Bull. Environ. Contam. Toxicol. (1998) 60:494-501

Bulletin of Environmental Contamination and Toxicology

Histopathological Changes Within the Testis Caused by Allyl Chloride Exposure in Mice

M. Zhao,[1,*] M. Omura,[1] S. Tokunaga,[2] Y. Romero,[1] N. Inoue[1]

[1]Department of Hygiene, Faculty of Medicine, Kyushu University, Fukuoka 812-82, Japan
[2]Department of Public Health, Faculty of Medicine, Kyushu University, Fukuoka 812-82, Japan

Received: 4 August 1997/Accepted: 1 December 1997

Allyl chloride (AC) is used in the production of epoxy resins, glycerol, sodium allylsulfonate, and other chemicals (IARC 1980). Polyneuropathy and liver dysfunction have been reported in workers exposed to AC (Häusler and Lenich 1968; He et al. 1980, 1985) and these adverse effects were reproduced in animal experiments (Adams et al. 1940; Torkelson et al. 1959; He et al. 1980, 1981 ; Lu et al. 1982). Recently, we found that AC was toxic to the male reproductive system when subcutaneously injected into mice (Zhao 1997). In our study, the number of homogenization-resistant testicular spermatids and the number of epididymal sperm began to decrease 4 days and 14 days after a single subcutaneous injection of 124 mg/kg of AC, respectively. Considering the time required for sperm to pass through the epididymis, it seems likely that AC damaged elongated spermatids and other spermatogenic cells in the testis within a few days after the injection. In addition, the frequency of sperm tail abnormalities increased moderately before epididymal sperm reduction became apparent. It would seem, therefore, that AC may perhaps damage sperm as they pass through the epididymis.

In this article, we report the histopathological changes within the testis caused by AC exposure in mice. We administered 124 mg/kg of AC to mice by a single subcutaneous injection and sequentially examined the histopathological changes within the testis over a period of 39 days.

MATERIALS AND METHODS

One hundred male ICR mice (Crj:cd-l) were purchased at seven weeks of age and were acclimatized to our animal facility for two weeks. Mice were then housed five per cage in a specific-pathogen-free room within the Laboratory of Animal Experiments belonging to the Faculty of Medicine, Kyushu University. The light cycle was 12 hours light: 12 hours dark, the temperature was 23 - 25°C, and the air humidity was 50 - 65%. Mice were provided with CE-2 (Clea Japan Inc., Tokyo, Japan) and tap water *ad libitum.* This experiment was reviewed by the Committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University and

Present address: Saga Research Institute, Otsuka Pharmaceutical Co., Ltd., Higashisefuri-son, Kanzaki-gun, Saga 842-01, Japan
Correspondence to: M. Omura

was carried out under Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No.6) of the Government.

All mice at nine weeks of age were randomly distributed into the AC group or the control group each comprising fifty mice. In the AC group, mice were administered 124 mg/kg of AC (the purity was 98.6%, Wako Pure Chemicals Industries Ltd., Osaka, Japan) by a single subcutaneous injection. This dose was one-fifth of the subcutaneous LD_{50} of AC (Omura et al. 1993). AC was dissolved in dimethylsulfoxide (DMSO, analytical grade, E. Merck, Darmstadt, Germany) just before the injection. Mice in the control group received an identical volume of DMSO in the same manner. Ten mice from each group were killed 4, 9, 14, 22, and 39 days after the injection and the right testis was dissected out. The duration of one cycle of the seminiferous epithelium is 8.6 days in mice (Clermont and Trott 1969). Therefore, these time intervals corresponded to 0.5, 1.0, 1.5, 2.5, and 4.5 cycles of the seminiferous epithelium, respectively. We chose 4.5 cycles of the seminiferous epithelium as the experimental period because it takes approximately 4.5 cycles of the seminiferous epithelium for stem cell spermatogonia to develop into mature spermatids (Amann 1982).

