CADMIUM EFFECTS OF METALLOTHIONEIN EXPRESSION IN EPIDIDYMAL EPITHELIUM OF RAT

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Cadmium (Cd) is a heavy metal with public health importance because it is known to present environmental and occupational hazards and a threatening to public health. Cd has negative effects on the liver, kidney, and male fertility. However, relatively little is known about the molecular toxicology of Cd in the epithelial cells of the male reproductive system. Thus, we studied the effects of Cd on the critical metal binging protein, metallothionein-1 (MT-1), in the epididymis of the intact rat. $CdCl_2$ (2 mg/kg/day) was administered for 10 days via minisomotic pumps implanted subcutaneously in mature male rats, and the rats were then sacrificed and their tissues, including the kidney and epididymis, were quickly frozen and examined via immunohistochemistry for MT-1, vacuolar H^+ - ATPase (V-ATPase) at confocal microscopic level or MT-1 (Western blot). We observed a significant upregulation of MT-1 expression in kidney proximal tubules by immunofluorescence and Western blot. MT-1 was positive over most of the basal cells (BC) along the epididymal duct and part of the clear cells (CC) in the caudal duct that co-localized with V-ATPase, a CCs marker. MT-1 expression was upregulated in distal caudal CCs after Cd treatment. However, the overall MT-1 expression assayed by Western blot in the epididymal duct decreased after treatment. This study indicated the validity of Cd administration via subcutaneous minipump. MT-1 was upregulated in caudal CCs after Cd treatment. The MT-1 change deserves further investigation.

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LIST OF ABBREVIATIONS

BC: basal cell

Cy3: cyanine 3

CC: clear cell

Cd: cadmium

DTT: dithiothreitol

FITC: fluorescein isothiocyanate

IF: immunofluorescence staining

MT: metallothionein

OCT: optimal cutting temperature

PBS: phosphate buffer solution

PC: principal cell

PLP: paraformaldehyde-lysine-periodate

SDS: sodium dodecyl sulfate

V-ATPas: vacuolar H^+-ATPase

VD: vas deferens

WB: western blot

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1.0 INTRODUCTION

1.1 CADMIUM

Cadmium (Cd) is one of the most important public health reltated heavy metal usually found in nature together with other metals such as zinc and lead. Occupational exposures to Cd occur when working with electroplating, batteries, melting, special steel, and pigments. Environmental exposures include smoking, pesticides, and fungicides [(1)]. When the environment is contaminated by these metal, humans can be over-exposed by ingesting contaminated foods. Cigarettes normally contain 1-2 μg of Cd per cigarette (up to 20 μg in sources heavily contaminated) due to naturally occurring Cd in tobacco leaves, of which approximately 5-10% can be absorbed by the respiratory tracts when people smoke. Significant increases of Cd in blood, placenta and seminal plasma were observed in smokers [(2-4)]. Cd should also account for the link between smoking and infertility, even though evidence of a direct causal link between cigarette smoke and such adverse effects is still unavailable [(5,6)] .

Cd toxicity includes acute and chronic clinical effects. The chronic effects, which are more common, include lung damage, kidney damage, and osteomalacia (Itai-Itai disease). Intensive studies have been addressed on the effects to these organs or systems [(6, 7)]. There have been few studies to date on the toxicity of cadmium on the male reproductive system [(8)], and the capacity of epididymal epithelial cells to detoxify cadmium and protect maturing spermatozoa.

1.2 METALLOTHIONEIN

Metallothioneins (MT) are small (6 kDa) cystein-rich proteins which can bind heavy metal (Zn, Cd, Cu, et al) [9]. In mice, there are four functional genes of MT family: MT-I, MT-II, MT-III and MT-IV. MT-III and MT-IV expressions are restricted to the central nervous system and the stratified squamous epithelium, and therefore MT-I and MT-II account for most of the systemic effects of functional MT [10]. Although precise physiological roles for the members of this multigene family are still unknown, it has been suggested that major pathophysiologic functions of MT-I and MT-II are to detoxify heavy metals, regulate heavy metal homeostasis, and scavenge reactive oxygen species [11, 12]. It is apparent that MT plays a critical role in protecting against heavy metals such as Cd [13], and MT is suggested to contribute to a cellular defense mechanism against partially reduced oxygen [9, 13-15] and nitrogen [16] species. *In vitro* data support the hypothesis that MT creates an important link between cellular redox state and metal ion homeostasis [17-19]. In this regard, cysteines of metal thiolate clusters facilitate for MT to participate in intracellular signal transduction pathways.

