Original Article

Mito-TEMPO, A Selective Mitochondrial Antioxidant Alleviates Acrylamide-Mediated Anterior 2/3 Lingual Damage in Rats. Biochemical, Histological and Immunohistochemical Study

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ABSTRACT

Background: Acrylamide (ACR) is a common toxicant. One of the most critical worldwide health issues right now is the exposure of individuals and animals to ACR through their diet. Dietary antioxidants have received attention as potential preventive strategy and as a nutritional supplement for addressing various ACR-induced toxicities.

Aim of work: Studying the potential protective effect of Mito-TEMPO (MT) a selective mitochondrial antioxidant on acrylamide induced lingual toxicity.

Study design: Thirty two adult male rats were separated into 3 sets. Group I (control group). Group II (ACR group): Rats were treated with 40 mg/kg/d acrylamide that was dissolved in physiological saline and given orally by gavage for 14 days. Group III (ACR+ MT group): Rats were treated as group II and were injected with 0.7 mg/kg of Mito-TEMPO (ip) once/day for 2 days before acrylamide and continued with acrylamide for another 14 days. It was given 30 minutes before acrylamide.

Results: Acrylamide intoxicated group revealed significant decline ($P<0.001$) in the antioxidant enzymes levels, with marked degenerative changes in the dorsal, ventral and muscle core at the level of light and electron microscopic examination involved degenerated papillae, thin keratin layer, dorsal epithelial metaplasia, disfigured muscle core, congested blood vessels and atrophied ventral mucous membranes. Moreover, there was significant rise ($P<0.001$) in COX-2, IL1-β and P53 immunoexpression in examined lingual tissues. Mito-TEMPO pretreated group showed significant rise ($P<0.001$) in the antioxidant enzymes with apparent improvement in the histological structure involved restored papillae, nearly normal muscle core and mucous membranes, with significant decline ($P<0.001$) in COX-2, IL1-β and P53 immunoexpression.

Conclusion: Mito-TEMPO revealed potential protective effect on acrylamide induced lingual toxicity.

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Key Words: Acrylamide, mito-TEMPO, lingual toxicity.

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BACKGROUND

The tongue is an anatomical site that indicates the state of the body's health. In many cases, the mouth cavity becomes a crucial diagnostic location[1]. It is supposed that the lingual papillae act as an indicator of overall health, therefore any nutritional shortage or medicine poisoning would manifest to abnormalities to these papillae[2].

Acrylamide (ACR) is α, β-unsaturated molecule, water-soluble, odorless crystalline solid[3]. It is primarily used to create polyacrylamide, which is used in personal care products, as well as in a number of chemical industries, chemical grouting, and soil conditioning[4]. However after the startling discovery that acrylamide was contained in several commonly consumed foods, public health worries over acrylamide increased[5]. Every country showed variable levels of ACR in foods, which was consistent with their dietary habits and methods for preparing food. People are subjected to ACR directly by the high temperature preparation of food, such as frying, baking, and roasting. Moreover, food packing with polyacrylamide exposes consumers indirectly to leftover ACR monomer[6].

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Additionally, concerns have been expressed that children may be at an increased danger for acrylamide exposure owing to their increased nutritional consumption[7].

Its monomeric state is very carcinogenic, teratogenic, and neurotoxic to rats and mice while its polymeric state is non-toxic[8]. Acrylamide is strongly related to the development of cancer in humans, particularly in the oral cavity, colon, prostate, rectum, esophagus, large intestine and larynx[9].

ACR is characterized by high water solubility and small molecular weight, so, it is able to permeate through cell membranes fast, quickly absorbed and widely distributed throughout the body after oral intake[10]. It is effortlessly absorbed by the digestive system. It has been discovered to cause harm to the gastrointestinal mucosa by increasing inflammatory responses, oxidative stress, and cell death[11]. From this angle, research is increasingly concentrating on finding ways to reduce toxicity in *vivo* by minimizing oxidative harm caused by the ACR.

Antioxidants were used to reduce the formation of waste metabolic products and enhance the removal of reactive oxygen species (ROS)[12]. While natural antioxidant cannot reduce oxidative stress, antioxidant supplements may improve the organism's ability to do so[13]. Owing to the fact that mitochondrial malfunction contributes to a number of common diseases, the use of mitochondria-targeted antioxidants as a therapy is becoming more widespread[14].

