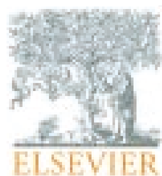

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Effects of low dose acrylamide on the rat reproductive organs structure, fertility and gene integrity

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ABSTRACT

Objective: To assesses the effects of long term exposure to low dose of acrylamide (0.4 μg /g) in post-weaning Sprague-Dawley rats on the structure of the reproductive organ as well as DNA integrity. **Methods:** The histological changes in the male and female reproductive organs the morphological changes in sperms as well as the genotoxic effect of acrylamide were assessed. The effect acrylamide on pregnancy outcome was evaluated. **Results:** Testes of acrylamide-fed rats showed decreased number of seminiferous tubules containing mature sperms and degenerative changes in sperm germ cell layers. Some sperms of epididymal cauda showed head deformity. In female, acrylamide included cystic ovarian changes, degenerative changes of zona pelluida, granulosa cells and oocytes. Post implantation loss and decrease in the number of full term fetuses were detected. Resorption sites showed necrotic fetal tissue with vacuolation of amniotic cells. **Conclusion:** Acrylamide cause harmful effect on the reproductive organ structure, fertility and cause extensive DNA damage in peripheral blood lymphocytes.

1. Introduction

Acrylamide (ACR) is carcinogenic in animal and probably carcinogenic to humans. It is carcinogenic in multiple organs in both sexes of several rodents. Glycidamide which is ACR metabolite is believed to be the cancer-risk agent in ACR exposure[1]. In rats, tumorigenesis occurs in several hormonally regulated tissues[2, 3]. Some previous studies showed that ACR is capable of inducing genotoxic, carcinogenic, developmental, and reproductive effects in tested animals. Since there is sufficient evidence of carcinogenicity in experimental animals as outlined under the U.S. environmental Protection Agency (EPA) proposed guidelines for carcinogen risk assessment, ACR is categorized as a 'B2' carcinogen and therefore be considered a 'probable human carcinogen[4].

Acrylamide is able to cross the placenta, reach significant concentrations in the conceptus and produce direct developmental and post-natal effects in rodent offspring. Acrylamide has an adverse effect on reproduction as evidenced by dominant lethal effects, degeneration of testicular epithelial tissue, and sperm-head abnormalities[5]. The finding that ACR is formed in carbohydrate rich food during preparation at high temperatures raised concern about cancer risks associated with the dietary intake of fried or backed carbohydrate food. Acrylamide is formed when frying, roasting, grilling or baking carbohydrate-rich foods at temperatures above 120 ℃ through interactions of amino acids with reducing sugar[6]. Acrylamide is thus found in a number of foods, such as bread, crisps, French fries and coffee. Tobacco smoking also generates substantial amounts of ACR. But it is the incidental formation during cooking of common starchy foods that leads to pervasive human exposure, typically in the range of 1 μg/kg body weight)/day[7]. Acrylamide neurotoxicity was reported in humans[8] and experimental animals[9]. Other toxic effects

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of ACR were reported such as chromosomal damage in somatic cells and mutagenesis[10] and disturbance in genomic imprinting during spermatogenesis[11], and recently male reproductive toxicity, prenatal lethality, and endocrine-related tumors in rodents[12-14]. The metabolism of ACR to its epoxide metabolite, glycidamide, which is mediated by cytochrome P450 2E1, is thought to be the active metabolite which plays a central role in ACR genotoxicity in experimental animals and humans[15].

The European Commission on acrylamide call for establishing an international network "Acrylamide in Food" inviting all interested parties to share relevant data as well as ongoing investigations[16]. The committee noted that the high levels of ACR formed by heat processing of food calls for urgent research to lower this formation and for research to understand the implications for human health [17]. Not enough information is available about levels of this chemical in different foods and the potential risk from dietary exposure. There is some consensus that low levels of ACR in the diet are not a concern for neurotoxicity or reproductive toxicity in humans. However, further research is needed to study the long-term, low-level cumulative effects of ACR exposure.

Although many previous researches have been conducted to study the impact of acrylamide on the different body organs, further research in this area focusing on better understand of the dose-response of health effects corresponding to dietary intake is recommended in recent studies[18].

