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Immunohistochemical study of serum albumin in normal and cadmium-treated mouse testis organs by "*in vivo* cryotechnique"

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Summary. The *in vivo* injection of cadmium (Cd) was reported to induce blood-testis barrier disruption, and assumed to be an experimental model to examine junctional structures in seminiferous tubules. The purpose of this study is to investigate time-dependent changes of albumin permeability in the normal or Cd-treated mouse testis by our "*in vivo* cryotechnique" with immunohistochemistry, reflecting tight junctional (TJ) barriers of Sertoli cells. The albumin in the seminiferous tubules was firstly immobilized by the cryotechnique, in which normal blood circulation was always kept. The cryofixed testicular tissues were then processed for freeze-substitution, and embedded in the paraffin wax. Serial sections were immunostained by anti-mouse albumin antibody with peroxidase immunostaining, and also stained with hematoxylin-eosine (HE) for morphological observation. In normal seminiferous tubules, the immunoreaction products were localized around peritubular myoid cells and between Leydig cells, as well as in blood vessels. They were also localized as arch-like patterns around some spermatogonia in basal compartments of seminiferous tubules. Twenty-four and 48 hrs after Cd-treatment, some enlarged spaces and vesicular formations in the seminiferous epithelium were observed on the HE-stained sections. The albumin immunolocalization was detected not only in the basal compartments, but also in the adluminal compartments between Sertoli cells and germ cells. Thus, the structural disruptions of inter-Sertoli TJ barriers could be clearly demonstrated by the "*in vivo* cryotechnique".

Key words: Immunohistochemistry, *In vivo* cryotechnique, Serum albumin, Mouse testis, Cadmium-treatment

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Introduction

In the animal testis, the blood-testis barrier (BTB) is constituted mainly by Sertoli cell's tight junctions (TJ). The functional and structural integrity of BTB plays a crucial role in maintaining the microenvironment of the seminiferous epithelium for both germ cell migration and normal spermatogenesis (Russell 1997; Wong et al., 2004). Cadmium (Cd) is already known to be a ubiquitous industrial and environmental metal pollutant, which has a long biological half-life and a cumulative toxic effect on both humans and experimental animals (Liao and Li, 1999; Benoff et al., 2004). It was also reported that Cd treatment caused biological damage to several organs, including kidney, liver, lung, bone and testis (Gubrelay et al., 2004). The probable damaging effect on the testis has been reported to disrupt the Sertoli cell's TJ-barriers, resulting in a decrease of germ cells of the seminiferous epithelium, and finally such a damaged testis becomes morphologically shrunken (Hew et al., 1993; Xu et al., 1999; Zhou et al., 1999; Chung et al., 2001). At a biochemical level, Cd treatment was reported to reduce the amount of Sertoli cell's TJ proteins, occludin and zonula occludens-1 (Fiorini et al., 2004).

For directly monitoring the *in vivo* immunolocalization of serum albumin and immunoglobulin proteins in living animal organs, we have already developed a new experimental protocol by our "*in vivo* cryotechnique" (Ohno et al., 1996) with immunohistochemistry (Zea-Aragon et al., 2004; Ohno et al., 2005). Although, the distribution of serum albumin in the rat testis lightly fixed by perfusion-fixation technique was already reported (Christensen et al., 1985), its *in vivo* localization has not yet been clarified in living animals. In the present study, the "*in vivo* cryotechnique" was used to the testis for the first time, followed by the freeze-substitution method, which is superior to the conventional chemical fixation and dehydration for preserving labile and soluble serum proteins *in situ*. We additionally used the routine

immunohistochemistry for serum albumin to evaluate time-dependent toxic effects of Cd on living mouse testis organs, especially on BTB permeability.