We are not aware of any studies that used the selective mitochondrial antioxidant Mito-TEMPO (MT) to counteract the cytotoxic effects of ACR on the rat tongue mucosa and underlying muscle. Thus, the goal of this research is to analyze the histological, immunohistochemical, and ultrastructural effects of ACR on the rat tongue in order to provide guidance to examine the potential protective impact of Mito-TEMPO co-administration.

MATERIAL AND METHODS

Chemicals

Acrylamide (99.9 purity) at a form of white, odorless crystals at room temperature. Its molecular formula is C3H5NO.

Mito-TEMPO (Mito-T): at a form of white powder. Empirical Formula: C29H35N2O2P · Cl.

Both drugs were got from Sigma-Aldrich Company, St. Louis, MO, USA)

Animals and Ethical approval

Ethics committee approval (No: BUFVTM 07-12-22) of the animal care and experimental protocols and procedures were revised and accepted for the study by ethical committee in the Faculty of veterinary Medicine, Moshtohor, Benha University.

The 32 adult, healthy male albino-type rats used in the current study ranged in weight from 180 to 220g. The animals were housed in controlled environments with a 12-hour light-dark cycle, controlled room temperature, and controlled humidity levels (30–35%). They were fed with regular pellet rat food, which gave them full access to water.

Experimental design (Figure 1)

The model size of the current research was divided into 3 sets after a week of adaptation.

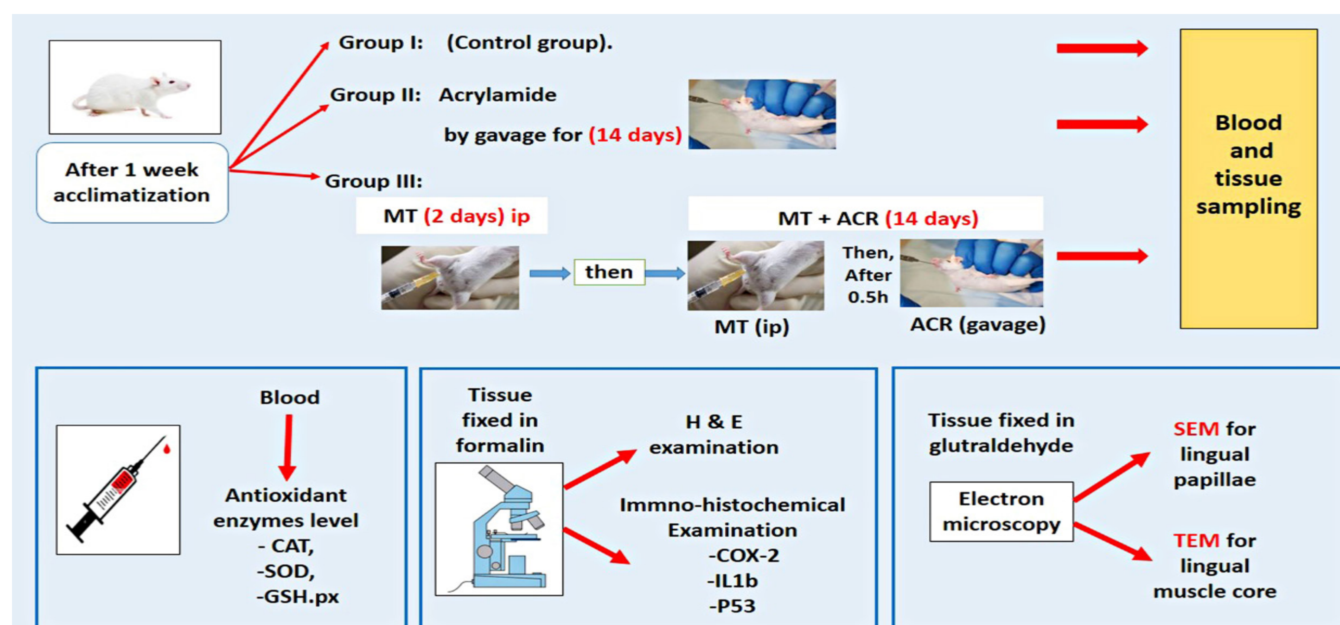

Fig. 1: Diagrammatic illustration summarizing the study design

Group I (Control group; 12 rats): Three subgroups of rats were similarly divided (each subgroup =4 rats).

- Ia: Rats were not given any drugs.
- Ib: Rats were treated orally by gavage with 0.5 ml/ rat of physiological saline for 14 days (vehicle for Acrylamide).
- Ic: Rats were injected with physiological saline intraperitoneally once daily for 2 days before beginning of oral saline and continued with oral saline for another 14 days.