The present work was designed to assess the cumulative effect of the exposure to low dose of ACR (0.4 μg acrylamide/gram of regular rodent food) via oral route early in post weaning period on the developing reproductive system of both male and female rat. The ACR-induced changes on the DNA integrity of lymphocytes were also assessed.

2. Materials and methods

2.1. Animals and acrylamide administration

This study was approved by the biomedical research ethics committee at King Fahed Medical research Center (KFMRC). Forty male and female Sprague-Dawley rats aged 3 weeks with an average weight of 30-35 g were used in this study. Animals were maintained on a 12 h light/dark cycle with food and sterile distilled water available ad libitum, the temperature range was 20 ℃ to 24 ℃, and the humidity range was 60% to 70%. After one week of acclimatization, animals were randomized into two main groups; control (n=10) and ACR-fed groups (n=30; 15 male and 15 female). Acrylamide ultra-pure electrophoresis grade was used in this study. The experimental ACR-fed group received diet containing ACR at a dose of 0.4 μg/g, while the control group received regular diet. The diet was prepared weekly. The total amount of acrylamide taken by the animals was equal to 60 μg/kg of body weight/daily. Exposure to ACR-contained diet was continued daily for the age of 60 and 90 days. The ACR dose and regimen adopted in the present study was according to the previous studies[19, 20].

2.2. Assessment of ACR-induced changes in reproductive organs

For sperm morphology assessment, sperms were collected from cauda in normal saline, killed by incubating the sperm suspension at 80 ℃ for 30 min. For morphological abnormalities a total of 300 sperms were smeared on glass slide examined under light microscopy at 400 x. Sperms lacking hook, banana-like head, and twin headed, and twin-tailed sperms were considered abnormal[21].

At age of 60 and 90 days, ten animals of each group were anesthetized, the chest was opened then pericardial perfusion with saline followed by 10% neutral buffered formalin was done to ensure in situ perfusion and avoid postmortem changes. The whole male and female reproductive system was removed as one block, re-fixed in 10% neutral buffered formalin. Testes, seminal vesicles, ovaries, fallopian and uterine horns were dissected out weighted and processed to obtain paraffin blocks for histological examination. The paraffin sections were stained with haematoxlin and eosin (H&E).

2.3. Assessment of ACR-induced changes on fertility outcome

Fifteen adult female rats (90 days) fed normal diets were housed with 5 ACR-fed male partner (3 female with one male in each cage). The females were checked every 24 hr for appearance of vaginal plug or the positive vaginal smear for sperm. If any appear, then this day was considered the day one of pregnancy. The number of pregnant animals was recorded. Pregnant rats were individually caged. At the day18 of pregnancy animals were sacrificed by decapitation. The abdomen was opened and pregnant uterine horns were removed and the numbers of full mature fetuses, site of resorption were recorded. The ovaries were inspected for presence of corpora lutea then processed for histological studies.

2.4. Assessment of ACR-induced genotoxicity and mutagenicity

To assess the genotxicity of pure ACR, the Single Cell Gel Electrophoresis (SCGE) or alkaline comet assay was performed according to the protocol of Hartmann and Speit[22], and was analyzed using Loat's comet assay software with extended dynamic range imaging (EDRI). A total of 2 of whole blood taken from the rats, initially collected in EDTA tubes, were used. Analysis of the comet tail was carried out using Loat's single gel comet assay software with EDRI and observed using a fluorescent microscope (Olympus BX-51; Japan). The positive control used in this assay was glycidamide (from LKT laboratories, St Paul, Minnesota, USA). It was reconstituted with distilled water. For the preparation of a negative control, control rat whole blood was used, the alkaline comet assay was performed, and the average tail moment was calculated. For the preparation of different glycidamide dilutions, glycidamide was incubated with the whole rat blood for 4 h at 37 ℃, and then the alkaline comet assay and analysis of comet tail were carried out. Slides were observed at 40× magnifications using a fluorescence microscope equipped with an excitation filter of BP 546 nm and a barrier filter of 590 nm. Images of 200 lymphocytes were randomly selected (100 cells from each of 2 replicate slides) and analyzed from each sample. Cells were automatically analyzed by Loats

comet assay software.

To assess the genotxicity of pure ACR, Salmonella typhimirium strains TA98, TA100 & TA1535 have been used. These strains are histidine requiring mutants and have been tested for its histidine requirement as[23]. Numbers of bacterial revertants for all concentrations were counted automatically by image pro-plus software (media cybernitics, USA).

