



## Effect of Thymoquinone on skeletal muscle regeneration via assessment of Pax-7 and Myo-D expression in the DMBA-treated hamster pouch

Efeito da Timoquinona na regeneração muscular esquelética através da avaliação da expressão das proteínas Pax-7 e Myo-D em bolsa jugal de hamsters tratados com DMBA

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### ABSTRACT

**Objective:** Pax-7 and Myo-D regulate satellite cells' activation and differentiation, thus muscle regeneration following damage. This research aimed to investigate the effect of Thymoquinone (TQ) on skeletal muscle regeneration following 7,12-dimethylbenz-(a)-anthracene (DMBA)-induced injury in the hamster buccal pouch via immunohistochemical assessment of Pax-7 and Myo-D expression. **Material and Methods:** 65 male golden Syrian hamsters were divided into 3 groups: Group 1: (n=5) received no treatment. Group 2: (n=20) served as a positive control. The left buccal pouches were painted with the carcinogen 3/week/ 6weeks. Group 3: (n=40) were subdivided into two equal sub-groups as follows: Group 3a: (n=20) were given one i.p. TQ injection. Group 3b: (n=20) were given two i.p. TQ injections. Five animals from each group (2 and 3) were euthanized at 24, 48 hrs, one, and two weeks after the last injection. A blood sample (2 ml) was withdrawn for assessment of TNF- $\alpha$  levels in serum. Serial sections of the pouches were examined histologically (H&E), and immunohistochemically (IHC) for the detection of Pax-7 and Myo-D proteins. **Results:** double i.p injections of TQ resulted in a significant elevation in the level of TNF- $\alpha$  from the second-day post-injection with a progressive formation of the muscle fibers (MFs) and mononuclear cells (MNCs) around the deeper blood vessels. At 14 days, no statistically significant difference was found between this group and group '2', while the difference remained significant compared to groups '1' and '3a'. The muscle fibers were more mature and compact. IHC results showed positive expression of the perivascular mononuclear cells (MNCs) to both Pax-7 and Myo-D with positive reactivity of the peripheral nuclei of muscle fibers to Pax-7 compared to the negative reaction in the positive control group. **Conclusion:** early and two TQ injections had a promising effect on the induction of striated muscle regeneration, mainly by non-myogenic stem cells.

### KEYWORDS

Thymoquinone; Pax-7; Myo-D; Buccal pouch; Muscle regeneration.

### RESUMO

**Objetivo:** Pax-7 e Myo-D regulam a ativação e diferenciação de células satélites durante a regeneração muscular pós-trauma. Assim, objetivamos investigar o efeito da timoquinona (TQ) na regeneração muscular esquelética após injúria causada por 7,12-dimetilbenzantraceno (DMBA) em bolsa jugal de hamsters, através da análise imuno-histoquímica de Pax-7 e Myo-D. **Material e Métodos:** 65 hamsters-sírios machos foram divididos em 3 grupos: Grupo 1: (n=5) controle negativo, sem tratamento. Grupo 2: (n=20) controle positivo. A bolsa jugal do lado esquerdo recebeu aplicação do DMBA por 3 e 6 semanas. Grupo 3: (n=40) receberam aplicação de

DMBA e foram então subdivididos em: Grupo 3a: (n=20) que recebeu 1 injeção intraperitoneal (ip) de TQ e Grupo 3b: (n=20) que recebeu duas injeções ip de TQ. Cinco animais dos grupos 2 e 3 foram eutanasiados em 24 horas, 48 horas, 7 dias e 14 dias após a administração de DMBA e da última injeção de TQ. Amostras de sangue (2 ml) foram coletadas para avaliação dos níveis séricos de TNF- $\alpha$ . Cortes seriados da bolsa jugal dos animais foram analisados histologicamente (H&E), e através de imunohistoquímica (IHC) para avaliação das proteínas Pax-7 e Myo-D. **Resultados:** duas injeções ip de TQ aumentaram os níveis séricos TNF- $\alpha$  à partir do segundo dia pós-administração com formação progressiva de fibras musculares (MFs) e células mononucleares (MNCs) ao redor dos vasos sanguíneos. No dia 14, não houve diferença estatística entre o grupo 3b e o grupo 2, enquanto a diferença permaneceu entre o grupo 1 e 3a. As MFs apresentavam-se mais maduras e compactas. A IHC mostrou expressão de Pax-7 e Myo-D nas MNCs ao redor dos vasos, e houve expressão nuclear de Pax-7 nas MFs no grupo 2. **Conclusão:** ambos regimes de administração do TQ, 1 ou 2 aplicações ip, apresentaram efeito promissor na indução da regeneração muscular esquelética, principalmente nas células não-miogênicas.

## PALAVRAS-CHAVE

Timoquinona; Pax-7; Myo-D; Bolsa jugal; Regeneração muscular.

## INTRODUCTION

The damaged skeletal muscle cells can regenerate through myogenesis [1]. The muscle-resident stem cells (i.e., satellite cells) are the key chain in the process of classic myogenesis. In brief, a comprehensive review by Musarò (2014) [2], had pointed out to the essential five inter-related and time-dependent phases for that classic way of muscle regeneration [2]. Starting with muscle fibers' necrosis (a transient inflammatory step), followed by regeneration through activation of stem cell populations (either satellite or other non myogenic stem cells). Then remodeling of the extracellular matrix and angiogenesis. Last phase is reinnervation of regenerated fibers [2].