The testis was fixed in Bouin's solution, embedded in paraffin, thinly sectioned, and then stained with periodic acid Schiff reagent (PAS) and hematoxylin. In some AC-treated mice, spermatids were lacking in seminiferous tubules and it was thus difficult to classify these tubules into specific stages. Therefore, in the histopathological examination of the testis, the stages of the seminiferous tubules were classified into four groups comprising stages I-VI, VII-VIII, IX-XI, and XII. These stage groups could be classified based on the type of spermatogenic cells situated at the base of the seminiferous epithelium (spermatogonia only for stages I-VI, preleptotene spermatocyte + spermatogonia for stages VII-VIII, and leptotene/zygotene spermatocyte + spermatogonia for stages IX-XI) or the appearance of meiotic figures (stage XII). Matsui et al. (1996) reported that the results of the histopathological examination in rats with classification of stages into four major groups (stages I-VI, VII-VIII, IX-XI, XII-XIV) were similar to those with strict stage classification. Therefore, we thought that the histopathological changes within the seminiferous tubules of mice could be evaluated using the four-stage classification described above. All round or ovoid cross-sections of the seminiferous tubules were examined in one transverse section of the testis. In addition, the frequencies of the seminiferous tubules with the following histopathological lesions were evaluated; empty tubules (tubules with no spermatogenic cells except for a few spermatogonia), sloughing of the seminiferous epithelium, appearance of multinucleated giant cells, and degeneration or lack of spermatogenic cells. The difference in the proportion of mice with the histopathological lesions was analyzed using Fisher's exact probability test. The results were interpreted as significant below a level of 0.05.

RESULTS AND DISCUSSION

Complete or partial lack of specific spermatogenic cells frequently occurred in AC-treated mice 4, 9, and 14 days after the injection. The type of spermatogenic cell which was lacking in the seminiferous tubule changed sequentially.

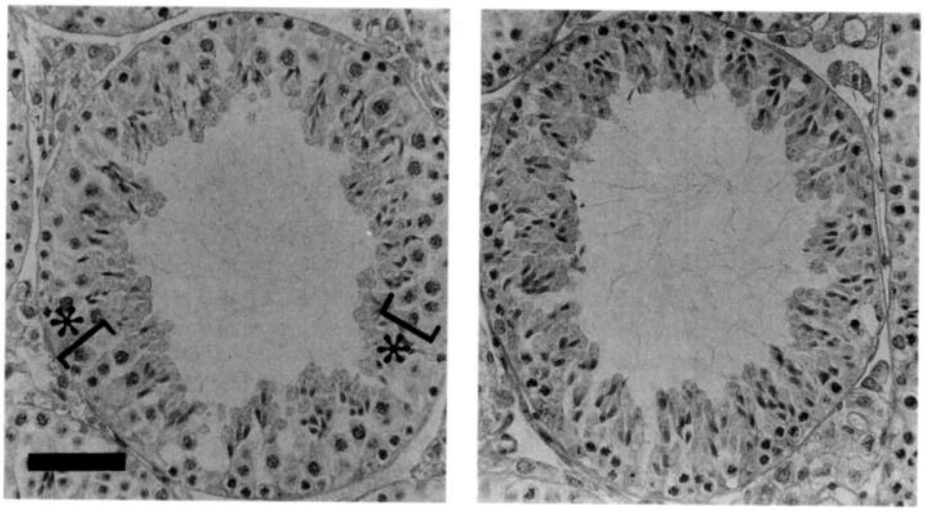

Figure 1. Stage X seminiferous tubule of a control mouse (left) and of a mouse treated with allyl chloride (right) four days after the injection. In the mouse treated with allyl chloride, the seminiferous epithelium lacks pachytene spermatocytes (indicated by asterisks in left figure. Bar indicates 50 μ m).