3. Results

3.1. Weight changes

The present study showed that ACR administrated with the ordinary rat food have induced adverse effects on male reproductive system. The absolute weights of testes and seminal vesicles were significantly reduced in group treated with ACR which more evident in the group of animals sacrificed at 90 days. Also both ovary and uterus weight were significantly reduced in groups received ACR (Table 1).

Table 1

Effect of acrylamide on number of fetuses and organ weights.

Parameters	Control group	AM-fed group scarified at 60 days	AM-fed group scarified at 90 days
Number of full term fetuses at 18 days	10	6**	3**
Testis weight (g)	2.80±0.70	2.20±0.50*	1.60±0.35**
Seminal vesicle weight (g)	0.23±0.06	0.18±0.02*	0.15±0.01**
Ovary weight (g)	0.08±0.02	0.06±0.02*	0.05±0.01**
Uterus weight (g)	0.50±0.02	0.30±0.01**	0.20±0.01**

*P<0.05 compared to the control; **P<0.01 compared to the control.

In non-pregnant animal scarified at 90 days, the ovarian and uterine tubes weights were decreased after ACR administration. Ovary weight decreased from 0.07 in the control to 0.05 and the uterus weight decreased from 0.4 to 0.2 g.

3.2. Morphological and histological changes in male reproductive organs

Caudal sperm count (sperm/mg of cauda) of the control rats was 60 ×10^3 while the sperm counts in the ACR-fed rats was 127×10^3. The sperms in the cauda epididymis from animals fed ACR showed head deformity, ill-defined neck, long middle piece marked by fusiform swelling and a short curved tails compared to control which showed normal sperm morphology (Figure 1).

After 60 days of feeding ACR, there was increase in peri-epididymal fat mass compared to the control. Seminal vesicles and testis appeared smaller in size, the seminal vesicle was less lobulated and the testis showed congested blood vessels compared to the control group. After 90 days of feeding ACR, all these changes were obviously observed (Figure 2).

Testis of animals fed ACR showed individual variation regarding the severity of histological changes. Some testis showed atrophy and separation of semniferous tubules and interstitial edema in the testicular parenchyma (Figure 3). Germ cell series showed various degrees of apoptotic cell death in the form of shrunken cells, dark stained nuclei and highly acidophilic cytoplasm. Most tubules exhibit loss of spermatid and spermatozoa stages. Some tubules showed large distorted non-nucleated elongated spermatid stages (Figure 4).

The seminal vesicle mucosa in ACR-fed groups showed significant decrease in mucosal folding. Its columnar epithelium cells appeared with decreased height and some of them showed dark small nuclei. Submucosal edema and mucosal separation from the underlying muscle layer were also observed (Figure 5).

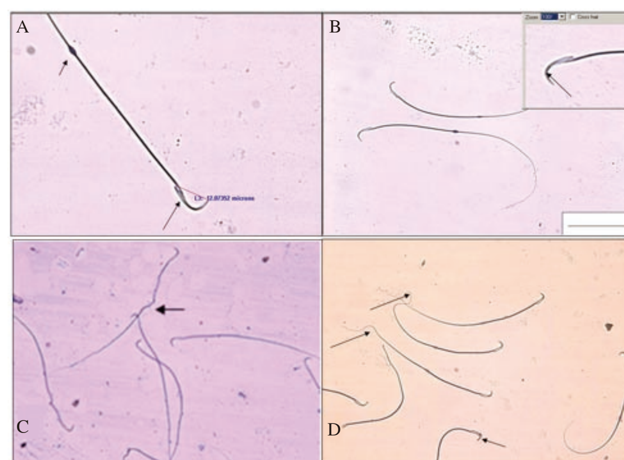

Figure 1. Caudal sperm morphology of (A, B) control rat with normal sperm heads and tails (arrow) (C, D) while ACR-fed rat showing twisted middle piece and short curved tails (black arrow) (× 40 Smears stained with Wright stain).

