Microcanalization in the epididymis to overcome ductal obstruction caused by chronic exposure to chromium – a study in the mature bonnet monkey (Macaca radiata Geoffroy)

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Abstract

In order to apprehend the toxic effects of chromium, an occupational/environmental pollutant, on the epididymis, adult bonnet monkeys were exposed to chromium (VI) in their drinking water at concentrations of 100, 200 and 400 p.p.m. for a chronic period of 180 days. At the end of the experimental period, testicles and segments of epididymis from control and treated monkeys were subjected to light microscopic (resin-embedded semi-thin sections) and transmission electron microscopic analyses. Among the various changes undergone by the epididymal epithelium, the present paper describes the origin of two different kinds of microcanals, probably caused by ductal obstruction. The first type of microcanal, which appears to provide passage for spermatozoa to bypass the obstructed main duct, is comparable with the one already reported in carbendazim-treated efferent ductules of the rat. The second type of microcanal, which is novel, consisted of a lumen in the epithelium enclosed by four to five cells, which are either modified basal cells, principal cells or a hitherto unknown cell type. This novel type of microcanal is suggested to be a device to entrap the spermatozoa which reach the core of the epithelium and may be a mechanism to prevent extravasation of sperm so as to avoid an autoimmune response of spermatic granuloma formation. Thus, the present study has shown that chronic exposure to chromium (VI) through drinking water can produce pathological manifestations in the epididymal epithelium but the epididymis, being a versatile organ, is capable of overcoming such adverse situations through novel devices.


Introduction

Several industrial chemicals, environmental pollutants, cytotoxic drugs and heavy metals are known to affect testicular structure, spermatogenesis and steroidogenesis in man and experimental animals, leading to infertility or sub-fertility (Cheek & McLachlan 1998).

The epididymis, present only in the amniotes, is formed of a single long convoluted and contorted duct, and plays an important role in post-testicular sperm maturation (Cooper 1998). It is differentiated anatomically, histologically and functionally into a number of segments along its length (Robaire & Hermo 1988). The success of in vivo or in vitro fertilization or intracytoplasmic sperm injection in assisted reproductive technologies, using spermatozoa collected from different segments of the epididymis, depends on the extent of epididymal maturation of sperm (Cooper 1993). There are also reports suggesting the epididymis as a target for certain toxicants (Gray & Kelce 1996, Mann 1997, Hess 1998, Klinefelter 2002). If that is the case, epididymal sperm maturation can be affected, leading to perturbation in the fertility of subjects exposed to such toxicants. Therefore, it would be pertinent to investigate the responses of the epididymis to more of the toxic substances, including environmental chemicals (Hess 1998, Klinefelter 2002).

Male reproductive toxicity of heavy metal pollutants such as cadmium, lead and mercury is well known (Zylber-Haran et al. 1982, Rodamilans et al. 1988, Vachhrajani & Chowdhury 1990). Chromium (Cr) is another occupational heavy metal pollutant to which workers in nearly 50 industries, including chrome-plating, stainless steel welding and
vulcanizing industries, ordnance factories and tanneries, are exposed (Burrows 1983). Further, emissions and effluents from these workplaces and industries contaminate the environment, which may affect man and animals living in the surrounding areas (Outridge & Schuehammer 1993). In the living system, Cr (VI) is rapidly converted to Cr (III), which is less toxic. However, recent studies have shown that even Cr (III) can damage DNA (DeFlora et al. 1990, Codd et al. 2001).

Dermatitis, cancer of the lung, nasal ulcers and kidney diseases are the well-known toxic effects of chromium in man (Barceloux 1999). Despite a few reports on rodent models, the male reproductive toxicity of chromium has not been viewed seriously so far. This is mainly due to the failure of a few studies to show any correlation between body chromium and fertility in men who are occupationally exposed to chromium in the welding industries (Bonde 1993). However, a recent study on metal welders suggested that the conclusions of Bonde may not be applicable to those exposed to a high level of welding fumes or other putative hazards (Hjollund et al. 1998). In a recent review, Bonde (2002) has also opined that evidence for the male reproductive toxicity of chromium is very limited.

Experiments in rat models have revealed that i.p. administration of Cr (VI) can result in testicular toxicity, manifested as premature release of germ cells from Sertoli cells, formation of multinucleate spermatids, decreased sperm counts and subnormal androgen secretion (Ernst 1990, Saxena et al. 1990, Ernst & Bonde 1992, Chowdhury & Mitra 1995). We have observed poor semen quality and disruption of spermatogenesis in monkeys (Macaca radiata) exposed to different doses of Cr (VI) through their drinking water for 6 months. This was attributed to the generation of reactive oxygen species in the testis and epididymis (Aruldhas et al. 2000).

