Genotoxicity and toxicological effects of acrylamide on reproductive system in male rats

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The toxicity of acrylamide was evaluated through mutagenicity of Salmonella, chromosome aberration of Chinese hamster lung fibroblasts, micronucleus formation in mice and reproductive toxicity in rats. Based on Ames test, acrylamide showed mutagenic potency for strains TA98 and TA100. Moreover, both chromosomal aberration assay and micronucleus assay indicated that acrylamide might have genotoxic potency; the chromosomal aberration frequencies were observed to be proportional to acrylamide concentrations of 5-50 mM, and acrylamide significantly increased micronuclei in peripheral blood cells of mice at doses of higher than 72.5 mg/kg. Male rats were treated with acrylamide at doses of 0, 5, 15, 30, 45, or 60 mg/kg/day for 5 consecutive days, and the toxicity of acrylamide was observed. In the group treated with the highest dose of acrylamide (60 mg/kg/day), the loss of body weight and reduced testis weight were observed. Also the epididymis weights were reduced significantly in all the groups treated with acrylamide. The number of sperms in cauda epididymidis decreased significantly in an acrylamide dose-dependent manner. Rats treated with 60 mg/kg/day of acrylamide showed several histopathological lesions in the seminiferous tubules. There were thickening and multiple layering of the tubular endothelium, and the formation of many multinucleated giant cells in seminiferous tubules. Taken together, acrylamide not only causes the genotoxicity of eukaryotic cells and mice but also shows the toxicological effects on reproductive system in male rats.

Key words: acrylamide, chromosomal aberration, genotoxicity, micronuclei, mutagenicity

Introduction

Acrylamide is a highly reactive and water-soluble polymer, which is commonly used in both industries and laboratories [16]. Individuals can be exposed to acrylamide either in their workplace [5] or in the environment [6]. A recent study reported the presence of acrylamide in heat-treated food products [11]. The formation of acrylamide is particularly associated with high temperature cooking process for certain carbohydrate-rich foods, especially when asparagine reacts with sugars [15]. Cooking at lower temperatures (e.g., by boiling) produces much lower level of acrylamide [19].

Exposure to acrylamide from foods is a growing concern because it causes cancer such as mammary adenomas, thyroid tumors, scrotal mesotheliomas in rats [3,7]. Furthermore, acrylamide is a possible human carcinogen with genotoxicity including micronuclei [10,22], chromosomal aberrations, sister chromatid exchanges, and mitotic disturbances in vitro [2] although it consistently exhibited negative results in bacterial gene mutation assays in strains of Salmonella [9,25]. Chromosomal aberrations were detected in spermatocytes, and micronuclei were observed in spermatozoa [13,28].

Reproductive toxicity of acrylamide has been extensively tested in mice including abnormal morphology of sperms [21], testicular damages such as vacuolation and swelling of the round spermatids [20], and DNA breakage during specific germ cell stages [23]. Male rats administered with acrylamide exhibited significant reductions of mating, fertility, and pregnancy indices as well as reduction of transport of sperms in uterus [27]. These studies suggest that acrylamide has the toxicity for male reproductive organs whereas female rodents seem to be resistant to the reproductive toxicity of acrylamide [4].

The present study was performed to evaluate the genotoxicity and male reproductive toxicity of acrylamide. To confirm the genotoxicity of acrylamide, Ames test, chromosomal aberration assay, and micronucleus assay were performed. To determine the toxicological effects of acrylamide on reproductive system in male rats, the sperm
reserves in cauda epididymidis were measured and histopathological lesions in the seminiferous tubules were observed.

Materials and Methods

Animals
Male ICR mice were purchased from Orient Co. Ltd. (Seoul, Korea). The mice, aged 45-50 days and weighing 25-30 g, were divided into six groups of 8 animals each. The animals were housed 4 per polycarbonate cage with wood shavings, and were maintained under a controlled environment with temperature at 23 ± 2°C, relative humidity at 55 ± 5%, and a 12 hrs/12 hrs light/dark cycle throughout the experiment.