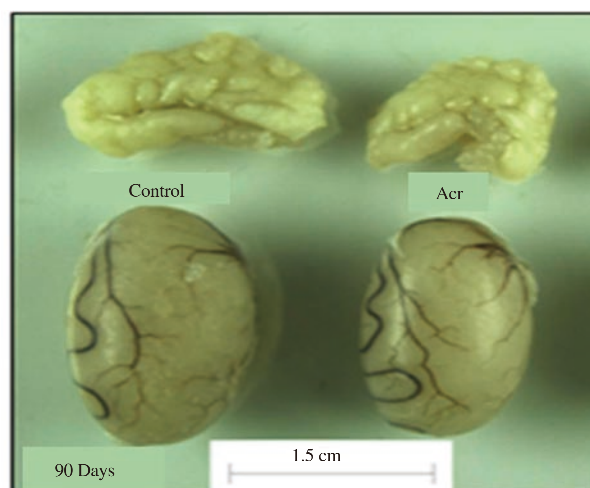

Figure 2. Morphological appearance of testes and seminal vesicles of ACR-fed rats at 90 days of age showing decreased size of both organs and less convolution of the seminal vesicle.

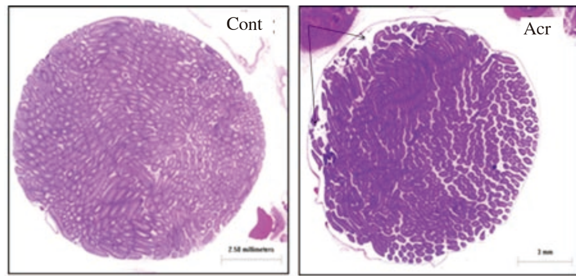

Figure 3. Comparison between testes of ACR-treated rats and control rats. Acrylamide resulted in atrophy and separation of semniferous tubules (arrows).

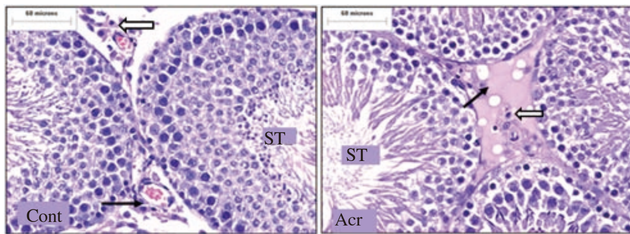

Figure 4. Testis sections from acrylamide treated and control rats (60 days). The control group showing normal semniferous tubules (ST) and interstitial tissue (arrow) compared to acrylamide group which shows absence of spermatid and sperm stages, edema of interstitial tissue (black arrow) and decrease Leydig cells (white arrows).

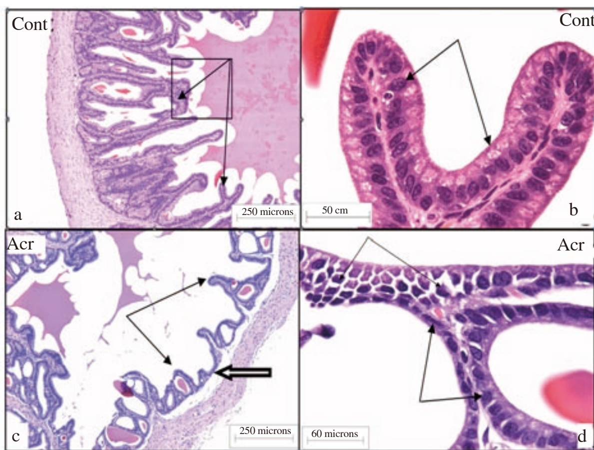

Figure 5. Seminal vesicle of control rat (a, b) showing elongated mucosal folds (square and arrows), and acidophilic secretory material within the lumen. Columnar epithelium with basal oval nuclei and supra-nuclear negative Golgi images (arrows) is seen. Seminal vesicle of acrylamide-fed animals (c, d) showing decrease mucosal fold length (arrows), submucosal edema and mucosal separation (white arrow) from the underlying muscle layer. Decreased height of lining cells with dark irregular shaped nuclei is observed (thin arrows).

3.4.Morphological and histological changes in female reproductive organs

Ovarian shrinkage, uterine horns irregularities and kinking were observed in most ACR-fed rats (Figure 6). Histological examination of ovaries in ACR-fed females revealed marked decrease in mature follicles. Some of the follicles observed showed cystic changes while others showed vacuolar degeneration of granulose cells and disorganization of both zona pellucid and corona radiate layers. Some oocytes appeared deformed and degenerated (Figure 7).