Studies suggested MT expression in the epididymis, although MT is mainly expressed in basal cells and regulated by testosterone [20]. There is no definitive evidence to support an association between cadmium exposure and MT expression, however.

1.3 MALE REPRODUCTIVE SYSTEM

1.3.1 Anatomy (overview)

The male reproductive tract consists of bilateral testes, the Vas deferens (VD), efferent ducts, and epididymides, as well as the accessory glands. The reproductive organs of the adult male rat, which is a species in which all the main accessory glands are present, are shown in Fig. 1

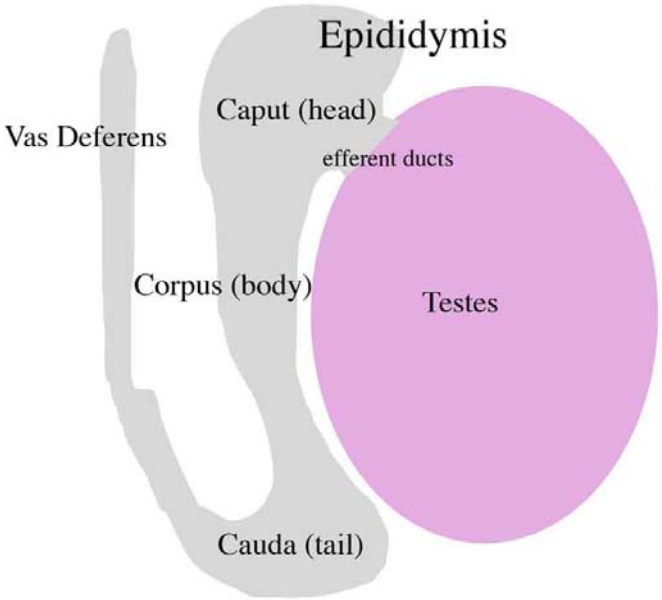


Figure 1. Scheme of the male reproductive tract with emphasis on the excurrent duct system (schematic from Pastor-Soler N)

The testes develop from primordial germ cells, which migrate from the yolk sac to the genital ridge of the mesonephros, then form the primary epithelial or medullary cords that are positioned

alongside the somatic cells from the genital ridge. The gonad then associates with the duct system of the generating mesonephros or Wolffian duct to form the epididymis, VD, ampulla, and seminal vesicles [21]. The prostate and bulbourethral (Cowper's) glands are derived from the urogenital sinus or urethra. The epididymis which developes from the main portion of the Wolffian duct, in most species, is a long, single tubule that coils onto itself and rests along one side of the testis. It carries sperms from the efferent ducts to the VD.

There is considerable variation between different species of mammals in terms of the types of accessory glands. In addition to the excurrent duct system of the testis (i.e., efferent ducts, epididymis and VD), which occurs in all species, the only other accessory gland present in virtually all mammals is the prostate gland. Virtually all other mammals have at least one pair of bulbourethral glands, which are particularly well-developed in certain insectivores (e.g., moles) and squirrels.

Now we will focus on the details of the epididymis.

1.3.2 Epididymis

The epididymal duct is a single though complicated duct in most of species, closely positioned to the surface of the testis to which it is connected via the efferent ducts, extending from the anterior to the posterior pole of the organ and attached to the tunica albuginea by connective tissue. The ductuli efferent empties into the initial segment. The remainder of the epididymis is divided into three parts: the caput, corpus, and cauda epididymidis (Fig 1) [5]. The epididymal duct is coiled into segments demarcated by the connective tissue septula and the organ is contained within a fibrous tissue capsule [21].

