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Assessment of DNA Damage in Multiple Organs from Mice Exposed to X-rays or Acrylamide or a Combination of Both Using the Comet Assay

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Abstract. *Background: X-rays and acrylamide (AA) are present in the general environment and workplace and are potential hazards for human health. Combined exposure to both agents is possible, especially at low doses. Materials and Methods: The induction of DNA damage after single or combined exposure to X-rays and/or AA was measured in multiple mice organs using a comet assay. Results: X-rays and AA alone induced generally dose-dependent increases in DNA damage of somatic and germ cells. Combined exposure to 0.10 Gy + 50 mg/kg bw AA induced higher DNA damage than each agent alone in the spleen, kidneys, lungs and testes. In bone marrow lymphocytes there was clear increase in DNA damage compared to that produced by X-rays only. Significant DNA damage was observed in liver cells only after combined exposure to 0.25 Gy + 50 mg/kg bw AA. Conclusion: Combined exposure to X-rays and AA enhanced DNA damage after single exposure to each agent.*

Acrylamide (AA) (CAS Registry Number 79-06-1) is a vinyl monomer from which polyacrylamides are synthesized. Polyacrylamides are used in the cosmetic, paper and mining industries, in the treatment of water and in underground pipe routing operations, as well as underground injection for soil stabilisation (1). In scientific research, acrylamide is used to selectively modify SH groups in proteins and as a quencher of tryptophan fluorescence. Polyacrylamide gels are used to separate compounds by electrophoresis (2). Occupational exposures to AA occur primarily in the production of acrylamide and polyacrylamide. AA exposures for the general population occurs *via* residual monomers in products such as cosmetics, toiletries and food packing. AA is also a component of tobacco smoke (1). In 2002 it was found that AA is formed during high temperatures heating food products rich in carbohydrates (3). It has been shown that AA is formed by the Maillard reaction between the amino acid asparagine and reducing sugars such as glucose or fructose (4, 5). AA is now known to be formed during industrial food processing, retail, catering and home food preparation (6). High concentration of AA were found in French fries, potato crisps, cereals, bread and crackers (3, 7, 8). The World Health Organisation estimated total daily intakes of AA from food to be in the range of 0.3-0.8 μg/kg of body weight (9).

Exposure to ionizing radiation comes mainly from natural (terrestial and cosmic), but also from man-made sources. The greatest source of human exposure from man-made devices is in medicine, where radiation is used for both diagnosis and treatment of diseases (10). Employees in the nuclear power industry can accidentally receive relatively large doses of radiation, whereas medical personnel generally receive relatively low doses.

Both acrylamide and X-rays are known to induce mutations in germ and somatic cells of laboratory animals.

Acrylamide has been shown to cause a distinct central-peripheral distal neuropathy, as well as affecting the central nervous system of experimental rats. Non-neurotoxic effects of AA exposure include genotoxic, carcinogenic, reproductive and developmental abnormalities (11-12).

Acrylamide produces chromosomal aberrations and micronuclei in somatic (13-17) and germ cells (15, 18-22) of male rodents, as well as AA inducing sperm head abnormalities (17, 23, 24).

Exposure to X-rays induces micronuclei in bone marrow polychromatic erythrocytes (17, 25-29). Radiation produces micronuclei in spermatids (19) and induces malformations in sperm heads (17, 30-31). It is known that occupational exposure to ionising radiation may cause chromosomal aberrations (32-34).

Combined exposure to low doses of X-rays and AA enhanced the frequency of sperm head abnormalities and micronuclei in polychromatic erythrocytes of bone marrow in comparison to results obtained after exposure to each agent alone in some cases (17).

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Key Words: Acrylamide, X-rays, combined exposure, DNA damage, comet assay.

