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Anti-inflammatory Thymoquinone and Muscle Regeneration in the Hamster Buccal Pouch-Induced Dysplasia

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Abstract

Aim of Study: To follow the sequential expression of Myo-D in the DMBA-painted hamster buccal pouches, following early thymoquinone intraperitoneal injections.

Material and Methods: Seventy five male golden Syrian hamsters were divided into 5 groups: G A: (5 animals) served as negative control, were euthanized before starting the experiment. G B: (10 animals) positive control ;the left buccal pouches were painted with the carcinogen 0.5% DMBA 3/wk/ 6 weeks, five hamsters were euthanized at second day of last painting and 5 hamsters 2 weeks later. Thymoquinone (TQ)-treated group: 60 animals were painted with DMBA, as in group B, then subdivided into three equal subgroups: G C: were given one i.p. injection of TQ (0.1 mg/kg). G D: were given two i.p. injections of TQ (every other day). G E: were given 3 ip injections of TQ (every other day). Blood samples were withdrawn for evaluation of TNF-α level (with light anaesthesia), before euthanization. Five animals, from the TQ-treated groups, were euthanized at 24, 48 hrs, one week and two weeks after the last injection. All buccal pouches were surgically excised, fixed, and processed for H&E stain, Cox-2, and Myo D (IHC) stains.

Results: The results showed significant elevation of serum TNF-α after 6 weeks of DMBA (G B) as compared to G A, and higher significant elevation, 48 hours, following one i.p. TQ injection as compared to all groups. Two and three i.p. TQ injections (Gs D &E) resulted in significant elevation from the second day of last injection, then declined to near that of G B level at end of the study. Grossly, following DMBA painting (G B), the left pouches were significantly reduced to about 2 cm, whereas the TQ injected groups, they showed gradual elongation from the 48 hours interval to end of the experiment, to near the normal control pouches' length (5 cm).

H&E and IHC Results: G B the epithelium showed variable dysplastic grades up to CIS, thick fibrotic lamina propria (l p), and at areas near the distal necrotic side, the newly formed muscle fibers (MFs) were multinucleated. Cox-2 was intense, diffuse along the epithelium, positive cells of l p, and mild diffuse MFs. Myo-D was negative in all mucosal layers. TQ-injected groups showed regressed dysplastic grades to mild dysplasia between one to three TQ injections, up to end of the experiment. Progressive increase of mature MF from 48 hours interval to end of experiment. Cox-2 had reduced expression in all mucosal layers from one to 3 injections, compared to G B. Myo D was negative in all MFs up to one week interval. However it was positive in perivascular mononuclear cells (MCs), at areas of increasing MFs' bulk, in the TQ- treated groups, from 48 hours of last injection. It was also expressed in fat cells and fibroblasts at the distal necrotic side (at an end to end with area of increased perivascular MCs), and fibroblasts of the lamina propria that became thinner where MFs are increasing. Of interest, nuclei of the bulk mature muscle fibers, from the first to the second weeks of injections, were positive, which may be an indication for remodeling (dedifferentiation) of the excessive muscle bulk.

Conclusion: Introducing new therapeutic perspective for skeletal muscle regeneration, by one, two and three ip TQ injections. It shows promising effect to induce rapid regeneration of the striated muscle layer independent of TNF-α/ Cox-2, or the muscle satellite cells, but due to other non-myogenic cell precursors. It also showed antifibrotic effect of the shortened pouches, which resulted from chronic irritation of the chemical carcinogen.

Keywords: Cox-2; Experimental Oral Carcinogenesis (DMBA/ HBP Model); Mesenchymal Stem Cells; Muscle Regeneration; Myo-D; Nonmyogenic Precursor Cells; Pericytes; Thymoquinone; TNF-α

Introduction

Of the most common sites for oral squamous cell carcinoma (OSCC) are the lip and tongue, which are muscular organs. The estimated cancer cases in Egypt from 2013-2050, was recorded by Ibrahim, *et al.* [1], as follows: In 2013: Lip: 262 cases, Tongue: 330 cases, in 2015: Lip: 281 cases, Tongue: 349 cases, in 2020: Lip: 342cases, Tongue: 417 cases, in 2025: Lip: 408 cases, Tongue: 515 cases, while in 2050: Lip: 866 cases, and Tongue: 1180 cases. Surgery, with safety margins, in most cases is the first line of treatment for these cancers, resulting in major health problem considering important functions as speech, chewing, and swallowing [2]. Most of these drawbacks are due to fibrosis in the resected area, especially if it is muscular. The hamster buccal pouch (HBP) / 7, 12 dimethyl benz-a-anthracene (DMBA) carcinogenesis model, is one of the most well characterized cancer-induction models, that opened a wide range of studies regarding oral carcinogenesis [3]. The early few DMBA paintings result in necrosis of the distal part, with reduction of its length from about 5-6 cm to about 2cm [4-6].

Even after carcinogen painting for 6 weeks, the shortened pouches fail to regenerate (elongate), with progression of the malignant process from severe dysplasia to squamous cell carcinoma [7]. The TNF-α- NF-kB pathway is one of the key mediators of the carcinogenesis process that shows mutual dependency, i.e. both affect each other, as reported by Hayden and Ghosh [8]. They stated that cytokines belonging to the TNF family induce rapid transcription of genes regulating inflammation, cell survival, proliferation and differentiation, primarily through activation of the NF-κB pathway. On the other hand, carcinogens are known to activate Nf-κB that in turn activates many genes, as pro-inflammatory genes, oncogenes and anti-apoptotic genes [9,10]. Most classic chemotherapeutics induce cell cycle arrest of malignant and normal cells that result in undesirable side effects. Phytochemicals as thymoquinone (TQ), showed reduced toxicity to normal cells while specifically affect transformed cells, mainly through suppression of the inflammatory reaction related to the malignant process, mediated by Cox-2, TNF-α, and NF-κB [4,11].

When TQ was used as therapeutic or protecting agent, in that model, was found effective in recovery of the pouches' shortening, and even lead to their elongation to near normal length [4,6]. This finding was documented by Hassan, *et al.* [4] mainly through suppression of the inflammatory state and regression of the malignant process with depletion of local inflammatory cells and negative expression of both TNF-α and Nf-kB. Furthermore, when TQ was loaded on gold nano-particles and given i.p. or topically, were also effective in pouch elongation, as well [12,13]. Another study showed that TQ and its nano-formulation in different concentrations, had similar effect [5]. Earlier, the anti-inflammatory effect of TQ was reported by Sethi, *et al.* [14] in a tissue culture experiment. The authors evaluated human myeloid leukemia cells (KBM-5) incubated with 25μ mol/L TQ for different times, then exposed to 0.1 nmol/L TNF for 30 minutes. The results showed that TQ for 6 hours did not activate NF-κB, but it abolished TNF-induced NF-κB activation maximally at 4 hours.