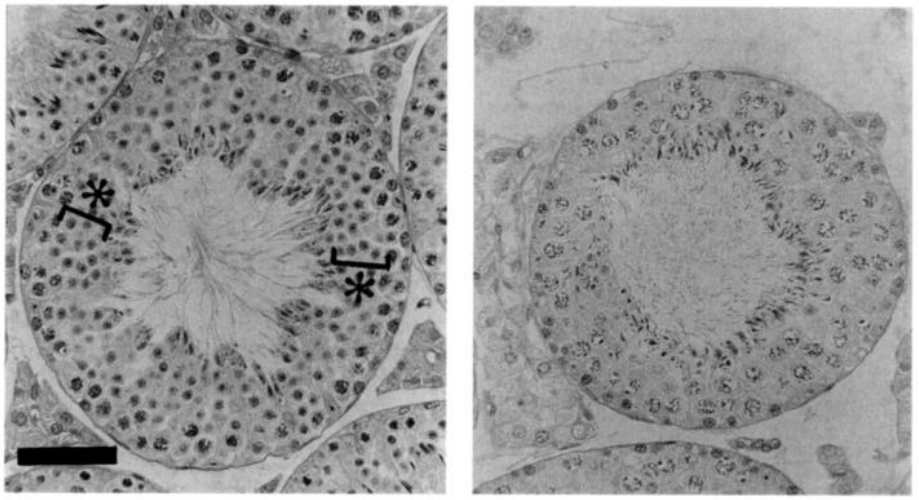

Figure 2. Seminiferous tubule of a control mouse (left) and of a mouse treated with allyl chloride (right) at stages VII-VIII nine days after the injection. In the mouse treated with allyl chloride, the seminiferous epithelium lacks round spermatids (indicated by asterisks in left figure. Bar indicates 50 μ m).

Four days after the injection, all the seminiferous tubules at stages IX-XI lacked pachytene spermatocytes in one AC-treated mouse. In this mouse, some tubules at stages VII-VIII also lacked pachytene spermatocytes. Figure 1 shows a stage X

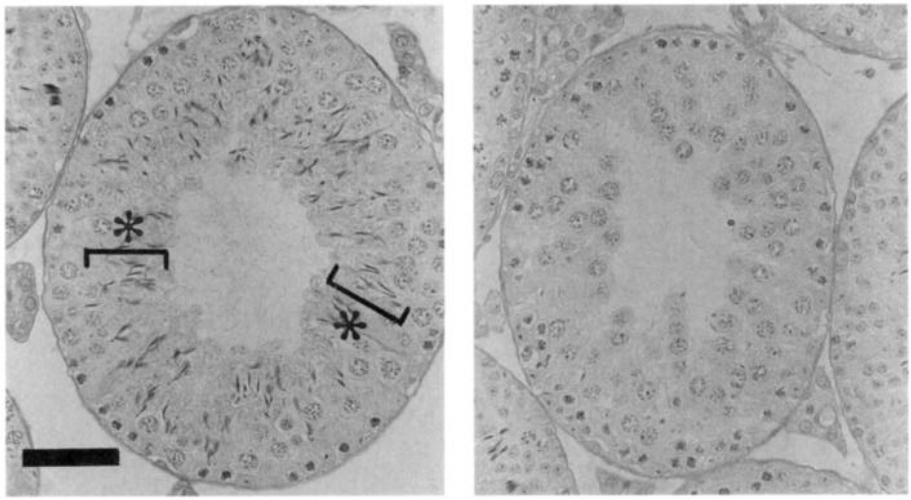

Figure 3. Seminiferous tubule of a control mouse (left) and of a mouse treated with ally1 chloride (right) at stages IX-XI 14 days after the injection. In the mouse treated with allyl chloride, the seminiferous epithelium lacks elongating spermatids (indicated by asterisks in left figure. Bar indicates 50 μ m).

Table 1. Proportion of allyl-chloride-treated mice in which more than 10% of the seminiferous tubules showed a lack of specific spermatogenic cells

Type of spermatogenic cell which was lacking	Days after injection				
	4 days	9 days	14 days	22 days	39 days
Pachytene spermatocyte at stages IX-XI	1/10	0/10	0/10	0/10	0/10
Round spermatid at stages I-VI and VII-VIII	0/10	7/10*	0/10	0/10	0/10
Elongating spermatid at stages IX-XI	0/10	0/10	5/10*	0/10	0/10

Statistical significance was analyzed with Fisher's exact probability test; * $p<0.05$.

seminiferous tubule of a control mouse (left) and of an AC-treated mouse (right) four days after the injection. In the control mouse, pachytene spermatocytes were situated between elongating spermatids and leptotene spermatocytes/spermatogonia. However, in the AC-treated mouse, pachytene spermatocytes were not observed and elongating spermatids were situated next to leptotene spermatocytes/spermatogonia.