Group II (ACR group; 10 rats): Rats were treated with 40 mg/kg/d acrylamide that was dissolved in saline and orally administered (0.5 ml/ rat) by gavage for 14 days[15].

Group III (ACR+ MT group; 10 rats): Rats in this group treated as group II and were injected with 0.7 mg/ kg of Mito-TEMPO dissolved in saline intraperitoneally[16] once daily for 2 days before beginning acrylamide and continued with acrylamide for another 14 days (Mito-TEMPO was given 0.5 hour before acrylamide).

Serological investigations

The following day after last ACR and Mito-TEMPO doses, ether was used to anesthetize rats. Blood samples (5ml) were taken from their hearts in heparinized tubes, centrifuged at (1500 × g for ten minutes) to obtain the serum, and stored at (−85°C) to analyze antioxidant parameters: catalase (CAT)[17], superoxide dismutase (SOD)[18] and glutathione peroxidase (GSH.Px)[19], using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). The samples were measured at the Central Research Lab, Faculty of veterinary Medicine, Moshtohor, Benha University.

Histological study

Half of the tongue samples were preserved in 10% neutral formaldehyde to prepare wax blocks. Paraffin sections of (5-7 µm) thickness were cut by leica ordinary microtome from the paraffin blocks, stained with Hematoxylin and Eosin (H&E) according to[20], and examined under a light microscope to study the histopathological variations in the study groups.

Immunohistochemical (IHC) Study

Additional sections were fixed on +ve charged slides for IHC staining:

1. Immuno-staining for cyclooxygenase-2 (COX-2) antibody (oxidative stress marker); using rabbit polyclonal antibody (Lab Vision, cat N: PA1-37505). Cox-II positive reaction is detected as brown cytoplasmic color.
2. Immuno-staining for IL1-β antibody (inflammatory marker); using a commercial kit (anti-IL-1β dilution (1/200) Cell Signaling Technology, Danvers, MA). IL-1β positive reaction is detected as brown cytoplasmic color.
3. Immuno-staining for P53 antibody (cell death marker); using rabbit polyclonal antibody (cat. N: ab1431, 1/100; Abcam, Cambridg, UK). P53 positive reaction is detected as brown nuclear reaction.

The immunostaining was done using the avidin-biotin complex technique[21]. Simply; the activity of endogenous peroxidase was inhibited by rehydrating deparaffinized sections and incubating them for 30 minutes with 0.01% H_2O_2 solution. To hide the antigenic location, the tissues were then incubated for an additional 10 minutes in ethanol before being placed in a 0.01-M citrate buffer at pH=6. After twenty minutes in the microwave, the antigen was removed. The 1ry antibodies were applied to sections all-night at 4 °C using diluted versions for each protein. The avidin-biotin complex (ABC) reagent was then reacted with the sections for 1h, followed by 6–10 minutes of peroxidase solution. Following that, hematoxylin was used as a counterstain. Negative control slides were prepared by using PBS instead of primary antibodies.

Electron microscopic study

The remaining tongue samples were preserved in glutaraldehyde and used for EM examination.

a- Transmission electron microscopy (TEM)[22].

The muscle core of the anterior 2/3 of tongue samples were cut into small parts (about one mm3) for electron microscopic examination, prefixed in 2.5% glutaraldehyde for 120 minutes, then post-fixed in 1% osmium tetra-oxide for 120 minutes. Then, specimens were dehydrated and inserted in epoxy resin to get resin blocks. A Leica ultracut (UCT) was used to cut semithin and ultrathin sections (Glienicker, Berlin, Germany). Toluidine blue was used to stain the semithin (0.5 µm thick) slices (1%) and viewed under a light microscope. A two-step staining procedure using uranyl acetate 5% for 15 minutes and lead citrate for eight minutes was used to color ultrathin sections, which were cut at a thickness of 80-90 nm, put on copper grids, and stained. Sections were inspected and electron microphotographs were taken by a transmission electron microscope (JEOL TEM; 100 CX; Japan) at the Faculty of Science, El-Shatby, Alexandria University, Alexandria, Egypt.

b- Scanning electron microscopy (SEM)[23]:

Axial tongue tissues were cut to preserve only the dorsum and were fixed in 4% glutaraldehyde, then post fixed in 1% osmium tetroxide, dehydrated in up-graded ethanol concentrations, placed into amyl acetate, dried with critical-point dryer (E-3000) and covered by gold particles by a sputter coater (SPI-Module). The specimens were stored over silica gel. The dorsum of the tongue samples was scanned under SEM (JEOL: JSM-636- OLA, accelerating voltage: 15kv) at the Faculty of Science, El-Shatby, Alexandria University, Alexandria, Egypt.

