**Table 2** Body and organ weights of young and adult rats given ACR in the drinking water for 4 weeks

| | | Acrylamide in the drinking water (ppm) | | | |
|---|---|---|---|---|---|
| | | 0 | 50 | 100 | 200 |
| *Young* | | | | | |
| Body weight | (g) | 287.4 ± 24.6[a] | 273.5 ± 15.7 | 263.7 ± 14.4* | 210.4 ± 24.3** |
| Brain | (g) | 1.97 ± 0.05 | 1.94 ± 0.09 | 1.83 ± 0.09* | 1.66 ± 0.04** |
| | (g%) | 0.69 ± 0.06 | 0.71 ± 0.05 | 0.69 ± 0.03 | 0.80 ± 0.08** |
| Testes | (g) | 2.57 ± 0.15 | 2.44 ± 0.22 | 2.39 ± 0.19 | 1.87 ± 0.36** |
| | (g%) | 0.90 ± 0.07 | 0.90 ± 0.09 | 0.91 ± 0.07 | 0.89 ± 0.12 |
| Epididymides | (g) | 0.40 ± 0.04 | 0.35 ± 0.02** | 0.37 ± 0.04 | 0.30 ± 0.02** |
| | (g%) | 0.14 ± 0.02 | 0.13 ± 0.01 | 0.14 ± 0.02 | 0.15 ± 0.02 |
| *Adult* | | | | | |
| Body weight | (g) | 444.3 ± 38.0 | 433.0 ± 42.0 | 426.7 ± 42.1 | 409.2 ± 45.5 |
| Brain | (g) | 2.07 ± 0.06 | 2.08 ± 0.11 | 2.02 ± 0.09 | 1.99 ± 0.07 |
| | (g%) | 0.47 ± 0.03 | 0.48 ± 0.04 | 0.48 ± 0.04 | 0.49 ± 0.06 |
| Testes | (g) | 3.30 ± 0.26 | 3.39 ± 0.39 | 3.25 ± 0.20 | 3.19 ± 0.24 |
| | (g%) | 0.74 ± 0.07 | 0.78 ± 0.08 | 0.77 ± 0.08 | 0.79 ± 0.09 |
| Epididymides | (g) | 0.97 ± 0.05 | 1.04 ± 0.09 | 0.97 ± 0.07 | 0.84 ± 0.06** |
| | (g%) | 0.22 ± 0.02 | 0.24 ± 0.02 | 0.23 ± 0.03 | 0.21 ± 0.02 |

10 animals per each group were examined

[a] Mean ± SD

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

**Table 3** Histopathology and morphometry of lesions developing in the nervous system

| | | Acrylamide in the drinking water (ppm) | | | |
|---|---|---|---|---|---|
| | | 0 | 50 | 100 | 200 |
| *Young* | | | | | |
| Trigeminal nerve | | | | | |
| No. of animals examined | | 10 | 10 | 10 | 10 |
| Central chromatolysis (+/++/+++)[a] | | 0 | 3 (3/0/0) | 10 (0/5/5)[##] | 10(0/0/10)[##] |
| Sciatic nerve (distal portion) | | | | | |
| No. of animals examined | | 10 | 10 | 10 | 10 |
| Density | (/100 μm$^2$) | 2.56 ± 0.32[b] | 2.73 ± 0.17 | 2.92 ± 0.25** | 2.42 ± 0.25 |
| Degenerated axons | (%) | 0.28 ± 0.15 | 0.39 ± 0.14 | 0.82 ± 0.19** | 7.51 ± 3.25** |
| Myelinated axons, <3 μm in diameter | (%) | 18.01 ± 3.45 | 16.74 ± 2.79 | 18.80 ± 2.73 | 21.57 ± 4.07 |
| Cerebellar cortex | | | | | |
| No. of animals examined | | 5 | 5 | 5 | 5 |
| SYP-immunoreactive aberrant dots | (/mm cortex) | 0.50 ± 0.20 | 0.41 ± 0.18 | 1.49 ± 0.59 | 6.09 ± 1.62* |
| *Adult* | | | | | |
| Trigeminal nerve | | | | | |
| No. of animals examined | | 10 | 10 | 10 | 10 |
| Central chromatolysis (+/++/+++)[a] | | 0 | 3 (3/0/0) | 10 (3/7/0)[##] | 10 (0/3/7)[##] |
| Sciatic nerve (distal portion) | | | | | |
| No. of animals examined | | 10 | 10 | 10 | 10 |
| Density | (/100 μm$^2$) | 2.10 ± 0.23 | 2.03 ± 0.15 | 2.10 ± 0.24 | 2.15 ± 0.24 |
| Degenerated axons | (%) | 0.39 ± 0.16 | 0.65 ± 0.27 | 0.96 ± 0.37* | 1.74 ± 0.77** |
| Myelinated axons, <3 μm in diameter | (%) | 13.96 ± 2.75 | 12.30 ± 2.39 | 13.45 ± 2.68 | 14.16 ± 2.82 |
| Cerebellar cortex | | | | | |
| No. of animals examined | | 5 | 5 | 5 | 5 |
| SYP-immunoreactive aberrant dots | (/mm cortex) | 0.54 ± 0.12 | 0.47 ± 0.09 | 1.71 ± 0.81 | 5.88 ± 2.61* |