TNF is expressed mostly by monocytes and macrophages, T-cells, natural killer cells, neutrophils [15], and myoblasts [16]. Serum TNF-α was found to be elevated in different chronic diseases as type 1 diabetes mellitus [17], Behçet's disease [18], and some cancers [19,20]. *In vivo* TNF can exist in membrane-bound as well as in soluble forms [21]. It was found to be expressed in damaged muscle fibers [22] and performs two different roles in muscle regeneration, depending on its concentration, as reported by Chen, *et al.* [16]. The authors found that when TNF-α-neutralizing antibody added to differentiation medium blocked p38 activation and suppressed the differentiation markers as myocyte enhancer factor (MEF)-2C, myogenin, p21, and myosin heavy chain, in C2C12 myoblasts. Conversely, recombinant TNF-α added to the differentiation medium stimulated myogenesis at 0.05 ng/ml while inhibited it at 0.5 and 5 ng/ml [16]. Another study showed that at high levels it suppresses myogenesis through proteolysis of Myo-D, or the NF-κB-TNF pathway [23]. The role of TNF in cancer was reported by Surh, *et al.* [24] and Xu, *et al.* [25]. They showed that TNF- promotes cancer growth, invasion and metastasis through induction of multiple inflammatory mediators, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).

Cox-2, another important pro-inflammatory cytokine, was shown to have a role in the myogenesis process [26]. COX-2 is low or nondetectable in most tissues, but can be readily induced in response to cell activation by cytokines, growth factors and tumor promoters [27]. On the other hand, NF-kB is a positive regulator of COX-2 expression in murine macrophages [28] and human colon cancer cell lines [29]. As well as in prostate cancer [30]. The relation between TNF-α and Cox-2 in muscle regeneration was documented by Lin, *et al.* [31]. The authors aimed to determine the mechanism of TNF-alpha-enhanced COX-2 expression associated with prostaglandin E2 (PGE2) synthesis in human tracheal smooth muscle cells (HTSMCs). They showed that TNF- α had markedly increased COX-2 expression and PGE2 synthesis in a time- and concentration-dependent manner, at least in part, mediated through NF-kB signaling pathways [31,32]. Moreover, the evidence for Cox-2 role in striated muscle regeneration was determined by Bondesen, *et al.* [26]. Their data suggest that COX-2-dependent PG synthesis is required during early stages of muscle regeneration. Prostaglandins (PGs) have been implicated in various stages of myogenesis, as myoblast proliferation, differentiation [33], and fusion [34,35].

These effects were found in vitro and in myofibers' growth during development [36,37]. Furthermore, through the use of selective COX-2 inhibitors, COX-2 pathway, was found crucial for normal muscle regeneration and in wound repair [38,39]. Mononucleated cells were isolated from regenerating tibialis anterior (TA) muscles, 3 days after injury from mice treated with vehicle or SC-236 (selective Cox-2 inhibitor) , plated, and immunostained for Myo-D and desmin. This time point corresponds to the peak of Myo-D expression in normal regenerating muscle [40]. Of interest, muscle regeneration was found to be impaired when inflammatory cells are depleted [41] and stimulated when they are increased [33,34], demonstrating the importance of inflammation after

muscle damage. Following satellite cells' proliferation and differentiation they fuse with formation of multinuclear myofibers, a process controlled by several basic helix loop expression including Myf5, Myf6, and Myo-D [40]. The sequence of mature muscle regeneration was tested, where the time of satellite cell activation was variable in different models [41,42]. In a rat model, activation of satellite cells starts 2-4 days post-injury (cardiotoxin- CTX- injections), followed by muscle fiber regeneration in 4-10 days. Maturation of regenerated muscles takes place in 10-15 days, while functional performance of injured muscles with innervation in 15-20 days [42].

In another study by Chargé and Rudnicki (2004), when 25 µl of 10 µM CTX was injected in adult mouse tibialis anterior muscle, mononuclear cells infiltrate in 1 day of injection up to 4 days. Myogenic differentiation and new myotubes formation from 5-6 days. By 10 days the overall architecture of muscles is restored, however the fibers were smaller in size with central myonuclei. Last stage, morphologically and histochemically, the normal mature muscles are seen at 3-4 weeks [43]. Other cells leading to myogenesis include the bone marrow (BM)-derived stem cells. They can differentiate into muscle cells in vitro, and contribute to muscle regeneration *in vivo* [44,45]. These cells could be the pericytes that are activated during local injury, after being released from their vascular wall [46]. Furthermore, the fibro/adipogenic progenitors (FAPs) are capable of giving rise to fibroblasts or adipocytes, and play a supportive role in the myogenic differentiation [47]. Lee, *et al.* [48], reported that fibroblasts can be differentiated by (-)-Epicatechin to the myogenic lineage, in a dose- dependent manner. This differentiation was evaluated through expression of Myo-D by Western blotting.

Aim of the Study

This study aims to look for the effect of TQ on the pro-inflammatory cytokines TNF-α and Cox-2 regarding induction of myogenesis (through expression of Myo-D marker), in the HCP/DMBA-induced dysplasia model.

Material and Methods

The experiment was carried out at the Animal House, Faculty of Dentistry, Suez Canal University. Chemicals: The carcinogen: 0.5% solution of 7, 12 dimethylbenza-(a)-anthracene (DMBA), dissolved in heavy mineral oil. Thymoquinone (0.1 mg/kg body weight) solution, dissolved in propylene glycol. (7) All from Sigma Chemical Co., St Louis, Mo, USA. ELISA kit for serum TNF-α: PicoKine™ ELISA, Catalog number: MBS175904, MyBioSource.com. IHC kit for Myo D: clone 5.8A & MYD712, Item #RA0233-C.5. Scy Tek laboratories, Utah 84323, USA. The used dilution was 1:50. The steps followed the manufacturer's instructions. IHC kit for Cox-2: Cox-2 antibody (rabbit polyclonal antibody) Cat #RB-9072-R7 (Thermo Fisher Scientific, Anatomical Pathology, UK).