Skeletal muscle injury can result from induction of epithelial dysplasia in hamster buccal pouch/7,12-dimethylbenz-(a)-anthracene(HBP/DMBA) model. It is one of the most well-characterized tumor-induction models that simulate that occurs in humans [3]. The early few DMBA paintings result in necrosis of the distal pouch, with a reduction of its length from about 5-6 cm to about 2 cm. The pouch does not regain its full length even after suspending DMBA painting [4].

Thymoquinone (TQ) [2-isopropyl-5-methyl-1, 4- benzoquinone (C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>)], is a major active constituent of *Nigella sativa* (NS) which is a dicotyledon of the *Ranunculaceae* family [5]. TQ has been extensively studied in vivo and in vitro and was proven to have many therapeutic properties including an anticancer activity [5,6].

TNF- $\alpha$  is expressed by many cells including monocytes, macrophages, natural killer cells, and myoblasts [7]. In the scope of muscle regeneration, TNF- $\alpha$  was found to be expressed in damaged muscle fibers and perform dual roles depending on its concentration [8]. At high concentration, it suppresses myogenesis via proteolysis of Myo-D, while inducing it at lower concentrations [9].

Myo-D is a member of the myogenic regulatory factors (MRFs) that include Myo-D, MRF4, myogenin, and Myf5 as well [10]. Early after muscle injury, Myo-D and Myf5 are upregulated in activated muscle stem cells (satellite cells) as a primary response to muscle injury; alternatively, they are not expressed in quiescent satellite cells [11,12].

The successive expression of key transcription factors drives satellite cell development through the myogenic pathway [2]. Pax7 is a paired box transcription factor that is found in all mature muscle satellite cells and is required for their function. Pax7 downregulation is required for terminal differentiation, whereas Pax7 elevation after proliferation favors a return to the rest state [13].

Quiescent satellite cells express the transcription factor Pax7, which co-expressed with Myo-D when the cell is activated. Then, most cells proliferate, downregulate Pax7, and differentiate. On the other hand, others keep Pax7 but lose Myo-D, returning to a condition that resembles quiescence [14]. Other non-myogenic cells were found to contribute to differentiating injured muscle cells. These nonmyogenic cells are

fibro-adipogenic precursors (FAPs), bone marrow-derived stem cells (mesenchymal stem cells), and pericytes [15]. In conclusion, this research aimed to prove the assumption that Thymoquinone (TQ) may stimulate skeletal muscle regeneration following DMBA-induced injury in the hamster buccal pouch via immunohistochemical assessment of Pax-7 and Myo-D expression.

## MATERIAL AND METHODS

### Chemicals and reagents

Dimethylbenz-(a)-anthracene [or 7,12-Dimethylbenz-(a)-anthracene (DMBA)] dissolved in heavy mineral oil (Sigma-Aldrich Company, Saint Louis, Mo, USA). DMBA (0.5%) solution was prepared by dissolving 1 gram in 200 mL of heavy mineral oil. The carcinogen was topically applied to the left hamster buccal pouches (HBPs) using number 4 camel hair brush. Thymoquinone (TQ) dissolved in propylene glycol (Sigma-Aldrich Company, Saint Louis, Mo, USA). TQ solution was dissolved in propylene glycol to get a solution of concentration 10mg/ml. one mg dose per hamster was injected intraperitoneally.

Sixty-five male Syrian golden hamsters (*Mesocricetus auratus*), 14 weeks old, with body weight 100-120 grams, were kept five per cage in a well-ventilated room with controlled temperature, 50-70% humidity, and 12 hours day/night cycle, in the animal house and were given water and recommended diet ad libitum. Ethical approval had been obtained from the institutional ethical review committee and the experiment followed the "ARRIVE" guidelines for handling and treating the experimental animals.

### Experimental design

The hamsters were divided into 3 groups:

- Group 1: 5 animals received no treatment and served as the negative control group. They were euthanized on the day of starting treatment of other groups.
- Group 2: 20 animals served as a positive control group. The left buccal pouches were painted with the DMBA (3 / week / 6 weeks).
- Group 3: 40 animals were painted with DMBA as in group 2, then

subdivided into two equal sub-groups as follows:

- Group 3a: 20 animals were given one intraperitoneal (i.p.) TQ injection.
- Group 3b: 20 animals were given two i.p. TQ injections every other day.

Five animals from each group were euthanized at 24, 48 hrs, one, and two weeks after the last injection (Figure 1).

Before the process of euthanization, the animals were anesthetized by a cotton-soaked with ether inside a firmly closed glass container. A blood sample (2 ml) was withdrawn from the retro-orbital venous plexus of the eye into a sterile tube with EDTA, for assessment of TNF- $\alpha$  levels. The data were statistically analyzed using One-Way Analysis of Variance (ANOVA) via SPSS® 22.

After euthanization, by inhalation of a lethal dose of ether, both right and left pouches were surgically excised, fixed in 10% neutral formalin, and embedded in soft paraffin wax. Sections of 5 $\mu$ m were cut using a rotary microtome then mounted on glass slides and stained for hematoxylin and eosin (for light microscopic study) and diagnosed by two pathologists.