[a] *Grade of change* + mild, ++ moderate, +++ severe

[b] Mean ± SD

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

[##] $P < 0.01$ vs. 0 ppm group (Fisher's exact test)

*SYP* synaptophysin

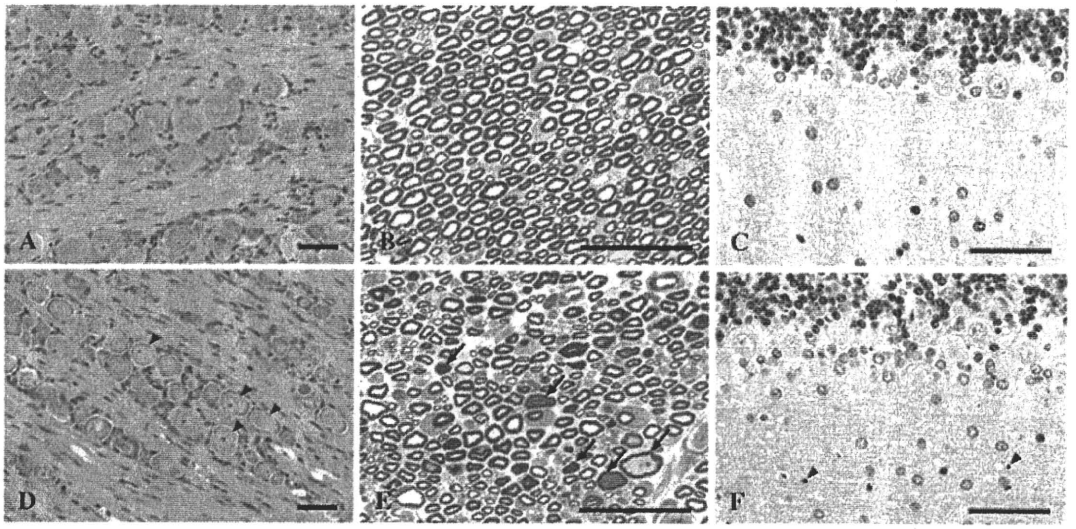


**Fig. 3** Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) in young rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of a young rat from the 0 ppm group. (**d–f**) At 200 ppm, central chromatolysis of ganglion cells (**d** *arrowheads*) in the trigeminal nerve was apparent. Increases in degenerated axons (**e** *arrows*) in the sciatic nerve and dot-like SYP-immunoreactive structures (**f** *arrowheads*) in the cerebellar molecular layer were also found. **a**, **d** hematoxylin and eosin. **b**, **e** resin-embedded semithin sections stained with toluidine blue. **c**, **f** immunohistochemical staining for SYP. *Bar* = 50 μm

