EFFECT OF CHRONIC LOW DOSE ACRYLAMIDE ON THE ULTRASTRUCTURE AND APOPTOSIS OF ADRENAL GLAND CORTEX IN ADULT FEMALE ALBINO RAT

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ABSTRACT

Introduction: Endocrine disturbances to the thyroid and adrenals were observed due to acrylamide exposure.

Aim of the work: to detect any alteration in the ultrastructure and apoptosis in the cortex of adrenal gland after exposure to acrylamide.

Material & methods: 16 female albino rats were separated into two groups, group I acting as control and rats in group II received 10 mg/kg body weight of acrylamide by oral gavages for 3 months. The adrenal glands were removed and processed for electron microscopy and histological examination. Sections were stained with Hx&E and Bax and Bcl-2 immunohistochemistry.

Results: In the ACR group, the rounded or arched clusters of ZG and the parallel columns of ZF were less apparent with narrowing of capillaries that may indicate the presence of swelling. By TEM, electron lucent areas were observed in the cytoplasm of ZG cells and many mitochondria showed defects. Many cells of ZF showed cytoplasmic vacuoles of irregular shape and electron lucent areas in the mitochondria. The expression of Bax in the cortex was significantly higher in the ACR group while the Bcl-2 expression was significantly decreased in the acrylamide treated group compared to the control group. Conclusion: The cellular and mitochondrial changes and the increase in the Bax to Bcl-2 expression ratio indicated increase in the direction of apoptotic pathway

in the cortex of adrenal gland due to exposure to ACR.

Key words: adrenals, acrylamide, ultrastructure, apoptosis, Bax, Bcl-2.

INTRODUCTION

Acrylamide (ACR) is an important industrial chemical with many uses in its polymeric forms [1]. Although accidental exposure to ACR can be significant for some occupations [2], the principal route of human exposure is through the diet because ACR is formed during the cooking of many common starchy foods [3].

Exposure to ACR is a toxicological concern because it is chemically reactive, particularly with thiol groups [4], causes peripheral neuropathy in laboratory animals [5] and humans [2], germ cell mutations and reproductive disorders in animals [6], and is carcinogenic in multiple tissues of chronically exposed rodents [1]. Endocrine disturbances to the thyroid and adrenals were also observed due to acrylamide exposure [7,8]. Some histological changes were noticed in the adrenal gland after acrylamide exposure [8]. Although some studies described ultrastructural changes induced by ACR on rat tes-

tis [9], human astrocytoma cells [10] and trophozoites [11], the effect on adrenal cortex was not described before.

Acrylamide was shown to induce apoptosis of cerebral cortex neurons [12], testis [13] and human astrocytoma cells [10]. Apoptosis, a selective process of physiological cell deletion, is induced by DNA damage or oxidative stress [13]. Members of the Bcl-2 family regulate apoptosis via two classes, anti-apoptotic (Bcl-2, Bcl-XL and Bcl-w) or pro-apoptotic (Bax, Bak and Bad) [14]. Imbalance between the proapoptotic and anti-apoptotic stimuli can direct the cell toward apoptosis.

So the aim of this study is to detect any alteration in the ultrastructure and apoptosis in the adrenal gland cortex.

MATERIAL & METHODS

Experimental animals:

Sixteen adult female albino rats weighing 150-180g were used in this study. They were kept in environmentally controlled room (22±2°C, 12h light/12h dark cycle) and allowed free access to food and water. All rats received care in according to

the rules and regulations of the Medical Research Ethics Committee of Mansoura Faculty of Medicine.

Experimental Design:

After an acclimatization period of one week, the rats were randomly separated into two groups of eight, with those in group I acting as the untreated controls and provided with tap water during the entire period of the study. Rats in group II received 10 mg/kg body weight of acrylamide (Sigma, Egypt) dissolved in tap water (1mg/ml) by oral gavages once in the morning for 3 months. The rats were isolated from males throughout the experiment. At the last four days of experiment, vaginal smears using cotton bud moistened with saline were done at 11 o'clock in the morning for oestrous cycle evaluations. The slides were examined and rats in the diestrous phase were sacrificed after ether anesthesia by decapitation and the adrenal gland was immediately removed and the right gland was processed for histology and the left for electron microscopy.