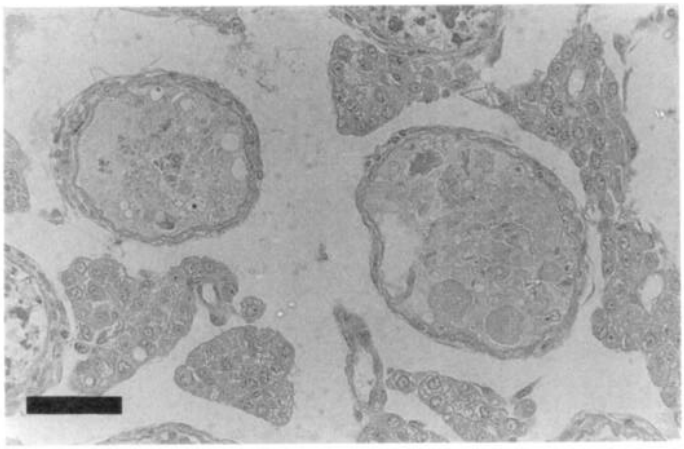

Figure 4. Seminiferous tubules of a mouse treated with allyl chloride four days after the injection. The seminiferous tubules are severely shrunken and contain no spermatogenic cells except for a few spermatogonia (Bar indicates 50 μ m).

Table 2. Proportion of mice in which more than 10% of the seminiferous tubules contained no spermatogenic cells except for a few spermatogonia (empty tubule)

Group	Days after injection				
	4 days	9 days	14 days	22 days	39 days
Control	0/10	0/9 [a]	0/10	0/10	0/10
Allyl chloride	3/10	4/10	5/10 *	2/10	6/10 *

[a] One animal was excluded because of unilateral atrophy of the testis.
Statistical significance was analyzed with Fisher's exact probability test; * $p<0.05$.

Nine days after the injection, some AC-treated mice showed a lack of round spermatids at stages I-VI and VII-VIII. Figure 2 shows a seminiferous tubule of a control mouse (left) and of an AC-treated mouse (right) at stages VII-VIII nine days after the injection. Normally, round spermatids are situated between elongated spermatids and pachytene spermatocytes at these stages. However, in the tubules of the AC-treated mouse, the seminiferous epithelium lacked round spermatids and elongated spermatids were situated next to pachytene spermatocytes.

Fourteen days after the injection, some AC-treated mice showed a lack of elongating spermatids at stages IX-XI. In these mice, a lack of elongated spermatids also occurred, but to a lesser degree, within the tubules at stages XIV and I-VI. Figure 3 shows a seminiferous tubule of a control mouse (left) and of an AC-treated mouse (right) at stages IX-XI 14 days after the injection. In the tubule of the AC-treated

mouse, no elongating spermatids were observed and pachytene spermatocytes were exposed to the tubular lumen.

Table 1 shows the proportion of mice in which more than 10% of the seminiferous tubules showed a complete or partial lack of the specific spermatogenic cells previously mentioned. In control mice, these histopathological lesions were never observed. This lack of specific spermatogenic cells occurred in pachytene spermatocytes at stages IX-XI, in round spermatids at stages I-VI and VII-VIII, and in elongating spermatids at stages IX-XI 4, 9 and 14 days after the AC injection, respectively. However, these histopathological lesions were not observed 22 days or 39 days after the AC injection. Even in AC-treated mice in which a lack of spermatogenic cells occurred, degenerated spermatogenic cells and multinucleated giant cells were rarely observed.