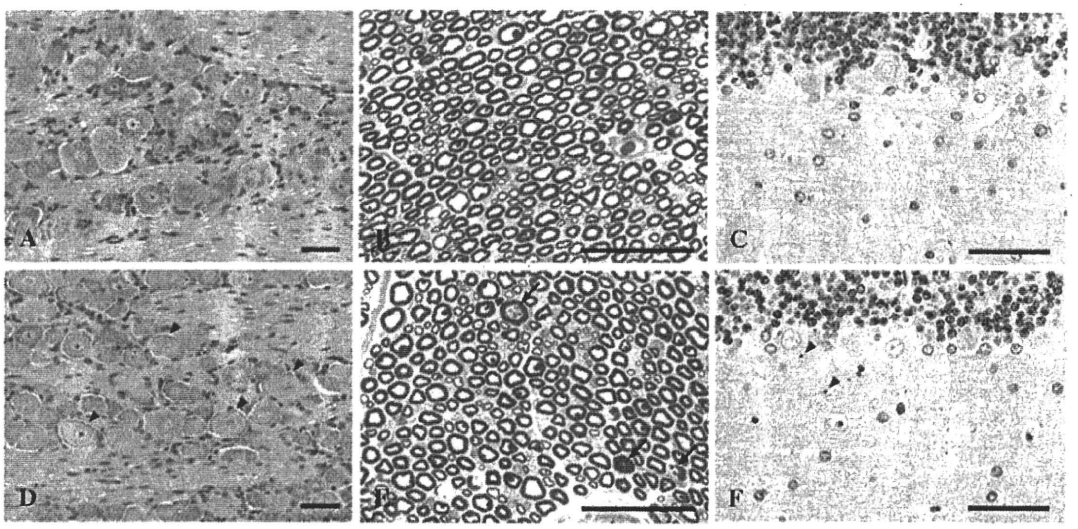


**Fig. 4** Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) of adult rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of an adult rat from 0 ppm group. (**d–f**) Similar to the young group, central chromatolysis of ganglion cells (**d** *arrowheads*) in the trigeminal nerve, increases in degenerated axons (**e** *arrows*) in the sciatic nerve and dot-like SYP-immunoreactive structures (**f** *arrowheads*) in the cerebellar molecular layer were observed at 200 ppm **a**, **d** hematoxylin and eosin. **b**, **e** resin-embedded semithin sections stained with toluidine blue. **c**, **f** immunohistochemical staining for SYP. *Bar* = 50 μm

axons in the sciatic nerve was increased only at 100 ppm in the young group, but without dose dependence. In both young and adult groups, significant increase in degenerated axons in the sciatic nerve was observed from 100 ppm, and increase in dot-like SYP-immunoreactive structures in the cerebellar molecular layer was also found at 200 ppm. Although not statistically significant, myelinated nerves <3 μm in diameter showed a tendency for increase at 200 ppm in both young and adult groups. At 200 ppm, most parameters were higher in young groups compared to adult counterparts.

In the testis, marked degeneration and loss of or decrease in spermatids was observed from 100 ppm in young animals (Fig. 5 and Table 4). Elongate spermatids