Histological processing:

The adrenal gland tissue was fixed in 10% buffered formalin, embedded in paraffin, cut into 3-5 μ thick

sections and stained with haematoxylin and eosin.

Transmission Electron Microscopy (TEM):

The adrenal gland tissue was cut into small pieces (approx. 1-mm³ cubes), fixed in 3% glutaraldehyde (in 0.1M phosphate buffer, pH 7.4) at 4°C for 1 h, washed and fixed in 1% osmium tetroxide (in phosphate buffer) at room temperature for 1 h. Tissue was then washed in water, dehydrated in graded ethyl alcohol and embedded in araldite. Ultrathin sections were cut, stained with uranyl acetate and lead citrate and photographed under a Jeol transmission electron microscope (JEM-2100, Japan) at the Electron Microscopy Unit, Mansoura University.

Immunohistochemistry:

Immunohistochemical staining to determine Bax or Bcl-2 expression was done according to the manufacturer instructions. Briefly, sections were deparaffinised in xylene and rehydrated by immersion in descending concentrations of ethanol. Sections were treated with 3% hydrogen peroxide to block endogenous peroxidases, subjected to routine heat antigen retrieval procedures then incu-

bated with a primary antibody raised against Bax (Abcam, Egypt) or Bcl-2 (Abcam, Egypt) overnight at 4°C. After washing with phosphate buffer saline (PBS) (PH7.4), the sections were incubated with secondary antibodies using ABC Detection IHC Kit (Abcam, Egypt). The cells that displayed brown precipitation were considered to be positive for Bax or Bcl-2 expression.

Morphometrical analysis:

The measurements were done on digital photomicrographs of the immunohistochemically stained sections for Bax and Bcl-2 using the software ImageJ programme. Two-three random fields per section and two sections per animal in each group were used. The colour deconvolution plugin feature of ImageJ programme was used to calculate the percentage of the color optical density.

Statistics:

All values were expressed as mean $\pm$ SEM. Significant differences between the groups were determined by performing one-way ANOVA followed by student t-test. The criterion for statistical significance was set at $p < 0.05$.

RESULTS

Adrenal gland histology:

Control group. The gland was formed of an outer cortex composed of three zones; zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) and inner medulla. The zona glomerulosa was the outermost zone and consisted of cells arranged in rounded or arched clusters. The zona fasciculata was the middle and thickest zone and was composed of parallel columns of cuboidal or polyhedral cells, one to two cells thick, separated by prominent capillaries. The cytoplasm appeared foamy due to dissolved intracellular lipid droplets. The zona reticularis was made of smaller cells forming a network of interconnected cords (**Fig. 1A&B**).

Acrylamide group. The rounded or arched clusters of ZG and the parallel columns of ZF were less apparent. There was narrowing of capillaries which may indicate swelling of its cells (**Fig. 2A&B**).

Adrenal gland ultrastructure:

Control group. The cells of ZG had round or oval euchromatic nuclei with some heterochromatin especially close to the nuclear membrane

and prominent nucleoli. The cytoplasm contained numerous mitochondria, dark lipid droplets and some lysosomes (**Fig. 3**). The cells of ZF had large euchromatic nuclei with prominent nucleoli. The mitochondria were more numerous than ZG while the lipid droplets were smaller. Lumina of numerous capillary vessels were visible in both zones (**Fig. 5**).

Acrylamide group. The nuclei of ZG contained more condensed chromatin compared to control. Electron lucent areas were observed in the cytoplasm and many mitochondria showed defects (**Fig. 4**). Vacuoles of irregular shape appeared in the cytoplasm of many cells of ZF. Electron lucent areas were also observed in the mitochondria. Lumina of the capillary vessels were narrower in the ZF (**Fig. 6**).

Immunohistochemistry:

Bax immunohistochemistry. Weak or moderate reaction was observed in many cells of ZF of the control adrenals with only few cells showed strong reactivity in their cytoplasm. In the ACR group, there was increase in the reactivity and many cells showed moderate or strong stain (**Fig. 7A&B**). The expression of bax was significantly higher in the ACR group as detected by optical density (**Fig. 8**).

Bcl-2 immunohistochemistry. The cells of the ZG showed more reaction for the prosurvival protein Bcl-2 than the ZF (**Fig. 9**). The reaction in the cortex was significantly decreased in the acrylamide treated group than the control group (**Fig. 10**).

