Effects of cadmium on survival and morphology of cultured rat Sertoli cells

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Summary. The toxicity of different metals on isolated Sertoli cells grown in culture has been investigated. Methyl mercury (CH_3HgCl) and mercury chloride (HgCl_2) were more toxic than cadmium (CdCl_2) which was slightly more toxic than arsenic (As_2O_3). Isolated peritubular cells and Sertoli cells were equally sensitive to cadmium. Cadmium reduced the Sertoli cell survival over the concentration range of 1–10 μM . Freeze-etch electron microscopy of cadmium-exposed Sertoli cells revealed circular areas of average diameter 500 nm devoid of intramembrane particles in the nuclear membrane, and general signs of degeneration such as vesiculation of the plasma membrane and intramembrane particle aggregation. However, cadmium did not dissolve junctional complexes between Sertoli cells. Isolated Sertoli cells were protected against cadmium-induced damage when the cells were preincubated for 48 h with selenium, zinc or low doses of cadmium. Preincubation with cobalt, FSH, testosterone or oestradiol did not protect against cadmium-induced damage. Cadmium bound to metallothionein had no toxic effects on isolated Sertoli cells.

Introduction

Parizek (1957) reported that cadmium salts have damaging effects on mammalian testes. The early injurious effects of this metal on the male gonad are on the vascular system (Meek, 1959; Kar & Das, 1960; Mason, Brown, Young & Nesbit, 1964; Chiquoine, 1964; Gunn & Gould, 1975; Aoki & Hoffer, 1978) and further cadmium-induced changes may be secondary to ischaemia (Aoki & Hoffer, 1978). Saksena & Lau (1979) demonstrated that testes from rats treated subcutaneously with 5 mg cadmium chloride (CdCl_2) regained steroidogenic capacity but the animals remained sterile for 120 days after treatment. Such information indicates that cadmium causes permanent damage of the germinal components of the testes.

Germ cells are closely associated with Sertoli cells (Fawcett, 1975). Since no information exists on the effects of cadmium on isolated Sertoli cells, we tested the survival of cadmium-exposed Sertoli cells and used freeze-etch electron microscopy to investigate how cadmium alters the morphology of isolated Sertoli cells grown in culture.

It has been demonstrated that agents like selenium can protect the vascular endothelium of the rat testis against cadmium damage (Niewenhuis & Fende, 1978). Protection against testicular damage due to cadmium appears to be mediated by metallothionein in mice (Nordberg, 1971). FSH, testosterone and oestradiol are known to interfere with Sertoli cell function *in vivo* (Verjans, de Jong, Cooke, van der Molen & Eik-Nes, 1972; Ahmad, Haltmeyer & Eik-Nes,

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1973) and *in vitro* (Dorrington, Roller & Fritz, 1975; Fritz, Rommerts, Louis & Dorrington, 1976; Rommerts, Krüger-Sewnarain, Van Woerkom-Blik, Grootegoed & van der Molen, 1978). Because of the expected biological activities of these agents it was considered important to investigate whether they interacted with the effect of cadmium on isolated Sertoli cells grown in culture.

Materials and Methods

Sertoli cells

Preparation. Testes with a mean weight of 65 mg were obtained from 20-day-old Wistar rats. Sertoli cell-enriched aggregates were prepared by the method of Fritz *et al.* (1976) except for the following modification: during preparation of cell aggregates, the tubular fragments were suspended in Eagle's minimum essential medium with Hepes buffer (Gibco) instead of Hank's balanced salt solution.

Culture conditions. The culture medium consisted of Eagle's minimum essential medium, supplemented with non-essential amino acids, L-glutamine, penicillin/streptomycin (100 µg/ml each) and fungizone (0.63 µg/ml). To prevent the possible effects of unknown serum factors, blood serum was not added to the medium. Aliquots of the suspension containing Sertoli cell-enriched aggregates were plated either on Thermanox plastic slides placed in Costar Petri dishes or in Falcon plastic bottles. About 2 bottles were obtained per testis. After addition of 5 ml culture medium the cultures were incubated in a water-saturated atmosphere of 95% air and 5% CO₂ at 32°C. The culture medium was changed after 48 h.

Peritubular cell preparation and culture

Peritubular cells were isolated as described by Rommerts *et al.* (1978). The isolated peritubular cells were cultured in the medium described above but supplemented with 1% chicken serum (Gibco). The peritubular cells were allowed to grow for 1 week before transfer to new culture flasks and growth for 1 additional week. During these 2 weeks of the experiment, the culture medium was changed every 48 h. For 2 days before the addition of cadmium chloride and during exposure to this metal the cells were grown in a serum-free medium.