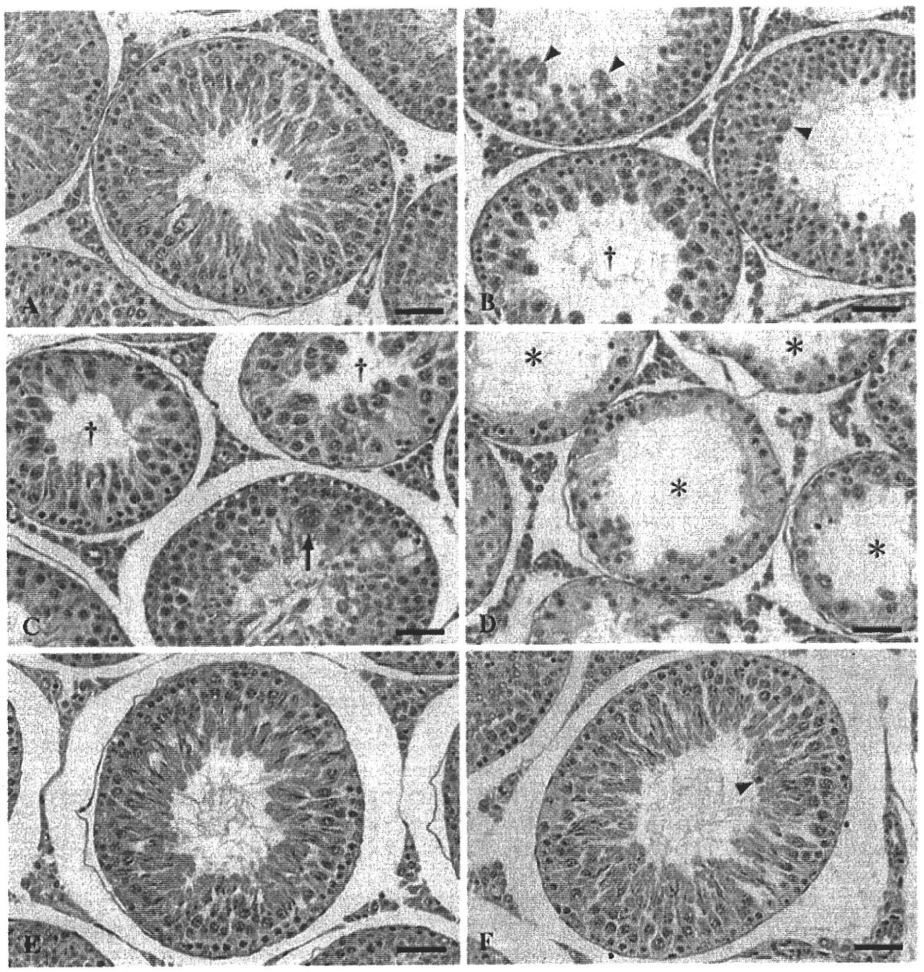


**Fig. 5** Histopathology of the testis of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. **a** Normal seminiferous tubules of a young rat from the 0 ppm group. (**b–d**) Degeneration of spermatids (*arrowheads*), loss of or decreased in elongated spermatids (†), and multinucleated giant cells (*arrow*) are apparent in a young rat at 200 ppm. In severely affected cases, many seminiferous tubules showed marked germ cell depletion (*). **e** Normal seminiferous tubules of an adult rat from the 0 ppm group. **f** Only a small number of exfoliated germ cells (*arrowhead*) was found in the lumina of tubules in a case of the adult group at 200 ppm. HE stain. All *bars* = 50 μm

appeared to be most vulnerable to ACR, and in severely affected cases, many seminiferous tubules showed marked germ cell depletion. In addition, exfoliation of germ cells and appearance of multinucleated giant cells were also found. Many exfoliated germ cells were observed in the epididymal duct. In the adult groups, only small numbers of exfoliated germ cells was found in lumina of seminiferous tubules. Sertoli cells were morphologically unaffected in both young and adult animals. Similar histopathological changes were also observed in the testis of each young and adult animal at 200 ppm in the satellite groups used for measuring GST activity and GSH contents.

Data for relationships between ACR intake per kg body weight and neurotoxicity parameters, including the gait score at week 4, the number of degenerated axons in the sciatic nerves, and the number of SYP-immunoreactive structures in the cerebellar molecular layer are shown in Fig. 6a–c. All these parameters increased in proportion to ACR intake. For testicular toxicity, the relationship between ACR intake per kg body weight and the percentage of affected seminiferous tubules is shown in Fig. 6d. With increase in ACR dose, affected tubules profoundly increased in the young group, while the magnitude of increase was very small in the adult group.