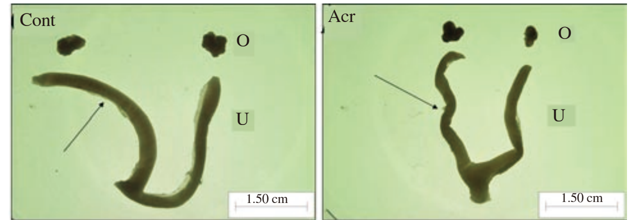

Figure 6. Morphology of the ovaries (O) and uterine horns (U) from control and acrylamide fed rat. the acrylamide fed rats shows shrinkage and irregularities of both ovarian uterine horns compared to the control.

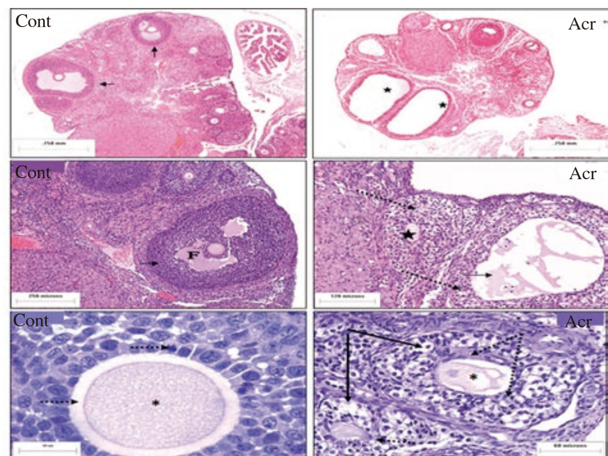

Figure 7. Marked cystic changes (black stars), marked degeneration of granulosa cell layers (dotted arrowsl) of growing follicles with fluid accumulation within the lumen (thin black arrow) as well as degenerated oocyte (asterisk), vacuolation of granulosa cells (black arrows) and disorganization of both zona pellucid and corona radiate layers (dotted arrows) are observed in acrylamide treated rats compared to control.

Ovaries of pregnant ACR-fed group showed vascularization of corpus luteum compared to the control ones (Figure 8). Histological -examination of endometrium of ACR-fed females which failed to conceive showed decreased height of lining epithelium. The uterine resorbed areas showed empty fetal sac, necrotic fetal debris and hypertrophy and vacuolation of amniotic cellular elements (Figure 9).

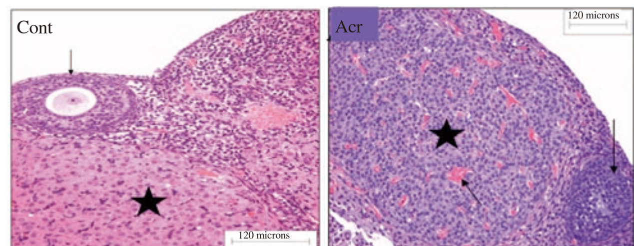

Figure 8. Part of an ovary of ACR-fed group showing vascularization (arrow) of corpus luteum (black star) in pregnant animals compared to the control ovary.

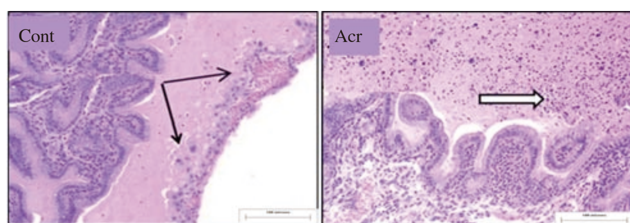

Figure 9. Section of uterine horns showing necrotic fetal tissue remnants at resorption sites in acrylamide fed rats (white arrow) compared to that of the control (black arrows).

3.5. Effect in pregnancy outcome

Significant differences were observed regarding the number and size of fetuses in the ACR fed rats compared to the control. In the untreated animals the number of full term fetuses at 18 days of gestation ranged from 10–12 per female. In animals receiving ACR, the number of fetuses was reduced, ranging from 3 to 6 per female. The lived fetuses looked abnormal in size (Figure 10).