Fig. 1. (A) A photomicrograph of an adrenal gland section from an adult female albino rat of the control group showing the three zones of the cortex (zona glomerulosa, ZG, zona fasciculata, ZF, and zona reticularis, ZR) and medulla (M). (B) A higher magnification viewing ZG that consisted of cells arranged in rounded or arched clusters. The ZF was the thickest zone and was composed of parallel columns of secretory cells with foamy cytoplasm (intracellular lipid droplets) separated by prominent capillaries (arrows). Hx&E; A x40, B x100

Fig. 2. (A) A photomicrograph of an adrenal gland section from an adult female albino rat of the acrylamide treated group showing its different layers. (B) The rounded or arched clusters of zona glomerulosa (ZG) and the parallel columns of zona fasciculata (ZF) are less apparent and separated by narrower capillaries (arrow).
Hx&E; A x 40, B x 100

Fig. 3. An electron photomicrograph of the zona glomerulosa of the control group showing cells with round or oval euchromatic nuclei (N). Dark lipid droplets (L) and numerous mitochondria (arrow heads) are seen in the cytoplasm.

Fig. 4. An electron photomicrograph of the zona glomerulosa of the acrylamide-treated group showing cells with round or oval nuclei (N) but with more condensed chromatin compared to control. Dark lipid droplets (L) are seen. Electron lucent areas are observed in the cytoplasm (arrows) and vacuolated mitochondria (arrow heads) are present.

Fig. 5. An electron photomicrograph of the zona fasciculata of the control group showing large euchromatic nuclei (N). The mitochondria (M) are more numerous while the lipid droplets (L) are smaller than those in zona gromerulosa. A lumen of capillary vessel (c) is visible.

Fig. 6. An electron photomicrograph of the zona fasciculata of the acrylamide-treated group showing vacuoles (arrows) in the cytoplasm and in the mitochondria (arrow heads).

Fig. 7. Photomicrographs of adrenal sections from control (A) and acrylamide (B) groups. Weak or moderate (arrow head) expression of Bax was observed in many cells of ZF of the control adrenals with only few cells showed strong reactivity (arrow) in their cytoplasm. In the ACR group, there was increase in the reactivity and many cells showed moderate (arrow head) or strong (arrow) stain.

Bax immunohistochemistry; x100

Fig. 8. A histogram showing the mean optical density of Bax expression in both ZG and ZF. Bax expression was significantly higher (*) in the ACR group as compared to control, $P < 0.05$

Fig. 9. Photomicrographs of adrenal sections from control (A) and acrylamide (B) groups. The reaction (arrows) of the prosurvival protein Bcl-2 in the cortex was significantly decreased in the acrylamide treated group than the control group.
Bcl-2 immunohistochemistry; x100

Fig. 10. A histogram showing the mean optical density of Bcl-2 expression in both ZG and ZF. Bcl-2 expression was significantly lower (*) in the ACR group as compared to control, $P < 0.05$

DISCUSSION

The adrenal gland is the most commonly associated with chemically induced lesions among the endocrine organs [15]. Previous works indicated that the adrenal gland could be affected by acrylamide (ACR) exposure. A significant decrease in serum corticosterone level [16,8] and histological changes in the form of cytoplasmic vacuolation of adrenal zona fasciculata were observed after 8 weeks of ACR treatment [8]. However, in the present study vacuolation was not visible in the histological sections but obvious reduction in the capillary size was seen in the zona fasciculata which may indicate swelling of its cells. Although some studies described ultrastructural changes induced by ACR on rat testis [9], human astrocytoma cells [10] and trophozoites [11], the effect on adrenal cortex ultrastructure was not described before. In this study, oral administration of ACR in a moderately low dose for 3 months resulted in more condensation of heterochromatin in the nuclei of both zona glomerulosa and fasciculata compared to control. The cytoplasm showed electron lucent areas especially in the ZF, fragmentation of endoplasmic reticulum and vacuolation of the mito-

chondria. Chen et al. [10] also observed mitochondrial changes in human astrocytoma cells in the form of marked vesicular matrix compartmentalization. Mitochondrial damage can result from oxidative stress which was observed to increase after ACR exposure [16,17,18]. ACR forms adducts with sulfhydryl groups on proteins with the major site of reaction is cysteine residue [4]. The sulfhydryl functional group of cysteine has a crucial role in protecting the cell from oxidative damage [19].