## Comet assays and MN tests

The comet assay revealed that ACR significantly induced DNA damage in a dose-dependent manner from 100 ppm

**Table 4** Histopathological data for the testes of young and adult rats given ACR in the drinking water for 4 weeks

| Findings (%)[a] | Acrylamide in the drinking water (ppm) | | | |
|---|---|---|---|---|
| | 0 | 50 | 100 | 200 |
| *Young* | | | | |
| Affected tubules[b] | 3.51 ± 1.68[c] | 9.03 ± 18.81 | 16.93 ± 12.23* | 66.59 ± 26.96** |
| Exfoliation of germ cells | 3.39 ± 1.61 | 3.93 ± 3.07 | 9.80 ± 6.22* | 10.44 ± 9.87 |
| Multinucleated giant cells | 0.02 ± 0.06 | 0.07 ± 0.14 | 0.57 ± 0.71 | 1.67 ± 3.06** |
| Degeneration of spermatids | 0.10 ± 0.18 | 0.84 ± 2.54 | 3.95 ± 6.47 | 20.90 ± 13.37** |
| Loss of or decrease in elongated spermatids | 0 ± 0 | 4.99 ± 15.74* | 5.62 ± 8.87** | 20.43 ± 14.61** |
| Loss of or decrease in round spermatids | 0.02 ± 0.06 | 0 ± 0 | 1.51 ± 3.19 | 12.68 ± 10.97** |
| Atrophic tubules[d] | 0 ± 0 | 0.17 ± 0.54** | 0.12 ± 0.38** | 24.03 ± 30.83** |
| Sertoli cell vacuolation | 0.60 ± 0.57 | 1.07 ± 0.55 | 0.99 ± 0.62 | 1.06 ± 0.92 |
| *Adult* | | | | |
| Affected tubules[b] | 0.47 ± 0.30 | 0.58 ± 0.23 | 1.17 ± 0.60* | 1.53 ± 0.67** |
| Exfoliation of germ cells | 0.45 ± 0.30 | 0.56 ± 0.23 | 1.17 ± 0.60* | 1.46 ± 0.71** |
| Multinucleated giant cells | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0.07 ± 0.17 |
| Degeneration of spermatids | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Loss of or decrease in elongated spermatids | 0.02 ± 0.06 | 0.02 ± 0.07 | 0 ± 0 | 0 ± 0 |
| Loss of or decrease in round spermatids | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Atrophic tubules[d] | 0 ± 0 | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Sertoli cell vacuolation | 0.62 ± 0.45 | 0.53 ± 0.50 | 0.72 ± 0.43 | 0.81 ± 0.53 |

10 animals per each group were examined

[a] Approximately 400–650 tubules/rat were examined

[b] Affected tubules represent total tubules with findings, except for tubules showing only Sertoli cell vacuolation

[c] Mean ± SD

[d] Atrophic tubules are those showing marked germ cell depletion

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

in young and adult groups (Fig. 7a). Although the values did not greatly differ between the groups, the values in the young group were higher than those in the adult group at 200 ppm. On the other hand, MN was clearly induced only in young group in a dose-dependent manner (Fig. 7b) with statistical significance at both 100 and 200 ppm ($P < 0.05$). ACR slightly induced MN in the adult group at 200 ppm.

GST activity and GSH contents in the liver and testis

In the liver, compared to the 0 ppm group, GST activity was significantly increased at 200 ppm in the young group (Fig. 8a). Although not statistically significant, GST activity in the adult animals at 200 ppm also showed a tendency for increase. GSH contents were unchanged in both groups. There were no differences in the level of GST activity and GSH contents in the liver between young and adult groups. In the testis, although GST activity and GSH contents in both groups were not changed by ACR treatment, the levels of GST activity in the young group were significantly lower than those in the adult group (Fig. 8b).

## Discussion

In the present study, ACR dose-related suppression of body weight, and food and water consumption was observed only in young rats. When gait abnormalities progressed, animals became unable to support their body weights, and it was difficult to take food and water from containers set in the upper part of the cage. Therefore, in the housing conditions designed for adult animals, the suppressions might be due to immature body size of young animals causing difficulty in access to food and water associated with the development of neurotoxicity.