Sertoli and peritubular cell survival

Sertoli cells were grown in culture for 48 h before CdCl₂, HgCl₂, CH₃HgCl, As₂O₃, NiSO₄·6H₂O, Mn(NO₃)₂·4H₂O, Pb(NO₃)₂, ZnCl₂, CoCl₂, Na₂SeO₃ or metallothionein were added to the incubation medium. All metals were obtained from Merck, except for CH₃HgCl which was a gift from Dr Tore Syversen, Institute of Pharmacology and Toxicology, University of Trondheim, Norway. Metallothionein of known purity (Nordberg, 1971) was kindly donated by Dr Monica Nordberg, Department of Environmental Hygiene, the Karolinska Institute, Stockholm, Sweden. Before the metals were added, 100–400 cells were selected and photographed with a Leitz inverted phase-contrast microscope. Further samples of cells were photographed and counted 24, 48 and 72 h after addition of the metals. Cell viability was estimated by the method of Rotman & Papermaster (1966), using fluorescein diacetate (Koch-Light Laboratories Ltd).

In some experiments Sertoli cell cultures were incubated with FSH (NIH-oFSH-S9), oestradiol (Seraloids Inc.) or testosterone (Seraloids).

Freeze-etch electron microscopy

Sertoli cells were cultured on Thermanox plastic cover-slips. After the appropriate culture period the cover-slips were quickly cut into 3 × 3 mm squares and the Sertoli cells frozen *in situ*

with a liquid propane jet-freezer according to Espevik & Elgsaeter (1981). The frozen specimens were fractured, etched for 1 min at -100°C , and replicated using a freeze-etch unit described by Elgsaeter (1978). The replicas were examined in a JEOL 100B or 100 CX electron microscope. The terminology of Branton *et al.* (1975) is used for designating the fracture faces. The shadows in the freeze-etch electron micrographs are white.

Results

Effects of different metals on Sertoli cell survival

Among the compounds tested only methyl mercury (CH_3HgCl) and mercury chloride (HgCl_2) were more toxic for the Sertoli cells than cadmium chloride (CdCl_2). Arsenic (As_2O_3) was slightly less toxic (Text-fig. 1). Sertoli cell survival decreased during exposure to CdCl_2 over the concentration range of 1–10 μM . For CH_3HgCl such effects were seen between 0.44 and 0.89 μM , for HgCl_2 between 0.44 and 8.9 μM , and for As_2O_3 between 4.45 and 17.8 μM (Text-fig. 1). At a fixed concentration of 8.9 μM ZnCl_2 , CoCl_2 , Na_2SeO_3 , $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, $\text{Mn}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ or $\text{Pb}(\text{NO}_3)_2$ no such effect on Sertoli cell survival could be detected after 72 h of exposure. Cadmium bound to metallothionein equivalent to a dose of 26.7 μM cadmium and 19.8 μM zinc had no effect on Sertoli cell survival after 72 h. If 26.7 μM CdCl_2 and 19.8 μM ZnCl_2 were added simultaneously to cultures, the cell survival was reduced to about 5% after 72 h.

To test whether cultured Sertoli cells were particularly sensitive towards CdCl_2 , isolated testicular peritubular cells were cultured and exposed to CdCl_2 under the same conditions as Sertoli cells. No significant difference was found between peritubular cell and Sertoli cell survival when CdCl_2 was added to cultures (data not shown).

Text-fig. 1. Effects of different metals on Sertoli cell survival over 72 h. Results are mean values $\pm$ s.e. of two determinations in duplicate cultures.

Protection against CdCl_2 -induced Sertoli cell damage

Cultures of Sertoli cells were preincubated with the test substance for 48 h. The medium was then changed and fresh medium containing 8.9 μM CdCl_2 was added to the cultures. Sertoli cell survival was recorded 24, 48 and 72 h after the latter addition.

This assay revealed that preincubation with 8.9 μM Na_2SeO_3 protected the Sertoli cells in the first 24 h after exposure to CdCl_2 . This protection effect was not present at 72 h (Text-fig. 2a).

Preincubation with $2.2 \mu\text{M}$ - CdCl_2 significantly protected the Sertoli cells over the whole 72 h period but had maximum effect after 24 h (Text-fig. 2b). The dose of $2.2 \mu\text{M}$ - CdCl_2 *per se* reduced Sertoli cell survival to $73 \pm 4\%$ after 48 h of preincubation. Sertoli cells preincubated with $17.8 \mu\text{M}$ - ZnCl_2 displayed significantly increased cell survival 24, 48 and 72 h after addition of CdCl_2 , but this dose of $17.8 \mu\text{M}$ - ZnCl_2 did not by itself affect Sertoli cell survival over 48 h.