Table I. *Score of DNA damage (±SD) in cells from different organs after exposure of male mice to X-rays.*

	Tissue					
Dose	Bone marrow	Spleen	Liver	Kidney	Lungs	Testes
0 (Control)	85.0±19.4	108.4±25.2	118.4±18.2	139.6±24.3	90.5±22.1	126.6±26.5
0.10 Gy	112.2±16.6[a1]	110.2±14.2	130.4±16.9	127.4±30.3	120.8±41.0	157.8±16.0
0.25 Gy	115.4±14.3[a1]	130.6±27.4	143.0±14.5[a1]	155.8±22.9	146.5±38.7[a1]	166.8±17.2[a1]
0.50 Gy	127.8±9.1[a2]	122.2±32.4	140.6±21.6	160.4±15.2	163.6±11.6[a2]	190.0±7.8[a3]
1.00 Gy	130.8±13.4[a2]	156.8±19.0[a1]	148.0±21.7[a1]	175.6±36.3[a1]	195.5±33.5[a2]	200.2±11.4[a3]
2.00 Gy	146.6±17.5[a3]	159.8±19.0[a2]	180.6±38.3[a1]	181.7±14.2[a1]	203.8±37.9[a2]	210.0±17.2[a3]

Statistical significance: [a1]$p<0.05$; [a2]$p<0.01$; [a3]$p<0.001$ compared to the to control (Student's *t*-test).

Table II. *Score of DNA damage (±SD) in cells from different organs after exposure of male mice to acrylamide.*

	Tissue					
Dose	Bone marrow	Spleen	Liver	Kidney	Lungs	Testes
0 (Control)	112.2±8.4	100.0±19.3	95.5±18.2	122.6±23.3	113.5±31.7	147.8±22.5
50 mg/kg	133.7±12.8[a2]	120.4±14.2	97.2±23.1	151.8±17.0	182.8±17.6[a2]	174.7±37.5
75 mg/kg	157.0±16.8[a3]	147.8±7.1[a3]	155.0±17.9[a3]	169.8±6.7[a2]	189.3±13.8[a2]	186.5±19.5[a2]
100 mg/kg	176.7±9.5[a3]	184.0±12.1[a3]	173.3±22.4[a3]	189.0±28.2[a1]	210.3±5.4[a3]	198.5±12.1[a3]
125 mg/kg	193.8±23.2[a3]	194.8±8.2[a3]	207.2±22.9[a3]	195.6±27.8[a2]	225.8±21.3[a2]	203.7±10.8[a3]

Statistical significance: [a1]$p<0.05$; [a2]$p<0.01$; [a3]$p<0.001$ compared to the control (Student's *t*-test).

X-rays and AA are potential hazards for human health because they cause mutagenic and carcinogenic effects. Both X-rays and AA are often present in environment and workplace, so combined exposure to both agents is possible, especially at low doses. In this paper, the effects of combined exposure to low doses of X-rays and AA on the induction of DNA damage measured using a comet assay in germ and somatic cells of laboratory mice were investigated. This is the first demonstration of the effect of DNA damage in different tissue following combined exposure to X-rays and AA measured using a comet assay.

Materials and Methods

Male mice. Pzh:SFIS male mice were obtained from the Laboratory of Animal Breeding of the National Institute of Hygiene (Warsaw, Poland). Animals had free access to a standard rodent diet and tap-water. They were housed in plastic cages in room designed for the control of temperature, humidity and light cycle (12 h light and 12 h dark). Males aged 8-9 weeks and weighted 28-32 g were assigned randomly to either control groups or exposure groups.

X-ray and chemical exposure. A therapeutic Roentgen unit Medicor type THX-250 was used as the X-ray source. It was operated with the following parameters: 175 kV, 20 mA, added filtration 0.5 mm Cu and HVL 0.8 mm Cu.

Male mice were subjected to whole body irradiation at a dose rate of 0.40 Gy/min. Mice were irradiated with total doses of 0.10 Gy, 0.25 Gy, 0.50 Gy, 1.00 Gy and 2.00 Gy.

AA monomer, >99% pure, obtained from Bio-Rad Lab (Hercules, California, USA) was dissolved in 0.9 % NaCl solution, and injected intraperitoneally (*i.p.*). The injection volume was 0.1 ml per 10 g body weight (bw). Males were treated with doses of 50 mg/kg bw, 75 mg/kg bw, 100 mg/kg bw and 125 mg/kg bw of AA. For combined exposure animals received a dose of 0.10 Gy + 50 mg/kg bw AA or 0.25 Gy + 50 mg/kg bw AA. A sample size of 4-6 animals was used in each group. Doses for combined exposure were chosen on the basis of the results from single exposure to each agent (Tables I and II). In groups receiving combined treatments mice were injected with AA immediately after irradiation. Control animals were injected with NaCl solution only.