On clinical observation, although both young and adult animals exhibited similar symptoms from 100 ppm, earlier occurrence and faster progression of the symptoms were

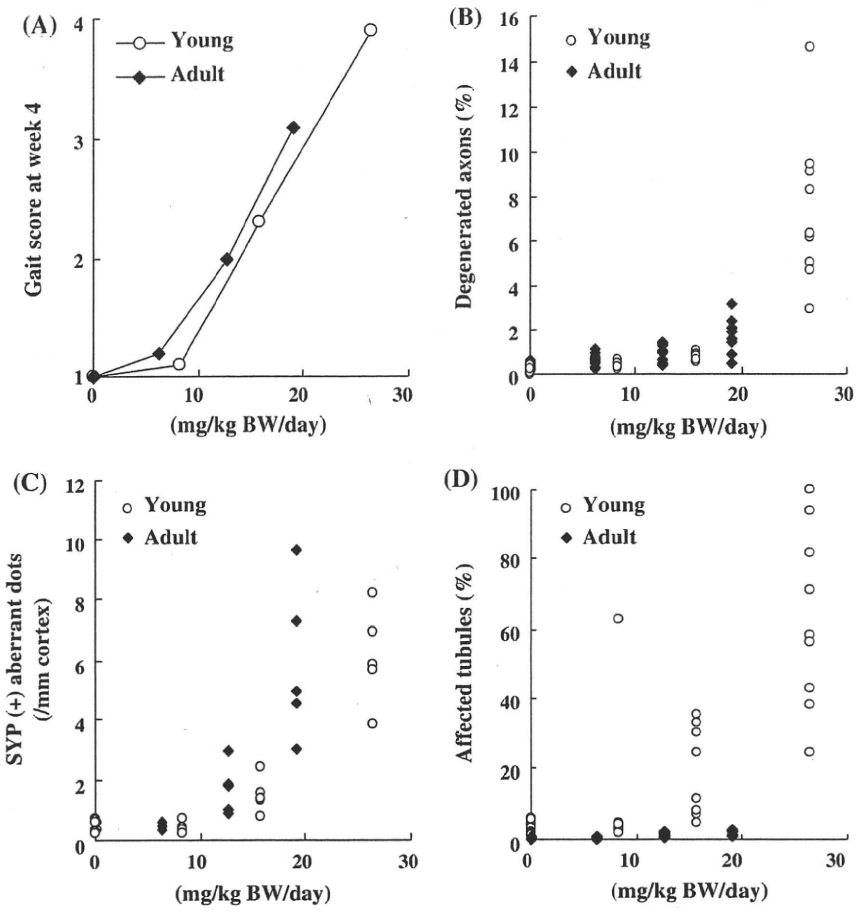


**Fig. 6** Relationship between ACR intake per body weight and changes in neurotoxicity and testicular toxicity parameters in young and adult rats

here observed in the young group. Also, neurotoxic lesions such as central chromatolysis of ganglion cells in the trigeminal nerves, degenerated axons in the sciatic nerve and dot-like SYP-immunoreactive structures in the cerebellar molecular layer, were evident from 100 ppm in both young and adult groups. The magnitude of changes in these parameters was higher in the young group than in the adult group, especially at the highest dose, and neurotoxicity appeared stronger in young animals, though the types of lesions observed were similar between the young and adult groups. Compared to adult animals, intake of ACR per kg body weight was higher in young animals at each dose and the parameters indicating the neurotoxicity increased in proportion to ACR intake. Accordingly, the stronger neurotoxicity in the young animals can be considered to be a reflection of larger amount of ACR intake per body weight. These results suggest that the susceptibility to ACR-induced neurotoxicity in young and adult rats is qualitatively similar under the given experimental conditions. As mentioned in the Introduction section, a few studies have demonstrated life stage-related differences in susceptibility to ACR neurotoxicity, though the experimental conditions, such as age of animals, dosing methods, and parameters examined, were different. While Suzuki and Pfaff concluded that suckling rats were more susceptible (Suzuki and Pfaff 1973), it seems that there was not much difference in number of injections to cause apparent symptoms and myelin degeneration between suckling and adult rats. In the report by Ko et al., earlier occurrence and faster progression of neurological abnormalities in young animals were similar to those observed in our study (Ko et al. 1999). Although the authors stated that the daily intake was not significantly different between the young and adult groups, intake of ACR per body weight at the beginning of the experiment might have been higher in the young group, because younger animals usually take more water than older ones. Taken together, clear evidence of the susceptibility difference in neurotoxicity between young and adults animals is considered to be undetermined.

