Acrylamide-induced adverse cerebellar changes in rats: possible oligodendrogeneric effect of omega 3 and green tea

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Background: Humans are widely exposed to acrylamide (ACR) and its neurotoxicity is a significant public health issue attracting wide attention. The aim of the study was to investigate ACR-induced adverse cerebellar changes in rats and study the possible oligodendrogeneric effect of omega 3 and green tea.

Materials and methods: Twenty-four adult albino rats weighing 150–200 g were randomly divided into four equal groups (6 rats each): control group (Group I), the rats that received ACR 45 mg/kg/day (Group II), the rats that received ACR concomitant with omega 3 at a dosage of 200 mg/kg/day (Group III), the rats that received ACR concomitant with green tea dissolved in drinking water at a dosage of 5 g/L (Group IV). The rats were euthanized after 8 weeks of the experiment. Malondialdehyde (MDA) and glutathione (GSH) were measured in cerebellar homogenates. Sections of 5 µm thickness from specimens from the cerebellum were stained with haematoxylin and eosin, silver stain and immunohistochemical stains: platelet-derived growth factor alpha (PDGFα; for oligodendrocytes), glial fibrillary acidic protein (GFAP; for astrocytes) and BCL2 (antiapoptotic).

Results: Omega 3 and green tea had improved MDA and GSH as compared to the ACR group. Histologically, the ACR group showed variable degrees of cellular degeneration. Omega 3 had induced oligodendrogenesis in Group III. The optical density of silver stain was significantly (p < 0.05) increased in Groups III and IV as compared to the ACR group. Area per cent of positive PDGFα was significantly increased in the ACR + omega 3 group as compared to the ACR group. Area per cent of positive GFAP was significantly decreased in Groups III and IV as compared to the ACR group. Area per cent of positive BCL2 was significantly increased in the omega 3-trated group as compared to the ACR group.

Conclusions: Concomitant administration of omega 3 or green tea with ACR might mitigate the adverse cerebellar changes caused by ACR thanks to an oligodendrogeneric effect of omega 3. (Folia Morphol 2019; 78, 3: 564–574)

Key words: acrylamide, cerebellum, omega 3, green tea, rats, oligodendrocytes
INTRODUCTION

Acrylamide (ACR) is an industrial neurotoxic chemical that has been found in carbohydrate-rich foods cooked at high temperatures such as potato chips [15]. Therefore, humans are widely exposed to ACR and its neurotoxicity in humans is a significant public health issue attracting wide attention [19]. It was also associated with carcinogenicity and reproductive toxicity [6]. The neurotoxicity of ACR has been known to affect nerve terminal and cysteine residues on the functionally important presynaptic proteins, resulting in inhibition of neurotransmitter release and eventual process degeneration [25, 26]. Cerebellum controls the maintenance of equilibrium (balance), influences posture and muscle tone as well as coordinating movement [8]. Cerebellar ataxia may be acquired from exposure to many toxic materials [39]. Myelin is an electrical insulator that increases conduction velocity of nerve fibres and is the physical basis for rapid saltatory conduction (in which impulses jump from one node of Ranvier to another) [29]. Inflammatory damage to the oligodendroglia and white matter is involved in the pathogenesis of demyelinating diseases such as multiple sclerosis [5]. Oligodendrocyte is the cell responsible for producing central nervous system (CNS) myelin by the concentric layers of its plasma membrane [34]. It is located in both the grey and the white matter of the CNS: interfascicular oligodendrocytes (white matter) and satellite oligodendrocytes (grey matter only) [14].

The observation that Eskimos had a very low incidence of cerebrovascular and its complications, had led to the first indication of a protective effect of fish oil on atherosclerosis. Today, fish oil is one of the most popular nutraceuticals available in health food stores [27]. Surprisingly, fish oil preparations are also anti-arrhythmic, particularly in patients who have already suffered a myocardial infarction (MI) [9]. In addition to the treatment of hypertriglyceridaemia, a preparation of omega 3-acid ethyl esters is licensed in the United Kingdom for the prevention of recurrent events after MI [32]. Recently, the combined treatment of fish oil dietary supplement and omega 3 polyunsaturated fatty acids injections had been proved to promote post-traumatic brain injury (TBI) restorative processes in the brain, including generation of immature neurons, microvessels, and oligodendrocytes, each of which was significantly correlated with the improved cognitive recovery [30].