Serial sections of 5  $\mu$ m were cut from each paraffin block and mounted on a positively charged glass slide (Super Frost Plus slides) for IHC staining to detect Pax-7 and Myo-D expression, following the manufacturer's instructions. Pax-7 polyclonal (Rhabdomyosarcoma Marker), (AB clonal, Catalog No. A7335), and Myo-D (Rhabdomyosarcoma Marker) (clone 5.8A&MYD 712, catalog No. RA0233-C.5), were used.

### Histopathologic analysis (H&E)

Oral epithelial dysplasia (OED) was graded, with modification, according Bánóczy and Csiba (1976) [16] classification into: mild: when fewer than three dysplastic parameters were present. Moderate: when three to seven dysplastic parameters were present. Severe: when more than seven parameters were present, and carcinoma in situ: when any number of dysplastic parameters are distributed from top to bottom or when all the parameters were seen with an intact basement membrane [16].

### Immunohistochemical analysis (IHC)

Sections were dewaxed by xylene, dehydrated in ethanol, then rinsed with distilled water.

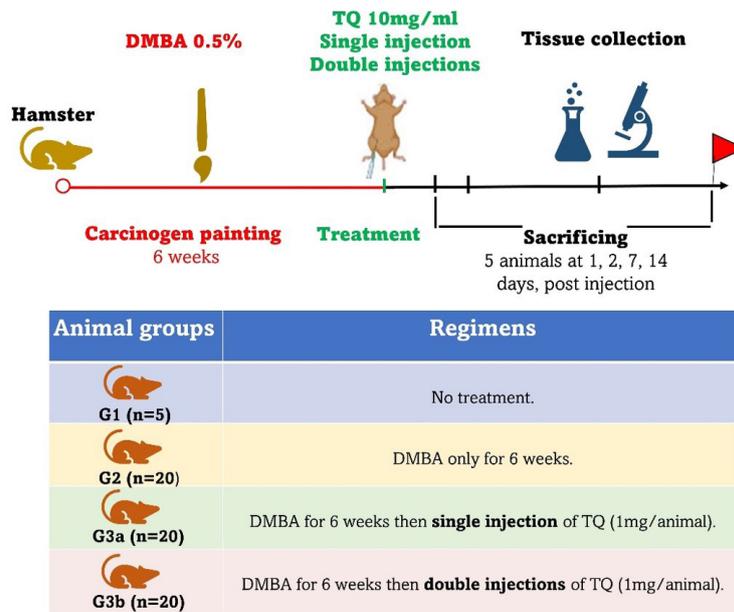


Figure 1 - Schematic representation of the experimental timeline and design.

The slides were immersed in 3% H<sub>2</sub>O<sub>2</sub> for 10 min and rinsed in distilled water for another 15 min. For antigen retrieval protocol, the slides were boiled in a microwave oven for 15 min with citrate buffer to unmask the antigenicity, then cooled at room temperature for 20 minutes. Primary antibodies were added to sections and incubated overnight at 4°C. They were then rinsed in PBS (phosphate-buffered saline) three times for two minutes each. Two drops (100ul) of poly HRP conjugate were added to cover the tissue, and incubated for 15 min. The sections were rinsed with wash buffer for ten min. The slides were then incubated with a DAB chromogen solution and incubated at room temperature for five min. The sections were counter-stained by dipping in Harris hematoxylin for one minute and rinsed under running water. After dehydration in ethanol, they were immersed in xylene, and coverslips were applied with synthetic adhesive resin (DPX).

### Digital image analysis

The slides were photographed by Olympus E-330 Evolt Digital Photography camera using Image Analyzing System (Olympus BX50 Microscope). Computerized image analysis of IHC staining "Image J, 1.41a, NIH, USA" software was used for quantitative IHC analysis of Pax-7 and Myo-D. A sample of the immune-positive area was chosen and applied to image J, then the image was adjusted to 8 bits followed by thresholding of the whole positive area.

Measurement of the thresholded area was finally done by the program and the area fraction was obtained. The mean fraction of the positive cells for each marker per group was calculated depending on the following key:

Score 0: Negative stain.

Score 1: when less than 15 positive cells were found.

Score 2: when 15 to 25 positive cells were found.

Score 3: when 26 to 50 positive cells were found.

Score 4: when more than 50 positive cells were found.

### Statistical analysis

The data were analyzed using [One-Way Analysis of Variance (ANOVA)] via SPSS® 22, for determination of relative protein expression and evaluation of the serum TNF-α levels. All values were expressed as mean ± standard deviation. The difference was considered significant at a p value ≤ 0.05.