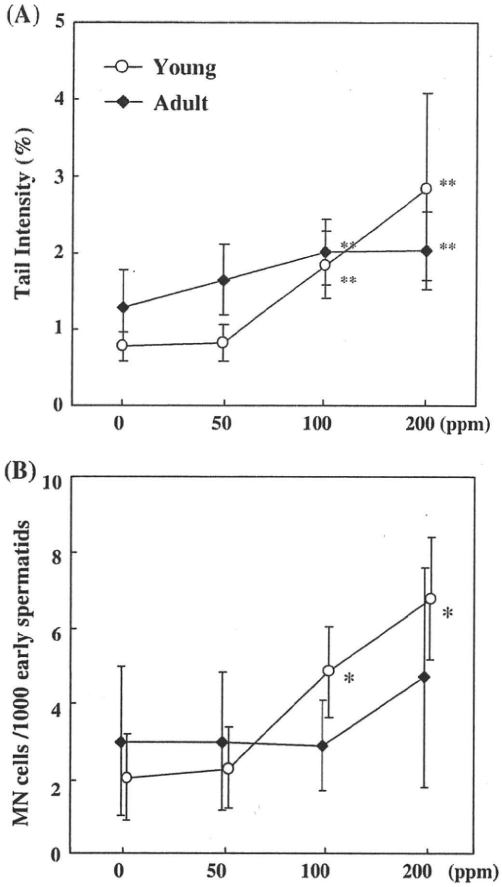


**Fig. 7** Tail intensity of the comet image (**a**) and micronuclei frequency (**b**) obtained from young and adult rats given ACR in the drinking water for 4 weeks. Data are mean ± SD. $^{**}P < 0.01$ vs. 0 ppm

Regarding the susceptibility to ACR testicular toxicity in the present study, young animals showed apparently diverse and more profound lesions exceeding the dose–effect relationship observed in adult animals. ACR is known to interfere with motor proteins such as kinesin found in the sperm flagellum and alkylate protein sulfhydryl groups in the sperm tail (Sickles et al. 2007; Friedman et al. 2008). Therefore, it is considered that elongate spermatids are highly susceptible to ACR. In the comet assay, although DNA damage in the young group was higher than that in the adult group at 200 ppm, the values were not greatly different. However, the MN test revealed that ACR clearly induced MN in the young group, but not in the adult group. These results well correspond with the observations on histopathological examination. Because the comet assay and MN test in the testis target spermatocytes and early spermatids, the late stage of spermatogenesis may be more susceptible to ACR-induced genotoxicity in young than in adult animals.

