



THERAPEUTIC EFFICACY OF CHRYSIN ON ACRYLAMIDE INDUCED STRIATED MUSCLES DYSTROPHY OF RAT'S TONGUE

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ABSTRACT

Objective: The aim of the present study was directed to evaluate the therapeutic efficacy of chrysin (CH) on acrylamide (ACR) induced striated muscles dystrophy of rat's tongue. **Subjects and methods:** Forty adult male albino rats (200–250 g) were divided into four equal group(s) (G(s)), used for two successive periods (20 days: A, 40 days: B). G1 were fed with standard diet, without receiving any kind of treatment and considered as negative control group. G2 were received 15 mg/kg body weight of ACR orally once daily for 20 days (G2A) and for 40 days (G2B) and considered as positive control group. G3 were treated as G2, in addition; they were received 50 mg/kg intraperitoneal of CH two weeks before, as well as during application of ACR on alternative days and considered as a chemopreventive group. G4 were treated as G2, in addition; they were received 50 mg/kg intraperitoneal of CH, at first, third and seventh days after application of ACR and considered as a treatment group. **Results:** G1 has no observable abnormalities. G2A and G2B revealed an increase in collagen fibers with degenerative changes. G3A and G3B revealed decreased collagen fibers with muscle regeneration. G4A revealed localized red swelling of tongue mucosa, diffuse in G4B. Both had limited areas of collagen fibers. **Conclusion:** Striated muscle damage in rat's tongue by ACR becomes worse with prolonged duration is realized. CH has a significant protective efficacy of when compared with that of treatment on ACR induced striated muscle injury of rat's tongue.

KEYWORDS: Muscle degeneration, chrysin, acrylamide.

INTRODUCTION

Adult muscle consists of multinucleated myofibers that can undergo changes in size (atrophy/hypertrophy) and type (slow-contracting, fatigue-resistant type/fast-contracting, fatigable type). Due to its daily mechanical work, muscle undergoes small tears and minor lesions. This leads to a slow turnover of constituent fibers, replacing no more than 1–2 % of myonuclei per week ⁽¹⁾. Oxidative

stress has been defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses, which may lead to tissue injury⁽²⁾. Oxidative stress has been recognized to play a major role in many chemical-induced cellular injuries, as well as a major contributor of muscle atrophy ⁽³⁾.

Acrylamide (ACR), is an unsaturated carbon-yl compound, with a significantly high chemical

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activity. It is extensively used in many fields from industrial manufacturing to laboratory personnel work, so it is often absorbed during occupational exposure⁽⁴⁾. ACR causes disturbances in the oxidative status and enzyme activities. The effect of ACR was pronounced with the high doses, this indicated a risk of organ damage during exposure to ACR. Glycidamide, a metabolite of ACR, binds to DNA and can cause genetic damage. Prolonged exposure has induced tumors in rats, but cancer in man has not been convincingly shown. The International Agency for Research on Cancer (IARC) has classified ACR as “probably carcinogenic to humans”⁽⁵⁾.

It has been found that muscle degeneration is followed by the activation of a muscle repair process. Cellular proliferation is an important event necessary for muscle regeneration⁽⁶⁾. Given the potential for tissue damage, it is perhaps not surprising that the body has evolved major antioxidant defense mechanisms to protect it from free radical attack. These defenses can be conveniently considered as cellular, membrane, and extracellular mechanisms. Guttridge has defined an antioxidant as any substance that, when present at low concentrations, compared with those of the oxidizable substrate, considerably delays or inhibits oxidation of the substrate⁽⁷⁾. Chrysin (CH), a flavonoid found in honey, propolis, and plant extracts, is a potent antioxidant that has been widely studied, this flavonoid has a protective activity against the damage caused by oxidative stress⁽⁸⁾. Thus, the goal of the current study was directed to investigate the effect of CH on ACR-induced striated muscle degeneration of rat's tongue.