Materials and methods

Treatment of mouse with cadmium

Nine adult male C57BL/6 mice, weighing 20-30g, were handled in accordance with the University of Yamanashi Guidelines for the use of animals. The cadmium chloride ($CdCl_2$) was prepared as 0.1% solution (wt/vol in physiological saline), and was administered to two groups of mice (three mice per each time point) by intraperitoneal injection at a single dose of 3 mg/kg body weight to induce their testis damage, as previously described (Wong et al., 2004). Thereafter, the mice were examined 24 and 48 hrs after the Cd-treatment. Three mice in a control group received the same volume of physiological saline.

"In vivo cryotechnique" for living mouse testis

Normal and Cd-treated mice were anesthetized with sodium pentobarbital, and their testis organs were surgically exposed without disturbing their blood supply. The "*in vivo* cryotechnique" was performed with a use of the "*in vivo* cryoapparatus" (Eiko Engineering Co. Ibaraki, Japan) (Ohno et al., 2004; Zea-Aragon et al., 2004). Briefly, a cryoknife edge precooled in liquid nitrogen (-196°C) was placed over the mouse testis. While the heart was normally beating, the testis was vertically cut and frozen with the cryoknife as fast as possible, and simultaneously the isopentane-propane cryogen (-193°C) was poured over, as reported before (Ohno et al., 1996). Lots of liquid nitrogen was additionally poured over the frozen testis, which was then carefully taken out for the following various steps.

Freeze-substitution and paraffin-embedding

The freeze-substitution solution consisted of absolute acetone containing 2% paraformaldehyde (PF). The frozen testis tissues were freeze-substituted at about -80 °C in dry ice-acetone for 48 hrs. They were then put into a freezer at -25°C for 2 hrs and a refrigerator at 4°C for 2 hrs, and finally at room temperature for 1 hr. They were washed in pure acetone, immersed in xylene, and routinely embedded in the common paraffin wax.

Immunostaining for albumin

The paraffin-embedded tissues were cut at 5 µm thickness, and de-paraffinized with a graded series of xylene and ethanol. For immunostaining, they were incubated with 1% hydrogen peroxide in phosphate buffered saline (PBS) for 60 min. Then, they were washed in PBS, incubated with 10% normal rabbit serum for 60 min, and individually incubated with avidin and biotin (Vector Laboratories Inc., Burlingame, CA, USA) in PBS for 1hr each. Furthermore, they were incubated with goat anti-mouse albumin antibody (dilution of 1:2000; Bethyl Laboratories INC. Montgomery, TX, USA) at 4°C for 12 hrs. Immunocontrol sections were incubated with PBS or normal goat serum instead of the primary antibody. Then, they were incubated with biotinylated rabbit anti-goat IgG antibody (Nichirei Co., Tokyo, Japan) at room temperature for 1hr, and with horseradish peroxidase (HRP)-conjugated avidin-biotin complex (ABC) for 1hr. They were routinely visualized by the peroxidase enzyme reaction with metal-enhanced 3, 3'-diaminobenzidine (DAB) (Pierce Co., Rockford, IL, USA) in buffer solution containing 0.02% hydrogen peroxide for 5 min (ABC-DAB method). Finally, they were incubated in 0.04% osmium tetroxide solution for 2 min to increase the contract of immunoreaction products.

Hematoxylin-eosine (HE) staining of testis tissues

The paraffin sections at 5 µm thickness were de-paraffinized as describe above. After being washed with distilled water for 10 min, they were individually stained with hematoxylin for 8 min, and eosine for 3 min, then routinely dehydrated and mounted on glass slides with cover glasses.

Results

Morphology of living mouse testis

By HE-staining of sections prepared after the combination of the "*in vivo* cryotechnique" with the freeze-substitution, well-preserved seminiferous tubules were obtained within 300-400 µm areas deep from the initially frozen tissue surface, where the isopentane-propane cryogen was directly poured. Artificial destruction of seminiferous tubular structures was not prominently recognized in such areas (Fig.1a,c,e). Twenty-four hours after the Cd-treatment (Fig. 2a), the number of germ cells, especially spermatids, had already decreased in some seminiferous tubules, and luminar spaces were relatively enlarged, as compared with those in normal testis. Finally, both definite atrophy of seminiferous tubules and marked decrease of their germ cells were always detected even with remaining Leydig cells, 48 hrs after the Cd-treatment (Fig. 3a).