As reported by others (Yousef and El-Demerdash 2006), the basal level of testicular GST activity in our cases was much lower than that in the liver. Although there were no life stage differences in the liver levels of GST activity, testicular GST activity in the present study was significantly lower in the young groups, irrespective of the ACR treatment. The activity of GST is low at birth and then increases gradually, but it has been known that the developmental profiles of antioxidant enzymes including GST in the testis differ greatly from those in the liver (Peltola et al. 1992). A study of the immunolocalization of GST-Yo, a member of the mu class expressed at high levels in the testis and epididymides, revealed that this enzyme was not detectable until 39 days of age and then appeared mainly in the elongate spermatids, with expression reaching maturity by day 49 (Papp et al. 1994). Therefore, the detoxification capacity of the testis in young animals was considered to be much lower than that in the adult animals during the experimental period in the present study, and such a difference might reasonably account for the high susceptibility to ACR-induced testicular toxicity observed in our young animals. In the liver, although GST activity was increased at 200 ppm, there were no apparent life-stage differences. Considering that the liver is the main organ involved in detoxification of ACR, similar level of GST activity may have contributed to the lack of differences in susceptibility to neurotoxicity between young and adult rats. Increase in GST activity in ACR-treated rats has been reported and considered to be due to increased formation of S-conjugates between ACR and GSH (Yousef and El-Demerdash 2006). ACR is known to cause GSH depletion (Zhang et al. 2009); however, decrease in GSH contents was not found in the present study. Because recovery or rather increase in liver GSH contents after depletion by treatment animals with acetaminophen has been reported (Ishii et al. 2009), the level of GSH in the present study might possibly have recovered after repeated treatment with ACR during the experimental period.

In summary, our results suggest that susceptibility to ACR neurotoxicity in young animals might not be different from that in adult ones when exposure levels are adjusted for the body weight. Regarding testicular toxicity, young animals proved more vulnerable than adults, and this might be due to a low level of testicular GST activity.

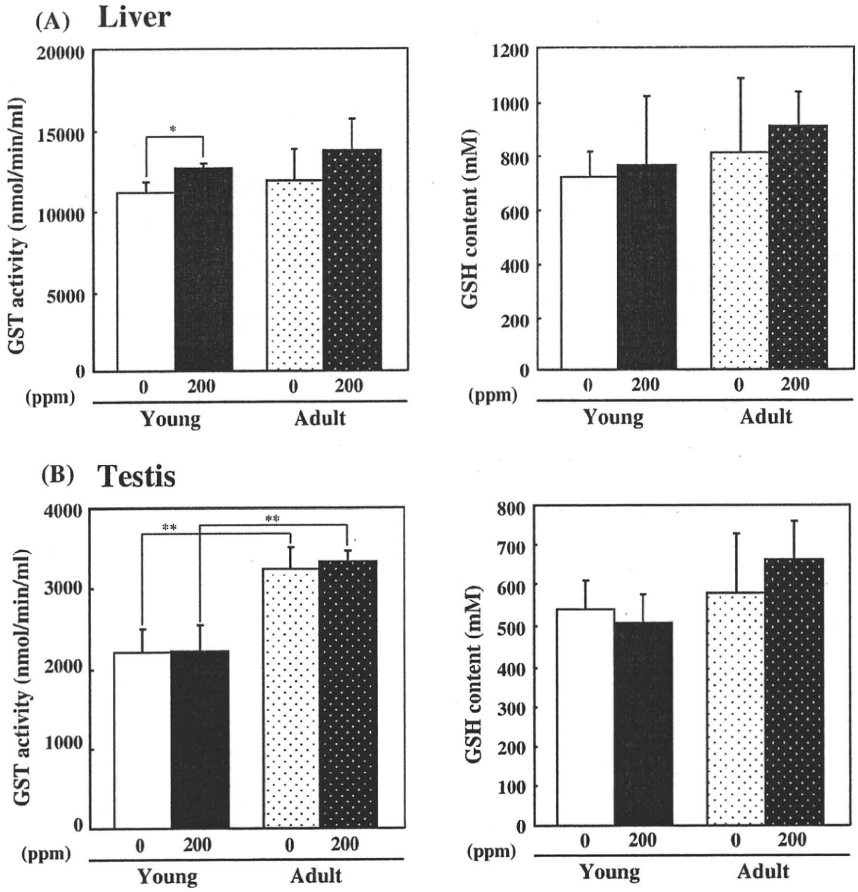


**Fig. 8** GST activity and GSH contents in the liver (**a**) and testis (**b**) of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. Data are mean ± SD. *, **$P < 0.05$ and $P < 0.01$

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