SUBJECTS AND METHODS

Animal model: Forty adult male albino rats (200–250 g) were obtained from an animal house, Cairo University (Cairo, Egypt). The experimental animals were housed in standard cages with sawdust bedding under controlled environmental conditions of humidity (30-40%), temperature ($20\pm 2^{\circ}\text{C}$),

and light (12-h light/12-h dark). All experimental animals were maintained on standard diet and water ad libitum. The experiment was conducted in the animal research unit of the Faculty of Pharmacy, Cairo, Boys, Al-Azhar University. The experiment was carried out according to the ethical and research committee protocol of the Faculty of Pharmacy, Cairo, Boys, Al-Azhar University. The procedures were conducted in accordance with the committee for the purpose of control and supervision on experiments on animals (CPCSEA Guidelines)⁽⁹⁾.

Experimental design: After a week of adaptation, the animals were divided into four equal groups, 10 each, and used for two successive periods (20 days: A and 40 days: B), five animals each, as follow:

The first group: In this group, animals were fed with standard diet, without receiving any kind of treatment and considered as negative control group.

The second group: In this group, animals received 15 mg/kg body weight of ACR orally once daily for the experimental period⁽¹⁰⁾, and considered as positive control group.

The third group: In this group, the animals were treated as the second group, in addition; they received 50 mg/kg intraperitoneal of CH⁽¹¹⁾ two weeks before, as well as during application of ACR on alternative days and considered as a chemopreventive group.

The fourth group: In this group, the animals were treated as the second group, in addition; they received 50 mg/kg intraperitoneal of CH⁽¹¹⁾, at first, third and seventh days after application of ACR and considered as a treatment group.

After termination of the experiment, the animals were sacrificed and the tongue tissues were excised, fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin blocks for sectioning and staining in order to be examined microscopically.

Investigations: Tissue sections were stained with the following: Hematoxylin and eosin (H&E) stain for recording the histopathological findings. Azophloxine GA stain was applied for demonstrate the histochemical reaction of both collagen and muscle fibers.

Statistical analysis:

The data was recorded and statistically analyzed. One way analysis of variance (ANOVA) test was used for comparison among the various groups used. In addition, Tukey's post hoc test was performed when ANOVA test reveals significant difference.

RESULTS

Group 1:

Gross observations: Gross observation of tongue mucosa of all animals in G1 showed no observable abnormalities, which appeared pink in color with smooth surface throughout the experiment period (Fig.1).

Histopathological results:

Histopathological section using H&E stain revealed vesicular, elongated and peripherally located nuclei under the sarcolemma (Fig.2). Azophloxine GA stain expression revealed well-arranged red

muscle fibers with dark blue nuclei which appeared in peripheral location inside the cells (Fig.3).

Group 2:

Gross observations: The tongue mucosa of all animals in G2A showed diffuse red swelling (Fig.4).

Histopathological results:

Histological section of the dorsal surface of the tongue using H&E stain showed fragmentation of the sarcoplasm with dense pyknotic nuclei and inflammatory cells infiltrate (Fig.5). Azophloxine GA stain expression revealed dense pyknotic nuclei, splitting of the myofibers, fragmentation of the sarcoplasm and yellowish collagen fibers (Fig.6).

Gross observations: The tongue mucosa of all animals in G2B showed diffuse red swelling (Fig.7).

Histopathological results:

Histological section of the dorsal surface of the tongue using H&E stain showed subepithelial mesenchymal tissue infiltrated by inflammatory cells, multiple spaces between the muscle fibers and fragmentation of the sarcoplasm (Fig.8). Azophloxine GA stain expression revealed separation of muscle fibers with yellowish color of collagen fibers and absence of nuclei (Fig.9).