We were not able to demonstrate protection against the standard dose of $8.9 \mu\text{M}$ - CdCl_2 when the cells were preincubated with $8.9 \mu\text{M}$ - CoCl_2 , $5 \mu\text{M}$ -FSH, $0.1 \mu\text{M}$ -testosterone or $0.1 \mu\text{M}$ -oestradiol.

Text-fig. 2. Sertoli cell survival as function of time after a $8.9 \mu\text{M}$ - CdCl_2 dose was added to cultures (O). (a) Pretreatment with $8.9 \mu\text{M}$ - Na_2SeO_3 for 48 h before addition of CdCl_2 (Δ). (b) Pretreatment with $2.2 \mu\text{M}$ - CdCl_2 for 48 h before addition of CdCl_2 (▲). (c) Pretreatment with $17.8 \mu\text{M}$ - ZnCl_2 for 48 h before addition of CdCl_2 (●). Results are mean values $\pm$ s.e. of two determinations in duplicate cultures. Where the s.e. is not indicated it is within the symbol. * $P < 0.025$ (Student's *t* test).

Alterations of Sertoli cell morphology after CdCl_2 treatment

Freeze-etch electron microscopy of CdCl_2 -exposed Sertoli cells often revealed well defined circular areas in the nuclear membrane devoid of intramembrane particles (Pl. 1, Fig. 1). These circular areas had a uniform size, about 500 nm. Such features in the nuclear membrane were not observed in control Sertoli cells or in cells exposed to 17.8 or $8.9 \mu\text{M}$ - ZnCl_2 . Sertoli cells subjected to CdCl_2 exhibited various degrees of intramembrane particle aggregation in the plasma membrane (Pl. 1, Fig. 2) although the pattern was different from that in the nuclear membrane. Vesiculation of the plasma membrane was a frequent occurrence and many vesicles were observed in the surrounding growth medium (Pl. 1, Fig. 2). In spite of these apparent signs of degeneration junctional complexes (mainly tight junctions) were still prominent between the Sertoli cells (Pl. 1, Fig. 2).

Discussion

It is difficult to study the specific effects of cadmium on testes *in vivo* because such studies involve problems of distinguishing between cadmium-induced injury and injury secondary to ischaemia (Aoki & Hoffer, 1978). Sertoli cells cultured under certain conditions maintain many of their

PLATE 1

Fig. 1. Freeze-etch electron micrograph of a nuclear membrane from a propane jet-frozen Sertoli cell exposed to $8.9 \mu\text{M}$ - CdCl_2 for 24 h, showing a circular area in the nuclear membrane devoid of intramembrane particles. Nuclear pores in the membrane are visible.

Fig. 2. Freeze-etch electron micrograph of plasma membranes from propane jet-frozen Sertoli cells exposed to $8.9 \mu\text{M}$ - CdCl_2 for 24 h. Junctional complexes, mainly tight junctions (arrows) are still intact. Notice vesiculation of the plasma membrane (arrow heads) and aggregation of intramembrane particles on the protoplasmic fracture face (PF). EF, extracellular fracture face.

(Facing p. 492)

physiological properties (Dorrington *et al.*, 1975; Fritz *et al.*, 1976; Gore-Langton, McKeracher & Dorrington, 1980; Elkington & Fritz, 1980; Kiergzenbaum *et al.*, 1980). In the present study we have cultured Sertoli cells under these conditions to examine the toxicity of metals on the cells. Only CH_3HgCl and HgCl_2 were more toxic for the Sertoli cells than CdCl_2 .

The LD_{50} for CdCl_2 -exposed rabbit alveolar macrophages *in vitro* is about $100\text{ }\mu\text{M}$ (Waters, Gardner, Aranyi & Coffin, 1975) and the LD_{50} for CdCl_2 -exposed NHIK 3025 cells *in vitro* is $30\text{ }\mu\text{M}$ (unpublished results). These values are considerably higher than the LD_{50} ($3.6\text{ }\mu\text{M}$) for cultured Sertoli cells (Text-fig. 1). The different serum concentrations required for proper growth of macrophages and NHIK 3025 cells *in vitro* could account for these differences in LD_{50} doses. Cultured Sertoli cells could, however, be particularly sensitive towards CdCl_2 . Peritubular cells were therefore isolated and cultured under the same conditions as Sertoli cells. No significant difference in cell survival following cadmium exposure was found between isolated Sertoli and peritubular cells. This fact may indicate that peritubular cells are also affected in acute testicular cadmium poisoning *in vivo*.

