



Toxicological Effects of Acrylamide on Testicular Function and Immune Genes Expression Profile in Rats

Wagdy K. B. Khalil^a, Hanaa H. Ahmed^b, Hanan F. Aly^{*c}, Mariam G. Eshak^a

^aCell Biology Department, National Research Center, Cairo, Egypt.

^bHormones Department of Cell Biology, National Research Center, Cairo, Egypt.

^cTherapeutic Chemistry Department, National Research Center, Cairo, Egypt.

*Corresponding author's E-mail: Hanan_abduallah@yahoo.com

Accepted on: 26-10-2013; Finalized on: 31-12-2013.

ABSTRACT

The potential toxicity of acrylamide in humans had arisen with the finding of acrylamide formation in some processed foods. This study was designed to evaluate the ability of acrylamide at the dose levels of 5, 10 and 15 mg kg⁻¹ b.w. to induce oxidative stress, testicular dysfunction and alterations in the expression of testicular and immune response genes in rats. Eighty adult male albino rats are enrolled in the present study. From which thirty rats were orally administered with acrylamide daily for four weeks. The other thirty rats were orally administered with acrylamide daily for 12 weeks. The last twenty rats were served as control. Blood, testis and liver samples were collected at the end of the experimental period. The present results clearly demonstrate, plasma total antioxidant capacity, total testosterone and free testosterone levels were decreased while, plasma malondialdehyde (MDA) level was increased in acrylamide-intoxicated group in a dose and time dependant manner. DNA damage and micronucleus formation were increased in acrylamide groups compared with control rats. mRNA analysis revealed that genes related to testicular hormones were down-regulated, however, immune response related genes were up-regulated more obviously in acrylamide-intoxicated rats for 12 weeks than those intoxicated for four weeks. Thus, it could be concluded that, acrylamide induces imbalance in the oxidant /antioxidant status, impairment in the testicular function and perturbations in the immune response genes in rats.

Keywords: Acrylamide, genes expression, antioxidant, genotoxicity, immune response, testicular hormones.

INTRODUCTION

Many types of food processing techniques have been employed throughout human history, mainly to ensure microbiological and chemical safety of foods and to improve palatability. Growing consumer demand for healthy nutritious and convenient food is a key driver for improvements and new developments in food processing. New processes or newly recognized compounds, often identified due to improved analytical capabilities, require careful evaluation of potential human health impact. As examples for processing-related contaminants, the risk assessments for 3-monochloropropanediol (3-MCPD) and acrylamide are discussed¹.

Since the finding that, acrylamide is formed in food during heat processing and preparation of food, much effort has been (and still is being) put into to understand mechanism of its formation, on developing analytical methods and determination of levels in food, and on evaluation of its toxicity and potential human health consequences. Although, several exposure estimations have been proposed, a systematic review of key information relevant to exposure assessment is currently lacking².

Acrylamide has been found in various heated carbohydrate-rich food and is now classified as a suspected human carcinogen by the International Agency for Research on Cancer³. Since, international organizations such as World Health Organization, Food

and Agriculture Organization of the United Nations and Joint Institute for Food Safety and Applied Nutrition/National Center for Food Safety and Technology⁴ have found acrylamide in various foodstuffs.

Acrylamide is a heat-induced contaminant naturally formed during home cooking and industrial processing of many foods consumed daily around the world. French fries, potato crisps, bread, cookies, and coffee exert the highest contribution to dietary exposure of acrylamide to humans⁵. Acrylamide is formed during frying, roasting, and baking and is not typically found in boiled or microwaved foods. The highest acrylamide levels have been found in fried potato products, bread and bakery wares, and coffee. It is also an industrial chemical used in polyacrylamide production⁶. Scientists demonstrated that acrylamide (AA, 2-propenamide, CAS RN 79-06-1), was produced when starchy foods (e.g., potatoes) were cooked at high temperatures⁷. The presence of acrylamide in the human diet and other sources, including industrial polyacrylamide production, generated concern worldwide about possible risks to human health^{8,9}. The daily intakes of dietary acrylamide for the general population and high consumers (including children) are estimated to be on average 1 and 4 mg/kg body wt, respectively. Children eat more acrylamide than adults probably because of their higher caloric intake relative to body weight as well as their higher consumption of certain acrylamide-rich foods, such as French fries and potato crisps¹⁰.



Furthermore, food safety international bodies and industrial sectors are very active for implementing strategies to minimize its formation during roasting, baking, frying, toasting, etc. Given the prevalence of acrylamide in the human diet and its toxicological effects, it is a general public health concern to determine the risk of dietary intake of acrylamide. However, associations between dietary acrylamide exposure and increase risk of different cancers are somewhat controversial and do not have a direct extrapolation to the global population. Accordingly, further long-term studies with a general view are ongoing to clarify the risk scenario and to improve the methodology to detect small increases in cancer incidence¹¹.

Shortly after its discovery in foods, it has been clearly established that the major pathway for acrylamide formation in foods is Maillard (MR) reaction with free asparagine as main precursor¹². 3-aminopropionamide is an intermediate in MR, can also form by enzymatic decarboxylation of free asparagine and yield acrylamide upon heating even in the absence of a carbonyl source¹³. Asparagine can thermally decompose by deamination and decarboxylation but when a carbonyl source is present the yield of acrylamide from asparagine is much higher explaining the high concentration of acrylamide detected in foods rich in reducing sugars and free asparagine such as fried potatoes and bakery products¹⁴. Other minor reaction routes for acrylamide formation in foods have been postulated, from acrolein, acrylic acid and wheat gluten¹⁵. Finally, acrylamide can be generated by deamination of 3-aminopropionamide (3-APA)¹³. A simple and cost-effective method using high-performance liquid chromatography coupled with a diode array detector has been applied for determination of acrylamide in baked and deep-fried Chinese foods¹⁶. The metabolism of acrylamide is important for the safety evaluation, and has been studied extensively, and glycidamide (its metabolite) has been shown to be a major reactive metabolite having a major role in acrylamide carcinogenicity in rodents¹⁷.