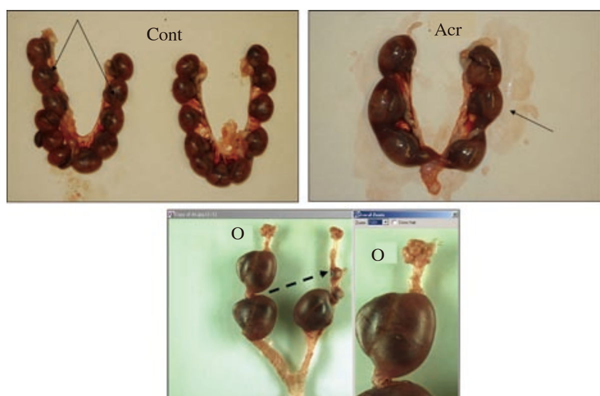

Figure 10. Uterine horns from pregnant rats showing decrease in number of the number of living fetuses (arrows) and increased number of resorption sites in acrylamide-fed group compared to the control group. Note, the normal number of corpora lute in the ovary (O).

3.6. Genotoxic Effect of ACR

Acrylamide was found to cause extensive DNA damage in lymphocytes of ACR-fed rats. This damage was evaluated by measuring the tail moment which determine the severity of the damage (tail length x the amount of DNA in the tail). The tail moment for lymphocytes of ACR-fed rats was 112.35 while that of the control was 0 and the mean amount of DNA in the tail of lymphocytes 86.7% while that of the control was 0.87% (Figure 11).

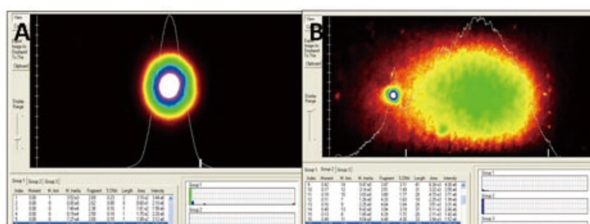

Figure 11. Photographic image of normal lymphocytes (A) (tail moment =0.00) and lymphocytes from ACR-fed rat (B) (tail moment = 112.35) subjected to single cell gel electrophoresis subsequently analyzed by image analysis system.

Acrylamide did not show any mutagenicity effect in the Salmonella mutagenicity test (Ames), with the three mutant strains TA98, TA100 and TA1535 in presence or absence of the metabolic activation system (S9), while glycidamide which is one of ACR metabolites showed clear mutagenicity with TA100 only at a doses of 1, 5 and 10mM/plate and in the presence of the metabolite activation fraction (S9) (Figure 12).

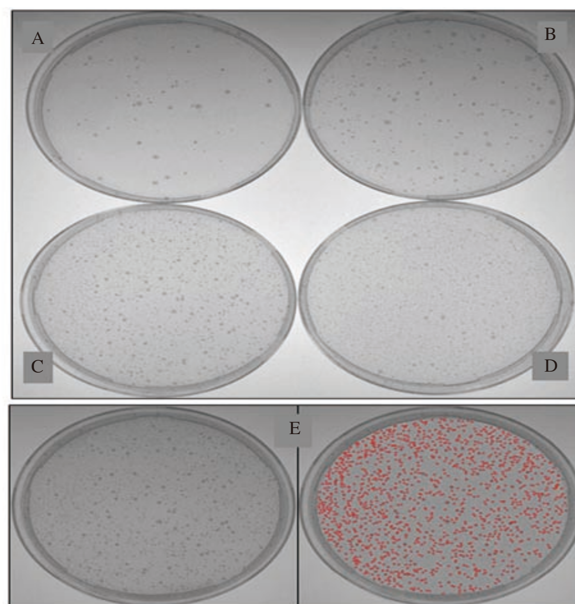

Figure 12. Mutagenicity of glycidamide in Ames test in the presence of the metabolite activating system (S9).

A- Negative Control plate containing salmonella typhimurium strain TA 100, (80 spontaneous revertants). B- Mutagenicity in the presence of 1mM glycidamide /plate, (305 revertants). C- Mutagenicity in the presence of 5mM glycidamide /plate, (1370 revertants). D-Mutagenicity in the presence of 10mM glycidamide /plate, (3800 revertants). E. Mutagenicity of glycidamide (5 mM/plate) in Ames test in the presence of activation liver fraction (S9): Number of bacterial revertants was counted automatically by image pro-plus software. The left plate is before counting automatically and the right one is after.