In addition to the lack of specific spermatogenic cells, severe atrophy of the seminiferous tubules was also frequently observed in the AC-treated mice. Figure 4 shows seminiferous tubules of an AC-treated mouse four days after the injection. The seminiferous tubules were severely shrunken. The tubules contained no spermatogenic cells except for a few spermatogonia (empty tubule) and it was impossible to classify them into specific stages. In the intertubular tissue, the number of Leydig cells appeared to increased. In AC-treated mice with severe atrophy of the seminiferous tubules, the volume of the testis was markedly reduced. Therefore, the increase in the number of Leydig cells in the AC-treated mice was probably a relative one. Table 2 shows the proportion of mice in which more than 10% of the seminiferous tubules were empty. In all the control mice, the frequency of tubules with this lesion was less than 10% (a maximum of 2.5%). Four days after the injection, three AC-treated mice had empty tubules within the testis. This tubular lesion was constantly observed in the AC-treated mice throughout the experimental period and six mice still had this lesion 39 days after the AC injection. In mice which had empty tubules, sloughin g of the seminiferous epithelium was also frequently observed.

In this study, two remarkable histopathological changes were observed in the AC-treated mice; a lack of specific spermatogenic cells and severe atrophy of the seminiferous tubules. This lack of specific spermatogenic cells was observed 4, 9, and 14 days after the AC injection, although the type of spermatogenic cell which was lacking in the seminiferous tubules changed sequentially: pachytene spermatocytes at stages IX-XI four days after the injection, round spermatids at stages I-VIII nine days after the injection, and elongating spermatids at stages IX-XI 14 days after the injection. The spermatogenic cells within the seminiferous tubule are developing continuously. In general, the time required for a spermatogenic cell to develop into another type of spermatogenic cell does not change. Therefore, we can specify the spermatogenic cell which was damaged by an agent at the time of injection based on the type of spermatogenic cell which is lacking at the time of death. Considering the length of time required for each stage of the seminiferous tubule in mice (Russell et al. 1990) pachytene spermatocytes at stages IX-XI four days after the AC injection had been pachytene spermatocytes at stages II-VII on day 0. Similarly, round spermatids at stages I-VIII nine days after the AC injection had been pachytene spermatocytes at stages I-IX, while elongating spermatids at stages IX-XI 14 days after the AC injection had been pachytene

spermatocytes at stages II-VII on day 0. Accordingly, it seems likely that pachytene spermatocytes were initially damaged by AC. This means that pachytene spermatocytes are probably the type of cell which is most susceptible to AC.

Severe tubular atrophy was observed four days after the AC injection. Even 39 days after the AC injection, this histopathological lesion was still present in more than half the AC-treated mice and no signs of any recovery of spermatogenesis, such as spermatogonia repopulation, were evident. This means that AC-induced damage to the seminiferous epithelium occurred within a few days after the injection and did not recover within the treatment-free period corresponding to 4.5 cycles of the seminiferous epithelium (39 days in mice). However, a treatment-free period corresponding to 12 cycles of the seminiferous epithelium is required in order to evaluate the reversibility of damage to the seminiferous epithelium (Amann 1982). We are therefore unable to make conclusions about the reversibility of AC-induced testicular damage from this study.

In this study, AC-treated mice showed a lack of specific spermatogenic cells due to damage to pachytene spermatocytes at the time of the injection. However, some AC-treated mice showed more serious histopathological lesions, such as severe atrophy of the seminiferous tubules, due to non-specific damage to the seminiferous epithelium. Why did these two histopathological lesions of differing severities coexist among the mice which received the same dose of AC? The ICR mouse is not an inbred strain but a closed colony strain. It therefore seems likely that a difference in susceptibility to the testicular toxicity of AC existed among the mice treated with AC. If AC had been given to mice which were more susceptible to AC, not only pachytene spermatocytes but also other spermatogenic cells which are less susceptible to AC would have been damaged, leading to even more serious histopathological lesions than the lack of specific spermatogenic cells. Accordingly, the difference in the severity of the histopathological lesions among the AC-treated mice could be explained by individual variations in susceptibility to the testicular toxicity of AC in ICR mice.