Animals and grouping: Seventy-five male golden Syrian hamsters (Mesocrietus auratus) (90-110 gms) were enrolled in the work. They were obtained from "The Holding Company for Biological Products and Vaccines (VACSERA)", Helwan, Egypt. The hamster colony was health monitored according to recommendations by Federation of European Laboratory Animal Science Associations (FELASA) [63]. They were kept 5/cage, in well ventilated room and supplied recommended food and water ad libitum. The hamsters were secured in environment that did not allow accidental escape. The surrounding temperature ranged from 16-22°C. Five animals served as the negative control group (A), were euthanized at beginning of the experiment, without any treatment. The rest of animals were painted, on the left buccal pouch only with the carcinogen, 3/wk./6wks [4]. Five animals were euthanized on the second day of last painting, and 5 at end of the study, serving as the positive control group (B). Twenty animals (group C) were injected intraperitoneally (i.p) one time with TQ, and euthanized after 24, 48 hrs, one and 2 weeks. Twenty animals (group D) were injected 2 times (every other day) with TQ, and were euthanized as in group C. Twenty animals (group E) were injected 3 times (every other day) with TQ, and were euthanized, as in groups C and D.

Euthanization by a heavy dose inhalation of ether (a piece of cotton soaked with ether in a tightly closed container). All pouches were surgically excised, fixed and processed for H&E, and IHC (Myo-D and Cox-2) stains. For TNF-α evaluation: Blood samples (2 ml) were withdrawn from the orbital sinus through the mesial angle of the eye, from all hamsters before euthanization (anaesthetized by light dose of ether inhalation). TNF-α was evaluated by ELISA technique, and were statistically analyzed. H&E was performed to follow muscle regeneration following TQ injections. IHC expression of both Cox-2 and Myo-D was represented as either expressed or not in all mucosal layers of the DMBA-painted pouches following TQ, to be compared with both negative (untreated animals) and DMBA-only painted pouches.

Results

Results of serum TNF-α:

Group	Number of animals	Mean and SD	Significance
Negative control (A)	5	1.67 ± 0.36	
Positive control (B)	5	36.28± 3.31	0.022*

*p value is significant at ≤0.05

Table 1: Comparison of TNF-a level between control groups using t-test (for Equality of Means)

Euthanisation time	TQ treatments	Number of animals	Mean and SD	Significance
24 Hours	one TQ	5	128.22 ±6.12	0.000
	two TO	5	60.98 ±4.37	
	three TO	5	31.51 ±2.83	
2 Days	one TQ	5	120.90 ±2.21	0.000
	two TO	5	51.61 ±2.92	
	three TO	5	24.31 ±2.25	
One week	one TQ	5	105.20 ±3.40	0.000
	two TO	5	49.23 ±2.25	
	three TO	5	20.20 ±1.81	
Two weeks	one TO	5	100.04 ±6.07	0.000
	two TO	5	38.53 ±2.28	
	three TO	5	19.43 ±1.81	

* The mean difference is significant at ≤0,05

Table 2: Comparison of TNF-a level between the three treated groups (Descriptives, using ANOVA test)

Dependent Variable	(1) group	(J) group Treatment	Mean Difference (1-J)	Significance
24 Hours	-ve control	one TQ	-126.55(*)	0.000
		two TQ	-59.31 (*)	0.000
		three TQ	-29.83 (*)	0.000
		+ve control	-34.61 (*)	0.000
	+ve control	one TQ	-91.94 (*)	0.000
		two TQ	-24.70 (*)	0.000
		three TQ	4.77	0.066
		-ve control	34.61 (*)	0.000
2 Days	-ve control	one TQ	-119.23 (*)	0.000
		two TQ	-49.94 (*)	0.000
		three TQ	-22.63 (*)	0.000
		+ve control	-34.61 (4)	0.000
	+ve control	one TQ	-84.62 (*)	0.000
		two TQ	-15.33 (*)	0.000
		three TQ	11.97 (*)	0.000
		-ve control	34.61 (*)	0.000
One week	-ve control	one TQ	-103.53 (*)	0.000
		two TQ	-47.55 (*)	0.000
		three TQ	-18.53 (*)	0.000
		+ve control	-34.61 (*)	0.000
	+ve control	one TQ	-68.92 (*)	0.000
		two TQ	-12.95 (*)	0.000
		three TQ	16.08 (*)	0.000
		-ve control	34.61 (*)	0.000
Two weeks	-ve control	one TQ	-98.37 (*)	0.000
		two TQ	-36.85 (*)	0.000
		three TQ	-17.75 (*)	0.000
		+ve control	-34.61 (*)	0.000
	+ve control	one TQ	-63.76 (*)	0.000
		two TQ	-2.25	0.302
		three TQ	16.85 (*)	0.000
		-ve control	34.61 (*)	0.000

* The mean difference is significant at the ≤0.05 level. TQ; thymoquinone

(-ve) control: negative control group (untreated hamsters)

(+ve) control: positive control group (6 weeks of DMBA painting)

Table 3: Comparison of serum TNF-a level between all groups, at different time intervals, in relation to the negative and positive control groups (Multiple Comparisons using LSD)

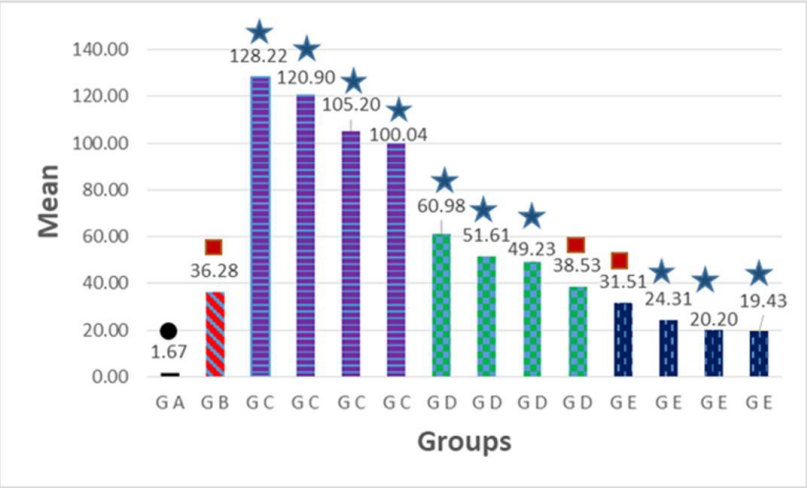

Chart: Showing the mean of serum TNF-α in all groups. Same shapes are not statistically significant. The star shape indicates statistical significance compared to groups A and B. Statistical significance was set at p value ≤ 0.05

Results of serum TNF-α were statistically analyzed using student T-test, and ANOVA test. The mean difference was set to be significant at $p \leq 0.05$ level, and highly significant at $p \leq 0.001$. In all experimental groups (B-E), and at all time points, serum TNF-α showed statistically significant elevation as compared to the untreated control group (group A).Highly elevated level of TNF-α was reported in group C (one i.p TQ injection) after 2 days of the injection, up to end of the experiment (2 weeks). Following 2 TQ injections (group D), TNF-α level was increased in a statistical significant level from 2 days to one week of the second injection, as compared to groups A and B. The level was decreased at week 2 to near group (B), however, was not statistically significant. On the other hand, group E (3 TQ injections) showed statistically significant elevation-compared to group A-at all time points, except at 24 hours interval, the level was not significant than group B. In all groups TNF-α level was steadily decreasing up to end of the experiment, but not to the level of group (A).