In the light of the above background in the literature, it is proposed that occupational or environmental exposure to Cr (VI) might, in addition to affecting the testis, also affect the epididymis. The hypothesis was tested in a non-human primate model, Macaca radiata, subjected to chronic chromium exposure through their drinking water for 180 days. In the present paper, we report the development of two different kinds of microcanals in the epididymal epithelium and the probable mechanisms underlying their development.

Materials and Methods

The experimental protocol of the present study on a non-human primate model (Macaca radiata) was approved by the Institutional Animal Ethics Committee for Studies on Experimental Animals and by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Experimental design

Adult male bonnet monkeys (Macaca radiata), trapped by the Department of Forest and Wild Life, Government of Tamil Nadu, India, for creating a public nuisance, were procured with permission from the Chief Wildlife Warden, Chennai, India. Animals were kept under quarantine and acclimatized to animal house conditions for 2 months before the experiment. They were kept individually in spacious cages built as per the specifications of CPCSEA (60 x 60 x 80 cm), under ambient temperature (28 ± 2°C) and light and dark schedules (12 ± 1 h) in a well-ventilated animal quarter. All animals were fed ad libitum with pellet diet for monkeys (Brooke Bond Lipton India Ltd, Calcutta, India), rice cooked with dhal, vegetables such as potato and beetroot and seasonally available fresh fruits such as banana and guava.

Treatment and analysis

The monkeys were divided into four groups, each consisting of three animals. Groups I to III were provided with drinking water containing Cr (VI) (potassium dichromate) at a concentration of 100, 200 and 400 p.p.m. respectively for 180 days. Group IV consisted of control animals, which were provided with chromium-free drinking water. At the end of the experiments, the monkeys were dissected under sodium pentathol anaesthesia (40 mg/kg body weight), and the testicles and epididymides were removed. As the tissues from the same animals were used for spermatological, biochemical, histological and ultrastructural studies, perfusion fixation was not practised. Slices of the testicles and the different segments of the epididymides were immersion fixed in 2.5% glutaraldehyde prepared in cacodylate buffer and post-fixed in 1% osmium tetroxide (Hess & Thurston 1977). Tissues were dehydrated in grades of alcohol and propylene oxide and embedded in thin viscosity resin (Sigma Chemical Company, St Louis, MO, USA). Semithin sections (1 μm thickness) obtained in an ultramicrotome (Leica Microsystems Nussloch GmbH, Nussloch, Germany) were stained in toluidine blue O (TBO) and used for light microscopic observations and histometry of the microcanals. Ultrathin sections were stained in uranyl acetate and lead citrate and observed in a Phillips 201C transmission electron microscope. Image analysis and processing were done using Axiovision Image Analysis software (Carl Zeiss GmbH, Jena, Germany). For histometric measurements of the microcanals, data were used to calculate the respective means and standard deviations.

Results

Microcanals in the cauda epididymidal epithelium

Among the various responses in the epididymis to chromium treatment, the appearance of microcanals in the cauda epididymidal epithelium of monkeys belonging to all the three treatment groups was of interest.
One kind of microcanal that was predominant consisted of irregular oval or oblong shaped lumina measuring $104 \pm 28\ \mu m$ along the longest axis and $52 \pm 11\ \mu m$ across. The lumen of this type of microcanal contained a few spermatozoa but cell debris was occasionally seen (Fig. 1). The microcanal was lined by short to tall columnar principal cells (Fig. 2). In all other respects, these principal cells were comparable with the principal cells of an unaffected cauda epididymidis. Although the roof of this kind of microcanal was also lined by principal cells whose nuclei were similar to those in the floor, these cells did not have any contact with the basement membrane, and appeared as floating cells. The lumen of the microcanal had a stellate appearance in view of the dome-shaped luminal ends of the lining principal cells.