Immunolocalization of albumin in normal testis

In normal seminiferous tubules (Fig. 1), albumin immunoreactivity was mainly detected around peritubular myoid cells, forming continuous immunostaining areas, as large ring-like patterns (Fig. 1b,d,f), as well as among interstitial Leydig cells and inside blood capillaries, although serum components in the interstitium tend to be movable near the tissue components probably due to high water content. Another

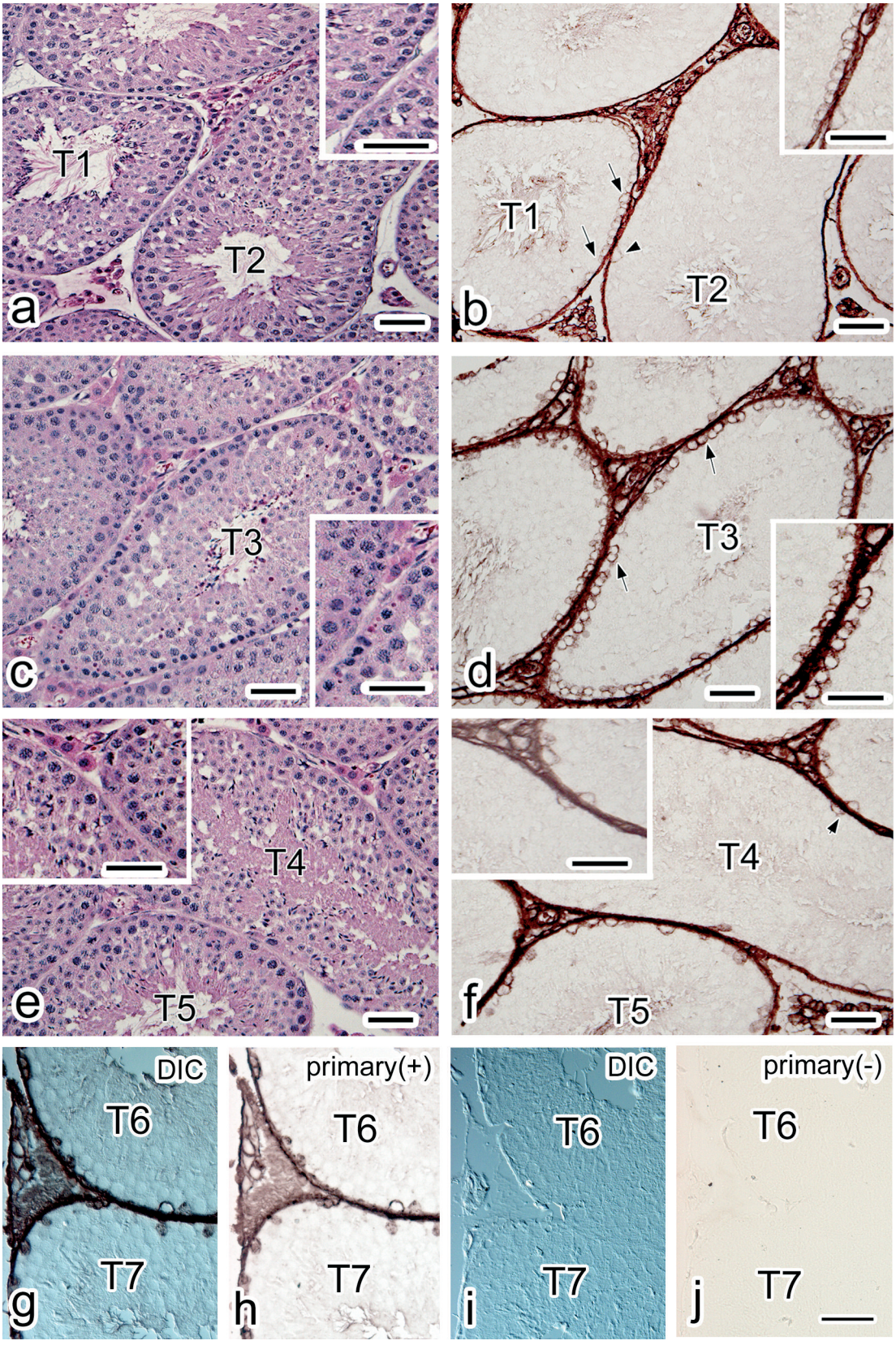

Fig. 1. Light micrographs of HE-staining **(a, c and e)** and immunolocalizations of albumin **(b, d and f)** in seminiferous tubules of the normal mouse testis. **(a, c and e).** Well-preserved seminiferous tubules without obvious ice crystals are seen by the HE-staining. Different stages of the spermatogenic cycle are recognized: T1 in **(a)** and T3 in **(c)** are at the stages VII-VIII (apical matured spermatozoa accumulation); T2 in **(a)** and T4 in **(e)** are at the stages I-II (spermatogenesis proceeding to the early spermatid stage). **(b, d and f).** The albumin immunoreactivity is mainly detected in the testicular interstitium as well as the peritubular myoid cells, forming a continuous immunostaining as ring-patterns. Typical arch-like immunostaining patterns are observed around spermatogonia in the basal compartment [insets in (**b**), (**d**) and (**f**)]. The amount of the arch-like immunostaining at the stages VII-VIII [arrows in T1 (**b**), T3 (**d**)] is obviously higher than that at the stages I-II [arrowheads in T2 (**b**), T4 (**f**)]. On serial paraffin sections, the immunostaining was performed with (**h**) or without (**j**) the primary antibody. (**g**) and (**i**): Corresponding differential interference contrast (DIC) images of (**h**) and (**j**), respectively. Bars: 40 µm.