## RESULTS

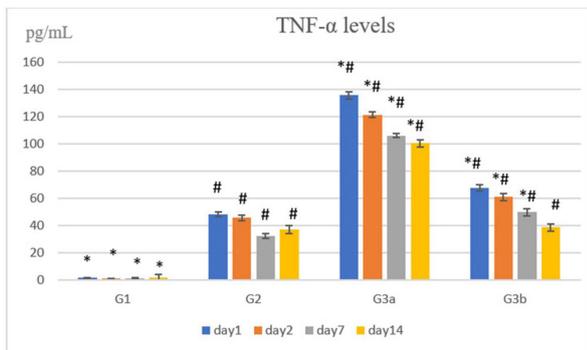
### Serum TNF-α level assessment

Statistically significant elevation (P=0.021) of the serum TNF-α level was detected in the

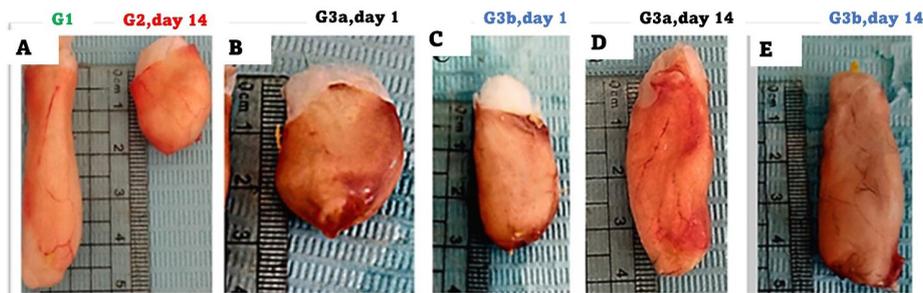
positive control group compared to the negative control group. Statistically significant elevation in the level of serum TNF- $\alpha$  one day following a single TQ injection was recorded between group '3a', compared to groups '1' ( $p \leq 0.002$ ), and '2' ( $p \leq 0.013$ ). The elevation remained statistically significant compared to groups '1' ( $p \leq 0.003$ ) and '2' ( $p \leq 0.024$ ) at 14 days post-injection. Regarding the levels of serum TNF- $\alpha$  in group '3b' one day post double TQ injections, statistically significant elevation was recorded in its level compared to groups '1' ( $p \leq 0.00$ ), and group '2' ( $p \leq 0.031$ ). At 14 days post-second injection, no statistically significant difference was found between this group and group '2' ( $P=0.631$ ), while the difference remained significant compared to groups '1' ( $p \leq 0.002$ ) and '3a' ( $p \leq 0.00$ ) (Figure 2).

### Clinical results

Both left and right pouches of the animals in group 1 (negative control) appeared healthy and normal, with a mean length of 5.2 cm (Figure 3A, left).



**Figure 2** - Representative graph of serum TNF- $\alpha$  levels in different groups at different time intervals: \* $p \leq 0.05$  compared to group "2"; # $p \leq 0.05$  compared to group "1".



**Figure 3** - Clinical presentation of the pouches' length of different groups at different time intervals. (A): (left) pouch length is almost 5cm in Group1, and (right) the left pouch of an animal from group 2 at end of the experiment. **(Day 1)**: (B & C): pouches of animals in groups 3a & 3b, respectively. **(Day 14)**: (D & E): pouches of animals in groups 3a & 3b, respectively, 14 days post TQ injections.

In group 2, the left pouches of the DMBA-treated animals showed exophytic masses and/or ulcers and severe inflammation with necrotic distal end. The left pouch's length was reduced to a mean of 2 cm (Figure 3A, right).

The left pouches of animals in group (3a) euthanized after one day of the single TQ injection, showed gross changes comparable to those observed in group B. The mean length of the pouches was 2.2 cm (Figure 3B) which increased to reach about 3.5 cm at 14 days post-TQ injection (Figure 3D).

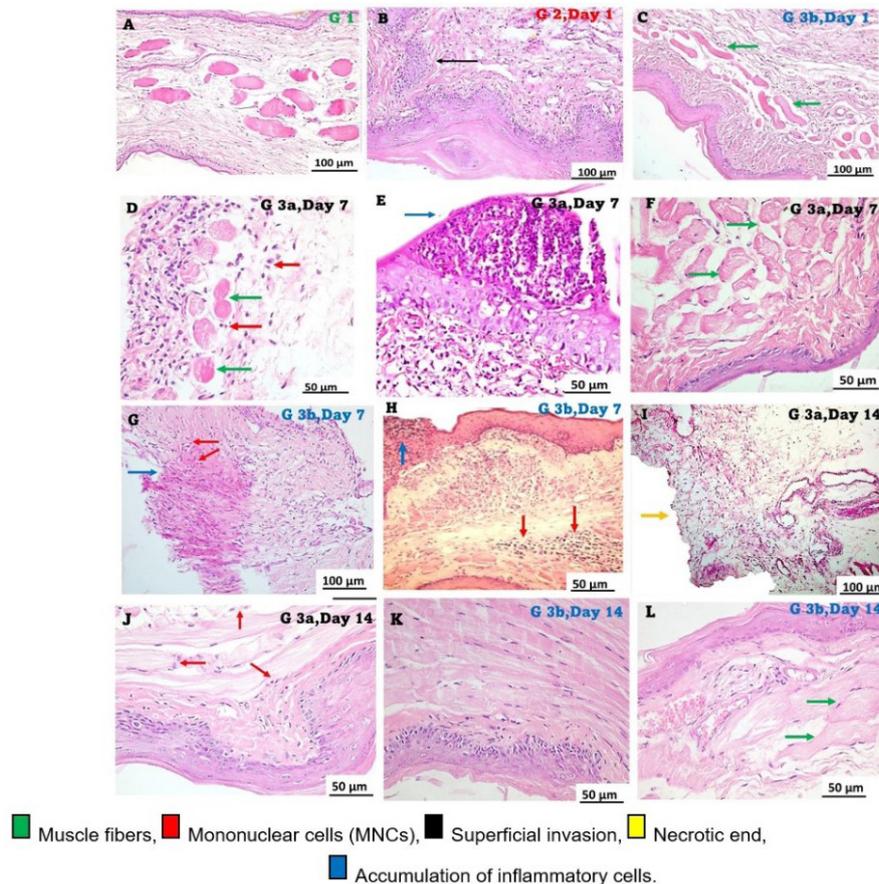
The left pouches of euthanized animals in group (3b) showed a reduced area of a distal necrosis and decreased number and size of the exophytic masses in comparison to groups 2 and 3a. The mean left pouches' length was 3cm, one day after the second TQ i.p. injection (Figure 3C) further increased to about 4.3 cm at 14 days post-second TQ injection (Figure 3E). The right pouches of animals in groups 2, 3a, and 3b appeared normal as in group 1.