The second kind of microcanal (Fig. 3) was rare, narrow and more circular in outline than the earlier one. In transection, they measured $37 \pm 4\ \mu m$ along the longest axis and $28 \pm 3\ \mu m$ along the shortest axis. Although low-power light micrographs suggested that they were lined by principal cells as in the earlier case (Fig. 3a), a careful examination revealed closely applied arcs of cytoplasm staining more intensely with TBO than the surrounding principal cells (Fig. 3b). Although principal cell nuclei were found on the sides and top of such microcanals, they were missing at the base. The nuclei of the cells immediately lining the microcanal were smaller in diameter (about $4–6\ \mu m$) than the nuclei of principal cells ($8–10\ \mu m$). The profile of this microcanal resembled a rosette. Microvilli, longer and more profuse than in the earlier type of microcanal, were seen to extend deep into the lumen, and their abundance was more at the junctional points of the rosette than in the other parts. Invariably, the lumen contained sperm and/or cell debris (Figs 3c and 4).
Critical ultrastructural analysis of this kind of microcanal revealed the lumen to be lined by cells that were different from the principal cells. These cells had a thin rim of cytoplasm, which distinguished them from the principal cells (Fig. 5a), and invariably the cytoplasm of these two cell types differed in electron density (Fig. 5b). The lining cells established tight junctions at the points of contact and produced tall, branching microvilli into the lumen (Figs 5a and 6a). A single cell, different in organization and electron density from the principal cells, contacted the basement membrane on one side and surrounded the basal portion of the microcanal above (Fig. 5). In such an organization, the cell had a stalk contacting the basement membrane and the apical portion extending around a portion of the microcanal-like arms. The cytoplasm of such cells possessed extensive saccular rough endoplasmic reticulum and abundant mitochondria with plicate cristae (Fig. 6b). The Golgi apparatus, which was prominent, closely approximated the nucleus and faced the luminal side of the cell (Fig. 6b). The cytoplasm also contained dense spherical granules that, from the morphology and electron density, could be inferred to be lysosomes (Fig. 6).

**Ductal obstruction and invasion of spermatozoa into the epithelium**

In order to find the basis of the development of such microcanals, an attempt was made to trace the causative factor for ductal obstruction, and the consequent attempt of sperm to enter the epithelium. Ductal obstruction of cauda epididymides was inferred from three observations. In the first instance, in the monkeys belonging to the 400 p.p.m. chromium-treated group, the epithelium of the cauda epididymidis was so intensely hypertrophied, pseudostratified and thrown into tall columnar villous folds that the ductal lumen was almost obliterated (Fig. 7). In the second instance, in the monkeys belonging to the 200 p.p.m. chromium-treated group, although the epithelium appeared pseudostratified, it did not form the obstructing villousis. However, the lumen was fully packed with immature germ cells and macrophages that had phagocytosed spermatozoa and cell debris. The luminal content was thus clearly obstructing the lumen (Fig. 8). Light micrographs of the testis of control and treated monkeys revealed that, in the latter, the seminiferous epithelium was thoroughly disrupted and multinucleate giant cells were generated. Immature germ cells and multinucleate giant cells were released into the lumen (Fig. 9) to arrive at the epididymis (Fig. 8). In the third instance, in the monkeys belonging to 100 p.p.m. chromium-treated group, the epithelium of the cauda epididymidis was pseudostratified, and the lumen contained an occluding density that was formed of neither sperm nor immature germ cells, but nuclei matching those of principal cells (Fig. 10a and b). Degeneration of the principal cells of caput and corpus epididymides of Cr (VI)-treated monkeys was noticed and the nuclei of such cells arrived...
at the lumen (Fig. 10c) to reach the cauda epididymidis (Fig. 10a and b).

The epithelium of the cauda epididymidis at the point ahead of the obstruction or at the point of the obstruction itself was seen to be ruptured at several points and spermatozoa were seen entering into the disrupted epithelium (Fig. 11a–c). The rupture could perhaps have begun at disintegrating principal cells (Fig. 11a–c) and such spermatozoa would have proceeded towards the basement membrane (Fig. 11d), where basal cells were invariably present (Figs 11d and e and 12). However, in spite of such extensive damage of the epithelium, spermatozoa were confined to the epithelium.

**Discussion**

We report, for the first time, the development of two types of microcanals in the cauda epididymidis of a non-human primate (*Macaca radiata*) in response to exposure to chromium dissolved in the drinking water. The results also indicated the obstruction of the distal portion of the cauda epididymidis in the chromium-treated monkeys, which may be due to (i) hypertrophy and hyperplasia of the epithelium to such an extent that it formed into villositis almost obliterating the lumen, (ii) accumulation of abnormal and/or immature germ cells arriving from the testis and macrophages arriving from the epididymal epithelium, and (iii) cell debris and nuclei of disintegrating principal cells of the proximal segments of the epididymis filling up the lumen. Quantification of the extent of prevalence of microcanals was not possible because of the limited number of sections of resin-embedded tissue. Serial paraffin sections would have overcome this limitation, but it was not done because of the restricted number of animals used for experimentation.