The epididymis is surrounded by connective tissue, which includes fibroblasts, macrophages, leucocytes, elastic fibers, collagen, smooth-muscle fibers, nerves, lymphatic and blood vessels. The thickness of the smooth-muscle layer surrounding the tubules increases from the initial segment to the cauda.

The epididymis has several roles include: transport of spermatozoa, creating a specialized luminal environment which allows for the maturation of spermatozoa. This environment is created through the absorptive and secretory activities of the epididymal epithelium.

As being summarized by Hermo & Robaire [22], the epididymal epithelium contains a variety of cell types. The major cell type is the principal cell (PC), which has apical stereocilia. Other types of cells include the apical cells, basal cells (BC), clear cells (CC), narrow cells (NC) and halo cells (intraepithelial lymphocytes). Some types of cells are located throughout the duct (e.g., PC), whereas others are found either exclusively or primarily in some specific regions (e.g., NC). Schematic representations of some of epithelial cells are shown in Fig.2 and Fig. 3.

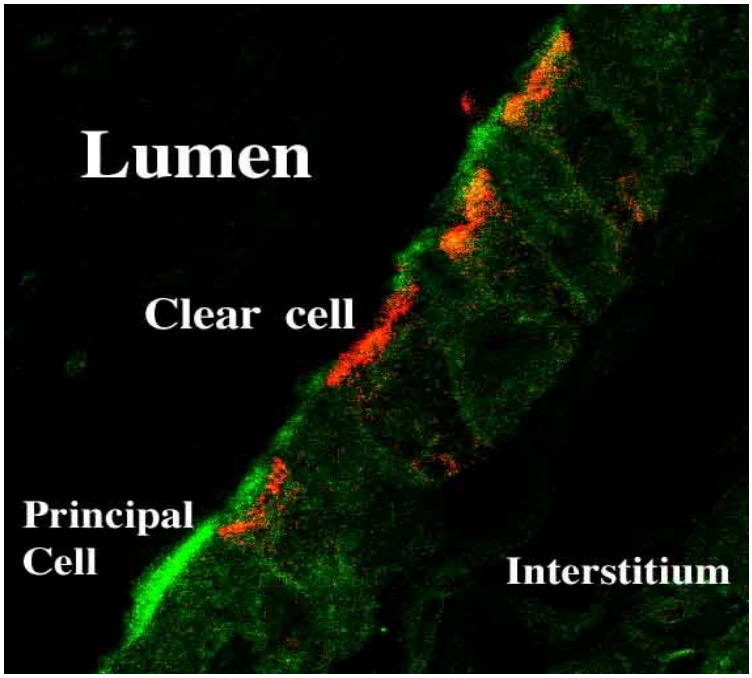


Figure 2. Cell types of adult rat epididymal epithelium shown by immunoflurent staining.