### Histopathologic results (H&E stain)

#### Day 1 post last injection

(Group 1): the mucosa of the pouch appeared normal with regular epithelial stratification, and normal underlying connective tissue. The muscle layer is formed of scattered mature muscle fibers. The same finding was seen in the right pouches of all other groups (Figure 4A).

(Group 2): the left pouches revealed degenerated distal end, with different grades of epithelial



**Figure 4** - H&E-stained sections from different groups at different time intervals. **(G1)**: (A) the normal pouch mucosa formed of thin keratinized epithelium, collagenous lamina propria, and loose muscular layer. **(Day 1)**: **(G2)** (B): showing superficial invasion. **(G3b)**: (C) thick, collagenous lamina propria and the MFs were progressively forming but loosely separated. **(Day 7)**: **(G3a)**: (D): the area near the necrotic end with MFs. MNCs were found between MFs and around the BVs. (E): accumulation of inflammatory cells in the superficial epithelium along the pouch length. (F): the MFs are mature and progressively forming but loosely separated by fat cells. MNCs are seen around the BVs. **(G3b)** (G): the healed necrotic area at the distal end with newly formed MFs and MNCs between them. (H): multiple perivascular MNCs in areas of muscle fiber's formation (arrow), and intraepithelial inflammatory cells. **(Day 14)**: **(G3a)** (I): remains of the distal necrotic end. (J): near the distal end, the MFs were more compact. MNCs were around the BVs, and between MFs. **(G3b)** (K): along the pouches' length, mature MFs were more compact. (L): normal-appearing epithelium with no necrosis at the distal end. The lamina propria was thin and free of inflammatory cells.

dysplasia from moderate dysplasia to carcinoma in situ (CIS). The underlying lamina propria was highly inflamed, with a loosely arranged muscular layer, due to degenerated fibers and widely infiltrating fat tissue (Figure 4B).

(Group 3a): the lamina propria was inflamed, thicker, and more fibrotic comparable to that of group 2.

(Group 3b): progressive formation of the MFs and MNCs around the deeper blood vessels (BV) (Figure 4C).

#### Day 2 post last injection

The histopathological picture was comparable to that in the previous section.

#### Day 7 post last injection

(Group 2): degeneration of the distal end with focal superficial invasion could be seen.

(Group 3a): the pouches showed moderate dysplastic epithelium with remnants of a necrotic area at the distal end (Figure 4D). An intra-epithelial accumulation of inflammatory cells was seen focally along the rest of epithelial lining (Figure 4E). Mesial to the necrotic area, the underlying muscle fibers (MFs) were progressively forming but loosely separated by fat tissue. There was an increase of mononuclear cells (MNCs)

around the blood vessels (BVs) under the forming MFs, along the pouches' length (Figure 4F).

- (Group 3b): healing of the distal necrotic end with mild to moderate dysplastic epithelium with a thinner fibrotic lamina propria could be seen. Newly formed MFs and MNCs between them and around blood vessels were prominent findings (Figure 4G). Along the pouch's length, MFs were more condensed and mature (with peripheral nuclei). The perivascular mononuclear cells were abundant in the areas of muscle formation. Aggregates of inflammatory cells could be seen extruded from the epithelium as a constant finding in the TQ-treated group (Figure 4H).

#### *Day 14 post last injection*

- (Group 2): severe dysplastic epithelium with superficial invasion. The underlying layer shows inflamed lamina propria and degenerated MFs.
- (Group 3a): the dysplastic criteria were mild to moderate with a decrease in the size of the distal necrotic end. (Figure 4I-4J).
- (Group 3b): normal-appearing epithelium with focal hyperplasia, and thinner inflammation-free lamina propria could be seen (Figure 4K). The necrotic tissue at the distal end had completely disappeared and the MFs were more mature and compact (Figure 4L).

### **Immunohistochemical results**

#### *Pax-7*

##### *Day 1 post last injection*

- (Group 1): IHC results of Pax-7 revealed mild positively stained nuclei of epithelial cells, fibroblast of the lamina propria, and endothelial

cells (score +1). Whereas the nuclei of MFs were negative (score 0) (Figure 5A).