**Ductal obstruction**

As discussed above, three different mechanisms of ductal obstruction were observed in the distal cauda epididymidis of chromium-treated monkeys. The first one pertains to hypertrophy of the epithelium and its formation into
villous folding of the epithelium has been reported in the corpus epididymidis of orchidectomized goats supplemented with testosterone, and in the cauda epididymidis of rete-ligated goats (Goyal et al. 1994). The situation in the present case may be due to a hormonal imbalance consequent to chromium treatment, as the epididymis is an

Figure 6 Different portions of MC2 in Fig. 5(a) seen at higher magnification. An occluding junction between MBCs (arrow, (a)) and cell organelles (b). G, Golgi apparatus; LY, lysosomes; M, mitochondria; RER, saccular rough endoplasmic reticulum. Scale bar represents 0.075 μm.

Figure 7 TBO–stained semithin sections of cauda epididymidis of a Cr (VI)-treated monkey showing ductal obstruction due to epithelium having been thrown into villous folds (V) consequent to hypertrophy of epithelium. Micrograph portions at low (a) and high (b) magnification. LP, lamina propria; SM, smooth muscle bundles; PT, Peritubular tissue. Scale bars represent 80 μm (a) and 40 μm (b).
androgen-dependent organ (Robaire & Hermo 1988). The arrival of a smaller number of spermatozoa at the cauda epididymidis as a result of disruption in spermatogenesis (Ernst 1990, Saxena et al. 1990, Murthy et al. 1991, Ernst &

Bonde 1992, Chowdhury & Mitra 1995) may be another reason. We have found decreased testosterone titre and sperm count in the chromium-treated monkeys of the present study (Aruldhas et al. 2000).

Obstruction due to the arrival of immature germ cells, multinucleate giant germ cells and macrophages, as
observed in the present study, is known in a few experimental situations such as carbendazim treatment of rats (Nakai et al. 1993), aflatoxin treatment of mice (Agnes & Akbarsha 2001), phosphamidon treatment of rats (Akbarsha & Sivasamy 1998) and in several cases of vasectomy (Akbarsha 2001), in which the debris was degenerating principal cells of the more proximal segments of the epididymis. A similar situation was also observed in the present study.

**Development of type 1 microcanal**

The first type of microcanal, lined all around by principal cells, appears to provide passage for spermatozoa to bypass the obstructed main duct, thus patenting the microcanal. The development of patenting microcanals has been reported in the occluded efferent ductules of carbendazim-treated rats (Nakai et al. 1993) and in the vas deferens of vasectomized men and experimental animals (Hayashi et al. 1983, Cruickshank et al. 1987, Freund et al. 1989). According to Nakai et al. (1993), microcanals are formed in the ductuli efferentes from the epithelium that has escaped inflammation. During this process, the edges of the proliferated epithelium migrate into the lumen and the sprouting epithelium from different directions meet on top of a canal. In this case, the original duct is partitioned into two or more canals, one or more of which transform into patenting microcanals and the other(s) retaining the occluded mass. The microcanal thus formed is lined by both migrated and native epithelium. Lateral and basal surfaces of the migratory epithelial cells are irregular in outline and without a basal lamina. Nakai et al. (1993) have observed microcanalization in the efferent ductules, whereas in the present study it had occurred in the cauda epididymidis. Patenting microcanals of the epididymis have not been reported elsewhere, to the best of our knowledge, although a kind of re-epithelialization process has been reported in human epididymis (Ball & Mitchinson 1984).

**Rupture of epithelium and entry of spermatozoa into the epithelium**

Obstruction due to immature germ cells arriving at the distal part of the epididymis and profuse periodic acid Schiff-positive granules released due to degeneration of principal cells at the intermediate zone of the epididymis of mice treated with aflatoxin has been recently reported (Agnes & Akbarsha 2001). Such an obstruction has been shown to result in degenerative changes in the principal cells in all the segments of the epididymis, establishing continuity between the ductal lumen and the principal cells; spermatozoa were seen in such damaged cells even up to or closer to the basement membrane. Although a comparable situation was obtained in the present study, epithelial degeneration in the cauda epididymidis was found in a large scale, providing scope for a more copious entry of spermatozoa into the epithelium. There are a few earlier reports on epididymal rupture, one in men following long periods of vas obstruction (Silber 1979), and the other in the mouse treated with testosterone where there was no ductal obstruction (Itoh et al. 1999). Thus, the present finding has shown the rupture of cauda epididymidal epithelium in monkeys, which were exposed to chromium.
for a chronic period, due to obstruction in the cauda itself forcing spermatozoa to enter into weaker patches in the epithelium.

