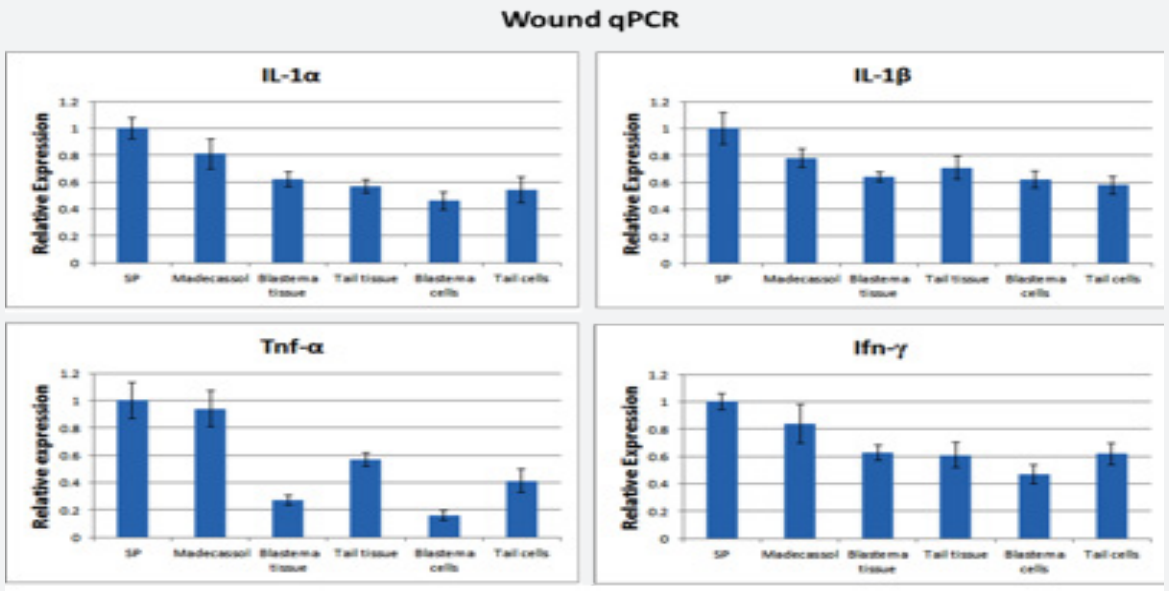

Fig. 4. Expression levels of cytokines
Expression levels of IL-1α, IL-1β , TNF-α and IFN-γ are significantly different among the control and treated groups. Animals treated with Axolotl samples display a significant decrease in pro-inflammatory cytokine expression. SP: Serum Physiologic, $p<0.05$

deposition of components in wound area is essential for functional repair. Therefore, histopathological examination provides valuable insights to follow healing success. As shown in fig. 3, samples from all groups were analyzed histologically to observe reconstitution levels at the wound site. Epidermal and dermal reorganization, thickness of the granulation tissue and angiogenesis were considered in evaluation. Regarding the histological staining and light microscopy, animals treated with Axolotl biological samples (Fig. 3; 1a-4b') fulfill these criteria excelling than the control groups (Fig. 3; 5a-6b'). Especially in Groups 1 and 3 an improved and complete epithelialization level is noted compared to control groups and even presence of hair follicles are observed. Formation of hair follicles is a noteworthy finding since its reappearance after an injury or burn is not very common. For all Axolotl samples treated animals epidermal and dermal structures are more regular particularly for the animals in group 1 and 3 in comparison to control group. Dermal papilla formation in group 1 and 3 indicates dermal and epidermal association. In higher magnification (x160), epithelial layer including basal lamina is noted to be in normal structure. In the epidermis, keratin layer was well arranged and epithelium had more cell layer. In the epithelial layer papilla structure were noted in between the connective tissue. Epithelium and connective tissue is noted to be connected.

VEGF is secreted by many type of cells to stimulate the endothelial cells for formation of new blood vessels. In hypoxia condition, expression of VEGF and VEGFRs is up regulated and this enhances the vascularization and angiogenesis levels during the embryogenesis, wound repair and tumorigenecity (Bao et al., 2009; Hoeben et al., 2004). Migration, proliferation and differentiation of endothelial cells are regulated by VEGF binding to its receptor (Koch and Claesson-Welsh, 2012; Marcelo et al., 2013). During wound repair epidermal keratinocytes secrete VEGF to induce angiogenesis around the wound zone for transportation of nutrients and blood to the damaged site and as a consequence wound healing is accelerated. Formation of new blood vessels and its high amount is considered as direct evidence of advanced wound repair. Wound healing success of Animals treated with Axolotl samples (Fig 4a-d) is in accordance with observed vascularization level compared to control groups (Fig4e-f). Nutrients and oxygen supply by circulation system is crucial for a complete and fast wound repair and therefore there is a correlation between wound healing capacity and newly formed blood vessels. VEGF immuno-staining results demonstrate that a blood vessel rich circulation network is formed at the restored wound area for experimental groups. Lack of adequate vascularization for control groups may explain the delay in wound repair for animals in control groups.

Significant down regulation of pro-inflammatory signals in Axolotl cells or tissues administrated animals may explain the enhancement of wound repair via immune-modulation. Pro-inflammatory signals such as IL-1α, IL-1β or TNFα are necessary to start initial phase of wound repair (Boyapati and Wang, 2007; Godbout and Glaser, 2006) however prolonged elevation of these cytokines elongate the healing process due to excessive inflammation signaling (Guo and Dipietro, 2010). Our data indicate the significant decrease in RNA levels of these genes at day 10 for the animals treated with Axolotl samples. Higher levels of pro-inflammatory signals at day 10 for SP treated animals and relatively low reduction for Madecassol treated animals may disclose the delay in wound repair in contrast to other experimental groups. Since there was no significant difference in expression levels of anti-inflammatory signals (IL-4 and IL-10) between the groups, regulation of pro-inflammatory signals may be the main immune-modulating mechanism by Axolotl samples. Another plausible explanation is chosen time point for RNA quantification was not optimal to examine the levels of anti-inflammatory cytokines. Analyzing the levels at an earlier time point might be more informative about the differential expression of anti-inflammatory signals among the various groups.

Taken together, this study demonstrates utilization potential of Axolotl's regeneration capacity in mammalian wound healing. A better wound repair might be achieved by the activity of Axolotl cells and tissues in improving reepithelization, advancing angiogenesis and regulating immunity. To explore the exact mechanism of facilitating wound healing, further studies, such as administration of subgroups of cellular components to wound area, analyzing of a wide range cytokine and growth factors panel (including TGFs, FGFs and EGF) and sampling at different time points for macroscopic/microscopic examinations, investigations of differences in gene expression and immune-staining assessment, should be performed.

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