albumin immunostaining was detected as small arch-like patterns within the seminiferous epithelium, and restricted in the basal compartment, locating around some spermatogonia (insets, Fig. 1b,d,f). Both appearance and disappearance of the arch-like patterns were closely related to the developmental stages of seminiferous epithelial cycles (T1-T5, Fig. 1b,d,f). The number of the arch-like patterns at stages VII-VIII of the seminiferous epithelial cycle [mature spermatozoon accumulated at the upper luminal part and ready to release (T1 in Fig.1b, T3 in Fig.1d)] was much higher than that at stages I-II [spermatogenesis proceeding to the early spermatid (T2 in Fig.1b, T4 in Fig.1f)]. Another weak albumin immunostaining was also observed in the luminal space of the seminiferous tubules. The immunoreactivity without the primary antibody (Fig. 1j) was completely eliminated, as compared to that with it (Fig. 1h).

Immunolocalization of albumin after Cd-treatment

The immunolocalization of albumin 24 and 48 hrs after the Cd-treatment (Figs. 2 and 3, respectively) were obviously different from that of the normal mice, as already shown in Figure 1. In 24 hrs (Fig. 2), the albumin immunostaining was detected not only in the basal compartment of seminiferous epithelium, but also in the adluminal compartment between supporting Sertoli cells and developing spermatogenic cells (Fig. 2b), indicating abnormal immunoreactivity of serum albumin through BTB structures. Forty-eight hours after the Cd-treatment, the immunoreactivity of albumin through BTB in some seminiferous tubules (T3 in Fig. 3b) was almost similar to that of the 24 hrs' group, as shown in Figure 2b. Other seminiferous tubules were shown to be typical atrophic structures (T2, T3 in Fig. 3a), by decreasing the number of germ cells. In such seminiferous tubules, the albumin immunostaining was also detected between the Sertoli cells (T2 in Fig. 3b). Without the primary antibody, its immunoreaction was not obtained (Fig. 3i), indicating no endogenous peroxidase activity after the cadmium treatment.