Oxidative stress and mitochondrial dysfunctions have been demonstrated to be key mechanisms in many chemical-induced cell injuries and neurodegenerative diseases. Oxidative stress refers to enhanced generation of reactive oxygen species/reactive nitrogen species and/or depletion of antioxidant defense system, causing an imbalance between pro-oxidants and antioxidants leading to apoptosis¹¹.

The early clinical symptoms following exposure to acrylamide include paraesthesia, muscle weakness, absence of tendon reflex, ataxic gait, foot drop, and muscular atrophy. Furthermore, increased body burden of acrylamide has been shown to retard the recovery from neurotrauma¹⁸.

Sub-chronic exposure to acrylamide could affect the normal development of sperm, cause changes of the activity of some enzymes in the testis and significantly

influence hind limb motor coordination. Acrylamide directly damages Leydig cells and affects the endocrine function of the testis¹⁹. In fact it is believed that acrylamide is clearly genotoxic both in somatic and germ cells¹⁸.

Acrylamide has been tested for its effects on reproductive parameters, including decreased sperm count and increased abnormal sperm morphology¹⁸. Song et al¹⁹, observed that sub-chronic exposure to acrylamide could affect the normal development of spermatozoa, especially decreased sperm vitality, and increased the percentage of abnormal sperm. It has toxic effects on seminiferous tubules and it decreases the production of sperm in male rats¹⁸. The results of Yang et al.²⁰ indicated that acrylamide induces histopathological lesions such as formation of multinucleated giant cells, vacuolation and production of high numbers of apoptotic cells in the seminiferous tubules of the rat. They also observed the dose-dependent effect of acrylamide on reducing serum testosterone level and Leydig cell viability, which resulted in diminished spermatogenesis; and the high doses of acrylamide can reduce the body weight, testis weight and epididymides weight¹⁸. Plasma membrane function activity is one of the most important aspects of sperm biology, involving metabolic exchanges with the surrounding medium which play an important role in several events during fertilization (e.g. capacitation, acrosome reaction and sperm-oocyte fusion)¹⁸. The present study is planned to explore, the toxicological effects of acrylamide on the oxidant /antioxidant status as well as testicular function of adult male rats. In addition to, we hypothesized that the differential expression of the genes involved in testicular function could contribute to the reproductive toxicity associated with acrylamide exposure. To the best of our knowledge, the toxicological effects of acrylamide on immune response genes expression profile in rat have not been reported in the literatures. Therefore, using on liver and testis isolated from acrylamide intoxicated rats, we examined the relation between potential reproductive toxicity and immune response differential gene expression profile in rats through RT-PCR analysis which has two key advantages; high detection sensitivity with wide dynamic range and quantitative data generation with good reproductively.

MATERIALS AND METHODS

Chemicals and kits

Acrylamide

Acrylamide was purchased from Merck-Schuchardt Chemical Co. (Hohenbrunn, Germany) with a molecular formula $\text{NH}_2\text{-C=O-CH-CH}_2$, the purity was about 99%.

Kits

Colorimetric kits for the determination of plasma malondialdehyde and total antioxidant capacity were purchased from Biodiagnostic Co. (Egypt). Enzyme Linked Immuno – Sorbent assay (ELISA) kit for the determination



of plasma testosterone was purchased from ADALTIS Co. (Italy) and for determination of plasma free testosterone was purchased from DiaMetra Co. (Italy).

Experimental Animals

Eighty adult albino male rats weighing 140-160 g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. The animals were kept individually in wire bottomed cages at room temperature ($25 \pm 2^\circ\text{C}$) under 12 h dark-light cycles. They were maintained on standard laboratory diet and water *ad libitum*. The animals were allowed to acclimatize their new conditions for one week before commencing experiment, and then they were allocated into eight groups (10 rats / group). All animals received human care in compliance with the guidelines of the Ethical Committee of Medical Research, National Research Centre, Egypt.

Experimental design

The doses of acrylamide and the route of administration were selected based on their citation in the literature. After an acclimatization period of one week, the animals were classified into the following groups: group (1) untreated control group (CONT1) for 4 weeks treatment period; group (2) orally treated with acrylamide dissolved in water ($5 \text{ mg kg}^{-1} \text{ b.w.}$) for 4 weeks²⁰; group (3) orally treated with acrylamide dissolved in water ($10 \text{ mg kg}^{-1} \text{ b.w.}$) for 4 weeks²¹; group (4) orally treated with acrylamide dissolved in water ($15 \text{ mg kg}^{-1} \text{ b.w.}$) for 4 weeks²²; group (5) untreated control group (CONT2) for 12 weeks treatment period; group (6) orally treated with acrylamide dissolved in water ($5 \text{ mg kg}^{-1} \text{ b.w.}$) for 12 weeks; group (7) orally treated with acrylamide dissolved in water ($10 \text{ mg kg}^{-1} \text{ b.w.}$) for 12 weeks; group (8) orally treated with acrylamide dissolved in water ($15 \text{ mg kg}^{-1} \text{ b.w.}$) for 12 weeks.

At the end of the experimental period, either 4 weeks or 12 weeks, fasting blood samples were withdrawn from the retro-orbital venous plexus under diethyl ether anesthesia. Blood samples were received in EDTA-containing tubes and centrifuged at $1800 \times g$ for 10 minutes at 4°C where plasma was separated and stored at -20°C for biochemical analyses.

After blood collection, the animals were rapidly sacrificed and both the liver and testes of each animal were dissected, thoroughly washed with isotonic saline, dried on filter paper and weighed. Immediately, each individual liver and testis were stored in liquid nitrogen and stored at -80°C for gene expression analysis of genes related to testicular-functions and immune response.