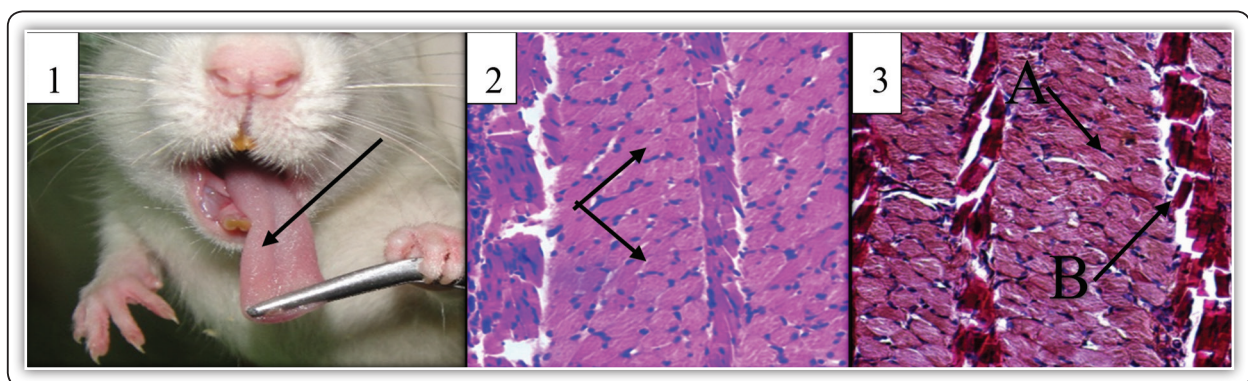


FIG (1): The tongue mucosa of the animals in G1 showing normal appearance of mucosal surface pink in color (arrow). **FIG (2):** Photomicrograph of tongue sections in G1 showing that the nuclei were vesicular, elongated and peripheral in position under the sarcolemma (arrow) (H&E stain X 200). **FIG (3):** Photomicrograph of tongue sections in G1 showing that the nuclei appeared dark blue with peripheral location in the cells (arrow A) of well-arranged red muscle fibers (arrow B), (Azophloxine GA stain X 200).

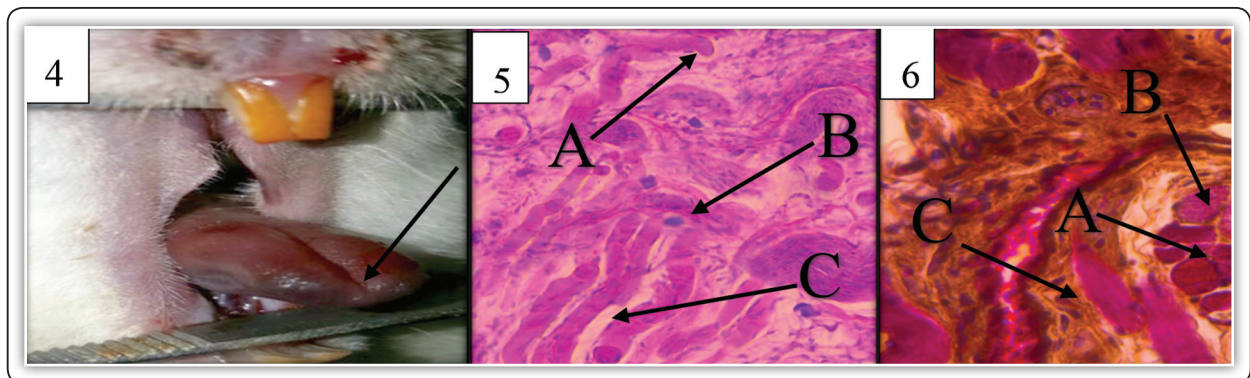


FIG (4): The tongue mucosa of the animals in G2A showed diffuse red swelling (arrow). **FIG (5):** Photomicrograph of the tongue tissue section in G2A showing dense pyknotic nuclei (arrows A), inflammatory cells (arrow B) and fragmentation of the sarcoplasm (arrows C) (H&E stain X 200). **FIG (6):** Photomicrograph of tongue sections in G2A showing dense pyknotic nuclei (arrow A), splitting of the myofibers, fragmentation of the sarcoplasm (arrow B) and yellowish collagen fibers (arrow c). (Azophloxine GA stain X 400).