- (Group 2): negatively stained nuclei of epithelial cells and MFs (score 0) till the end of the experiment (Figure 5B).
- (Group 3a): negatively stained nuclei of epithelial cells and MFs (score 0).
- (Group 3b): negatively stained nuclei of epithelial cells and MFs. Mild positive reaction of MNCs (score +1) (Figure 5C).

##### *Day 2 post last injection*

- (Group 3a): the positive reaction of MNCs' and MFs' nuclei to Pax-7 (score +1) was first recorded (Figure 5D).
- (Group 3b): positive stain of the lamina propria cells, the perivascular MNCs, and some MFs peripheral nuclei (score +2) was recorded. (Figure 5E).

##### *Day 7 post last injection*

- (Group 3a): the positive reaction to Pax-7 was more intense (score +2) (Figure 5F).
- (Group 3b): The positive stain intensity of MNCs & MFs to Pax-7 was sustained. (Figure 5G)

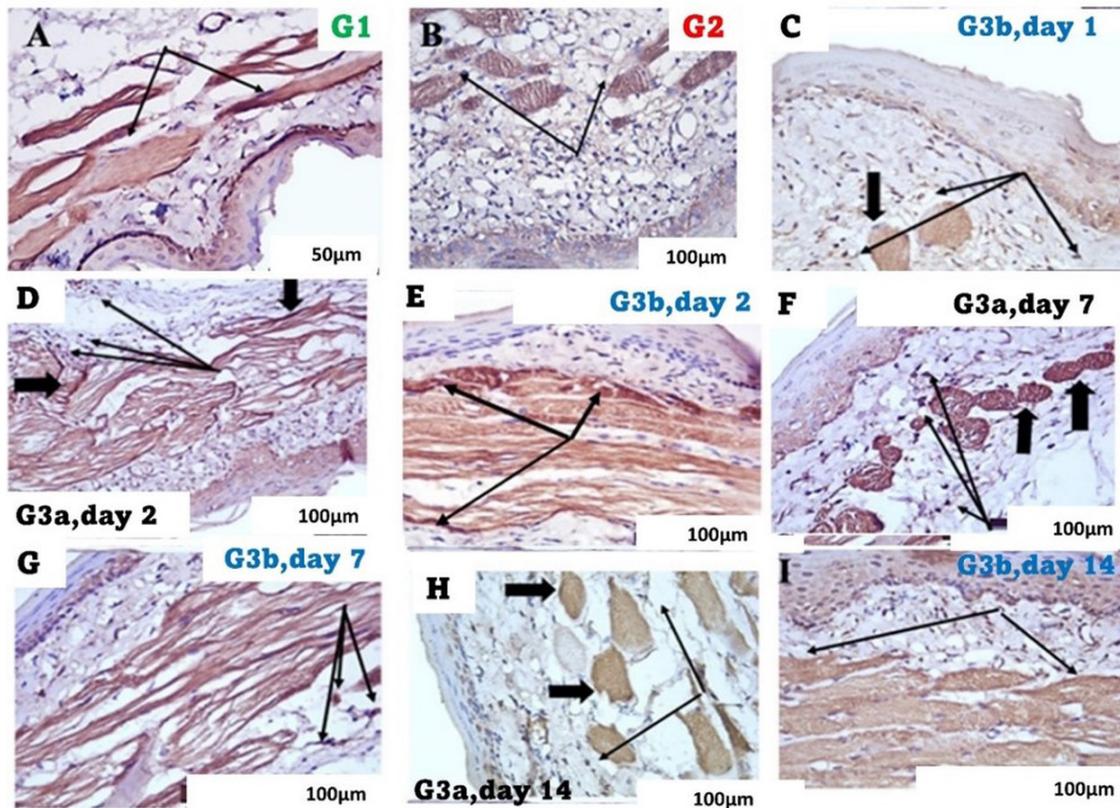
##### *Day 14 post last injection*

- (Group 3a): milder immunoreactivity of the peripheral nuclei of MFs and MNCs to Pax-7 was recorded (score +1) (Figure 5H).
- (Group 3b): The positive reactivity to Pax-7 declined to score +1 (Figure 5I).