Discussion

The "*in vivo* cryotechnique" has been developed to simultaneously perform morphological and immunohistochemical studies, by which all features to be examined should reflect the physiological or pathological significance under different experimental conditions *in vivo* (Ohno et al., 1996). The common aldehyde fixative for cross-linking soluble molecules with a perfusion or immersion technique takes a considerable time to complete the cross-linking

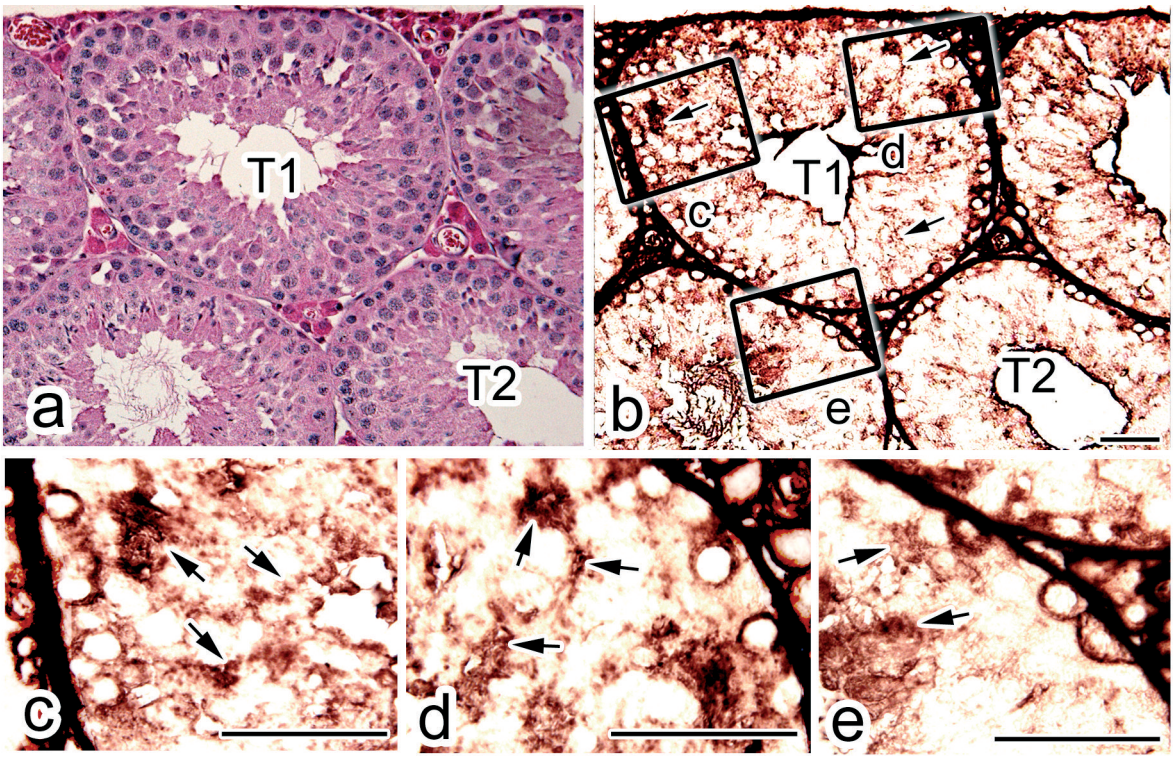

Fig. 2. Light micrographs of HE-staining **(a)** and immuno-localization of albumin **(b)** in seminiferous tubules 24 hrs after the Cd-treatment. **a.** A decreased number of spermatids and enlargement of luminar spaces (T1, T2) are observed on thin sections by HE-staining. **b.** The albumin immunostaining is not only in the basal compartment, but also in the adluminal compartment between Sertoli cells and germ cells [arrows in **(b)**] on the serial section of **(a).** At higher magnification [**(c), (d) and (e)**], corresponding to the squares in **(b)**, many dotted and lining immunostaining patterns are detected in the adluminal compartment (arrows). Bars: 40 µm.