Biochemical analysis

Plasma malondialdehyde (MDA) was assayed colorimetrically according to the method described by Satoh²³. Plasma total antioxidant capacity was determined colorimetrically according to the method of Koracevic et al.²⁴ Quantitative measurements of plasma

total testosterone and free testosterone were performed by enzyme linked immunosorbent assay (ELISA) procedure according to the methods of Granoff and Abraham²⁵ and Rydberg et al.²⁶

Genetic analysis

a) Comet assay for DNA strand break determination

Isolated hepatic cells of all groups of rats were subjected to the modified single-cell gel electrophoresis or comet assay^{27,28}. To obtain the cells, a small piece of the liver was washed with an excess of ice-cold Hank's balanced salt solution (HBSS) and minced quickly into approximately 1 mm^3 pieces while immersed in HBSS, with a pair of stainless steel scissors. After several washings with cold phosphate-buffered saline (to remove red blood cells), the minced liver was dispersed into single cells using a pipette. In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a fluorescence microscope (Olympus BX60 F-3) with a green filter at $\times 40$ magnifications. For each experimental condition, about 100 cells (about 25 cells per fish) were examined to determine the percentage of cells with DNA damage that appear like comets. The non-overlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between $1\times$ and $2\times$ the nuclear diameter; and class 3 = tail longer than $2\times$ the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus²⁹. A total damage score for each slide was derived by multiplying the number of cells assigned to each class of damage by the numeric value of the class and summing up the values. Slides were analyzed by one observer to minimize the scoring variability.

b) Micronucleus test

The bone marrow cells of rats re-suspended in a small volume of fetal calf serum on a glass slide were used for smear preparation. The smear of bone marrow cells was prepared from each rat. After air-drying, the slide was fixed in methyl alcohol for 10 min and stained with 5% Giemsa stain for 10 min. Three slides were prepared for each animal and were coded before observation and one was selected for scoring. From each coded slide, 2,000 polychromatic erythrocytes (PCEs) were scored for the presence or micronuclei under oil immersion at high power magnification. In addition, the percentage of micronucleated polychromatic erythrocytes (%MnPCEs) was calculated on the basis of the ratio of MnPCEs to PCEs³⁰.

c) Semi-quantitative RT-PCR

RNA extraction

Total RNA was isolated from 100 mg of tissue samples by the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 µl molecular biology grade water. In order to remove any possible genomic DNA contamination, the total RNA samples were pre-treated using DNA-free™ DNase removal reagents kit (Ambion, Austin, TX, USA) following the manufacturer's protocol.

Reverse transcription

The complete Poly(A)+RNA samples were reverse transcribed into cDNA in a total volume of 20 µl using 1 µl oligo(dT) primer. The composition of the reaction mixture, termed as master mix (MM), consisted of 50 mM MgCl₂, 10x reverse transcription (RT) buffer (50 mM KCl; 10 mM Tris-HCl; pH 8.3; Perkin-Elmer), 10 mM of each dNTP (Amersham, Brunswick, Germany), and 50 µM of oligo (dT) primer. The RT reaction was carried out at 25°C

for 10 min, followed by 1 h at 42°C, and finished with denaturation step at 99°C for 5 min. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through polymerase chain reaction (PCR).

Polymerase chain reaction (PCR)

The first strand cDNA from different samples was used as templates for RT-PCR with a pair of specific. The sequences of specific primer and product sizes are listed in Table 1. β-Actin was used as a housekeeping gene for normalizing mRNA levels of the target genes. The reaction mixture for RT-PCR was consisted of 10 mM dNTP's, 50 mM MgCl₂, 10x PCR buffer (50 mM KCl; 20 mM Tris-HCl; pH 8.3; Gibco BRL, Eggenstein, Germany), and autoclaved water. The PCR cycling parameters were one cycle of 94°C for 3 min, 35 cycles of 94 °C for 30 s, 42 °C to 58 °C for 30 s, 72 °C for 90 s, and a final cycle of 72 °C for 7 min. The PCR products were then loaded onto 2.0% agarose gel, with PCR products derived from β-actin of the different samples.

Table 1: Primers and reaction parameters in RT-PCR

Target cDNA	Primer name	Primer sequence (5'–3')	Annealing temperature (°C)	PCR product size (bp)
β-Actin	Act-F	CCCCATCGAGCACGGTATTG	57	189
	Act-R	ATGGCGGGGGTGTGAAGGTC		
CAG	CAG-F	GTG CGC GAA GTG ATC CAG A	57	178
	CAG-R	GTT TCC TCA TCC AGG ACC AGGTA		
SIMP	SIMP-F	ACCCCAGTCCCAGCGTAGTG	58	331
	SIMP-R	ATGTCGTCGCCTGAGTATCCAATC		
GARP	GARP-F	CCTTCACGGCAACAACATCC	54	1,656
	GARP-R	ATCACGCTAATCCACAACAC		

Table 2: Plasma MDA level in the different studied groups

Treatment duration	Groups	Malondialdehyde (MAD) mM/mL	P values
4 Weeks	Control	19.06 ± 0.583	
	5mg kg ⁻¹ AA	21.80 ± 0.406	≤ 0.0001
	10mg kg ⁻¹ AA	22.26 ± 0.19	0.32*
	15mg kg ⁻¹ AA	22.88 ± 0.49	0.072**
12 Weeks	Control	19.55 ± 0.30	
	5mg kg ⁻¹ AA	22.16 ± 0.191	≤ 0.0001
	10mg kg ⁻¹ AA	22.50 ± 0.19	0.342*
	15mg kg ⁻¹ AA	23.43 ± 0.211	0.064**

P ≤ 0.05 is considered significant, P > 0.05 is considered insignificant; P values control P* versus 5 mg/kg AA group, P** versus 10 mg/kg⁻¹.

Statistical analysis

Data are presented as mean ± standard error and were analyzed statistically by student's t-test³¹ with a preset probability level of p < 0.05 that was considered to be statistically significant and p < 0.01 which was considered to be highly significant.