Green tea extract had been partially efficacious in preventing neurodegeneration in the brain of lead-treated rats, which resulted from its inhibitory effect on free radical chain reactions generated during oxidative stress caused by lead and from an increase in antioxidant enzyme capacity [23]. Over the last two decades polyphenols, one of green tea constituents, have drawn attention as promising natural dietary molecules for the prevention of ageing and neurodegenerative diseases [22]. Recently, dietary polyphenols, endowed of antioxidant and anti-inflammatory properties, had been reported to extend brain health span as they were actively investigated as potential adjuvants to support proliferation and survival of neural progenitors, and counteract age-dependent neurogenic decline [36].

The present study deals with the influence of green tea extract and omega 3 fatty acids in ACR-induced adverse cerebellar changes in rats.

MATERIALS AND METHODS

Animals

The experiment was ethically approved by the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) under the number of CU/III/F/48/18. The present study was carried out on 24 male albino rats weighing 150–200 g. The animals were housed in cages, under standard laboratory and environmental conditions with free access to food and water at a temperature of (20 ± 2°C) with a natural 12-h light/dark cycle and free access to standard pellet chow and drinking water ad libitum. Animals were obtained from Animal House, Faculty of Medicine, Cairo University. The rats were set in the laboratory for a period of 2 weeks for acclimatisation before carrying out the experiment.

Chemicals

Acrylamide. The dose of ACR used was 45 mg/kg/day [31]. Acrylamide (99% pure) was purchased from Sigma Chemical Company (St. Louis, Missouri, USA). It was dissolved in distilled water and administered every day by oral gavage.

Omega 3 plus. Omega 3 fatty acids (Sedico, Egypt) was given in a dosage of (200 mg/kg/day; DHA 100 mg/kg/day + EPA 100 mg/kg/day) which was administered every day by oral gavage [38].

Green tea. Green tea. Royal Regime Tea, packed in Egypt by Royal Herbs, was dissolved in the drinking water at a concentration of 5 g/L. Tea was prepared freshly 3 times per week and stored at 48°C until use. The content of drinking vessels was renewed every day [23].
Experimental design
The rats were randomly divided into four equal groups (6 rats each) as follows:
— Group I (control group): the rats received no medications;
— Group II: the rats received ACR;
— Group III: the rats received ACR concomitant with omega 3;
— Group IV: the rats received ACR concomitant with green tea.
All animals were sacrificed after 8 weeks (the end of the experiment) by decapitation using guillotines to avoid brain injury [10]. Just before sacrifice, the body weight of all animals was measured. After euthanasia of animals, the cerebellum of all animals was immediately removed and underwent the following:

Biochemical analysis
The cerebellum of all animals was immediately washed in ice-cold glass slides, homogenised separately in 10 volumes (w/v) of 0.1 M phosphate buffer, pH 7.4 using a polytron homogenizer for 1 min. The homogenates were centrifuged at 4000 rpm for 20 min and refrigerated at 4°C. The supernatant was used for estimation of the quantitative activities of both malondialdehyde (MDA) and the total glutathione (GSH).

Histological and immunohistochemical study
Specimens from the cerebellum were fixed in 10% neutral-buffered formalin. Sections of 5 µm thickness were prepared from each specimen. The sections were stained with haematoxylin and eosin (H&E) as well as the silver stain for histological assessment. For silver staining [2], sections were deparaffinised, treated with 1% potassium permanganate, bleached in 1% oxalic acid, treated with 2.5% iron alum, placed in a Coplin jar of silver solution, reduced in 10% aqueous formalin, treated in 0.2% gold chloride solution, treated with 5% sodium thiosulfate, counterstained with eosin, dehydrated through ascending grades of alcohol then cleared in xylene.
Deparaffinised sections were prepared for immunohistochemical study [24, 35]. They were mounted on positively charged slides for staining with platelet-derived growth factor alpha (PDGFα; as a marker for oligodendrocytes), glial fibrillary acidic protein (GFAP; to assess for astrocytic activity) and BCL2 (as a measure for antiapoptosis). The deparaffinised sections underwent rehydration, heat-induced epitope retrieval (HIER). The slides were placed on Dako autostainer instrument, EnVision Flex peroxidase blocked. Immune reactions were evaluated with various primary antibodies PDGFα (Genetex, USA), BCL2 (Dako, Denmark), GFAP (DAKO, Denmark). Secondary antibodies used were Dako EnVision Flex/HRP. Visualisation had been done with EnVision FLEX DAB + chromogen. The prepared sections were examined and photographed using a Canon digital camera (Canon, Japan) attached to the IBM computer system.