The entry of spermatozoa into the epithelium can be viewed from another perspective. Aruldhas et al. (2000) have observed an increased concentration of electrophiles and decreased activities of antioxidant enzymes in the caput and cauda epididymides of the chromium-treated monkeys used in the present study. This could also be a consequence of the ductal obstruction. Thus, the sperm in the cauda epididymis of chromium-treated monkeys would have been under severe stress, and they tended to escape through weak patches in the epithelium.

**Type 2 microcanals as a protective device to prevent extravasations of spermatozoa entering through the weak patches in the epithelium**

Spermatozoa can evoke an autoimmune response if they are extravasated (Nashan et al. 1990, Flickinger et al. 1995, 1998, 1999) as seen after vasectomy (McDonald 2000, McGinn et al. 2000), and can result in provocation of an autoimmune response of extravasated sperm granuloma formation. Autoimmune response to sperm antigens is an established fact, and is the basis for infertility in men undergoing vasectomy (Alexander 1975, Alexander & Anderson 1979, Rose & Lucas 1979, Tung & Menge 1985, Flickinger et al. 1986).

Agnes & Akbarsha (2001) proposed that, on entry of spermatozoa into the epithelium through degenerating principal cells, extravasation of spermatozoa is prevented by the underlying basal cell, which develops into a pale vacuolated epithelial cell (PVEC) and encloses such spermatozoa in a lumen within the cell. The type 2 microcanal observed in the present study fulfils this role, as the spermatozoa reached up to the basement membrane through the weaker patches in the epithelium where basal cells are present. The structural organization of the cells lining this kind of microcanal in the monkey epididymis is comparable with that of PVEC in the mouse epididymis, although it forms a single cell in the mouse epididymis. The cauda epididymidal epithelium in the monkey being about three times taller than that of the mouse (Alsum & Hunter 1978), a single basal cell may not be sufficiently adequate to form a structure to enclose the spermatozoa.
sperm which would extravasate. Therefore, four or five basal cells might have grown around the damaged epithelium and encircle spermatozoa in the lumen. The suggestion that the participating cells are basal cells is based on the observation that they are different in ultrastructural organization from the principal cells and clear cells. Clear cells have been recently reported to be present in the cauda epididymal epithelium of the monkey (Bomgardner et al. 1999). Further, the principal and clear cells have not so far been reported to transform into any other cell type. The role of basal cells in producing structures which seclude the spermatozoa arriving at the site of epithelial ruptures to prevent their extravasation, and thereby avoiding the generation of antibodies, may be justified in the light of the fact that basal cells consistently express F4/80 and mac-1 antigens. Basal cells are possibly tissue-fixed macrophages, and provide immune defence against sperm antigens (Seiler et al. 1998, 2000, Hoischbach & Cooper 2002). However, since our inference is based purely on histological evidence, the possibility that the cells lining the type 2 microcanals are modified principal cells or even yet another cell type hitherto unknown cannot be ruled out at present. Immunocytochemical tests for specific markers for the various cell types in the epididymal epithelium need to be carried out to identify the cell type lining this microcanal. Until confirmation, the lining cells may be designated as microcanal boundary cells.

**Relationship between dose and response**

The histopathological changes in the testis and epididymis, and also the incidence of microcanalization in the cauda epididymal epithelium were dependent on the concentration of chromium in the drinking water. Although the amount of chromium in the experimental animals was not quantified, the impact was most acute in the animals exposed to water containing 400 p.p.m. chromium, followed by the groups exposed to 200 p.p.m. and 100 p.p.m.

Thus, the present study has revealed that the epididymis could be a target organ for chromium toxicity, and also be a versatile organ capable of overcoming adverse situations by diverse devices.

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**References**


Asum DJ & Hunter AG 1978 Regional histology and histochemistry of the ductus epididymis in the rhesus monkey (*Macaca mulatta*). *Biological of Reproduction* 19 1063–1069.


Burrows D 1983 Chromium Metabolism and Toxicology. Florida: CRC Press.


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