Morphometric study

Two slides from each rats of each group were

assessed. Two random fields/slide from each animal were appraised. The mean area percentage (area %) of positive immunoreaction of COX-2, IL1-β and P53 were assessed in the non-overlapping fields of each section at (X200) magnification. The immune-sections were photographed by camera (Olympus; model: E24-10 M pixel, China) fixed on an Olympus microscope with a × 0.5 photo adaptor. The resulting photomicrographs were analyzed at central research lab, Moshtohor faculty of veterinary medicine, Benha University.

Statistical results evaluation

All data were statistically estimated by statistical Package for Social Science software program version N: 23 (IBM SPSS, Inc., USA). One-way analysis of variance (ANOVA) followed by "Tuckey" post-hoc test was used to conclude the statistical significance between different groups (*p value* <0.001 was considered to specify statistical significance). Statistics were presented as mean ±standard deviation (SD).

RESULTS

Histo-pathological studies

The normal tongue panoramic morphology showed the different parts of the ant 2/3 of rat tongue. The dorsal surface, muscle core and the ventral surface. (Figure 2)

Mito-TEMPO protected the tongue structure from the toxic effect of acrylamide:

On exposure to acrylamide for (14 days), noticeable degenerative changes that involved the dorsal, muscle core as well as the ventral surface were observed compared to control group. These degenerative changes were improved in co-treatment with MT.

The dorsal surface

Two forms of papillae were studied in the dorsal surface (Filiform papillae and fungiform papillae).

Filiform papillae

In H&E results the dorsum of control group showed many regular filiform papillae with tips directed backwards. The different cells showed vesicular nuclei (Figure 3a). In ACR-group (II), degenerated filiform papillae with broken tips and disfigured detached keratin layer. Some areas of the epithelial covering displayed focal metaplasia. Dilated blood vessel of the lamina propria with shallow epithelial ridges were apparent (Figure 3b). Co-treatment with MT showed nearly normal dorsal surface with few vacuolated cells (Figure 3c).

Fungiform papillae

Control group also showed normal appearance of fungiform papilla with characteristic mushroom-shape containing taste bud (Figure 4a). In ACR group we noticed thin atrophied fungiform papillae with absent taste bud. The adjacent filiform papillae showed lost tips and disfigured keratin covering (Figure 4b). Co-treatment with MT improved the fungiform papillae morphology with apparent taste bud (Figure 4c).

Muscle core

In control group; the muscular core was formed of striated muscle fibers oriented in longitudinal and tranvrse directions with peripheral vesicular nuclei (Figure 5a). On exposure to acrylamide for 14 days, the muscle core revealed highly degenerative changes with obvious fibers fragmentation. Some fibers were pale acidophilic, others were deep acidophilic with pyknotic nuclei. Huge congested blood vessels were remarkable (Figure 5b). In MT pre-treated group; the muscle showed nearly normal muscle fibers that were oriented in different directions with fatty infiltration in the connective tissuein-between the fibers (Figure 5c).

Ventral surface

The ventral mucous membrane of control group was smooth formed of stratified squamous keratinized epithelium with underlying lamina propria (Figure 6a). The ventrum showed apparent thinning with thin, detached keratin layer. The Lamina propria revealed congested blood vessels (Figure 6b). In MT pretreated group, the ventral surface showed improved histological appearance except for some vacuolated cells (Figure 6c)

Mito-TEMPO protected the tongue tissue via antioxidant, anti-inflammatory and anti-apoptotic effects

COX-2 immuno-expression

Examination of COX-2 immunostained sections of tongue of control groups exhibited negative staining reactivity of cells of the dorsal, muscle core and ventral surface (Figures 7 a,d,g). In contrast, lingual tissues of rats treated with ACR for short period (14 days) demonstrated A robust positive staining reactivity especially in the lamina propria of the dorsal and ventral surfaces (Figures 7 b,h) in addition to in the muscular core (Figure 7e). Lingual mucosa of dorsal and ventral surfaces of rats pretreated with MT as protective drug displayed mild positive staining reactivity in lamina propria (Figures 7 c,i.) along with the muscle core (Figure 7f)