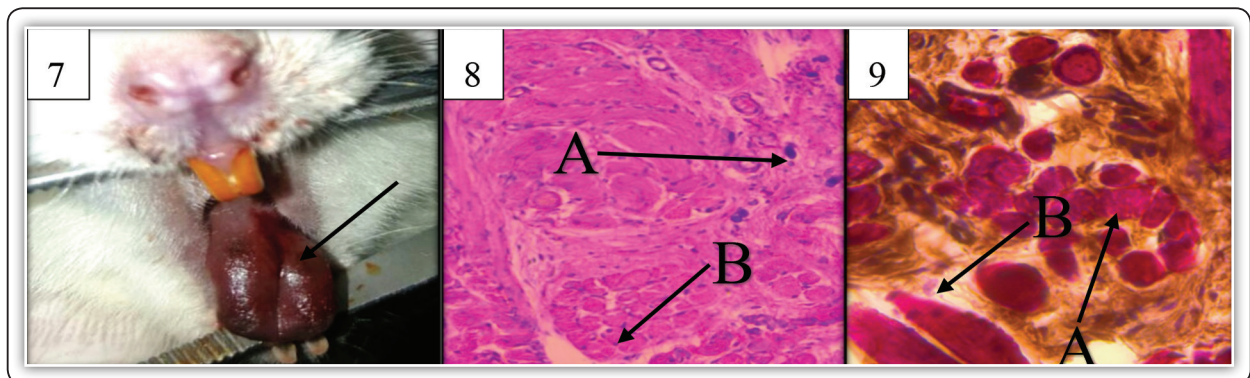


FIG (7): The tongue mucosa of the animals in G2B showed diffuse deep red swelling (arrow). **FIG (8):** Photomicrograph of tissue section of the tongue in G2B showing subepithelial mesenchymal tissue infiltrated by inflammatory cells (arrow A). Multiple spaces between the muscle fibers and fragmentation of the sarcoplasm (arrows B) (H&E stain X 200). **FIG (9):** Photomicrograph of tongue section in G2B showing absence of nuclei (arrow A), and separation of muscle fibers were seen (arrow B) (Azophloxine GA stain X 400).

Group 3:

Gross observations: The tongue mucosa in the animals of G3A showed localized red swelling, in 2 out of 5 animals (Fig.10), while the remaining (3 animals) appeared without observable abnormalities.

Histopathological results:

Histological section of the dorsal surface of the tongue using H&E stain showed abnormal wavy course of muscle fibers (Fig.11). Azophloxine GA stain expression revealed limited areas of yellowish collagen fibers with deeply stained peripherally located nuclei (Fig.12).

Gross observations: The tongue mucosa in the animals of G3B showed localized deep red swelling in 4 out of 5 animals (Fig.13), while the remaining (1 animal) appeared without observable abnormalities.

Histopathological results:

Histological section of the dorsal surface of the tongue in G3B using H&E stain showed dense pyknotic nuclei, irregular pattern of wavy course muscle fibers (Fig.14). Azophloxine GA stain expression revealed limited areas of yellowish collagen fibers deeply stained with peripherally located nuclei (Fig.15).