RESULTS

Biochemical data

The results presented in Table 2 illustrated the effect of different treatment with AA for four and 12 weeks on plasma lipid peroxidation level represented by MDA. Significant increase in plasma MDA level was detected in AA treated group as compared to the corresponding

control group in a dose dependant manner and with slight increase in MDA level was noticed goes from 4 to 12 weeks.

The total antioxidant capacity decreased significantly in animals treated with AA at different doses for 4 and 12 weeks compared with the corresponding control animals in a dose dependant manner, with slight decrease in the total antioxidant capacity goes from 4 to 12 weeks (Table 3).

The results depicted in Table 4 showed the effect of different treatments with AA for 4 and 12 weeks on

plasma total and free testosterone levels. Significant decrease in total testosterone level was demonstrated in AA treated animals as compared to the corresponding control rats in a dose dependant manner with slight decrease in plasma testosterone goes from 4 to 12 weeks. In addition, free testosterone level showed significant decline in AA treated animals as compared to the corresponding control rats in a dose dependent manner with slight decrease in plasma testosterone level goes from 4 to 12 weeks.

Table 3: Plasma total antioxidant capacity in the different studied groups

Treatment duration	Groups	Total antioxidant mM/L	P values
4 Weeks	Control	3.50 ± 0.20	
	5mg kg ⁻¹ AA	3.24 ± 0.20	≤0.156
	10mg kg ⁻¹ AA	2.90 ± 0.26	0.305*
	15mg kg ⁻¹ AA	2.71 ± 0.15	0.446**
12 Weeks	Control	3.59 ± 0.16	
	5mg kg ⁻¹ AA	2.98 ± 0.15	0.0149
	10mg kg ⁻¹ AA	2.68 ± 0.15	0.212*
	15mg kg ⁻¹ AA	2.50 ± 0.17	0.454**

P ≤ 0.05 is considered significant, P > 0.05 is considered insignificant; P values control P* versus 5 mg/kg AA group, P** versus 10 mg/kg⁻¹.

Table 4: Plasma total and free testosterone level in the different studied groups.

Treatment duration	Groups	Total testosterone (ng/mL)	P values	Free testosterone (ng/mL)	P values
4 Weeks	Control	3.50 ± 0.30		30.90 ± 2.28	
	5mg kg ⁻¹ AA	1.34 ± 0.18	≤0.0001	10.50 ± 0.28	≤0.0001
	10mg kg ⁻¹ AA	1.15 ± 0.10	0.397*	7.97 ± 0.30	0.012*
	15mg kg ⁻¹ AA	0.90 ± 0.14	0.186**	6.80 ± 0.26	0.216**
12 Weeks	Control	3.60 ± 0.29		31.00 ± 2.09	
	5mg kg ⁻¹ AA	1.10 ± 0.07	≤ 0.0001	10.06 ± 0.95	≤ 0.0001
	10mg kg ⁻¹ AA	0.90 ± 0.08	0.237*	7.00 ± 0.91	0.0039*
	15mg kg ⁻¹ AA	0.70 ± 0.07	0.147**	6.10 ± 0.32	0.1052**

P ≤ 0.05 is considered significant, P > 0.05 is considered insignificant; P values control P* versus 5 mg/kg AA group, P** versus 10 mg/kg⁻¹.

Table 5: Visual score of DNA damage in male rats treated with different doses of acrylamide for different time intervals

Treatment duration	Groups	No. of cells		Class*				DNA damaged cells (% mean)
		Analyzed	Comet	0	1	2	3	
4 weeks	Control	100	4	96	4	0	0	4
	5mg kg ⁻¹ AA	100	12	88	9	2	1	12*
	10mg kg ⁻¹ AA	100	18	82	10	5	3	19**
	15mg kg ⁻¹ AA	100	26	74	5	9	12	26***
12 weeks	Control	100	5	95	4	1	0	5
	5mg kg ⁻¹ AA	100	16	84	9	4	3	16*
	10mg kg ⁻¹ AA	100	23	77	12	5	6	23**
	15mg kg ⁻¹ AA	100	35	65	8	13	14	35***

: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. Data are presented as mean ± SEM. Values marked with an asterisk () are significantly different (P < 0.05), (**) are significantly different (P < 0.01), and (***) are significantly different (P < 0.001).

DNA strand break determination using comet assay

Results of the comet assay for DNA strand break in individual liver cells of male rats exposed to several doses of acrylamide for different time intervals are summarized

in Table 5. The results showed that considerable DNA damage was observed in a dose-dependent manner. These results showed that in comparison to control rats DNA damaged cells were significantly higher in rats

exposed to low, medium and high doses of acrylamide ($P < 0.5$, $P < 0.01$, $P < 0.001$, respectively). These effects of acrylamide induced DNA damage were showed at the both time intervals, however, the effect of acrylamide was higher in the period of 12 weeks than for 4 weeks of the exposure.

Additionally, the DNA damaged cells categorized in class 3 defined with long tail of damage were also higher in rats exposed to high dose of acrylamide than low or medium doses of acrylamide groups. Moreover, rats treated for 12 weeks showed more damage in the DNA categorized in class 3 than those treated with 4 weeks of acrylamide (Table 5).

MnPCEs formation

The effect of different doses of acrylamide for different time intervals (4 and 12 weeks) on MnPCEs formation in the bone marrow cells of male rats is summarized in Figure 1). The results revealed that formation of MnPCEs in the bone marrow cells increased in a dose-dependent manner. MnPCEs formation induced by acrylamide was lower in the 4 week treatment than in the 12 weeks treatment (Fig. 1a & 1b). Moreover, at the 4 weeks treatment, exposure of the male rats with low dose of acrylamide did not increase significantly the MnPCEs formation compared with control group (Fig. 1a). However, this increase in the MnPCEs formation with the low dose of acrylamide was significantly at the 12 weeks treatment (Fig. 1b). Additionally, the MnPCEs formation increased significantly in the male rats treated with medium and high doses of acrylamide compared with control group either in the 4 or 12 weeks treatment (Fig. 1a&1b).