Sertoli cells pretreated with Na_2SeO_3 , ZnCl_2 or a low dose of CdCl_2 were not as sensitive towards a standard high dose of CdCl_2 as Sertoli cells not exposed to these pretreatments (Text-fig. 2), suggesting that isolated Sertoli cells are able to synthesize substances protecting them against cadmium-induced damages. Chen, Wagner, Hoekstra & Ganther (1974) reported that pretreatment with NaSeO_3 , zinc acetate or low dose of CdCl_2 resulted in different patterns of cadmium binding to proteins in rat testes. It is therefore possible that the testes are able to synthesize cadmium-binding proteins and that the Sertoli cells are possibly involved in this process. Cultured HeLa cells (Rudd & Herschman, 1979), human fibroblasts (Lucis, Shaikh & Embil, 1970) and epithelial cells (Rugstad & Nordseth, 1975) have all been shown to produce metallothionein. However, metallothionein containing the equivalent of $26.7\text{ }\mu\text{M}$ -cadmium and $19.8\text{ }\mu\text{M}$ -zinc did not affect the survival of Sertoli cells over a 72 h period and cadmium bound to this substance was not able to reduce the Sertoli cell survival. Niewenhuis & Fende (1978) reported that pretreatment with Na_2SeO_3 prevented the progressive time-related changes in endothelial cell junctions in testes following injection of CdCl_2 to the animal. They suggested that this apparent inactivation of CdCl_2 must take place in the blood or at the surface of the endothelial cells rather than in the testis itself. We have demonstrated that Sertoli cells pretreated with Na_2SeO_3 are protected against CdCl_2 -induced Sertoli cell damage in the first 24 h (Text-fig. 2a). This result therefore indicates that the Sertoli cells may be involved in the inactivation of cadmium after selenium pretreatment. The ability of the Sertoli cells to synthesize substances protecting them from cadmium injury (Text-fig. 2) seems not to be regulated by FSH, testosterone or oestradiol in the doses and regimens used in our study. FSH and testosterone are known to stimulate both protein and steroid synthesis in culture (Dorrington *et al.*, 1975; Fritz *et al.*, 1976; Rommerts *et al.*, 1978).

Fende & Niewenhuis (1977) reported that the primary ultrastructural effects of CdCl_2 on cryptorchid and scrotal testes of rats consisted of changes in the endothelial cell junctions of intratesticular capillaries. Niewenhuis & Fende (1978) suggested that an interaction between cadmium and calcium could be responsible for the breakdown of the cell junctions between endothelial cells. Our freeze-etch electron microscopic study of CdCl_2 -exposed Sertoli cells demonstrated that the junctional complexes between Sertoli cells were hardly affected by CdCl_2 (Pl. 1, Fig. 2). Nevertheless, the micrographs suggest that CdCl_2 interferes with the nucleus. Bell, Masters, Ingram, Waters & Shelburne (1979) documented the localization of cadmium in nuclear inclusions in cultured rabbit alveolar macrophages, observing lamellar substructures with an average diameter of 500 nm in the nucleus of frozen-sectioned macrophages. We observed circular areas devoid of intramembrane particles in the nuclear membrane of about the same diameter in the CdCl_2 -exposed Sertoli cells. Such features were never observed in ZnCl_2 -exposed or in control Sertoli cells and are therefore not likely to be an effect of cooling the cells before freezing (Wunderlich, Wallach, Speth & Fisher, 1974).

In conclusion, isolated Sertoli cells can be protected against cadmium toxicity by exogenous substances, and our results suggest that they also have the ability to produce endogenous protective substances but in the absence of such substances they are very sensitive to the effects of cadmium, undergoing extensive alterations during the source of exposure.

We thank the Institute of Botany, University of Trondheim, and Section of Electron Microscopy, Trondheim Regional Hospital, for use of their electron microscopes; K. Jacobsen for the work on CdCl_2 effects on NHIK 3025 cell survival; Professor J. Lamvik for his generous help; and Mrs R. Grasdalen for technical assistance. T.E. is a research fellow of the Norwegian Society for Fighting Cancer, and M.K.L. is a research fellow of The Royal Norwegian Council for Scientific and Industrial Research.

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Received 9 December 1981