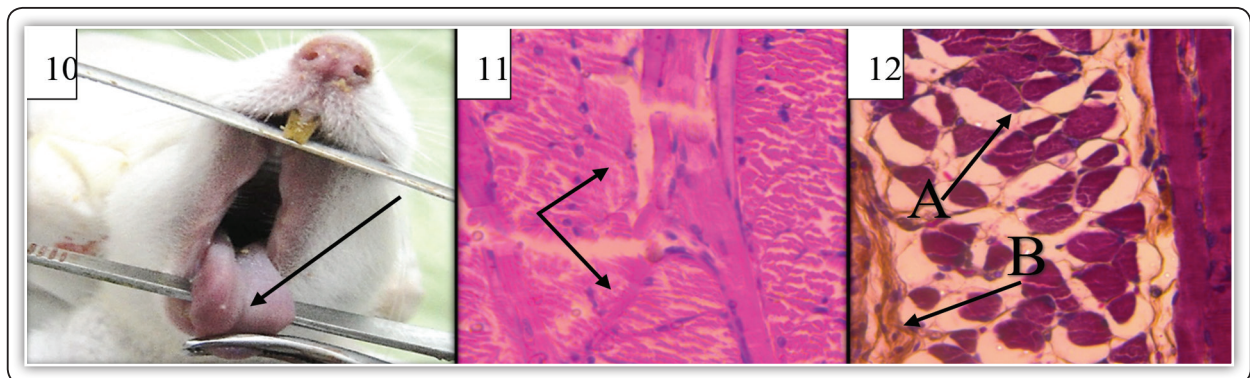


FIG (10): The tongue mucosa of the animals in of G3A showed localized red swelling (arrow). **FIG (11):** Photomicrograph of tongue section in G3A showing abnormal wavy course of muscle fibers with limited areas of the spaces in between (arrows) (H&E stain X 400). **FIG (12):** Photomicrograph of tongue section in G3A showing deeply stained peripherally located nuclei (arrow A), Limited areas of yellowish collagen fibers (arrow B) (Azophloxine GA stain X 400).

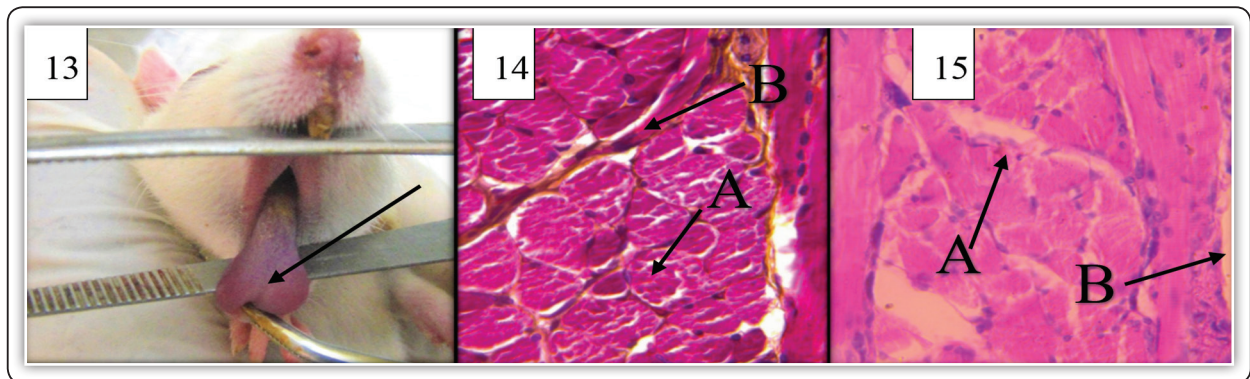


FIG (13): The tongue mucosa of the animals in G3B showed localized deep red swelling (arrow). **FIG (14):** Photomicrograph of tongue section in G3B showing there are dense pyknotic nuclei (arrow A) and Irregular pattern of wavy course muscle fibers with variable sized spaces in between was seen. (arrow B) (H&E stain X 400). **FIG (15):** Photomicrograph of tongue section in G3B showing there are deeply stained peripherally located nuclei (arrow A) and limited areas of yellowish collagen fibers (arrow B) (Azophloxine GA stain X 400).

Group 4:

Gross observations: The tongue mucosa in the animals of G4A showed localized red swelling in 2 out of 5 animals (Fig.16), while the remaining (3 animals) showed a slight decrease in the amount of redness.