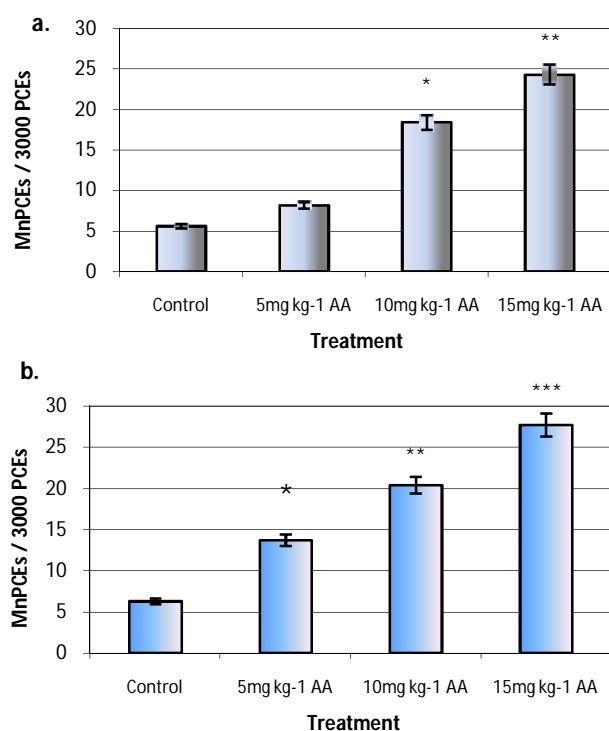


Figure 1: Micronucleated polychromatic erythrocytes (MnPCEs) of male rats treated with different doses of

acrylamide (AA) for 4 (a) or 12 weeks (b). Values marked with an asterisk (*) are significantly different ($P < 0.05$), (**) are significantly different ($P < 0.01$), and (***) are significantly different ($P < 0.001$).

Semi-quantitative RT-PCR

The effect of different doses of acrylamide on the gene expression profile of several genes related to reproduction; cytochrome aromatase gene (CAG) and immune system response such as: glycoprotein A repetitions predominant (GARP) and immune-dominant MHC-associated peptides (SIMP) was investigated. RT-PCR assay was conducted to perform the expression of these genes in the liver and testis tissues of male rats treated with different concentrations (5, 10 and 15 mgkg⁻¹ b.w) of AA for 4 and 12 weeks.

The expression of CAG gene in liver and testis tissues of control and treated group with low dose (5 mg kg⁻¹ b.w.) of AA for 4 weeks was up-regulated (Fig. 2). However, treatment of male rats with medium and high doses of acrylamide for 4 weeks down-regulated the expression of CAG gene in liver and testis tissues. Moreover, CAG gene expression within liver and testis tissue samples of rats treated with all doses of AA for 12 weeks was down-regulated (Fig. 3).

On the other hand, SIMP and GARP genes expression were up-regulated in liver and testis samples isolated from rats treated only with 10 and 15 mg kg⁻¹ b.w. of AA at 4 weeks (Fig.2). While, at 12 weeks treatment the expression of SIM and GARP genes in liver and testis tissues collected from rats treated with low, medium and high doses of AA was highly up-regulated (Fig. 3).

DISCUSSION

In the current study, the level of lipid peroxidation product (MDA), in the plasma of rats treated with 5, 10 and 15mg kg⁻¹ day of acrylamide increased from 4 to 12 weeks than control. In contrast, the levels of total antioxidant capacity in the plasma of rats treated with 5, 10 and 15 mg kg⁻¹ day⁻¹ of acrylamide decreased from 4 to 12 weeks than control. These findings could be attributed to the metabolism of acrylamide in the body causing the generation of reactive oxygen species (ROS), which play a pivotal role in the oxidative stress of acrylamide causing oxidative DNA damage^{11,32}.

In the present study, we have found that acrylamide induced DNA damage and increased the micronucleus formation especially with the high dose and for long term treatment.

It has been reported that acrylamide is a weak clastogen which acts through protein binding rather than DNA binding³³. However, data in the *in vivo* micronucleus test estimated that acrylamide induces micronucleus formation starting from a dose of 4 mg/kg/day³³. In agreement with these finding our data reported that acrylamide began to induce significantly DNA damage the micronucleus formation starting from the dose of 5 mg/kg

and increased continuously with the higher doses. To explain the mechanism of acrylamide inducing genotoxicity, it has been reported that most sensitive endpoints of genetic toxicity for acrylamide are kinesin inhibition³⁴ and oxidative stress³⁵. These enzymes centre and then segregate the chromosomes and then depolymerise the mitotic spindles. Inhibition of kinesin is consistent with the mitotic inhibition and the aneuploidy observed *in vitro* in fibrosarcoma cells³⁴.

Acrylamide has toxic effect on seminiferous tubules and it decreases the production of sperm in male rats³⁶. Also, acrylamide has been found to induce histopathological lesions in testes such as formation of multinucleated giant cells and vacuolation as well as apoptosis in the cells of the seminiferous tubules¹⁸. cDNA microarray analysis revealed that genes related to testicular-functions, apoptosis, cellular redox, cell growth, cell cycle, and nucleic acid-binding are up/down-regulated in testes due to acrylamide treatment which affects motor proteins and therefore, sperm function, while the dominant lethal mutations resulted from interaction of the metabolite glycidamide with chromatin, leading to clastogenesis^{19,20}. Additionally, acute and sub-chronic acrylamide intoxication caused a major depletion in plasma level of testosterone which resulted from the elevation in the levels of beta-endorphin and substance P in the hypothalamus and met-enkephalin levels in the frontal cortex³⁷.