Clinical Observations: (Gross Results of Myo-D and Cox-2)

Group A: (negative control group): the hamsters were healthy, and both pouches appeared normal, measuring about 5-6 cm in length. Same pouches' length was recorded for all right (un-painted) pouches of other groups (Figure 1a). Group B (positive control): the hamsters were skinny, and debilitated. The DMBA-painted left pouches were reduced in length to about 2 cm due to necrosis of the distal end. After two weeks (end of the experiment), these pouches did not increase in length and showed exophytic masses and multiple ulcerations (Figure 1b). Following TQ injection(s) (groups C-E), gradual elongation of the DMBA-painted pouches was significant from 48 hours' interval to end of the experiment. The pouches were comparable to the untreated pouches, in gross appearance and length (about 5 cm) (Figure1c & 1d).

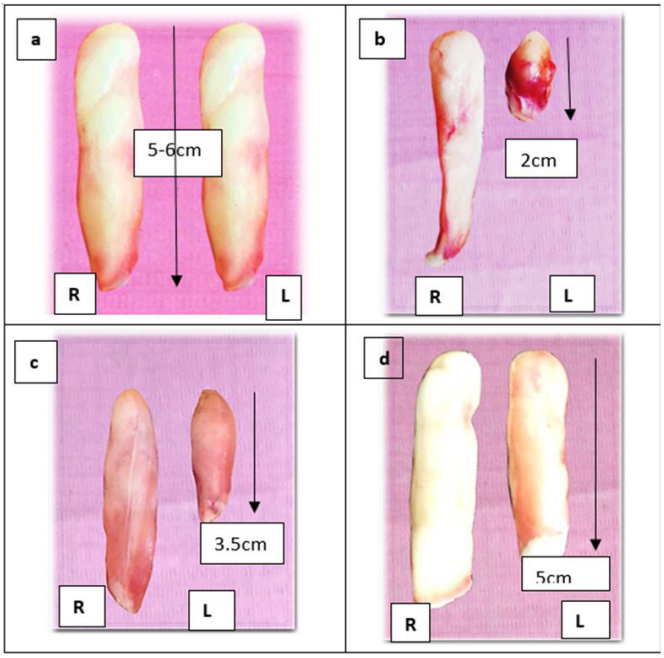

Figure 1: (a)-Normal cheek pouches (left and right) about 5-6 cm in length. **(b)**: DMBA-only painted group, the left pouch showed significant length reduction (2cm). Note: apparent redness and exophytic lesions. **(c)**: Pouches from TQ-treated groups, (2 days after the second and third TQ injections) the left pouch showed marked elongation (3.5cm). **(d)** Pouches after 2 weeks of 2 and 3 TQ injections. The left pouch length is about 5 cm, with almost normal appearance as the untreated right pouch

Histopathological and IHC results: Left and right pouches of the negative control group (G A), and right pouches of other groups showed normal appearing hamster buccal pouch (HBP) mucosa (Figure 2a). The IHC results revealed negative Myo-D expression of the mature muscle fibers (MFs) (Figure 2b). Cox-2 expression was diffuse in all nucleated epithelial cells, endothelial cells and MFs (Figure 2c).

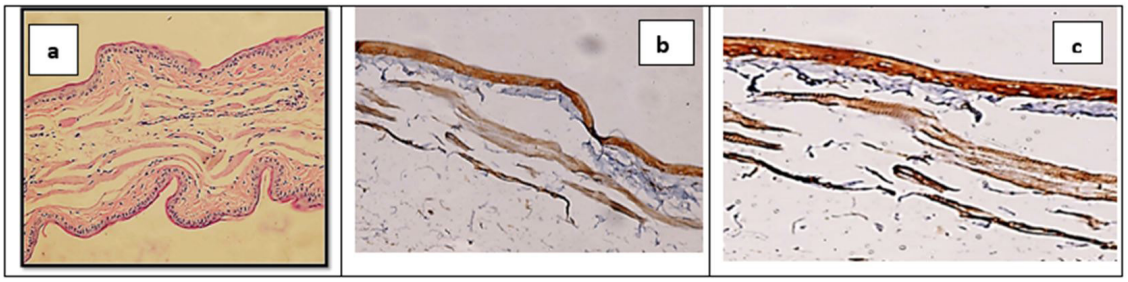

Figure 2: Histologic sections from untreated pouches (negative control) **(a)**: The lining mucosa is covered by thin keratinized stratified squamous epithelium with no rete ridges, thin collagenous lamina propria, free of inflammatory infiltrate and thin muscle fibers (H&Ex10). **(b)**: Myo-D stained section showing negative reaction in all layers [Myo D x10]. **(c)**: Cox-2 stained section from G A, showing moderate diffuse reaction of the epithelial layer, negative lamina propria and mild diffuse reaction of the muscle fibers (Cox-2 x20)

In G B, the distal end of the pouches were severely necrotic, bordered by granulation tissue and fibro-adiposic tissue (Figure 3a). The rest of the pouches' mucosa showed different degrees of epithelial dysplasia up to CIS (Figure 3b). At areas of new muscle formation, the MFs were multinucleated and showed negative Myo D stain (Figure 3c). After 2 weeks, these pouches showed exophytic and endophytic growths with severe dysplasia and carcinoma in situ. The lamina propria was more fibrotic and increased in thickness. Healthy muscle layer was lost near the necrotic end, and under the fibrotic lamina propria. Cox-2 expression was diffuse in all nucleated cells of surface epithelium, endothelial cells, as well as MFs (Figure 3d, 3e & 3f).