Histopathological results:

Histological section of the dorsal surface of the tongue using H&E stain showed abnormal wavy course of muscle fibers with areas of variable-sized the spaces in between (Fig.17). Azophloxine GA stain expression revealed limited areas of yellowish collagen fibers with deeply stained peripherally

located nuclei (Fig.18).

Gross observations: The tongue mucosa in the animals of G4B showed diffuse swelling in 4 out of 5 animals (Fig.19), while the remaining (1 animal) showed slight decrease in the amount of redness.

Histopathological results:

Histological section of the dorsal surface of the tongue using H&E stain showed abnormal wavy course of muscle fibers with variable sized spaces in between them (Fig.20). Azophloxine GA stain expression revealed fragmentation of the sarcoplasm and areas of yellowish collagen fibers with dense pyknotic nuclei (Fig.21).

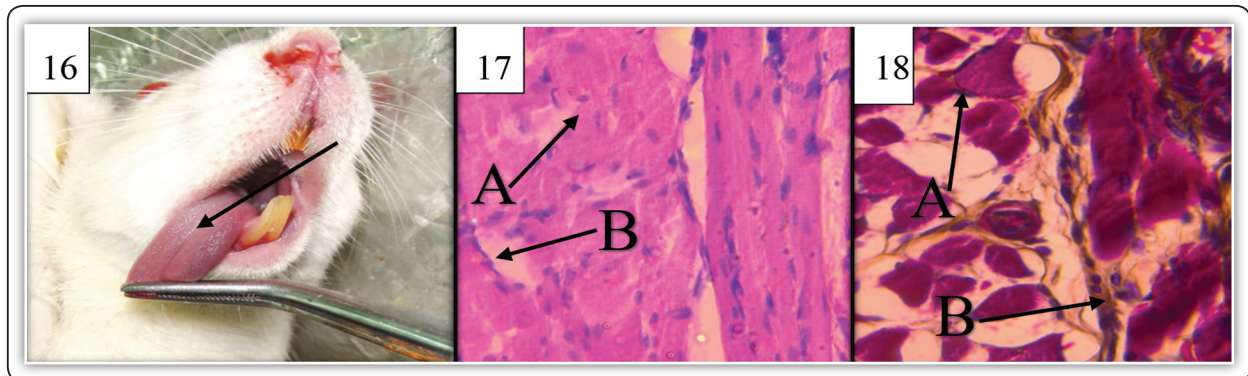


FIG (16): The tongue mucosa of the animals in G4A showed localized red swelling (arrow). **FIG (17):** Photomicrograph of tongue section in G4A showing abnormal wavy course of muscle fibers (arrow A), with areas of variable-sized the spaces in between (arrow B) (H&E stain X 400). **FIG (18):** Photomicrograph of tongue section in G4A showing deeply stained peripherally located nuclei (arrow A), And limited areas of yellowish collagen fibers were seen (arrow B) (Azophloxine GA stain X 400).