Comet assay. Animals were sacrificed 24 h after irradiation and/or acrylamide treatments. Femora, testes, liver, kidneys, spleen and lungs were removed from each animal. Bone marrow was flushed from the femora with RMPI 1640 medium. The liver, kidneys, spleen, lungs and testis were placed in RMPI- medium and minced with scissors. Tissue pieces were filtered through a mesh. Finally, single cells remained in the suspension. For use in the comet assay, depending on the organ, 5-10 μl of cell suspension were mixed in an Ependorff tube with 75 μl low melting point agarose (LMPA).

The basic alkaline technique of Singh *et al.* (35) was followed. Cells were mixed with 75 μl of 0.5 % LMPA and pipetted onto microscope slides previously covered with normal melting point agarose (NMPA). Slides were then covered with cover slips and put

Table III. *Score of DNA damage (±SD) in cells from different organs after combined exposure to X-rays and acrylamide.*

Dose	Tissue					
	Bone marrow	Spleen	Liver	Kidney	Lungs	Testes
0 (Control)	96.4±21.9	97.2±26.1	108.6±18.6	118.2±6.0	100.6±32.2	145.4±22.9
0.10 Gy	112.2±16.4	118.4±34.6	128.4±9.5	138.6±22.6	127.8±28.0	161.6±17.3
0.25 Gy	127.8±11.3^{a1}	133.0±20.5^{a1}	142.2±24.8^{a1}	145.6±20.5^{a1}	139.2±34.9	177.4±12.1^{a1}
50 mg/kg AA	140.8±19.3^{a2}	131.8±23.0	144.2±31.2	165.8±18.0^{a3}	155.2±26.0^{a1}	174.0±16.9^{a1}
0.10 Gy+50 mg/kg AA	143.3±28.3^{a1}	158.6±27.7^{a2}	140.0±39.9	180.8±15.5^{a3,b2}	173.4±17.8^{a2,b2}	192.2±22.9^{a1,b1}
0.25 Gy+50 mg/kg AA	175.2±23.8^{a3}	171.2±18.4^{a3,c1,d1}	169.6±21.8^{a2}	191.8±6.9^{a3,c2,d1}	188.4±12.2^{a3}	202.6±20.6^{a2,c1,d1}

Statistical significance: $^{a1}p<0.05$, $^{a2}p<0.01$, $^{a3}p<0.001$ compared to the control; $^{b1}p<0.05$, $^{b2}p<0.01$ compared to 0.10 Gy; $^{c1}p<0.0$, $^{c2}p<0.01$ compared to 0.25 Gy; $^{d1}p<0.05$ compared to 50 mg/kg AA (Student's *t*-test).

in the fridge for 5 min to solidify the agarose. After removing the cover slips, another layer of LMPA was added, spread using cover slips and allowed to solidify at 4˚C for 5 min. After removal of the cover slips, the slides were immersed in freshly prepared cold lysing solution overnight at 4˚C. The following morning the slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side, leaving no spaces. The slides were incubated in a fresh alkaline electrophoresis solution (pH<13) for 20 min at 4˚C allow the unwinding of DNA and expression of alkali labile damage before electrophoresis. Electrophoresis was conducted at 4˚C for 20 minutes using 19 V and approximately 300 mA. After electrophoresis slides were neutralised 3 times for 5 min using Tris buffer. Cells were stained using EtBr (20 μl/ml) and slides were covered with cover slips and analysed within 3-4 h.

Slides were coded and examined using a fluorescence microscope. Images of 200 randomly selected cells from each organ (100 cells from each of two slides) were analysed from each mouse. According to the method described by Kumaravel and Jha (36), cells were graded by eye into five categories, based on the distance of migration and preceived proportion of DNA in the tail, and given a value of 0, 1, 2, 3 or 4 (from undamaged – 0 to maximally damaged – 4). The percentage of cells with each level of damage were calculated. In this way the total score could range from 0 (all undamaged) to 400 (all maximally damaged). The same person categorised DNA damage in all groups. Statistical analyses were performed using Student's t-test.