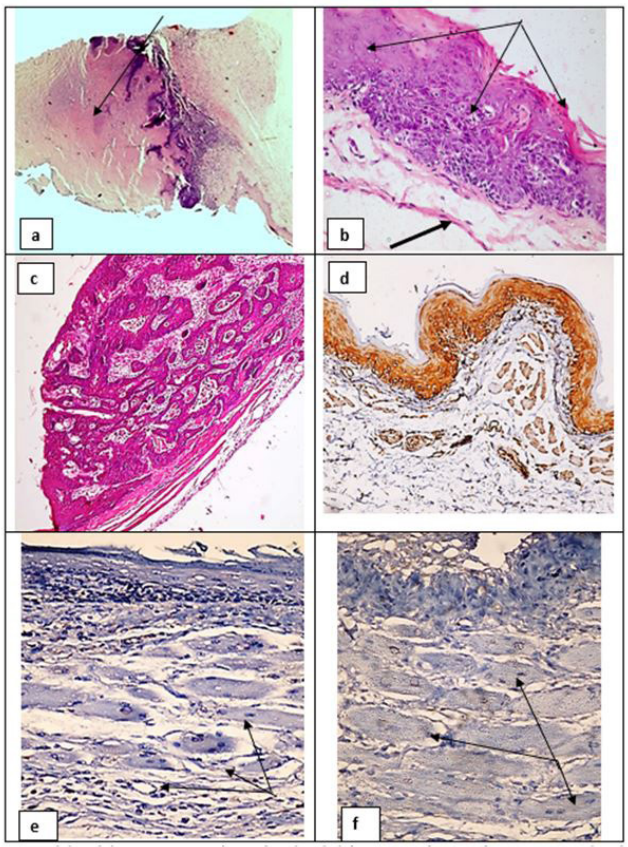

Figure 3: From **(a)** to **(e)** DMBA-painted pouches **(G B).** **(a)**: Section showing large necrotic distal end (arrow) (H&E x4). **(b)**: the rest of the pouches' mucosa showed hyperplastic, hyperkeratinised epithelium and different grades of epithelial dysplasia (arrows). Note there is almost no muscle fibers in these areas (thick arrow) (H&E x20). **(c)**: After 2 weeks, exophytic and endophytic growths with severe dysplasia and carcinoma in situ. The muscle layer was lost under a fibrotic lamina propria (H&E x10). **(d)**: Showing the epithelium is positive for C0x-2, cells of the lamina propria, the endothelial cell lining, and the muscle fibers showed mild diffuse cytoplasmic reaction (Cox-2 IHC x20). **(e)**: Myo-D stained section, showing negative expression in the multinucleated MFs (Myo-D IHC x40). **(f)**: Section from G C: the same presentation as in Fig **(e)**, second day of one TQ injection, near the necrotic end

After 24 hours of the first TQ injection (G C), there were no much differences than in G B. After two days, the distal necrosis began to regress in size, and the nearby epithelium was hyperplastic, hyperkeratinised with focal areas of mild/ moderate dysplasia. From the 48

hours to the first week, post TQ injection(s), focal accumulation and extrusion of inflammatory cells, from the surface epithelium, was a constant observation (Figure 4).

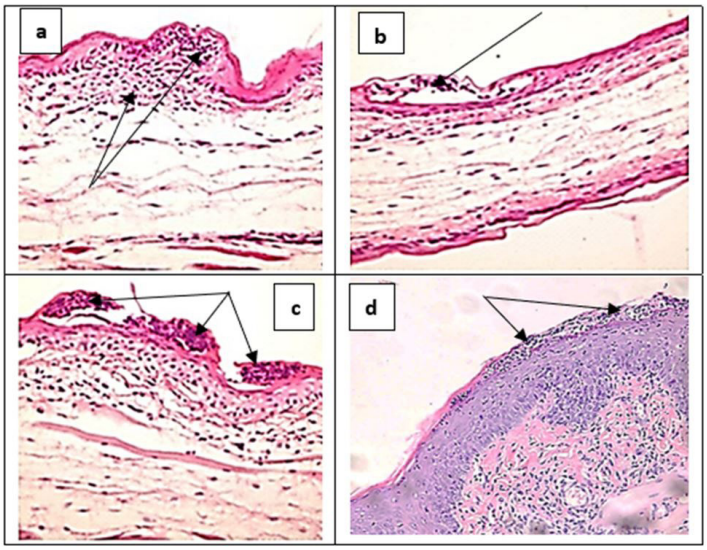

Figure 4: Sections from pouches after two days of TQ-injection (s), showing focal subepithelial accumulation of inflammatory cells, then their expulsion/ extrusion from the surface epithelium (arrows) (H&E x20)

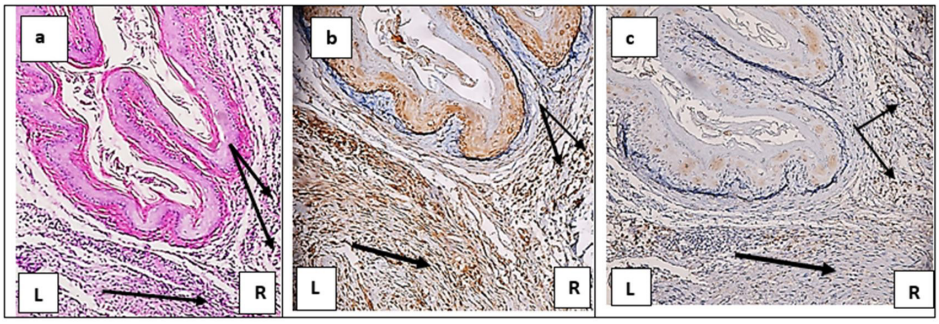

Figure 5: Sections from pouches one day after the second and third TQ injections, showing the point of transition of muscle fibers formation. In the (R) side: muscle formation is through perivascular MNs (arrows), while in the (L) side: muscle formation is through transition of fibroblasts to the myogenic lineage (arrow) (H&E x 10). **(b)**: Section from the same groups, showing Myo-D positive fibroblasts (L side), at the point where perivascular mononuclear cells (R side) appears (Myo-D x 10). **(c)**: Same field showing positive Cox-2 cells of both cell types as in (b) (Cox-2 x10)