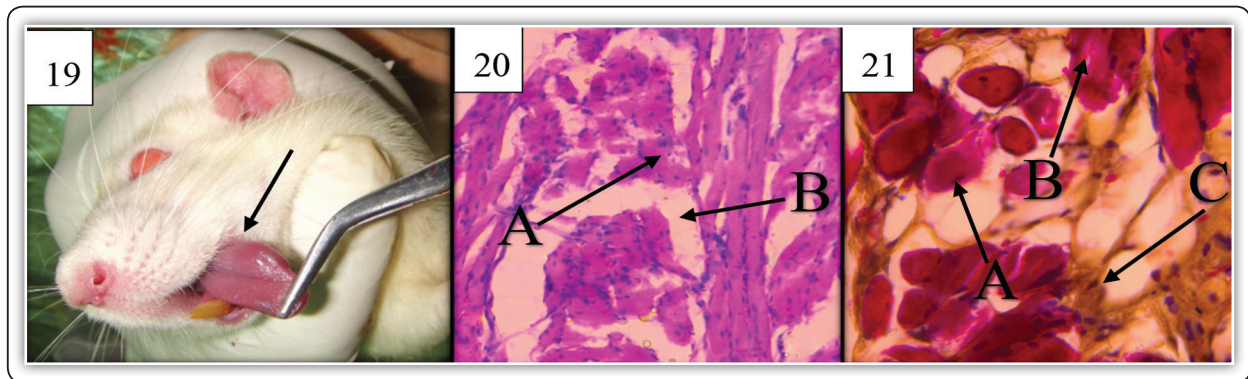


FIG (19): The tongue mucosa of the animals in G4B showed diffuse red swelling (arrow). **FIG (20):** Photomicrograph of tongue section in G4B abnormal wavy course of muscle fibers (arrow A) with variable sized spaces in between (arrow B) (H&E stain X 400). **FIG (21):** Photomicrograph of tongue section in G4B showing there are dense pyknotic nuclei (arrow A), with fragmentation of the sarcoplasm (arrow B), and areas of yellowish collagen fibers (arrow C) (Azophloxine GA stain X 400).

Statistical analysis results:

Statistical analysis results of Azophloxine GA staining were obtained by comparing the percentage of area distribution in the groups used (Table: 1 and Fig.22).

Table {1} Statistical analysis results of Azophloxine staining % and one way ANOVA between all groups.

	Mean ±SD	Range	P-value vs group 1	P-value of A vs B
Group 1	11.59 ± 3.31	7.49 – 15.44	-	
Group 2A	40.62 ± 6.17	32.65 – 46.25	<0.001	0.007
Group 2B	59.51 ± 9.80	48.37 – 69.04	<0.001	
Group 3A	16.45 ± 5.39	11.84 – 25.60	0.124	0.764
Group 3B	17.34 ± 3.51	12.79 – 22.42	0.028	
Group 4A	20.45 ± 2.87	17.30 – 23.93	0.002	0.001
Group 4B	36.40 ± 6.22	26.83 – 43.42	<0.001	
2A vs 3A			<0.001	
2A vs 4A			<0.001	
3A vs 4A			0.181	
2B vs 3B			<0.001	
2B vs 4B			0.002	
3B vs 4B			<0.001	

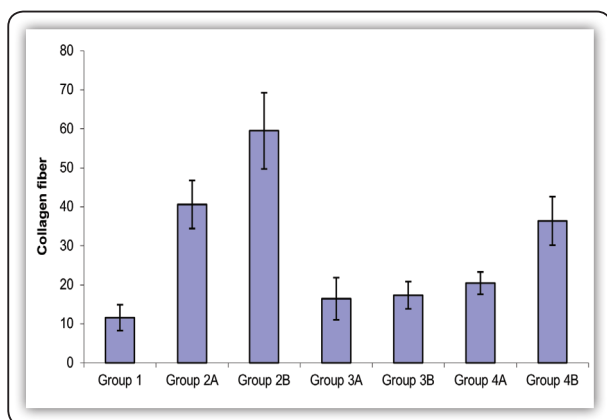


FIG (22) Bar chart representing mean area % results of collagen fibers in all groups

DISCUSSION

The present study revealed the valuable investigation of the effect of CH on ACR-induced striated muscle degeneration of rat's tongue when utilized routine examination by H&E stain as well as when applying Azophloxine histochemical staining. In the current study, the effects of ACR on the striated muscle fibers of the adult male albino rats tongue showed variable changes in both gross finding as well as on submicroscopic observation when utilizing either H&E stain or Azophloxine GA stain.