Results

X-rays exposure. Results of the DNA damage in cells from different organs of irradiated mice are shown in Table I. In non-irradiated animals the highest DNA damage was noted in the testes and kidneys. In irradiated animals, the DNA damage in cells from examined organs generally increased with dose. In spleen and liver the dose of 0.25 Gy of X-rays induced slightly greater DNA damage than the dose of 0.5 Gy. The most radioresistant occurred kidneys and spleen, which showed significant increased DNA damage at 1.00 Gy. In testes, lungs and liver cells, statistically significant results in comparison to corresponding control at 0.25 Gy were noted. The most radiosensitive tissue appeared to be the bone marrow lymphocytes, with the statistically significant effect being observed at the lowest dose. The greatest differences in DNA damage score between the lowest and the highest dose were noted in lung cells.

Acrylamide exposure. Results of the effects induced in somatic and germ cells following exposure to AA are presented in Table II. After exposure to AA, DNA damage increased with dose in the majority of organs. In the spleen, liver, kidneys and testes significant DNA damage was observed at a dose of 75 mg/kg bw AA. The lungs and bone marrow lymphocytes appeared to be more sensitive, with statistically significant results being observed from 50 mg/kg bw AA. The greatest difference in the score of DNA damage between the lowest and the highest dose of AA were found in liver and lung cells.

Combined exposure to X-rays and acrylamide. The results of combined exposure compared to effects caused by each agent are shown in Table III. In cells from the majority of organs, combined exposure to low doses of X-rays and AA induced greater DNA damage than each agent alone. Following the doses of 0.10 Gy + 50 mg/kg bw AA, clear effects of DNA migration from head to tail were observed in the spleen, kidneys, lungs and testes. Results for kidney, lung and testes cells were statistically significant compared to effects obtained after exposure to 0.10 Gy alone. In bone marrow lymphocytes, there was a slight increase in DNA damage in comparison to the effect produced by AA alone and a clear increase in comparison to X-rays alone. In liver cells a statistically significant increase in DNA damage was only noted after combined exposure to 0.25 Gy + 50 mg/kg bw AA. Results observed after combined exposure to 0.25 Gy + 50 mg/kg bw AA in spleen, kidney and testes were statistically significant in comparison to both 0.20 Gy and 50 mg/kg bw AA alone.

Discussion

The alkaline comet assay is capable of detecting DNA single-strand breaks (ssb, alkali labile sites, DNA-DNA, DNA-protein, cross-linking and ssb associated with incomplete repair sites (37). The comet assay is known to be useful for investigating primary genotoxic effects and in identifying possible human mutagens and carcinogens, although a perfect correlation between a positive test in the comet assay and carcinogenicity is not expected (37-39).

Although it is recognised that repair of DNA damage can take place immediately after treatment, the results presented in this paper demonstrate effects still present 24 h after treatment.

The first experiments with the comet assay involved an evaluation of the ability of X-rays and hydrogen peroxide to induce DNA damage (35). The authors observed a significant increase in the length of DNA migration in human lymphocytes exposed to ionizing radiation at doses of 25 rads or higher. They also observed an approximately linear increase in the length of DNA migration for doses between 25 and 100 rads.

DNA damage caused by X-rays was also observed in laboratory animal cells. Our results for bone marrow, liver and testes of male mice confirmed results of animal studies performed by other authors. Carrera *et al.* (40) observed a statistically significant enhancement in the level of DNA damage in liver after irradiation of mice with 0.5 Gy and 1.00 Gy of γ-rays. The induction of comets in murine peripheral blood lymphocytes following exposure of mice to 1.00 Gy of γ-rays from a ^{137}Cs source were observed by Mendiola-Cruz and Morales-Ramirez (41). A significant increase of DNA damage in bone marrow lymphocytes after irradiation with doses of 0.10 Gy - 2.00 Gy and in male mice haploid germ cells following X-rays exposure to 0.50 Gy - 2.00 Gy were observed by Dobrzynska (42), however then DNA damage score was calculated in a different way. Studies performed by other groups showed that, in sperm, comet length and moment were increased following treatment of male mice with the isotope Indium-114 (1.85 MBq) which was localised in testes. Irradiation of spermatids, spermatocytes, differentiating spermatogonia and stem cell spermatogonia of male mice with dose of 4.00 Gy caused an increase in DNA damage present in spermatozoa collected at different time points. The maximum increase was seen in differentiating spermatogonia which were also sensitive to irradiation with lower (0.5 Gy-2.00 Gy) doses (42, 43). There are no papers described DNA damage measured using the comet assay in spleen, kidney or lungs following irradiation.