Statistical analysis
Statistical analysis was performed using statistical package for the social sciences (SPSS) version 21.0 (IBM Corporation, Somers, NY, USA) statistical software. The data were expressed as means ± standard deviation (SD). Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by post hoc Tukey test. Significance was considered when p value was less than 0.05.

RESULTS
Clinical observations and rats body weight
The acrylamide group of rats became progressively less active and showed general weakness with a decrease in their appetite from the first time of treatment.
with the drug. Although no mortality was recorded, this group showed a decline in their body sizes. The ACR + omega 3 and ACR + green tea groups were more active with slight decrease in their appetite with no mortality recorded. The body weight of the ACR and ACR + green tea groups was significantly decreased as compared to the control group. It was significantly increased in the ACR + omega 3 group as compared to the ACR and ACR + green tea group (Table 1).

**Biochemical results**

The mean GSH of the ACR group was significantly decreased as compared to the control group. It was significantly increased in the ACR + omega 3 and ACR + green tea group as compared to the ACR group (Table 1). The mean MDA of the ACR group was significantly increased as compared to the control group. It was significantly decreased in the ACR + omega 3 and ACR + green tea group as compared to the ACR group (Table 1).

**Histological results**

On H&E (Fig. 1) staining, the control group showed regularly arranged Purkinje cells and granular cells. The ACR group exhibited regular Purkinje cells alternating with swollen degenerated ones with pale chromatins materials in their nuclei. Some Purkinje cells were seen displaced in the granular layer. The ACR group also exhibited degenerated Purkinje cells surrounded with empty spaces. The granular cells were markedly diminished in number with clumps of pyknotic cells with intercellular eosinophilic areas (necrosis). The ACR + omega 3 group showed largely preserved Purkinje cells with apparently intact all layers of the cortex. Many specimens of this group showed multilayers of Purkinje cells surrounded by many vacuolated cells (clear cells) mostly oligodendrocytes. The ACR + green tea group showed largely preserved Purkinje cells with all layers of the cortex apparently intact.

On silver staining (Fig. 2) of the cerebellar cortex, the ACR group revealed marked loss of Purkinje cells with nuclear degeneration in the remaining ones. The ACR + omega 3 group revealed a multilayer deposition of regular Purkinje cells with oligodendrocytes inbetween. Granular cells appeared regular despite their decreased staining. The ACR + green tea group revealed largely preserved Purkinje and granular cell layers. On silver staining of the cerebellar medulla, the control group exhibited normal silver staining of the axons while the ACR group exhibited markedly decreased axonal staining. The ACR + omega 3 group exhibited mildly decreased axonal staining while the ACR + green tea group exhibited normal axonal staining.

**Immunohistochemical results**

On PDGF immunostaining (Fig. 3), the control group showed many oligodendrocytes with a positive PDGF reaction while the ACR group showed few oligodendrocytes with decreased reaction. The ACR + omega 3 group exhibited an increased number of oligodendrocytes with a positive PDGF reaction, especially at the junction between molecular and granular cell layers, while the ACR + green tea group exhibited a considerable amount of oligodendrocytes with a positive reaction.

On GFAP immunostaining (Fig. 4), the control group showed a considerable number of astrocytes with a positive GFAP reaction while the ACR group showed a huge number of astrocytes with a positive reaction. The ACR + omega 3 group exhibited a considerable number of astrocytes with a positive GFAP reaction while the ACR + green tea group exhibited mild increase in the number of astrocytes with positive GFAP reaction.

On BCL2 immunostaining (Fig. 5), the control group showed mild positive BCL2 reaction while the ACR group showed a scanty BCL2 reaction. The ACR + omega 3 group showed mild increase in BCL2 reaction while the ACR + green tea group exhibited mild positive BCL2 reaction.
Histomorphometric results

The mean number of Purkinje cell in the ACR group was significantly decreased as compared to the control group. It was significantly increased in the ACR + omega 3 and ACR + green tea group as compared to the ACR group (Table 1).

The optical density of silver stain was significantly decreased in the ACR group as compared to the control group. It was significantly increased in the ACR + omega 3 and ACR + green tea group as compared to the ACR group (Fig. 6).

The area per cent of PDGF positive reaction was significantly decreased in the ACR group as compared to the control group. It was significantly increased in the ACR + omega 3 as compared to all groups. The ACR + green tea group showed significant increase as compared to the ACR group (Fig. 6).

The area per cent of GFAP positive reaction was significantly increased in the ACR group as compared to the control group. It was significantly decreased in the ACR + omega 3 and group IV as compared to the ACR group (Fig. 6).