In this study, there were alterations between G1 and G2A, through histological alterations which showed abnormal wavy course of the muscle fibers with splitting of the myofibers and fragmentation of the sarcoplasm. Moreover, dense pyknotic nuclei were detected, these results coincides with the study of Al-Serwi et al (14). The early phase of muscle injury is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and myogenic cells. It has been suggested that factors released by the injured muscle activate inflammatory cells residing within the muscle, which in turn provide the chemotactic signals to circulating inflammatory cells (12,13).

In the present study, in regard to Azophloxine GA stain there were high significant differences among the mean area percentage of Azophloxine GA in G2A compared to G1 and p value ($p \leq 0.01$). These results were found to be due to deposition of collagen fibers in degenerative muscles. The degenerative findings of the current study are mostly related to the oxidative stress effect of ACR on the striated muscle, this result coincides with the study of Allam et al (15) and Rodrigo et al (16).

Also, the results of gross and histological alterations in G2B compared to G1 and G2A were also seen and increased which coincides with the study of Al-Serwi et al (14) whom stated that by increase time of ACR administration splitting of the myofibers was increased. Fragmentation of the sarcoplasm, dense pyknotic nuclei and remnants of nuclei were also seen. This results also was confirmed by Shinomol et al (2013)(17) as they stated that the toxic effects of ACR in different tissues is a dose dependent which increased by increasing the dose or the duration. In the present study, in regard to Azophloxine GA stain there were high significant differences among the mean area percentage of Azophloxine GA in G2B compared to G1 and p value ($p \leq 0.01$). These results were found to be due to increase in the deposition of collagen fibers in degenerative muscles.

The current study reflected the positive effect of CH as chemopreventive measure on animals in G3A which were supported by Azophloxine GA stain analysis as indicated by area percentage as well as p value. Moreover, there was no significant differences among the mean area percentage of Azophloxine GA stain in G3A as compared to G1 and p value ($p > 0.05$). These findings may be due to the protective effect of CH against muscle degeneration. CH has been found to possess antioxidant properties(18). Malarvili et al (2014) (19) stated that the capability of CH to enhance the levels of antioxidants along with its anti-LPO activity indicates that this compound may be useful

in counteracting free radical-mediated injury which is involved in the development of tissue damage. There were high significant differences among the mean area percentage of Azophloxine GA in G3A as compared to G2A and p value ($p \leq 0.01$). These results were found to be due to decrease of collagen fibers in regenerated muscles.

In the present study, in regard to Azophloxine GA stain, there were no significant differences among the mean area percentage between G1 and G3B and p value ($p > 0.05$). These results were found to be due to decrease of collagen fibers in regenerated muscles. The current study reflected the effect of CH as therapeutic measure on animals in G4A. The histological results revealed some alterations showed irregular pattern of muscle fibers with deeply stained peripherally located nuclei. Limited areas of yellowish collagen fibers were seen. These results were explained by Balta et al⁽²⁰⁾ whom stated that CH has an antifibrotic effect as CH treatment induced downregulation of collagen type 1 (Col I) at transcriptional and translational levels in a dose-dependent manner. This was obvious in G4B with more progressive manner. These results were, also, explained by Balta et al⁽²⁰⁾ whom stated that the antifibrotic effect of CH is associated with its ability to modulate extra cellular matrix (ECM) by tissue inhibitors of metalloproteinase 1 TIMP/ MMP rebalance and decrease collagen deposition in a dose-dependent manner. These results were supported by Azophloxine GA staining analysis as indicated by area percentage as well as p value. There was high significant differences among the mean area percentage and p value ($p \leq 0.01$), of Azophloxine GA staining in G4B as compared to those of G2B.

CONCLUSIONS

Striated muscle damage in rat's tongue by ACR which becomes worse with prolonged duration is realized. CH has a significant protective efficacy when compared with that of ACR-induced striated

muscle injury of rat's tongue. However, CH has a significant protective efficacy when compared with that of treatment on ACR induced striated muscle injury of rat's tongue.

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