The results of the comet assay for the AA treatments presented in this paper are similar to those obtained by Andrews *et al.* (45). Following treatment with 100 mg/kg bw AA they observed a significant increase in DNA migration in the blood, brain, liver and spleen, with liver cells exhibiting the greatest response. Similarly, in this study after treatment of mice with doses 75 - 125 mg/kg bw AA, liver cells showed higher DNA damage than spleen cells and lymphocytes. In contrast, Klaunig and Kamendulis (46) observed no increase in DNA damage in liver cells of rats exposed to AA (15 mg/kg/day). Tice *et al.* (47) showed that 100 mg/kg AA induced a significant increase of DNA damage in liver, spleen, blood and testes diploid cells 6 h after treatment, while only the spleen exhibited a significant increase of DNA damage at 10 mg/kg AA. Results presented here did not confirm the high sensitivity of spleen, with significant increases in DNA damage after treatment of mice with doses of 75-125 mg/kg bw of AA, but not following exposure to 50 mg/kg bw AA. Maniere *et al.* (48) observed increased DNA migration in brain and testes, but none or weak in liver, bone marrow and adrenals after a single oral dose of AA (18-54 mg/kg bw) in rats. In other papers, it has been established that following chronic exposure F344 rats exhibited an increase in the incidence of adrenal pheochromocytomas, testicular mesotheliomas, thyroid adenoma and mammary neoplasmas (11, 46). There are no papers describing the effect of DNA damage caused by AA on lung and kidney cells measured using the comet assay.

The effects of combined treatments with X-rays and AA on the DNA damage measured with the comet assay have not previously been described. The results presented here showed an increase in DNA damage in cells from all investigated organs. In comparing the response of various organs after exposure to each agent alone, acrylamide showed the greatest induction of DNA damage, but X-ray irradiation was able to enhance the effects caused by AA in a dose-dependent manner. After combined exposure to 0.10 Gy + 50 mg/kg bw AA, the DNA damage in bone marrow lymphocytes and in liver cells were similar to those from exposure to AA alone, but higher than after exposure to X-rays alone. In spleen, kidney, lung and testes, DNA damage after combined exposure was higher than the effects of single exposure to X-rays or AA. Following combined treatment with 0.25 Gy + 50 mg/kg bw AA, DNA damage in cells from all organs was significantly higher than that after exposure to each agent alone. Research conducted previously in our department showed that combined exposure to X-rays and acrylamide, even at low doses, can lead to an enhanced level of mutation in germ and somatic cells of laboratory mice. Combined exposure to both agents enhanced the frequency of micronuclei and dominant lethal mutations (17, 49).

Nowadays, one of the important sources of exposure of the general population to AA seems to be the consumption of high-acrylamide food. In addition, as a consequence of human activity or radiation accidents, the environment is a source of artificial radionuclide. Some of which have a long half-life and, are accumulated in the food chain. Combined exposure to low doses of irradiation and AA can occur not only in the

workplace and in the environment, but also through food. On the other hand, other agents can also enhance DNA damage caused by acrylamide. Processed food may contain potentially toxic compounds or protective compounds, which could have additive, synergistic or antagonistic effects on the biological actions of acrylamide (2). It would be interesting to find agents able to protect DNA against such damage. Recent results suggest that acrylamide-induced oxidative DNA damage, which may be reduced by antioxidants like spin traps and vitamins C and E (50).

As mentioned before, the presented results show that X-rays enhanced DNA damage in somatic and germ cells caused by acrylamide. Such damage in somatic cells, if not repaired or if repaired incorrectly can lead to mutation, cancer transformation or cell death in target organs. Damage in germ cells can affect the process of fertilisation and lead to spontaneous abortion, congenital malformations and in heritable diseases, including cancer in the offspring (51-52). In particular, the integrity of germ cell DNA plays an important part in the transmission of genetic information to the offspring. DNA damage measured using the comet assay in human spermatozoa has been shown to be associated with infertility (53). The negative effect of combined exposure of males to irradiation and AA on the developing foetus is already known (49). Recently, Sorgel *et al.* (54) found that from 10% to 15% of dietary acrylamide present in pregnant women is transferred *via* the blood and placenta to the foetus. To protect the foetus prospective parents should minimise their consumption of high-acrylamide food before and after conception.

Human biomonitoring studies could be useful in investigating the effects of combined exposure to low doses of both radiation and AA on somatic and germ cells.

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