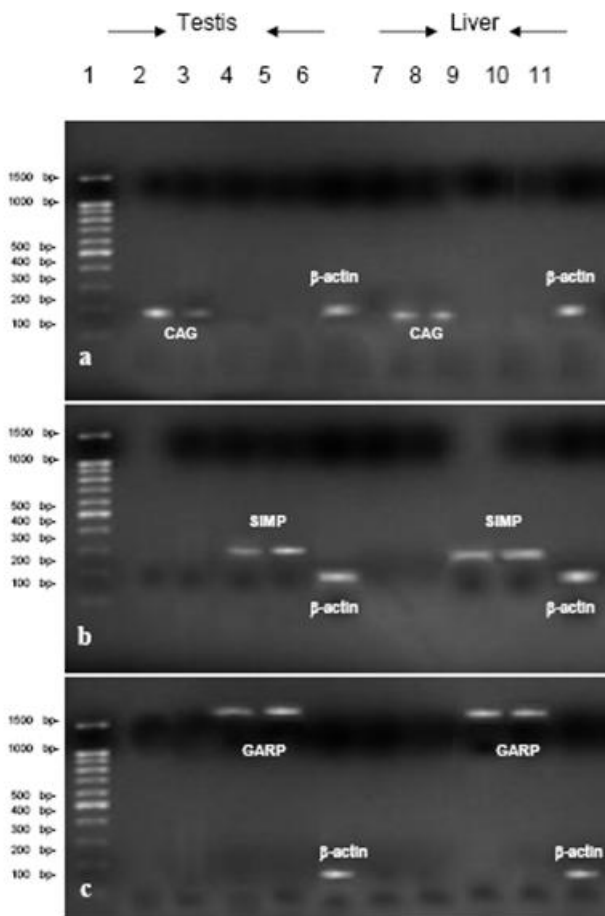


Figure 2: RT-PCR confirmation of one reproductive gene (CAG) and two different expressed immune genes (SIMP and GARP) in male rats treated with acrylamide (5 mg kg⁻¹ in lanes 3&8; 10 mg kg⁻¹ in lanes 4&9; 15 mg kg⁻¹ b.w. in lanes 5&10) for 4 weeks. Control (lanes 2 & 7) and treated samples were normalized on the bases of β-actin expression (lanes 6 & 11).

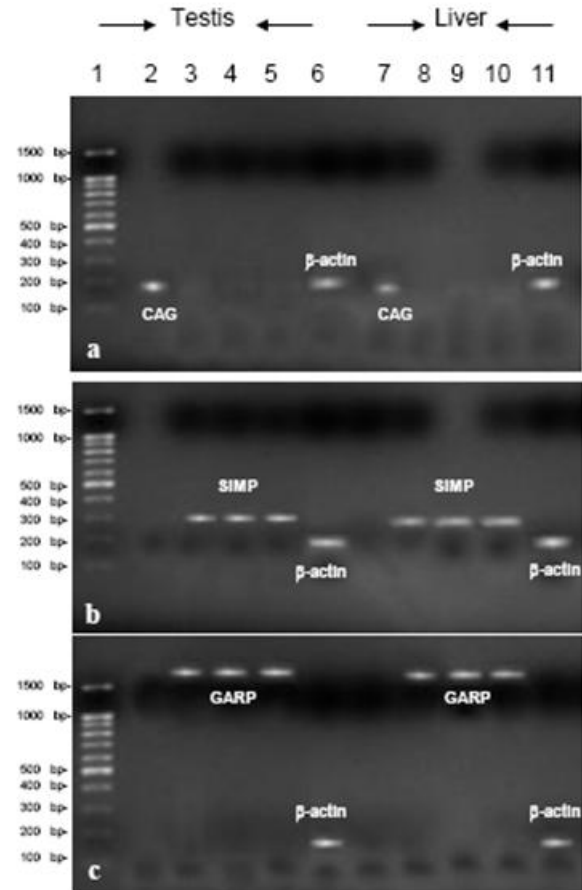


Figure 3: RT-PCR confirmation of one reproductive gene (CAG) and two different expressed immune genes (SIMP and GARP) in male rats treated with acrylamide (5 mg kg⁻¹ in lanes 3&8; 10 mg kg⁻¹ in lanes 4&9; 15 mg kg⁻¹ b.w. in lanes 5 & 10) for 12 weeks. Control (lanes 2&7) and treated samples were normalized on the bases of β-actin expression (lanes 6 & 11).

The present study indicate that testis considered to be a target organ of acrylamide action as it caused significant decrease in plasma free and total testosterone in a dose dependant manner and these findings are in agreement with Adler et al.³⁰ and Yang et al.²⁰ concluded that, the dominant lethal effects resulted from a different mechanism in spermatids of male mice than the other parameters due to acrylamide toxicity. One mechanism suggested, transcriptional profiling of 542 mitochondria – related genes indicated a significant down- regulation of genes associated with the 3-beta hydroxyl steroid dehydrogenase family in acrylamide treated rats³⁸. In addition, acrylamide significantly induced expression levels of genes associated with lipid metabolism and oxidative phosphorylation in particular ATP synthase,

which correlated with elevated ATP levels, indicating an increased energy demand in liver during acrylamide exposure³⁹.

The findings of the current study are consistent with previous findings. In this study, an attempt was made to evaluate the relationship between toxicological effects of AA and differential gene expression profiles in acrylamide treated rats. The toxic influence of AA on the reproductive and immune response genes was conducted in testis and liver of male rats. The results of the present study indicated that expression of CAG gene, which plays an important role in rodent reproductive performance, was down-regulated in testis tissues of all of samples exposed to AA for 12 weeks. In agreement with these findings, Yang et al.²⁰ found reduction in sperm concentration in cauda epididymis, as well as increase in morphological abnormalities of sperm of acrylamide-treated rats.

Tyl and Friedman⁴⁰ summarized the mechanisms of AA toxicity in which AA binding to motor proteins (e.g. kinesin and dynein) causes distal axonopathy, and affects sperm motility. In addition, up-regulation of dynein-associated protein RKM23 gene was observed in testes isolated from the AA treated rats²⁰. This protein is closely associated with dynein heavy chains and may directly regulate motor functions⁴¹. The potential function of dynein-associated protein RKM23 gene in spermatid transportation in Sertoli cells⁴¹ suggested that its differential expression could contribute to the reproductive toxicity associated with AA exposure. On the basis of the previous findings, we can suggest that AA could inhibit the reproductive activity of male rats through decrease the expression of the CAG gene at 12 week treatment.