Male Sprague-Dawley rats were purchased from Orient Co. Ltd. (Seoul, Korea). The rats, aged 50-60 days and weighing 200-250 g, were divided into six groups of 8 animals each. The animals were housed and maintained under a controlled environment as above throughout the experimental period.

Ames test
Ames test was performed to evaluate the mutagenicity of acrylamide in a bacterial reverse mutation system [14]. Briefly, the strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) were grown overnight in the mixture was prepared as follows: 0.1 M phosphate buffer, pH 7.4, 8 mM KCl salt solution, and 4% of S9 fraction. Each culture medium was cultured at 37°C in an incubator with 8% CO₂ under saturated humidity. The cells were subcultured and maintained every 3-5 days using 0.05% trypsin-EDTA solution (Gibco, USA). The microsomal S9 fraction was prepared from the livers of mature male Sprague-Dawley rats as described above. The concentrations of acrylamide used in *vitro* chromosome aberration assay were decided based on results of a preliminary toxicity study. Cells were plated in a disposable 24-well plate with 1 × 10⁴/well and incubated for 2 days, and were exposed to acrylamide of 5 concentrations ranging from 1 to 400 mM. After acrylamide exposure for 24 hrs at 37°C, media was discarded and the cells were rinsed twice with 0.5 ml Dulbecco’s phosphate buffered saline. Cells were fixed with methanol for 10 minutes and were stained by 5% Giemsa staining solution (in phosphate buffer, pH 6.8). The concentration of 50% cytotoxicity was determined by microscopic observation. Based on the preliminary toxicity study, the acrylamide concentrations used in chromosome aberration assay were decided ranging from 1.25 to 50 mM. The cells were exposed to 5 levels of concentrations in this range. Positive control cultures were treated with either 0.05 µg/ml of mitomycin C or with 0.02 mg/ml of benzo(a)pyrene in the absence or presence of metabolic activation system, respectively. Negative control cultures were treated with physiological saline solution. At approximately 22 hours after dosing, colcemid (0.2 µg/ml; Gibco, USA) was added to the cultures in each treatment group to arrest the dividing cells in metaphase.

Metaphase cells were collected by shake-off approximately 2 hrs after addition of colcemid. The cells were centrifuged at about 180 × g (1,000 rpm) for 5 minutes. The supernatant was discarded, and the cells were resuspended in 10 ml of hypotonic solution (0.075 M KCl) for 15 minutes at 37°C. Cells were centrifuged at 180 × g (1,000 rpm) for 5 minutes and the hypotonic solution was discarded. The pellet was resuspended in 4 ml of fixative (3 : 1, methanol:glacial acetic acid) and washed 3 times with the fixative. After last removal of the fixative, a small portion of fixative was added and the pellet was resuspended. One drop of cell suspension was placed onto a clean cold slide. Slides were air-dried, and stained in 5% Giemsa in phosphate buffer (pH 6.8) for 15 minutes and dried in air. The results were judged according to the percentage of average chromosome aberrations: less than 5%; negative (-), 5-10%; false positive (+), 10-20%; positive (+), 20-50%; positive (++), and more than 50%; positive (+++) (n = 3).
**In vivo micronucleus assay**

Administration doses were determined based on LD$_{50}$ value. Acrylamide was administered to each group of mice at doses of 0, 18.13, 36.25, 72.5, 100, or 145 (LD$_{50}$) mg/kg by oral gavage with single dose. For control group, mitomycin C (Sigma-Aldrich, USA) was administered intraperitoneally at a dose of 1 mg/kg. After 48 hrs of acrylamide treatment, blood sample was collected from peri-orbital blood vessel of each mouse for micronucleus assay.

Blood sample was collected from peri-orbital blood vessel of each mouse after 48 hrs of acrylamide treatment, and slides were prepared for micronucleus assay. For acridine orange (AO; Sigma-Aldrich, USA) staining of blood cells, 5 µl of blood sample was placed on an AO-coated slide (1 mg/ml, 15 µl per slide), and covered with a coverslip. Stained blood cells were examined by dark field fluorescent microscopy (Axioskop; Carl Zeiss, Germany). The frequency of micronucleated polychromatic erythrocytes was counted based on the observed number of 1,000 polychromatic erythrocytes (PCE). The results were analyzed according to the method of Sugihara et al. [24].