4. Discussion

In the present study, it was observed that oral administration of low dose of ACR produced structural changes in reproductive in male and female rats. Histological changes revealed decrease in seminiferous tubules that contain mature sperm stages, disruption and degenerated of germ cell layers were also observed. Seminal vesicle, the main supplier of sperm nutritive material showed decreasing mucosal folds and epithelial lining height. These finding were in line with some previous results. Shipp *et al.*, [24] reported negative impact of ACR at 0.83 μg/kg/day, double the present study dose, on the reproductive system. Other studies have reported that only the high doses of ACR and/or its metabolite glycidamide can affect the male

rat reproduction via binding to dopamine receptors and spermatid protamines and inhibits activity of kinesin and dyneine, resulting in interference with intracellular transport and sperm morphology and motility[11]. Other investigators reported some toxicological effects of ACR on the reproductive system of weaning male rats and the presence of abnormal sperm morphology as well as hormonal disturbance in the level of follicle-stimulating, luteinizing hormone and testosterone hormone[14].

These results are in accordance with a recent study that indicated that maternal ACR and alcohol exposure during the gestation and/or lactation period reduce testis weight, and causes pathological changes in the testes. Additionally ACR decreases the number of spermatogenic cells, while it increases the number of degenerated cells[25].

A decrease in male rat fertility was observed in this study. It seems to be multifactorial. The decreased in sperm count degenerative and morphological changes in sperms and seminiferous tubule germ cell degeneration especially spermatid stages were reported by some researchers[26]. Other investigators reported decrease in grip strength due to distal muscle weakness associated with distal axonopathy of sciatic nerve[27]. In the present study, changes in sperm morphology and degenerative changes in seminiferous tubules precedes the appearance of hind limp splay thus the ACR-induced negative impact on sex organs in both male and female seems to be the factors responsible for decrease fertility reported in the present study.

In the ACR treated group, an interstitial edema was observed among seminiferous tubules of animals indicating an inflammatory response, associated with increased vascular blood flow and permeability and subsequent transudation. This edema compressed the adjacent seminiferous tubules which might resulted in increase in migration of sperms into coda, with subsequent increase in its number. Investigators studying the relationship between ACR reproductive and neurotoxicity in male rats found that ACR at a dose of 15 to 60 mg/kg/day exhibited significantly reduced weight gain, reduced mating, fertility, and pregnancy indices as well as increased postimplantation loss, at dose 45 and 60 mg/kg/day[11]. In the present study low doses of ACR giving daily to female rats starts in post weaning period affect developing reproductive organs. Cystic changes, vacuolation and degenerative changes in growing follicles with absence of mature follicles denote effect on ovarian parenchymal elements. These finding are in contract with those previously reported that ACR does not affect female reproduction and found that females exhibit only neurotoxicity[11].

Some previous studies have dealt with the ACR-induced histological testicular changes[28–30]. In the present study, the reduction in the number of pregnant females could be attributed to both male and female factors. Cystic changes in the ovaries, reduction in the number of mature follicles, degeneration of oocytes and their surrounding zona pellucida and follicular cells may all count for decreased fertility in those animals. The increased incidence of fetal loss, and resorption could be explained in view of the degenerative changes in fallopian tube lining epithelium, hypertrophy and degeneration of muscular wall that would interfere with transport of fertilized eggs. Vacuolar changes of endometrial epithelial linings and decrease stromal cellularity and presence of lymphocyte infiltration may be another factor decreasing implantation rate or result in fetal resorption. Although ovaries showed normal number of corpora, they seemed to be histologically degenerated with vaculation of such corpora, abnormal congestion and vaculation of lutein cells adding to that the decrease endometrial cellularity and lymphocyte infiltrations which all could account for the fetal loss.

In this study, ACR induced significant genotoxicity detected by using the alkaline comet test and this findins is in line with the previous study done on in C. auratus peripheral blood cells[31]. In this study, acrylamide did not show any mutagenicity effect, while glycidamide showed clear mutagenicity in the Salmonella mutagenicity test. This result was in partial agreement with the study of Mei *et al*. [32] who reported that both ACR and glycidamide generate mutations through a clastogenic mode of action in mouse lymphoma cells. They added that GA induces mutations via a DNA adduct mechanism whereas ACR induces mutations by a mechanism not involving the formation of GA adducts.

Conflict of interest statement

All authors reported no conflicts of interest.

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