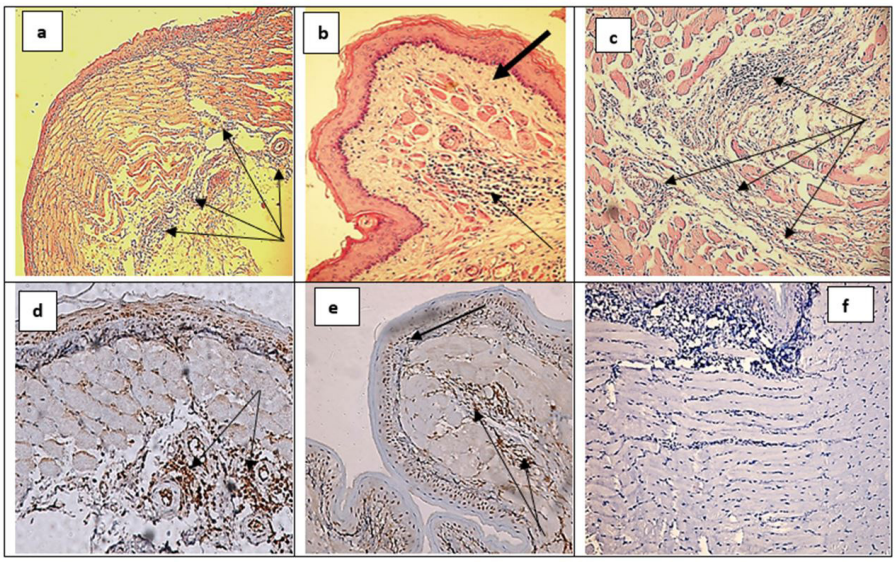

Figure 6: (a-c): Sections from pouches after TQ- injections, showing increased perivascular mononuclear cells (MNs). **(a)**: At areas of new muscle formation (arrows) (H&E x 10). (b): Heavy infiltration of perivascular MNs (arrow) with increasing muscle fibers (arrow), note: thicker fibrotic lamina propria (H&E x20). (c): Increased MNs with increasing MFs (H&E x10). (d): Section from a pouch one week after 2 TQ injections, showing mature, bulk, compact muscle fibers, note: MNs are increased where MFs are forming (Myo-D IHC x20]. (e): Myo-D positive MNs (arrows), and fibroblasts of the lamina propria (arrow) (Myo-D IHC x20). (f): Section from TQ-injected groups after one week showing bulky muscle layer, the MFs are mature and were Myo-D negative (Myo-D IHC x10)

These fibroblasts meet the area where perivascular mononuclear cells (MNs) were dominant at areas of new muscle fibers' formation. In all TQ-injected hamsters, increasing number of perivascular mononuclear cells (MNs) was prominent observation from day 2 to end of the first week (Figure 6a, 6b & 6c). Progressive increase of MFs from loose fibers to thick compact bulky layer that all were mature (peripherally located nuclei) and were Myo-D negative (Figure 6d, 6e & 6f).

With elongation of the DMBA-painted pouches, and following TQ-injections, the positive Myo-D cells were the perivascular mononuclear cells (MNs), spindle shape cells (fibroblasts) and fat cells at the necrotic side, as well as fibroblasts of the lamina propria (Figure 7).

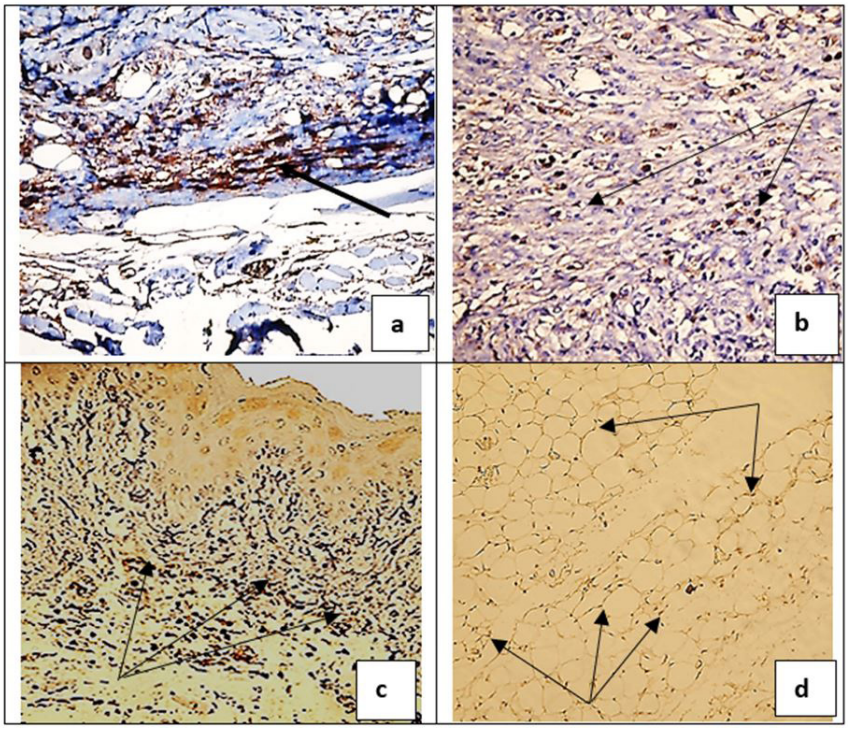

Figure 7: (a, b): Sections from pouches one day after the second and third TQ injections, from the distal necrotic side, showing Myo-D positive cells at the fibro-adipose area (arrows) (Myo-D x10 and x20). (c): Myo-D positive cells in the lamina propria (arrows) (Myo-D IHC x40). (d): Section near the necrotic side characterized by increased fat tissue, Myo-D positive nuclei of fat cells (arrows) (Myo-D IHC x10)

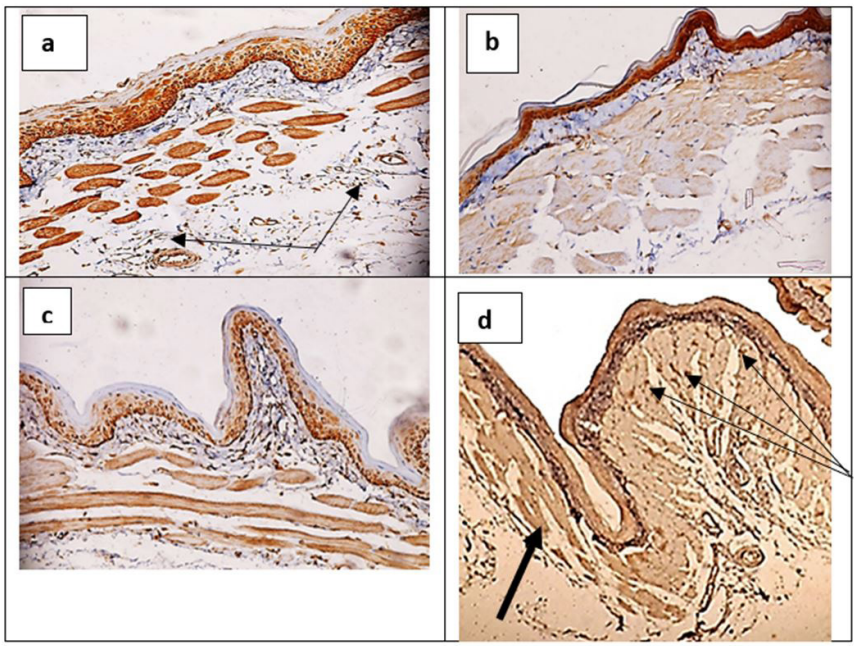

Figure 8: Sections from pouches after TQ injection(s), (a): One day after 2 TQ injections showing positive Cox-2 stain of all nucleated hyperplastic epithelium, reducing thickness of the lamina propria and increasing muscle fibers where Cox-2 reaction is diffuse (Cox-2 IHC x20). (b): Two weeks after two TQ injections showing diffuse Cox-2 reaction of all normal appearing epithelium, negative lamina propria, and light diffuse stain of reduced muscle bulk (Cox-2 x10). (c): Two weeks after 3 TQ injections, showing moderate stain of the lower epithelial layers, positive cells of the lamina propria, mild/ moderate diffuse stain of more reduced bulk of loose muscle fibers (Cox-2 x20). (d): Myo-D stained section, 2 weeks after 2 TQ injections, showing positive nuclei of MFs (arrows) that are reduced in thickness (thick arrow)(Myo-D IHC x10)