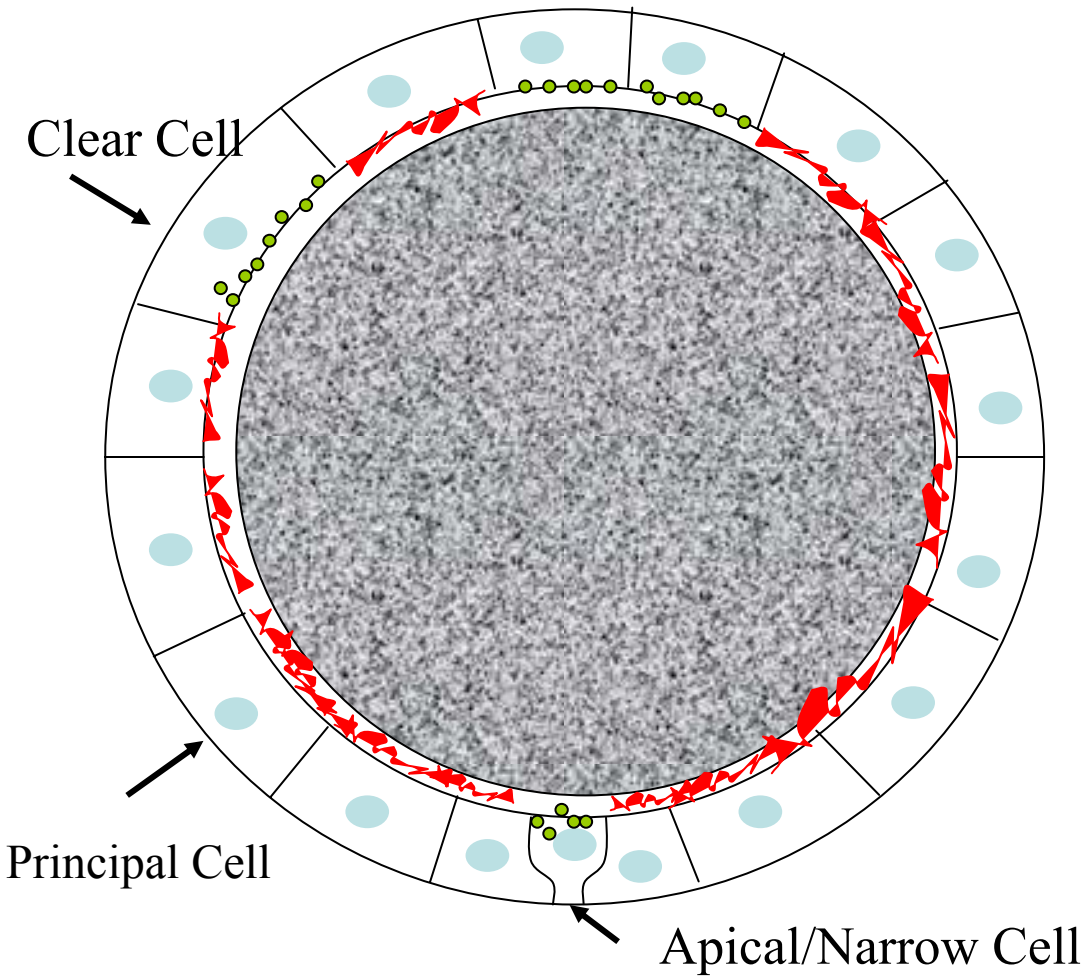


Figure 3. scheme of the cell types of epididymal epithelium

1.3.2.1 Principal cells (PC)

PC is the main cell type in the epididymis of all mammals, which comprises approximately 65% to 80% of the total epithelial cell population of this organ. PCs synthesize a large number of proteins, which either remain in the cells or are actively secreted into the lumen. They also play an active role in endocytosing luminal proteins. PCs have abundant aquaporin 9 (AQP9) staining at their apical pole. AQP9 play an important role in the transmembrane water movement as well as a conduit for other solutes [23].

1.3.2.2 Apical cells (AC), Clear cells (CC), and Narrow cells (NC)

AC is found primarily in the epithelium of the initial segment. These cells have a characteristic apically-located spherical nucleus and do not contact the basement membrane. Little is known about the specific functions of AC, aside from their ability to endocytose substances from the lumen. Apical cells express the V-ATPase [24, 25] which may be involved in the acidification mechanism of the male reproductive ducts.

CCs are large, active endocytic cells found in the caput, corpus, and cauda regions. CCs are characterized by an apical region containing endosomes, coated pits, multivesicular bodies, vesicles, and lysosomes and a basal region containing the nucleus and a changeable amount of lipid droplets [26]. The acidification of the luminal fluid is the other major function of CC. The key proteins for this process (vacuolated-ATPase, carbonic anhydrase II, and soluble adenylate cyclase) are specifically localized to CCs [24,25,27]. V-ATPase is widely used as a CC marker in epididymis research.

NCs appear only within the epithelium of the initial segment in the rat and mouse. These cells are attenuated and are narrower than the adjacent PCs, and send a thin process of cytoplasm to the basement membrane. NCs also express V-ATPase in their apical pole [27].

1.3.2.3 Basal cells (BC)

BCs, present in all species studied to date, are semi-circular cells attach themselves to the basement membrane and do not have direct access to the lumen, although their processes extend occasionally toward the lumen. Basal cells have thin and attenuated processes that extend along the basement membrane to cover most part of the circumference of the epididymal tubule. BCs have a few known functions, including the endocytosis of factors derived from the blood or principal cells. BCs also have secretory functions. It has been suggested that basal cells may act

as immune cells. Some studies also suggest that BCs have a role in regulating electrolyte and water transport by principal cells [21].