According to the gene expression of some immune response genes, the results of the present study indicated that GARP and SIM genes were up-regulated in testis and liver samples treated with medium and high doses of AA at 4 weeks treatment and were more highly up-regulated with all doses of AA at 12 weeks treatment. To our knowledge, the toxicological effects of AA on immune response genes expression profile in rat have not been reported in the literature.

Assessment of health hazards arising from occupational exposure to chemicals is based on the estimation of levels of known toxic chemicals in the environment. It is expected that pollution including toxic chemicals in animals would evoke an immune response. In the present study, exposure to AA stimulated the immune response in male rats. Our previous study indicated that immune response genes (GARP and SIMP) were up-regulated due to different stress agents such as environmental pollution in *Solea aegyptiaca* and stannous chloride treatment in mouse dams and fetuses^{42, 43}. Chang et al.⁴⁴ found a few protein components of acute-phase response (APR) in grass carp in response to parasite infection as part of the innate immune defense. APR was usually characterized

with the change of plasma proteins which are referred to as acute-phase proteins (APPs), and also characterized with the secretion of some other innate defense molecules⁴⁵. Bayne et al.⁴⁵ also, identified most of these components in the up-regulated libraries from infected livers.

We could also suggest that the up-regulated expression of GARP and SIMP genes in male rats may also indicate that APPs are expressed and capable of up-regulation during the AA treatment. In addition, the identification of known or novel genes involved in the pollution provides the foundation for further study on the immunological interaction between the animal and toxic chemicals, and it appears possible that construction of subtractive library may be useful for studying immune mechanisms to toxic chemicals.

REFERENCES

1. Nordin AM, Walum E, Kjellstrand P, Forsby A, Acrylamide-induced effects on general and neurospecific cellular functions during exposure and recovery. *Cell Biol Toxicol*, 19, 2003, 43–51.
2. Tritscher AM, Human health risk assessment of processing-related compounds in food, *Toxicology Letters*, 149, 2004, 177–186.
3. International Agency for Research on Cancer (IARC) some industrial chemicals, Lyon: IARC monographs on the evaluation of carcinogenic risk to humans, 60, 1994, 389-441.
4. WHO World Health Organisation, Health implications of acrylamide in food, Geneva: Report of a Joint FAO/WHO Consultation, 2002, June 25-27.
5. Friedman M, Chemistry, biochemistry, and safety of acrylamide, A review, *J Agric Food Chem*, 51, 2003, 4504–4526.
6. Amrein TM, Lukac H, Andres L, Perren R, Escher F, Amadò R, Acrylamide in roasted almonds and hazelnuts. *Journal of Agricultural and Food Chemistry*, 53, 2005, 7819-7825.
7. Tareke E, Heinze TM, Da Costa GA, Syed A, Acrylamide formed at physiological temperature as a result of asparagine oxidation, *Journal of Agriculture and Food Chemistry*, 13, 2009, 517- 522.
8. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm RA, van den Brandt PA, Dietary acrylamide intake is not associated with gastrointestinal cancer risk, *Journal of Nutrition*, 138(11), 2008a, 2229-2236.
9. Hogervorst JG, Schouten LJ, Konings EJ, Goldbohm R A, van den Brandt PA, Dietary acrylamide intake and the risk of renal cell, bladder, and prostate cancer, *American Journal of Clinical Nutrition*, 87(5), 2008b, 1428-1438.
10. Dybing E, Farmer PB, Andersen M, Fennell TR, Lalljie SPD, Muller DJG, Olin S, Petersen BJ, Schlatter J, Scholz Scimeca JA, Slimani N, Toironqvist M, Tuijtelars S, Verger P, Human exposure and internal dose assessments of acrylamide in food, *Food and Chemical Toxicology*, 43, 2005, 365–410
11. Arribas-Lorenzo G and J. Morales F, Recent Insights in Acrylamide as Carcinogen in Foodstuffs, *Molecular Toxicology*, 6, 2012, 1872-0854.
12. Stadler, RH, Acrylamide formation in different foods and potential strategies for reduction, In Friedman M, Mottram DS (Eds.), In chemistry and safety of acrylamide in food, 561, 2005, 157–169.
13. Granvogel M, Schieberle P, Quantification of 3-aminopropionamide in cocoa, coffee and cereal products. Correlation with acrylamide concentrations determined by an improved clean-up method for complex matrices, *European Food Research and Technology*, 225, 2007, 857-863.