Groups D and E, Cox-2 stain results did not show marked differences (Figure 8). It shows positive diffuse Cox-2 expression of all nucleated hyperplastic epithelium, reducing thickness of the lamina propria and increasing muscle fibers. Perivascular MNs were seen below the increasing MFs (Figure 8a). Two weeks after two TQ injections showing Cox-2 positive stain of normal appearing epithelium, negative lamina propria, and light diffuse stain of reduced muscle bulk, there were no MNs in these areas (Figure 8b). Two weeks after 3 TQ injections, moderate stain of the lower epithelial layers, positive cells of the lamina propria, mild/ moderate diffuse stain of more reduced bulk of muscle fibers, with no MNs as well (Figure 8c). However, from the first to the second weeks, the MFs showed nuclear positive expression with progressive decrease in their bulky thickness (Figure 8d).

Discussion

The present work aimed to look at the early events of muscle regeneration under the influence of thymoquinone (TQ), following shortening of the hamster buccal pouches due to DMBA painting for 6 weeks. TQ, was used for its strong anti-inflammatory effect [49]. The time of euthanization was determined according to a classic sequence of differentiation of muscle satellite cells (Myo-D expression), starting from 2-4 days post injury, and their maturation by 10-15 days [40].

By 48 hours, after one and two TQ injections there were significant elevation of serum TNF-α, compared to groups A and B. It was reduced to near G B level by 2 weeks, indicating a time- and dose-dependent effect of TQ on TNF-α. Furthermore, confirming the time- and dose-dependent effect of TQ, is that after 2 days of the third injection (G E), it was less than in the DMBA-only group, however in a non-statistically significant level. As in groups C and D, it continues to decrease steadily till end of the study, but not to the level of negative control group. This finding confirms the strong anti-inflammatory effect of TQ, in another mechanism that was not reported before. Increase of serum TNF- α following early TQ injections was reported in a comparable research thesis by Algharyni [50]. The origin and fate of the serum TNF-α needs to be explored. One explanation is that DMBA for six weeks, in the present work, results in toxicity to many tissues /organs in the body [51,52]. In that model, severe necrosis and inflammation of the painted pouches indicate its toxicity and induction of severe inflammatory effect. TQ appears to result in expulsion of the formed TNF- α in the affected cells/ tissues to the circulation. It remains to follow-up the serum TNF-α level for longer time, as well as its fate, and whether it reaches the normal level or completely disappear.

The pouches of untreated control group (G A), and right pouches of other groups showed normal mucosa with thin loosely arranged MFs. As they are mature, they were negative for Myo-D. Whereas Cox-2 was expressed in all epithelial layers, and was faint in the MFs. Its expression in the untreated pouches' epithelium could be the result of continuous daily physiologic insult of chewing resembling physiologic exercise, which promotes inflammatory reactions and cytokines' production [38]. Grossly, the left DMBA-painted pouches of G B were significantly reduced in length (about 2cm), due to necrosis of its distal end, following the early carcinogen paintings. This observation was reported by other studies [5,7]. Histologically, the epithelium showed different dysplastic grades up to CIS. Then progressed to small nodules with superficial invasion, by end of the experiment. Progression of the carcinogenesis process, in that model was confirmed in a study by El-Dakhakhny, *et al.* [7]. At the area of distal necrosis, the prominent structures around the necrotic area, were granulation tissue and fibroadiposic tissue. The new formed MFs were multinucleated and negative for Myo-D, where Myo-D is reported to be expressed in satellite cells before differentiation to myoblasts, and not expressed in proliferating / fusion stage (multinucleated fibers), or in mature MFs (peripherally located nucleus) [40-42].

The negative expression of Myo-D in all MFs, in G B, could be due to the time of euthanisation (second day of last DMBA painting), where Myo-D expression may appear during the carcinogen painting, or earlier than the time of euthanisation, or due to increased Cox-2 and/ or TNF- α in these pouches resulted in destabilization of Myo-D in a NF-kB-dependent manner, as reported by Langen, *et al.* [53]. This classic pathway for muscle repair, depends in part, on the TNF-NF-κB pathway [22], as well as TNF-α concentration [16]. Furthermore, in G B, muscle formation that follows the classic pathway, depends on the muscle satellite cells (multinucleated cells' formation), and the local inflammatory environment [41,42]. The distal necrotic end of the DMBA-painted pouches surrounded by granulation tissue, apparent fat and fibrous tissue that could be the cause of its fibrosis, i.e. inhibition of elongation of these pouches by new muscle fibers. Of interest, no perivascular mononuclear cells (MCs) were seen in any section of this group, compared to the TQ-injected groups. With TQ injection(s), there were steady elongation of the left pouches from 48 hours interval to end of the experiment (2 weeks) up to near normal length (5cm). They were also normal appearing without redness, ulcerations, or nodules.

In groups D and E, along the two weeks of the experiment, progressive elongation of the DMBA-painted normal appearing pouches was noted grossly. Histologically, the pouches showed progressive increase of muscle layer bulk, under normal appearing epithelium and non-inflamed thinning lamina propria. The depletion of inflammatory cells, in these pouches, of the present study, was reported in a comparable study, when focal aggregation and extrusion of inflammatory cells through the surface epithelium was a constant finding. In that study, TQ was injected at the same time of DMBA painting, and animals were euthanized every 2 weeks. Furthermore, IHC expression of TNF-α and NF-κB was negative in the expelling inflammatory cells, and surface epithelium of the TQ-treated hamsters [4]. While in the present work, focal aggregation and extrusion of inflammatory cells was a constant finding after 2 days of TQ injection(s) up to the first week. The mechanism/ molecular events of the local aggregation and extrusion of the inflammatory cells, needs further investigation.

In the present study, in G C and the group treated only with the carcinogen (G B), it appears that new muscle repair, takes place through muscle satellite cells, where multinucleated cells are seen only in these groups at the nearest area of necrosis, and at 24 and 48 hours in G C. These cells in both groups were Myo-D negative. Starting from the 48 hours period, (in all TQ-injected

hamsters), perivascular MNs predominate the area of increasing muscle layer bulk. These MNs were Myo-D positive, while the new mature fibers (peripherally located nucleus) were Myo-D negative. None of the MFs at area of perivascular MNs showed a stage of multinucleated cells' formation. This could endorse the origin of these new muscle fibers to be the perivascular mesenchymal stem cells. This finding would indicate that each MN directly forms one MF, as all newly formed MFs were mononuclear with peripherally-located nucleus. Cox-2 expression in the loose MFs in normal pouches was diffuse and moderate / intense. That could be a normal response to the chewing activity of these pouches.