DISCUSSION

Human beings and animals are exposed every day simultaneously and concurrently to environmental contaminants. Acrylamide is known to exert its toxicity through oxidative stress by generating reactive oxygen species [21]. Body weight is an important marker in toxicology experiments. In our study, ACR admin-
Figure 2. A. Control group showing regularly arranged granular cells (arrow head) and flask shaped Purkinje cells (black arrows); B. Acrylamide (ACR) group showing loss of Purkinje cells with nuclear degeneration in the remaining ones (black arrow). The granular cells (arrow heads) appear with decreased staining and wide intercellular spaces; C. Omega 3-treated group showing multilayers of regular Purkinje cells with oligodendrocytes (red arrows) inbetween. Granular cells appear regular despite their decreased staining; D. Green tea-treated group showing largely preserved Purkinje cells and granular layer; E. Control group cerebellar medulla showing normal silver staining of axons (blue arrows); F. ACR group cerebellar medulla showing markedly decreased silver staining of axons; G. Omega 3-treated group cerebellar medulla showing mildly decreased silver staining of axons in the cerebellum; H. Green tea-treated group cerebellar medulla showing normal silver staining of axons; silver ×400.

Figure 3. A. Control group (cerebellar cortex and medulla respectively) showing many oligodendrocytes (arrows) with positive platelet-derived growth factor (PDGF) reaction; B. F. Acrylamide (ACR) group cerebellar (cerebellar cortex and medulla respectively) showing few oligodendrocytes (arrows) with positive reaction; C, G. Omega 3-treated group (cerebellar cortex and medulla respectively) showing many oligodendrocytes with positive reaction especially at the junction between molecular and granular cell layers; D, H. Green tea-treated group (cerebellar cortex and medulla respectively) showing a considerable amount of oligodendrocytes with positive reaction; PDGF; ×400.
istered to adult rats reduced the body weight. This might be due to a decrease in food and water intake by adult rats due to decreased appetite [1]. Moreover, ACR toxicity could affect absorption or metabolism of the food, resulting in a decrease in body weight [37].

The significant increase in MDA levels in the ACR group might be due to high content of lipids in the cerebellum which makes lipid peroxidation the main landmark of brain oxidative stress [42]. The decline in GSH levels in the ACR group might reflect its consumption through oxidative stress [7, 12, 41]. Supporting our results, it was reported that ACR had enhanced oxidative stress through increased formation of reactive oxygen species and lipid peroxidation while decreased superoxide dismutase activity and glutathione levels [19].

Histologically, the ACR group exhibited variable degrees of cellular degeneration, especially Purkinje cells, confirmed by the significant decrease in their number as compared to the control group. In agreement to these results, it was observed undifferentiated Purkinje cells with frequent pyknotic ones, a loss of their number and also marked oedema in the upper part of the internal granular layer in the cerebellum of drug-treated rats [15, 18]. The observed migration of Purkinje cells into the granular layer in this study might be due to the fact that ACR delays the cell proliferation in the granular layer as well as migration and differentiation [1]. These changes might be explained by the possible action of ACR to induce alterations in the cytoskeleton, membrane necrosis, free radicals, oxidative stress and mitochondrial dysfunction [41].
In this study, some specimens in Group II showed clumping of the granular layer with areas of necrosis inbetween the cells. Partially supporting these findings, it was revealed that chronic ACR administration had led to thinning of the external granular layer, which in turn delays the proliferation of the cells of this layer [1].

On GFAP immunostaining, Group II showed strong positive GFAP reaction in our study. GFAP is an important skeleton protein of astrocytes [37]. Up-regulation of GFAP is associated with proliferation and activation of astrocytes. Almost all types of brain injury may stimulate the upregulation of GFAP in reactive astrocytes, so GFAP could be used as a marker of CNS injuries. ACR had been reported to upregulate the expression of GFAP in the cerebellum in response to the Purkinje cells damage [40]. On BCL2 immunostaining of this work, although mild reaction was observed in all groups, the area per cent of positive reaction in ACR group was significantly decreased as compared to the control group. Supporting this result, it was reported that BCL2 mRNA expression had significantly decreased in PC12 cells treated with ACR implicating increased apoptosis [19].