De Rooij et al. (1996) administered AC to rats by intraperitoneal injection and found that two urinary metabolites of epichlorohydrin, α-chlorohydrin and 3;chloro-2-hydroxypropyl mercapturic acid, were excreted in urine. From this result, they speculated that epichlorohydrin is formed by epoxidation of AC *in vivo*. Epichlorohydrin and α-chlorohydrin are both well-known male reproductive toxicants (Kluwe et al. 1983). Acrolein and ally1 alcohol are also putative metabolites of AC (De Rooij et al. 1996), although their male reproductive toxicities have not been reported. Therefore, epichlorohydrin and α-chlorohydrin formed from AC *in vivo* may be responsible for the testicular toxicities of AC. A comparison of the testicular toxicities of AC and its metabolites is needed in order to clearly define their contribution to AC-induced testicular damage.

Acknowledgments

We thank Mrs. Y. Hirose for her technical assistance and Miss K. Miller (Royal English Language Centre, Fukuoka, Japan) for correcting the English used in this manuscript. This study was partially supported by Sasakawa Health Science

Foundation.

REFERENCES

Adams EM, Spencer HC, Irish DD (1940) The acute vapor toxicity of allyl chloride. J Ind Hyg Toxicol 22: 79-86

Amann RP (1982) Use of animal models for detecting specific alterations in reproduction. Fund Appl Toxicol 2: 13-26

Clermont Y and Trott M (1969) Duration of the cycle of the seminiferous epithelium in the mouse and hamster determined by means of ^{3}H-Thymidine and radioautography. Fertil Steril 20: 805-817

De Rooij BM, Commandeur JNM, Groot El, Boogaard PJ, Vermeulen NPE (1996) Biotransformation of allyl chloride in the rat, influence of inducers on the urinary metabolic profile. Drug Metab Dispos 24: 765-772

Häusler M, Lenich R (1968) Zur Wirkung von Allylchlorid bei chronischer gewerblicher Exposition. Arch Toxikol 23: 209-214 (in German)

He, F, Shen D, Guo Y, Lu B (1980) Toxic polyneuropathy due to chronic allyl chloride intoxication: A clinical and experimental study. Chin Med J Engl 93: 177-182

He F, Jacobs JM, Scaravilli F (1981) The pathology of allyl chloride neurotoxicity in mice. Acta Neuropathol (Berl) 55: 125- 133

He F, Zhang S (1985) Effects of allyl chloride on occupationally exposed subjects: Scand J Work Environ Health 11 (suppl 4): 43-45

IARC (1980) IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, Vol.36, Allyl compounds, aldehydes, epoxides and peroxide. IARC, Lyon, pp. 39-54

Kluwe WM, Gupta BN, Lamb JC 4th (1983) The comparative effects of 1,2-dibromo-3-chloropropane (DBCP) and its metabolites, 3-chloro-1,2-propaneoxide (epichlorohydrin), 3-chloro-1,2-propanediol (alphachlorohydrin), and oxalic acid, on the urogenital system on male rats. Toxicol Appl Pharmacol 70, 67-86

Lu B, Dong S, Yu A, Xian Y, Geng T, Chui T (1982) Studies on the toxicity of allyl chloride. Ecotoxicol Environ Saf 6: 19-27

Matsui H, Toyoda K, Kawanishi T, Mitsumori K, Takahashi M, Fukuhara K, Miyata N (1996) Advantages of simplified quantitative morphometry using stage grouping analysis of spermatogenic cycle for evaluation of the testicular toxicity of ethylene-1,2-dimethanesulfonate in rats. J Toxicol Pathol 9: 285-292

Omura M, Itonaga Y, Komatsu H, Zhao M, Hirata M, Tanaka A, Inoue N (1993) The acute toxicity of allyl chloride by subcutaneous injection in mice. Fukuoka Acta Medica 84: 427-432

Russell LD, Ettlin RA, Sinha-Hikim AP, Clegg ED (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, pp. 119-161

Torkelson TR, Wolf MA, Oyen F, Rowe VK (1959) Vapor toxicity of allyl chloride as determined on laboratory animals. Am Ind Hyg Assoc J. 20: 217-223

Zhao M (1997) Testicular toxicity of allyl chloride. Fukuoka Acta Medica 88: 49-55 (in Japanese)