The adrenal gland is subjected to balanced dynamic structural changes including cellular proliferation and death to ensure integrity and functionality of the adrenal gland [20]. Apoptosis is a natural cell elimination process, important during tissue turnover [21] characterized by activation of caspases and execution of cell death. Members of the Bcl-2 family proteins direct the fate of a cell towards either survival or death by the opposing action of the anti-apoptotic (Bcl-2, Bcl-XL and Bcl-w) or pro-apoptotic proteins (Bax, Bak and Bad) [14]. In the mitochondrial pathway of apoptosis, the cytosolic harmless monomer Bax becomes changed into deadly oligomer that

translocates to mitochondria upon exposure to various cytotoxic agents [22,23]. This results in permeabilization of the mitochondrial outer membrane and release of proapoptotic factors such as cytochrome c to the cytoplasm that initiates a proteolytic cascade [23]. Members of the anti-apoptotic family, Bcl-2 and Bcl-xL, can inhibit or delay this release thus significantly prevent cell death [24]. In the present study, the expression of Bax was significantly higher in the ACR group as detected by optical density. This was accompanied with a significant reduction in Bcl-2 expression indicating increase in the direction of apoptotic pathway. In the cerebral cortex of rats exposed to ACR, similar effects were also observed by [12].

Conclusion: Acrylamide induced damage to the adrenal gland cortex probably through increasing oxidative stress that targets the mitochondria as shown from the electron microscopic changes and the expression of Bax and Bcl-2.

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الملخص العربى

التأثير المزمن لجرعة منخفضة من الأكريلاميد على التركيب

الدقيق والموت المبرمج فى قشرة الغدة الكظرية فى

إناث الجرذان البيضاء البالغة

أمنية عرفان ومنى الشحات

قسم التشريح ، كلية الطب ، جامعة المنصورة

المقدمة: لقد لوحظ وجود اضطراب فى نشاط الغدد الصماء مثل الغدة الدرقية والكظرية بعد التعرض للأكريلاميد.

الغرض من البحث : اكتشاف أى تغيرات فى التركيب الدقيق والموت المبرمج فى قشرة الغدة الكظرية نتيجة التعرض للأكريلاميد.

المواد والطرق المستخدمة : لقد تم استخدام ١٦ أنثى فأر أبيض فى هذه التجربة تم تقسيمهم الى مجموعتين الاولى قياسية و الثانية أخذت الأكريلاميد بجرعة ١٠ مج/كجم من وزن الجسم عن طريق الفم لمدة ٣ شهور. وتم استئصال الغدة الكظرية ومعالجتها للميكروسكوب الإلكتروني والفحص الهيستولوجى. وتمت صباغة الشرائح بالهيماتوكسلين والإيوسين والصبغة المناعية الهيستوكيميائية باكس و بى سى ال تو.

النتائج : فى مجموعة الأكريلاميد لوحظ عدم وضوح التجمعات القوسية فى المنطقة الحبيبية واعمد المتوازية من الخلايا فى المنطقة الحزمية كما وجد ضيق فى الاوعية الدموية وهذا قد يشير الى وجود تورم فى الخلايا. بالميكروسكوب الالكترونى وجدت فراغات فى سيتوبلازم

الخلايا فى منطقة المنطقة الحبيبية وفراغات فى كثير من الميتوكوندريا. وكثير من خلايا المنطقة الحزمية كان يوجد بها فراغات غير منتظمة الشكل فى السيتوبلازم وأجزاء خالية فى الميتوكوندريا. , أظهرت الصبغات المناعية زيادة فى الباكس فى مجموعة الاكريلامايد بالمقارنه بالمجموعة القياسيه وكانت صبغة البى سى ال تو اقل فى مجموعة الاكريلامايد عن المجموعة القياسيه.

الاستنتاج : التغيرات التى لوحظت فى الخلايا والميتوكوندريا مع الزيادة فى نسبة الباكس الى البى سى ال تو تشير إلى زيادة فى الإجهاد الموت المبرمج للخلايا فى قشرة الغدة الكظرية نتيجة للتعرض للأكريلامايد.