IL1-β immuno-expression results

Regarding the results of lingual sections immunostained with IL1-β antibody, lingual sections of control groups exhibited minimal staining reactivity in the dorsal surface and muscle core as well as the ventral surface (Figures 8 a,d,g). Moreover, exposure to ACR, lingual sections demonstrated strongly positive staining reactivity in the epithelial lining, lamina propria of the both dorsal and ventral surfaces (Figures 8 b,h) as well as in most areas of the muscular core (Figure 8e). Lingual tissues of rats pretreated with MT as protective drug displayed mild positive staining reactivity in the underlying lamina propria of dorsal and ventral surfaces (Figures 8 c,i.) as well as in the muscle core (Figure 8f)

Taken together, these results show that Mito-TEMPO may keep the mitochondrial integrity that could enhance cell survival by hindering the formation of ROS-associated cell death[63].

CONCLUSION

Acrylamide is toxic to the rat tongue mucosa and underlying muscle, which results in considerable serological, histological, immunohistochemical, and ultrastructural changes. Therefore, we think Mito-TEMPO has excellent protective potential and should be included in preventive and therapeutic approaches to ACR toxicity. Further researches are required to clarify the time dependence and, in particular, the long-term effects of using Mito-TEMPO after chronic ACR exposure. Also, we need to demonstrate the therapeutic effect of Mito- TEMPO in different animal models of ACR-induced toxicity.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

الميتوتمبو: أحد مضادات الأكسدة الانتقائية للميتوكندريا، يخفف من الضرر الناتج عن ماده الأكرالاميد في الثلثين الأماميين من اللسان في الجرذان. دراسة كيميائية وهستولوجية وهستوكيميائية مناعيه

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المقدمة: مادة الأكريلاميد هي مادة سامة شائعة. ويعد تعرض الانسان و الحيوان للأكرالاميد خلال النظام الغذائي أحد أهم القضايا الصحية في جميع أنحاء العالم في الوقت الحالي. و قد حظيت مضادات الأكسدة الغذائية بالاهتمام كاستراتيجية وقائية محتملة وكمكمل غذائي لمعالجة مختلف السموم التي يسببها الأكرالاميد.

هدف البحث: دراسة التأثير الوقائي المحتمل لعقار الميتوتمبوعلى سمية اللسان الناجمة عن مادة الأكريلاميد.

المواد و الطرق المستخدمة: تم تقسيم اثنين وثلاثين من ذكور الجرذ البالغة إلى ٣ مجموعات. المجموعة الأولى (المجموعة الضابطة). المجموعة الثانية (مجموعة الأكرالاميد): عولجت الجرذان بـ ٤٠ ملجم / كجم / يوميا من ماده الأكريلاميد مذاب في محلول ملحي عن طريق الفم بالتزقيم لمدة ١٤ يومًا. المجموعة الثالثة (مجموعة المايتوتمبو+ الأكرالاميد): عولجت الفئران مثل المجموعه الثانيه بالاكرالاميد. حُقنت بـ ٠,٧ مجم / كجم من المايتوتمبو داخل الصفاق مرة واحدة يوميًا لمدة يومين قبل مادة الأكريلاميد واستمرحقنها مع مادة الأكريلاميد لمدة ١٤ يومًا أخرى. (يعطى الميتوتمبو قبل ماده الأكرالاميد ب ٣٠).

النتائج: أظهرت المجموعة المعالجة بالأكريلاميد انخفاضًا ملحوظا (P <٠,٠٠١) في مستويات الإنزيمات المضاده للاكسده، واظهر الفحص بالمجهر الضوئي والالكتروني تغيرات مرضية ملحوظة في كلا السطحين والعضلات الأساسية والذي شمل تدهور حليمات اللسان وتشوه العضلات وضمورالأغشية المخاطية. كانت هناك زيادة ملحوظه (P<٠,٠٠١) في التفاعل المناعي لبروتين سيكلواوكسيجيناز ٢ و انترلوكين ١- بيتا و بي ٥٣ في جميع مناطق اللسان التي تم فحصها. أظهرت المجموعة المعالجة مسبقًا بعقار الميتوتمبو زيادة (P <٠,٠٠١) في الإنزيمات المضاده للاكسده مع تحسن واضح في التركيب النسيجي وانخفاض التفاعل المناعي (P <٠,٠٠١) لبروتين سيكلواوكسيجيناز ٢ وانترلوكين ١- بيتا و بي ٥٣.

الإستنتاج: كشفت الدراسه عن تأثير وقائي محتمل لعقار الميتوتمبو على سمية اللسان الناجمة عن مادة الأكريلاميد.