**Toxicity on reproductive system**

The body weights of rats before and after administration of acrylamide were measured and compared. Initially, mean body weights were evenly set for all groups right before the first administration, and rats were administered with acrylamide at doses of 0, 5, 15, 30, 45, or 60 mg/kg/day for 5 consecutive days by oral gavage. After 72 hrs of last administration, the body weights gained after administration of acrylamide were measured and compared. Rats were sacrificed by decapitation, and testes were removed and weighed. After isolation of left epididymis from each testis, the tail region of each epididymis was removed and weighed.

Cauda epididymis were minced with ophthalmologic scissors, and were homogenized for 1 min in 5.0 ml of physiological saline solution [17]. The homogenate was filtered through a nylon mesh and then 0.1 ml of filtrate was diluted with 2.0 ml of saline solution containing 4% trypan blue. From this solution, 20 µl aliquots were placed on the Neubauer hemacytometer for counting the number of sperms/mg of cauda epididymis tissue.

The excised testes were fixed in Bouin solution, and processed using standard laboratory procedures for histology. The tissue was embedded in paraffin blocks, sectioned perpendicular to the longest axis of the testis with 3 µm thickness, and stained with hematoxylin and eosin. Stained section were mounted with dextran-plasticizer xylene and examined using light microscopy.

**Statistical analysis**

Data were analyzed by one-way analysis of variance (ANOVA) followed by two-tailed t-test when the ANOVA test yielded statistical differences (p < 0.05 or 0.01). A value of p < 0.05 was used as the criterion for statistical significance. All data were expressed as the mean ± SE.

| Test article | Dose (µg/plate) | S9 mix | Number of revertant colony/plate (mean ± SE) |
|--------------|-----------------|--------|-----------------------------------------------|
|              |                 |        | TA 98                                         | TA 100 | TA 1535 | TA 1537 |
| Acrylamide   | 5,000           | +      | 84.5±7.8                                      | 294.0±2.8** | 37.0±14.0 | 31.5±10.6 |
|              | 2,500           | +      | 93.0±1.4*                                     | 189.5±10.6** | 29.0±4.2 | 12.0±5.7 |
|              | 1,250           | +      | 86.5±6.4                                      | 122.5±14.9 | 18.5±3.5 | 19.5±3.5 |
|              | 625             | +      | 77.5±3.5                                      | 121.5±29.0 | 16.5±5.0 | 13.5±2.1 |
| DMSO         | 100 µl/plate    | +      | 45.0±9.9                                      | 65.5±12.0 | 23.5±5.0 | 13.5±7.8 |
| NaN3         | 1.5             | +      | -                                             | 466.5±87.0* | 420±43.8* | -        |
| 2-AF         | 10.0            | +      | 1,421±43.8**                                   | -      | -      | -        |
| ICR-191      | 0.1             | +      | -                                             | -      | -      | 55.5±14.9 |

| Test article | Dose (µg/plate) | S9 mix | Number of revertant colony/plate (mean ± SE) |
|--------------|-----------------|--------|-----------------------------------------------|
|              |                 |        | TA 98                                         | TA 100 | TA 1535 | TA 1537 |
| Acrylamide   | 5,000           | -      | 46.0±2.8**                                    | 56.0±5.7 | 21.5±5.0 | 2.5±0.7 |
|              | 2,500           | -      | 40.5±3.5*                                     | 44.0±9.9 | 24.0±2.8 | 4.5±0.7 |
|              | 1,250           | -      | 10.5±0.7                                      | 53.0±4.2 | 32.0±1.4 | 0.5±0.7 |
|              | 625             | -      | 10.5±2.1                                      | 58.5±24.8 | 20.5±2.1 | 6.5±5.0 |
| DMSO         | 100 µl/plate    | -      | 11.5±0.7                                      | 31.0±9.9 | 16.5±9.2 | 7.5±2.1 |
| NaN3         | 1.5             | -      | -                                             | 171±17.0* | 167±15.6* | -        |
| 2-AF         | 10.0            | -      | 108±14.1*                                     | -      | -      | -        |
| ICR-191      | 0.1             | -      | -                                             | -      | -      | 159.5±31.8* |

Acrylamide was dissolved in DMSO. Asterisks indicate significant differences from vehicle group, *p < 0.05; **p < 0.01.