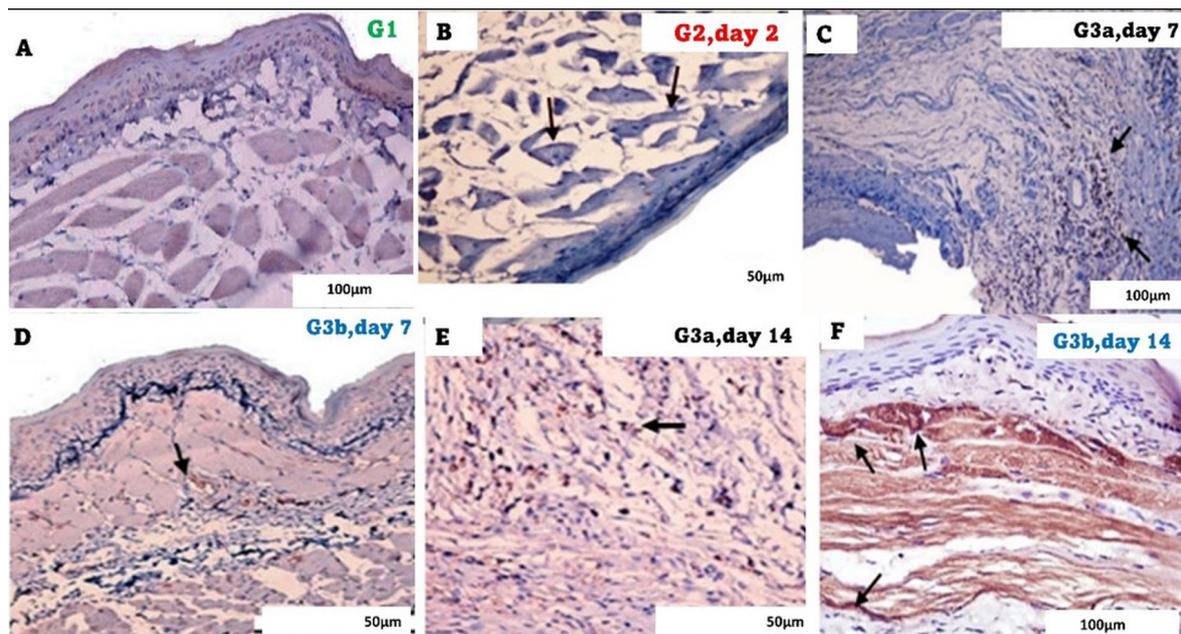
#### *Myo-D*

##### *Day 1 post last injection*

- (Group 1&2): Negative expression of mature muscle fibers was recorded in every time interval till the end of the experiment (score 0) (Figure 6A-6B).



**Figure 5** - Pax-7 IHC stained sections from different groups. **(G1):** (A): mild positively stained nuclei of epithelial and connective tissue cells. Negatively stained nuclei of MFs (arrow). **(Day 1): (G2):** (B): negatively stained nuclei of MFs (arrow) and nuclei of epithelial cells. **(G3b):** (C): negatively stained nuclei of epithelial cells, and MFs (broad arrows). The nuclei of perivascular MNCs were positively stained (thin arrows). **(Day 2): (G3a):** (D): positive stain of MNCs (thin arrows) and the peripheral nuclei of some MFs (wide arrows). **(G3b):** (E): negatively stained nuclei of upper epithelial cells, positively stained nuclei of perivascular MNCs, and some MFs peripheral plaque (arrows). **(Day 7): (G3a):** (F): positive stain of MNCs (narrow arrows) and peripheral nuclei of some MFs (wide arrows). **(G3b):** (G): positive reaction of MNC & MFs to Pax-7 (arrows). **(Day 14): (G3a):** (H): positive stain of MNCs (narrow arrows), and peripheral nuclei of some MFs (wide arrows). **(G3b):** (I): positive staining of the peripheral nuclei of some MFs (arrows).



**Figure 6** - Myo-D IHC stained sections from different groups. **(G1):** (A): negative Myo-D reaction. **(G2):** (B): negative expression of the multinucleated muscle fibers (arrows). **(Day 7): (G3a)** (C): positive reaction of mononuclear perivascular cells near the area of necrosis (arrows). **(G3b)** (D): positive reaction of perivascular cells (arrow). **(Day 14): (G3a)** (E): positive immunoreaction of MNCs (arrow). **(G3b)** (F): positive immunoreaction of MNCs and muscle fibers (arrows).

(Group 3a&3b): All the newly formed MF & MNCs were negative to Myo-D (score 0).

*Day 2 post last injection*

(Group 3a): The deeper MNCs around blood vessels are positive for Myo-D (score +1).

(Group 3b): Positive Myo-D expression of MNCs was recorded (score +1).

*Day 7 post last injection*

(Group 3a): The deeper MNCs around blood vessels were positive for Myo-D (score +1) (Figure 6C).

(Group 3b): A positive reaction of the MFs to Myo-D was first recorded (score +1) (Figure 6D).

*Day 14 post last injection*

(Group 3a): The deeper MNCs around blood vessels were still positive to Myo-D (score +1) (Figure 6E).

(Group 3b): Both MNCs and MFs were positively stained to Myo-D (score +2) (Figure 6F).

## DISCUSSION

The present work aimed to evaluate the influence of TQ on skeletal muscle regeneration following DMBA-induced muscle injury. Shortening of the buccal pouch was induced when DMBA was painted for consecutive 6 weeks. The muscle regeneration potentiality of TQ was assumed based on its documented anti-inflammatory effect [17]. The animals were euthanized on the 1st, 2nd, 7th, and 14th days post single and double TQ injections. This sequence was determined based on the classic order of muscle satellite cells' differentiation as early as 2 days post-injury till maturation after two weeks, with a focus on Myo-D and Pax-7 expression sequence [1,2].

TNF- $\alpha$  is normally produced from activated macrophages, CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and other stromal cells where it performs diverse cellular events resulting in acute phase reactions [9]. This can explain the significant elevation of TNF- $\alpha$  in group '2' following DMBA painting

for 6 weeks compared to the negative control group. This elevated level of TNF- $\alpha$  following DMBA application was reported in comparable research [18]. DMBA, despite being applied topically has a systemic effect and is known to have toxic effects on multiple organs [19]. This toxicity and severe inflammatory effect are presented in the current study by severe necrosis of the painted animals' pouches.