Whereas, in G B, Cox-2 was mild / moderately expressed, mostly due to the carcinogen-degenerative effect. According to Lin, *et al.* [31]. TNF- α had markedly increased COX-2 expression and PGE2 synthesis in a time- and concentration-dependent manner, at least mediated through NF-kB signaling pathways. In all TQ-injected groups, of the present work, Cox-2 expression was diffuse and moderate in the loosely arranged MFs, at the early follow-up intervals. This could be a reflection of serum TNF-α level at these points where it was not fully expelled from the body cells/ tissues. Whereas after one week, serum TNF-α level was significantly decreased as most of it was expelled from the body cells/ tissues, resulting in the mild/ moderate Cox-2 expression. The loosely arranged MFs, at these early time points reflects their developing stage, where Cox-2 is required [24,26], while when they are compact, with complete depletion of TNF-α and NF-κB, Cox-2 is downregulated.

In groups D and E, depletion of inflammatory cells as well as TNF-α and Cox-2, muscle regeneration with a very small TQ concentration and dose(s) appears to be through three non-myogenic lineages that were Myo-D positive. First, is stimulation of FAP cells (near the area of necrosis) to differentiate to the myogenic lineage [47]. As the nuclei of fat cells and interstitial cells, were positive for Myo-D, as well as the fibroblasts (spindle-shape cells) originating from the necrotic side towards the increasing muscle bulk. Hassan, *et al.* [4] reported the same finding regarding transition of fibrotic to myogenic lineage by Masson's trichrome stain from the necrotic side. Second, is stimulation of fibroblasts, in the thick fibrotic lamina propria, to be differentiated to the myogenic lineage, as they were positive for Myo-D. This would explain reduction of the thicker, dense lamina propria following DMBA treatment, to thinner normal-appearing thickness (TQ-injected groups), i.e. replaced by increasing muscle bulk.

It was reported that Myo-D was expressed in fibroblasts during its differentiation to the myogenic lineage [48,54,55]. Third, is perivascular mononuclear cells (MNs) that were Myo-D positive from the 48 hours follow-up period, in groups given TQ. These MNs are expected to be either bone marrow-derived-mesenchymal stem cells (BMMSCs) [56-59] or pericytes [60-61] due to their persistent relation to the local vessels in close proximity to the newly forming muscle fibers. In the review by Cappellari and Cossu [61], they indicated that pericytes, in inflammatory conditions and signaling mediators would be prevented from their regenerative effect to a fibrotic effect.This observation would explain fibrosis and aborted elongation of the distal necrotic end, while TQ injections had stimulated pericytes to regain their regenerative effect towards muscle formation, i.e. elongation of the DMBA-painted pouches in TQ-injected groups.

At the necrotic end, fat cells appear to have a remarkable effect on new muscle formation, according to de la Garza-Rodea, *et al.* [62]. They showed that adipose tissue drived mesenchymal stem cells (AT-MSCs) appear to be the best choice in view of their efficient contribution to myoregeneration, than that of bone marrow (BM)- or synovial (SM)-MSCs. The reason of undetected multinucleated MFs in the TQ-injected groups, can be attributed to the observation reported by Natsu et al (2004). They stated that BMMSCs in the rat model, contributed to the regeneration of skeletal muscle by mechanisms other than fusion to myofibers (i.e. multinucleation of MFs) after differentiation [44]. In that hamster carcinogenesis model, and with TQ injection, the Myo-D positive nonmyogenic cells that results in new muscle formation, were expressed within the reported cascade of classic muscle regeneration / repair in other rodent species (rats and mice) [63].

Myo-D expression in these cells appeared at 48 hours. In a rat model activation of satellite cells starts 2-4 days post-injury (cardiotoxin-CTX- injections), followed by muscle fiber regeneration in 4-10 days. Maturation of regeneration takes place in 10-15 days. In the present study the increased muscle bulk took place from 48 hr to 1-2 weeks. Whereas, functional performance of injured muscles with innervation in 15-20 days [40]. The author indicated that from day 10-15 (post cardiotoxin injury), remodeling of muscle fibers takes place. (40) In the present work, after 1 and 2 weeks of TQ injections, the nuclei of mature, compact fibers were Myo-D positive that could be the stage of remodeling through dedifferentiation of these fibers. Where at the second week the bulky muscle layer was decreasing. In that carcinogenesis /TQ model, El-Sherbiny, *et al.* [6] reported that the elongated hamster pouches, showed near normal thickness of the muscle layer, but were compact than the control untreated pouches, after 6 weeks of TQ injections.

In general, the slight differences in timing of new muscle formation between the present study and others [40,42,43] could be due to many factors: First: the animal model, especially the nature of the hamster pouches' muscles, and type of muscle injury. Second: depletion of the local environment (non inflamed and carcinogenesis background). Third: is the time of euthanisation and marker expression. Fourth: TQ, in the defined doses and concentration, could be the stimulatory agent for nonmyogenic stem cells to form new MFs. The classic pathway of muscle regeneration by satellite cells, is known to be impaired when inflammatory cells are depleted, and stimulated when they are increased [41,43], as in group B, demonstrating the importance of inflammation after muscle damage in normal circumstances. The carcinogenesis process, stimulated by DMBA for 6 weeks, allows persistent inflammatory environment with expected increase of local TNF-α and Cox-2. Depletion of this inflammatory environment by TQ aborts the classic pathway of muscle regeneration, while stimulating other non-myogenic stem cells to repair/ regenerate new muscle layer, as well as reverting fibrosis to new muscle formation and so elongation of the shortened pouches.

Furthermore, the local concentration of TNF-α supports both pathways [16]. In group B, increased inflammatory cells, Cox-2 and TNF-α supports satellite cells' activation, differentiation and maturation. Whereas, in TQ-injected groups followed by local depletion of inflammatory cells and TNF-α / Cox-2 mediators, might be the reason of stimulating non-myogenic cells to form new muscle fibers and revert the fibrotic end to MFs'formation. It remains to evaluate the functionality of these new MFs, by the nonmyogenic cells, through formation of neuromuscular junction and its mediator(s). This is the point of a current research in our lab.

Conclusion

Muscle regeneration, in the present model, appears to be through non myogenic stem cells, after one, two or three ip TQ injections. It appears to be through its strong anti-inflammatory effect, independent on TNF-α or Cox-2.

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