DMSO: dimethylsulphoxide, NaN3: sodium azide, 2-AF: 2-aminofluorene.
Ames test

Numbers of *Salmonella typhimurium* revertants induced by acrylamide with or without metabolic activation are shown in Table 1. Increased numbers of revertants were observed in TA98 at higher concentration of acrylamide (2,500 and 5,000 µg/plate) in the presence and absence of S9 mixture. Moreover, numbers of revertants in TA100 increased significantly \((p < 0.01)\) at higher concentration of acrylamide (2,500 and 5,000 µg/plate) in the presence of S9 mixture, which suggests the formation of mutagenically active metabolite(s) of acrylamide.

Chromosomal aberration

The results of the chromosomal aberration test are summarized in Table 2. CHL fibroblasts treated with 5 mM of acrylamide significantly increased the frequencies of chromosomal aberration in the presence or absence of S9 mixture. Moreover, numbers of revertants in TA100 increased significantly \((p < 0.01)\) at higher concentration of acrylamide (2,500 and 5,000 µg/plate) in the presence of S9 mixture, which suggests the formation of mutagenically active metabolite(s) of acrylamide.

Micronucleus

The result of the micronucleus test in peripheral blood cells of acrylamide-administered mice is shown in Table 3. The number of naturally-occurred micronucleus was less than 2 out of 1,000 PCE. Acrylamide did not induce micronuclei in peripheral blood cells of mice at doses of below 36.25 mg/kg, whereas it significantly \((p < 0.01)\) increased micronuclei at doses of 72.5, 100, and 145 mg/kg.

Toxicity on reproductive system

The body weights gained after administration of acrylamide were measured and compared. After 72 hrs of last administration of acrylamide, the gained body weights decreased significantly \((p < 0.01)\) at dose of 45 mg/kg/day compared with vehicle control group (Fig. 1). In the group treated with the highest dose of acrylamide (60 mg/kg/day), the loss of body weight \((p < 0.01)\) (Fig. 1) and reduced testis weight \((p < 0.05)\) (Fig. 2) were observed. The epididymides weights were reduced significantly \((p < 0.01)\) in all groups.
treated with acrylamide (Fig. 3), which suggests that acrylamide has the toxicity to reproductive organs.

Most striking feature of the reproductive toxicity of acrylamide was reduced sperm reserves in cauda epididymidis isolated from rats treated with acrylamide (Fig. 4). Even the lowest dose of acrylamide (5 mg/kg/day) reduced the number of sperm in left cauda epididymidis to half level. The sperm reserves further decreased in an acrylamide dose-dependent manner.

Most of the rats in the treatment groups showed some evidence of morphological changes in the testicular histology when compared with the vehicle control group (Fig. 5). All rats in the control group showed normal histological pattern (Fig. 5A), whereas rats treated with 60 mg/kg/day of acrylamide showed histopathological changes in the seminiferous tubules (Fig. 5B). There were thickening and multiple layering of the tubular endothelium, degeneration of germ cells, and the formation of many multinucleated giant cells in atrophied seminiferous tubules (Fig. 5B).

Discussion

In the present study, we evaluated the genotoxicity and reproductive toxicity of acrylamide. Based on Ames test, acrylamide showed mutagenic potential for strains TA98 and TA100, which is contradict to previous observation [9]. We also observed micronuclei and chromosomal aberrations at high concentrations of acrylamide as reported by Higashikuni et al. [10] and Adler et al. [2]. Although the highest dose (60 mg/kg/day) of acrylamide decreased testes weights, epididymides weights of rats were greatly reduced from the lowest dose (5 mg/kg/day). Most striking feature of this study is the effect of acrylamide on sperm reserves in cauda epididymidis. The number of sperms in cauda epididymidis was reduced to half level even with the lowest dose (5 mg/kg/day) of acrylamide. Rats treated with 60 mg/kg/day of acrylamide showed several histopathological lesions in the seminiferous tubules. There were thickening
and multiple layering of the tubular endothelium, degeneration of germ cells, and the formation of many multinucleated giant cells in atrophied seminiferous tubules. Overall, acrylamide causes diverse toxicity through genotoxicity and reproductive toxicity.