mechanism, during which morphological changes easily occur (Ohno et al., 2004; Zea-Aragon et al., 2004), resulting in the loss of soluble components (Moreira et al., 1998). On the contrary, the physical cryofixation to immobilize cells and tissues can be much more rapidly completed within milliseconds (Ohno et al., 1996). The following freeze-substitution method can also stabilize the frozen soluble components in the cells and tissues under a vitreous ice condition, during which diffusion of the soluble components would be limited in cold acetone containing fixatives. Therefore, it has been accepted that the various cryotechniques combined with the freeze-substitution are better than the conventional chemical fixation in preserving diffusible and soluble components for immunohistochemistry by light or electron microscopy (Werner et al., 1991; Shiurba, 2001; Terada and Ohno, 2004).

In this study, some immunostaining patterns of the albumin were arch-like within the seminiferous epithelium, restricting especially basal compartments of the normal living mouse testis. The amount of the arch-like immunostained tissue areas was differently detected, depending on developing stages of the seminiferous epithelial cycle, as shown in Figure 1. Some relationship between spermatogenic cells and extracellular components in the living mouse testis is supposed to be important for germ cell proliferation and differentiation (Mruk and Cheng, 2004; Siu and Cheng, 2004). We assume that the extracellular spaces filled with serum components, such as albumin, may reflect the physiological significance of functional structures during the processes of spermatogonium proliferation and migration in the basal compartment. However, such *in vivo* morphological features were easily distorted by the conventional fixation procedures (Ohno et al., 1996). A further detailed study is now being undertaken to examine the spermatogonia surrounded by the extracellular wide spaces with albumin in the basal