14. Yaylayan VA, Wnorowski, A, Perez-Locas C, Why asparagine needs carbohydrates to generate acrylamide. *Journal of Agriculture and Food Chemistry*, 51, 2003, 1753-1757.
15. Claus, A, Weisz G M, Schieber A, Carle R, Pyrolytic acrylamide formation from purified wheat gluten and gluten-supplemented wheat bread rolls, *Molecular Nutrition and Food Research*, 49, 2006, 87-93.
16. Wang H Feng F, Guo Shuang S, Choi MMF, HPLC-UV quantitative analysis of acrylamide in baked and deep-fried Chinese foods, *Journal of Food Composition and Analysis*, 31, 2013, 7–11.
17. Tardiff RG, Gargas ML, Kirman CR, Carson ML, Sweeney LM, Estimation of safe dietary intake levels of acrylamide for humans, *Food and Chemical Toxicology* 48, 2010, 658–667.
18. Kermani M, Anvari M, Talebi A and Amini-Rad O, The effects of acrylamide on sperm parameters and membrane integrity of epididymal spermatozoa in mice. Effects of acrylamide exposure on serum hormones, gene expression, cell proliferation, and histopathology in male reproductive tissues of Fischer 344 rats, *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 153, 2010, 52–55.
19. Song HX, Wang R, Geng ZM, Cao SX and Liu TZ, Subchronic exposure to acrylamide affects reproduction and testis endocrine function of rats, *Zhonghua Nan Ke Xue*, 14(5), 2008, 406-410.
20. Yang HJ, Lee SH, Jin Y, Choi JH, Han DU, Chae C, Lee MH and Han CH, Toxicological effects of acrylamide on rat testicular gene expression profile, *Reprod Toxicol*, 19(4), 2005, 527-534.
21. Garey J, Ferguson SA, Paule MG, Developmental and behavioral effects of acrylamide in 344Fischer rats. *Neurotoxicol Teratol*, 27, 2005, 553-563.
22. Klaunig J E and Kamendulis LM, Mechanisms of acrylamide induced rodent carcinogenesis. *Adv Exp Med Bio*, 561, 2005, 49-62.
23. Satoh K, Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method, *Clin Chim Acta*, 90(1), 1978, 37-43.
24. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S and Cosic V, Method for the measurement of antioxidant activity in human fluids, *J Clin Pathol*, 54, 2001, 356-361.
25. Granoff AB, Abraham GE, Peripheral and adrenal venous levels of steroids in a patient with virilizing adrenal adenoma, *Obstet Gynecol*, 53(1), 1979, 111-5.
26. Rydberg P, Eriksson S, Tareke E, Karlsson P, Ehrenberg L, Tornqvist M, Investigations of factors that influence the acrylamide content of heated foodstuffs, *J Agric Food Chem*, 51, 2003, 7012–7018.
27. Fairbairn DW, Olive PL, O'Neill KL, Comet assay: A comprehensive review, *Mutat Res*, 339, 1995, 37-59.
28. Singh NP, McCoy MT, Tice RR, Schneider EL, 1988, A simple technique for quantitation of low levels of DNA damage in individual cells, *Exp Cell Res*, 175, 1995, 184-191.
29. Collins A, Dusinska M, Franklin M, Somorovska M, Petrovska H, Duthie S, Fillion L, Panayiotidis M, Raslova K, Vaughan N, Comet assay in human biomonitoring studies: Reliability, validation, and applications, *Environ Mol Mutagen* 30, 1997, 139-146.
30. Adler ID, Baumgartner A, Gonda H, Friedman MA and Skerhut M, 1- Aminobenzotriazole inhibits acrylamide-induced dominant lethal effects in spermatids of male mice, *Mutagen*, 15, 2000, 133-136.
31. Woolson R F and Bean J A, Mantel-haenszel statistics and direct standardization, *Statistics in Medicine*, 1(1), 2006, 37–39.
32. Patel HC, Boutin H and Allan SM, Interleukin-1 in the brain: mechanisms of action in acute neurodegeneration, *Ann N Y Acad Sci*, 992, 2003, 39-47.
33. Davis J and Recio L, Determination of a Micronuclei Frequency Peripheral Blood of B6C3F1 Mice Exposed Acrylamide for Four Weeks, *ILS Report*, 2007, C155-01.
34. Sickles DW, Sperry AO, Testino, A, Friedman MA, Acrylamide effects on kinesin-related proteins of the mitotic/meiotic spindle, *Toxicol Appl Pharmacol*, 222(1), 2007, 111-121
35. Yousef MI and El-Demerdash FM, Acrylamide-induced oxidative stress and biochemical perturbations in rats, *Toxicolog*, 219, 2006, 133-141.
36. Wang H, Ge JY, Zhou ZQ, Wang ZC, Shi FX, Oral acrylamide affects the development and reproductive performance of male rats, *Zhonghua Nan Ke Xue*, 13(6), 2007,492-497.
37. Camacho L, Latendresse JR, Muskhelishvili L, Patton R, Bowyer JF, Thomasc M and Doerge DR, Effects of acrylamide exposure on serum hormones, gene expression, cell proliferation, and histopathology in male reproductive tissues of Fischer 344 rats, *Toxicology Letters*, 211, 2012, 135– 143.
38. Lee T, Manjanatha MG, Aidoo A, Moland CL, Branham WS, Fuscoe JC, Ali AA, Desai VG, Expression analysis of hepatic mitochondria – related genes in mice exposed to acrylamide and glycidamide, *J Toxicol Environ Health*, 75(6), 2012, 324-39.
39. Teodor V, Cuciureanu M, Filip C, Zamostean N and Cciurean R. Protective effects of selenium on acrylamide toxicity in the liver of the rat. Effects on the oxidative stress, *Rev Med Chir Soc Med Nat lasi*, 115(2), 2011, 612-8.
40. Tyl RW, Friedman MA, Effects of acrylamide on rodent reproductive performance, *Reprod Toxicol*, 17, 2003,1–13.
41. Ye F, Zangenehpour S, Chaudhuri A, Light-induced down-regulation of the rat class 1 dynein-associated protein robl/LC7-like gene in visual cortex, *J Biol Chem*, 275, 20002, 7172–27176.
42. Ali F K, El-Shafai S A, Samhan F A, Khalil W K, Effect of water pollution on expression of immune response genes of *Solea aegyptiaca* in Lake Qarun, *African Journal of Biotechnology*, 7 (10), 2008, 1418-1425.
43. El- Makawy A, Girgis SM, Khalil WK, Developmental and genetic toxicity of stannous chloride in mouse dams and fetuses, *Mutation Research*, 657(2), 2008, 105-110.
44. Chang MX, Nie P, Liu GY, Song Y, Gao Q, Identification of immune genes in grass carp *Ctenopharyngodon idella* in response to infection of the parasitic copepod *Sinergasilus major*, *Parasitol Res*, 96, 2005, 224–229.
45. Bayne CJ, Gerwick L, Fujiki K, Nakao M, Yano T, Immune relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. *Dev Comp Immunol*, 25, 2001, 205–217.

Source of Support: Nil, Conflict of Interest: None.