Acrylamide is metabolized by cytochrome P450 to the epoxide glyciamide, which is then the ultimate DNA-reactive clastogen in mouse spermatids [1]. Therefore, chromosome aberration by acrylamide might result from direct binding of glyciamide to DNA by making DNA adducts. Also Tyl and Friedman [26] observed that acrylamide and/or glyciamide binding to spermatid protamines causes dominant lethality of gonadal cells and morphological abnormalities of sperms. One of the histopathological lesions observed in the present study was the formation of many multinucleated giant cells in atrophied seminiferous tubules. The giant cells result from the inability of primary 4N spermatocytes to undergo meiotic divisions to generate haploid sperm cells, which undergo additional DNA replication giving rise to multinucleated giant cells [18]. Gassner and Adler [8] reported that cell proliferation and cell cycle delay were found in spermatocytes by acrylamide treatment.

Our current study observed the regulation of the genes by acrylamide in rat testis using cDNA microarray [29]. Testis isolated from acrylamide-treated rat showed up/down-regulated genes related to the function of testis, apoptosis, cellular redox, cell cycle, and nucleic acid binding [29]. Especially, testis-specific transporter 1 (TST 1) gene and steroid receptor RNA activator 1 gene which are important for the regulation of sex steroid transportation and spermatogenesis were up-regulated in acrylamide-treated rat testis [29]. Therefore, acrylamide disturbs the gene expression related to spermatogenesis, which might result in reduced sperm reserves in cauda epididymidis. Moreover, acrylamide perturbs the gene levels related to cell proliferation and cell cycle, which might result in abnormal histopathological features in reproductive organs observed in this study.

Since there is no information of bioavailability based on its biomarkers, it is hard to define the sensitivity of acrylamide in human being. Also no study observed the difference of sensitivity between animal and human being. In the present study, the reproductive toxicity of acrylamide was observed at doses from 5.0 mg/kg/day for 5 days. The doses of acrylamide significantly reduced the sperm concentration in cauda epididymidis, which suggests that no observable effect level (NOEL) for the reproductive toxicity is less than 5.0 mg/kg/day. Tyl et al. [27] observed that rats exposed to acrylamide in drinking water for 10 weeks showed 2.0 mg/kg/day of NOEL for the prenatal (dominant) lethality and the reproductive toxicity. Since the sperm concentrations in cauda epididymidis decreased in an acrylamide dose-dependent manner, we observed the histopathological lesions under the extreme condition, which is a dose of acrylamide at 60 mg/kg/day.

In summary, we have evaluated the genotoxicity and the toxicological effects of acrylamide on reproductive system in male rats. Both chromosomal aberration assay and micronucleus assay indicated that acrylamide might have genotoxic potency. Acrylamide reduced the sperm reserves in cauda epididymidis, and induced several histopathological signs in rat testis. Taken together, acrylamide not only causes genotoxicity but also shows the toxicity on reproductive system in male rats. Even though many previous studies observed the toxicity of acrylamide, the basic mechanisms of the toxicity were not understood thoroughly. Our future study will be focused on the regulation of the genes by acrylamide in rat organs using cDNA microarray analysis, which might explain the mechanisms of acrylamide toxicity.

Fig. 5. Histopathological lesions of testes. Testes were isolated from the vehicle control rat (A) and the acrylamide (60 mg/kg/day)-treated rat (B). Thickening and multiple layering of the tubular endothelium (arrow), and the formation of many multinucleated giant cells (arrow heads) in seminiferous tubules. H & E stain, ×50.
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