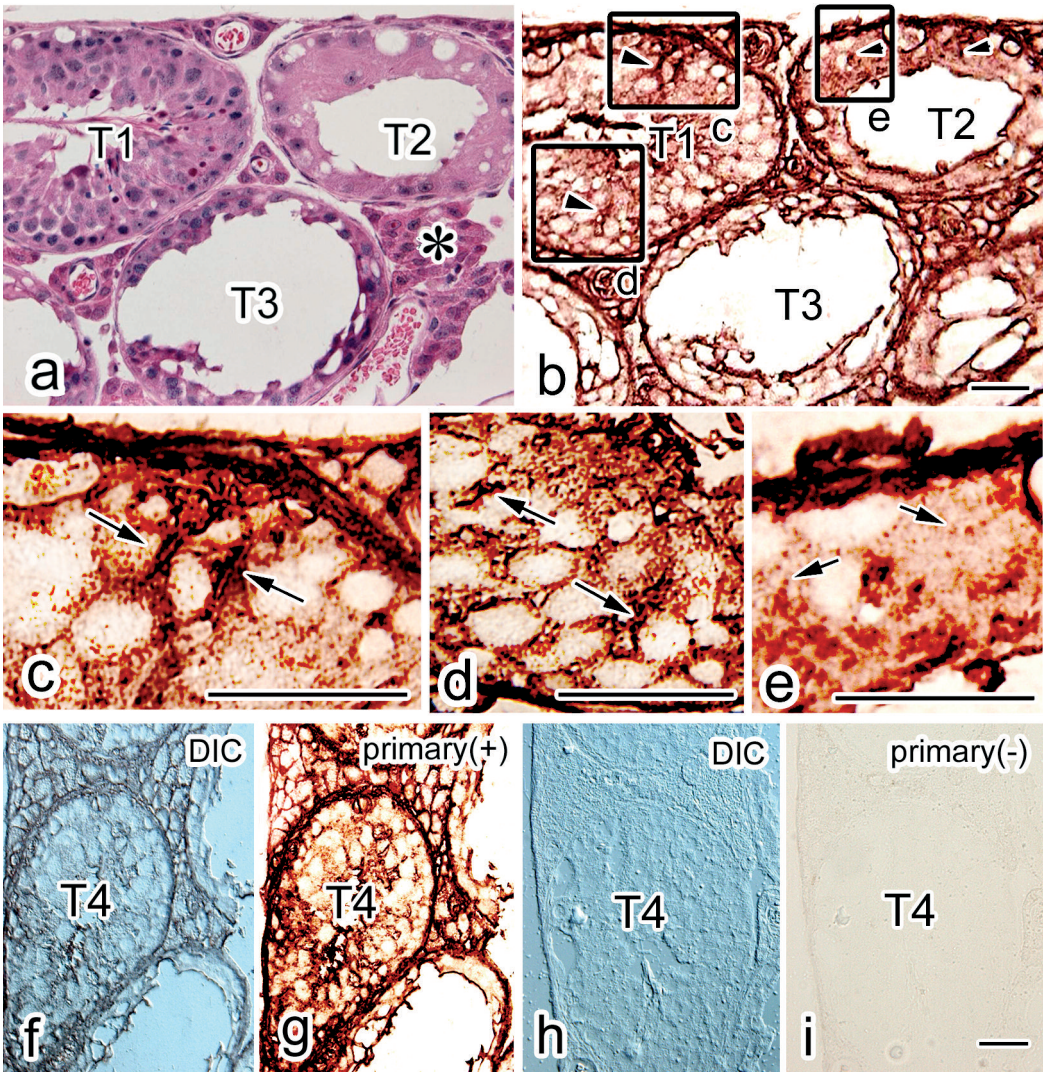

Fig. 3. Light micrographs of HE-staining **(a)** and immunolocalization of albumin **(b)** in the seminiferous tubules 48 hrs after the Cd-treatment. **a.** Markedly decreased number of germ cells and some vesicular formation in atrophic seminiferous tubules (T2, T3), as well as prominently remaining Leydig cells [asterisk in **(a)**], are observed on thin sections by HE-staining. **b.** The leakage of serum albumin through BTB in the seminiferous epithelium appears to be obvious in all seminiferous tubules, (arrowheads in T1), and the albumin immunostaining is also detected among the remaining Sertoli cells (arrowheads in T2). **c - e.** At higher magnification, corresponding to the squares in **(b)**, lining immunostaining patterns are detected in the adluminal compartment [arrows in **(c)**, **(d)**], and also dotted immunostaining ones in Sertoli cells [arrows in **(e)**]. On serial paraffin sections, the immunostaining was performed with **(g)** or without **(i)** the primary antibody. **f and h.** Corresponding differential interference contrast (DIC) images of **(g)** and **(i)**, respectively. Bars: 40 μm.

compartment by means of electron microscopy and fluorescence microscopy after intratesticular injection of a fluorescence-labeled probe, followed by the "*in vivo* cryotechnique" (Terada et al. 2005).

The present findings also confirmed that the immunointensity of leaked albumin through BTB in the basal compartments was obviously stronger in the seminiferous epithelium of the living mice at 24 hrs after the Cd-treatment. The different immunolocalizations of serum albumin in the seminiferous tubules between normal and Cd-treated mouse testis organs support an idea that the TJ structure against protein permeability between Sertoli cells is the most vulnerable portion affected by Cd toxicity, as reported before (Lui et al., 2003; Fiorini et al., 2004). In this study, the weak albumin immunoreactivity was observed in the luminal space in the seminiferous tubules (Fig. 1). By mapping of seminal plasma proteins with two-dimensional gel electrophoresis, some proteins, which are present in the serum, such as albumin, transferrin, lactoferrin and α1-antitrypsin, have been also characterized in the seminal plasma (Edwads et al., 1981; Starita-Geribaldi et al., 2001). It is still another question how such proteins are naturally leaking out in the normal seminiferous tubules.

In summary, the time-dependent morphological changes of seminiferous tubules in the Cd-treated living mice, including different immunostaining patterns of albumin in the basal compartment with BTB disruption, can be clearly detected by the "*in vivo* cryotechnique" combined with both freeze-substitution and immunohistochemistry.

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