Levels of TNF- $\alpha$  were further elevated following TQ injections in comparison to the DMBA-only group. This can be possibly explained by the effective systemic anti-inflammatory activity of TQ, which resulted in the expulsion of the formed TNF- $\alpha$  from the affected tissue cells to the circulation. This explanation is demonstrated in the present research via histological sections of pouches from the TQ-treated groups, showing absence of inflammatory cells (TQ-treated groups), and necrosis in the DMBA-only painted group. This finding was further supported by a study in which the IHC staining technique was used to detect the presence of the TNF- $\alpha$  and NF- $\kappa$ B in the surface epithelium and inflammatory cells in the pouches of TQ-treated animals. The researchers reported negative expression of both markers in the surface epithelium and inflammatory cells as well [20].

Histologically, pouches of the negative control group and right pouches of other groups showed normal-appearing mucosa and loosely arranged mature muscle fibers (peripherally located nuclei) were Myo-D negative. The left pouches of the DMBA-painted pouches (positive control) showed a necrotic distal end, that resulted in a significant reduction in the length of the pouch (average 2cm). This observation was reported in comparable studies [4,20]. On the other hand, animals that received one and two i.p. TQ injections, after cessation of DMBA application, showed gradual elongation in pouches' length with no ulceration or inflammation. They showed grossly and histologically, normal-appearing epithelium with inflammatory-free collagenized lamina propria. The muscle layer showed progressively increased bulk till fourteen days after two TQ injections. This result was reported in other studies [4,20,21].

Myo-D was negatively expressed in group "2", one possible explanation may be based on the histopathological picture of the painted pouches that revealed degenerated muscle fibers, with hyperplastic and hyper-keratinized epithelium

close to the necrotic end, with highly inflamed lamina propria. At the mesial end of the pouches, the newly formed MFs were multinucleated, so were Myo-D negative. Myo-D may have been expressed during the carcinogen painting (i.e. during the 6 weeks of DMBA painting). The negative expression may be regarded to destabilization of Myo-D caused by increased TNF- $\alpha$  levels in NF-kappa B-dependent manner, as reported by Langen et al. [22]. The authors reported that TNF- $\alpha$  / NF- $\kappa$ B signaling pathways play an essential role in the process of myogenesis [22]. Comparable studies had demonstrated the effect of elevated TNF- $\alpha$  on the expression of Myo-D and the process of myogenesis. When TNF- $\alpha$  was added to proliferating myoblast, it inhibited myoblasts' differentiation and downregulated Myo-D and myogenin expression [22,23]. Coletti et al. [24] reported muscle wasting and impaired muscle regeneration following TNF- $\alpha$  gene transfer [24]. However, in the present study, Myo-D was positive in MNCs, connective tissue cells near the necrotic area, and lamina propria, suggesting that these cells had the main role in myogenesis and elongation of the pouches due to TQ injection(s).

In groups, 3a/3b, Myo-D negative expression of the peripheral nuclei of newly formed muscle fibers, in early timing following TQ injection. This possibly could be regarded to the non-myogenic origin of these fibers that were positive for Myo-D in early timings. However, the positive reactivity of the nuclei of fat cells, interstitial cells, and fibroblasts suggests stimulation of FAPs cells' differentiation into a myogenic lineage. A comparable study suggested a possible transition of fibrotic to myogenic lineage as evidenced by Masson's trichrome stain [20]. The transition of thick bulky fibrotic lamina propria into myogenic cells, as they were positive to the proposed markers, may explain the reduction of the thick lamina propria after DMBA treatment to thinner lamina propria in the TQ- treated groups. This finding indicates that the lamina propria was replaced by the increased muscle bulk. Other studies reported positive reactivity of fibroblasts to Myo-D during differentiation into myogenic lineage [25,26].

The third proposed non-myogenic lineage is the perivascular mononuclear cells (MNCs) that were positive to Pax-7 and Myo-D at 24- and 48-hours post-TQ injection(s), respectively. The origin of these MNCs is questionable. Based on their location in proximity to the local vessels of the newly formed muscle fibers, it is suggested

that their origin is either bone marrow-derived mesenchymal stem cells or pericytes [27,28].

Positive expression of Pax-7 in the TQ-treated groups was recorded in the nuclei of MNCs and muscle fibers starting from the 2nd day post-injection. This observation could indicate the activation of satellite cells from the second day after injection and their proliferation at about seven days post-injection. It was found that following activation of satellite cells, Pax7 is activated and then progressively became transcriptionally inactive in most satellite cell progeny as they are committed to differentiation [1,29]. This can explain the decline of the staining intensity by end of the experiment (at 14 days).

## CONCLUSION

The present protocol introduces a novel mechanism for skeletal muscle regeneration, depending on non-myogenic cells, as revealed by early maturation of new fibers (peripheral nuclei), as well as different sequences and localization of Myo-D and Pax-7 expression (MNCs, and FAPs) following muscle degeneration. This mechanism was mainly due to the absence of TNF- $\alpha$  and inflammatory cells, following TQ injection.

## Author's Contributions

SAS: Conceptualization. SAS: Methodology. SAS: Software. SAEA: Investigation. SAEA, MMAH: Data Curation. SAS, MMAH: Writing – Original Draft Preparation. SAEA, MMAH: Writing – Review & Editing. SAEA: Visualization.

## Conflict of Interest

No conflicts of interest declared concerning the publication of this article.

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## Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of: 265/2020

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