The omega 3-treated group showed significantly improved results in comparison with those in the ACR group. Histochemically, there was significant decrease in cerebellar MDA level of omega 3-treated rats as compared to the ACR-treated ones. Also, there was significant increase in cerebellar glutathione level of omega 3-treated rats as compared to the ACR group. Concordantly, omega 3 fatty acid supplementation elevated superoxide dismutase and catalase activity in various organs such as the kidney, liver, and intestine [4]. In addition, omega 3 had significantly increase GSH and neuroprotectin D1 (NPD1) in the brain of posttraumatic brain injury in rats [37].

Oligodendrocytes precursor cells are present in the adult CNS and have NG2 and PDGFrα receptors [13]. Omega 3 had promoted oligodendrogenesis in the cerebellar cortex in Group III of this work especially at the junctional area between molecular and granular cell layers, confirmed by their positive PDGFrα immunostain and negative GFAP immunostain to exclude
ACR-induced Bergmann astrocytosis. Confirming these findings, the area per cent of PDGF positive reaction in this work was significantly increased in Group III as compared to all groups. Supporting our results, the combined treatment of fish oil dietary supplement and omega 3 polyunsaturated fatty acids injections had been proved to promote post-TBI restorative processes in the brain, including generation of immature neurons, microvessels, and oligodendrocytes, each of which was significantly correlated with the improved cognitive recovery [30]. Oligodendrocytes were abundant in H&E sections of omega 3 plus-treated rats in this work. Oligodendrocytes are identified histologically by having condensed, rounded nuclei and unstained cytoplasm due to very abundant Golgi complexes, which stain poorly [28]. The observed oligodendrocytes in this work were in the grey matter of cerebellum in its molecular layer (i.e. satellite oligodendrocytes). It was suggested that the function of this type of oligodendrocytes is to monitor the extracellular fluid around neuronal cell bodies, act in a reserve capacity, and, if the need arises, they may migrate into the white matter to replenish interfascicular oligodendrocytes [14].

Omega 3 polyunsaturated fatty acids (n-3 PUFAs) exist abundantly in the brain and play a crucial role in essential neuronal functions, such as axonal guidance, synapse and dendrite formation, neurotransmission, etc. [17, 20]. Other mechanisms by which omega 3 polyunsaturated fatty acids exert potent protective effects following experimental TBI had been reported, for example, amelioration of oxidative stress [33], mitigation of endoplasmic reticulum stress [3] and modulation of microglial activation [16].

The present work showed improvement in the histopathological findings with the co-administration of green tea extract with ACR. Interestingly, the protective effects of green tea extract against neurotoxicity caused by ACR exposure, both in vivo and in vitro, was attributed to antioxidant effects of this extract [11]. Both epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), which are green tea catechins, showed inhibitory effects on ACR neurotoxicity. They increased GSH level and decreased lipid peroxidation in rat cerebral cortex [12]. A study on the PC12 cell line of pheochromocytoma revealed that EGCG attenuates ACR-induced neurotoxicity in PC12 cells by maintaining mitochondrial function and regulating the expression of BAX and BCL2 mRNA and redox state [19].

Optical density of silver staining in this work was significantly lower in the ACR-treated group as compared to the control. This might indicate the demyelinating effect of ACR. Having the major role in myelinating central axons, each oligodendrocyte can myelinate individual intermodal segments of an average of 30 separate axons (as high as 60 axons); adjacent internodal segments are myelinated by different oligodendrocytes [13]. This pattern of central myelination leaves periodic nodes of Ranvier bare, separating between adjacent segments, with sodium channels, at which action potentials are reinitiated as they travel down the myelinated axon and its branches (called saltatory conduction) [8, 13]. Both omega 3 and green tea significantly increased the optical density of silver staining of the cerebellar axons as compared to ACR-treated rats in the current work; this might suggest their myelinating role and their potential oligodendrocytes recruitment. Oligodendrocytes can be attacked by antibodies directed at specific oligodendrocyte proteins in multiple sclerosis, leading to oligodendrocyte death and axonal dysfunction [13]. Also, they could be attacked by a polyoma virus (JC virus) causing demyelination of axons especially in the occipital and parietal lobes of the brain leading to progressive multifocal leukoencephalopathy [14].

Clinically implicated from the current work, omega 3 might have an oligodendrogenic role and might be used in CNS injury to help myelin sheath regeneration. However, the exact mechanism by which omega 3 induces oligodendrogenesis is not clear and still needs further investigations. Also, this oligodendrogenic role after already established CNS injury should be thoroughly investigated.

**CONCLUSIONS**

In conclusion, ACR causes adverse cerebellar changes. Concomitant administration of omega 3 or green tea with ACR might mitigate these adverse changes thanks to an oligodendrogenic effect of omega 3.

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