1.3.2.4 Halo cells (HC)

HCs are small cells with a thin rim of clear cytoplasm, and are present throughout the epididymal epithelium. Usually found at the base of the epithelium, HCs contain variable numbers of dense core granules with unknown functions. Study indicated that HCs are the primary immune cells in the epithelium of the epididymis, also named as intraepithelial lymphocytes [28].

1.4 TOXICITY OF CADMIUM IN MALE REPRODUCTIVE SYSTEM

The toxic effects of Cd in human have been known for more than 100 years, although few human cases have been documented. Animal research mainly focused on Cd effects on renal and liver function and testes structural damage [29]. Exposure to Cd, whether through the environment or industry, can directly harm the Sertoli cells and epithelial cells of the testes, especially in the Sertoli cells. A World Health Organization (WHO) study suggested that even low-level exposure (10 μg/L) to Cd can determinably reduce the quality of semen [30]. Toxicology of the epididymis has received less attention than other regions of the male reproductive tract. It is important to understand the regulation of MTs in the epididymis and whether they can be induced by heavy metal Cd and whether they contribute to the protection of spermatozoa in condition of toxic Cd exposure.

1.5 AIM OF THIS STUDY

Despite the myriad of studies done to test the toxic effects of Cd on kidneys and lungs, relatively little is known regarding its effects on the male reproductive system, especially its association with the critical metal binging protein MT-1 in the epididymis. Given that heavy metals such as Cd act on the male reproductive tract, it is important to study the regulation of MT in these tissues, since MT may protect the cells from such toxicants. The aim of this study was to test the validity of the rat Cd exposure model and to investigate the effects of Cd on MT-1 in the epididymis epithelial cells of the intact adult rat.

2.0 MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Animals experimental protocols were approved by the University of Pittsburgh ACUC (Animal Care and Use Committee), in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult Wistar male rats (12 week old, body weight: 314.3 ± 19.0, n = 3 for both control and experimental groups) were used in this study.

2.2 CADMIUM TREATED ADULT RATS

Three rats were treated (s.c.) with an aqueous solution of $CdCl_2$ via implanted osmotic minipumps (Alzet, Cupertino, CA) at a dose of 2 mg/kg/day for 10 days. The minipumps were placed under anesthesia using sterile technique as previously described [23]. The control animals received vehicle alone also via osmotic minipump for the same period of time.

2.3 TISSUE FIXATION AND IMMUNOCYTOCHEMISTRY

2.3.1 Tissue preparation

As described in Pastor-Soler's lab protocol [23], rats were anesthetized using with sodium pentobarbital (65 mg/kg body weight i.p.) and the male reproductive organs (and the kidneys) were perfused via left cardiac ventricle with 1x PBS pH7.4, followed by paraformaldehyde-lysine-periodate (PLP) fixation for 5 min. The organs were removed and placed in PLP buffer at room temperature for 4-6 hours or overnight at 4 ^{o}C. Tissue was then washed three times in PBS and kept in PBS containing 0.02% sodium azide at 4 ^{o}C prior to use.

2.3.2 Antibodies

Monoclonal mouse anti-MT (Dako Corporation, Carpinteria, CA) was used for IF and WB. This antibody recognizes both MT-I and MT-II [31]. V-ATPase antibody against the C-terminal 14 amino acids of the 31 kDa subunit of the bovine kidney medulla proton pump was raised in chicken, and affinity-purified [23]. The β–actin is from Sigma Chemical Company (St. Louis , MO).

2.3.3 Immunofluorescence (IF)

PLP-fixed tissues were cryoprotected in a solution of 30% sucrose in PBS for at least 2 hours at room temperature. They were embedded in OTC Compound 4583 (Tissue-Tek, Miles Inc.,

Torrance, CA) and mounted on a cutting block. After freezing in a Reichert Frigocut microtome, the tissue was cut at 4-5 μm thickness and sections were placed onto Fisher Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Tissue sections were rehydrated in PBS container at room temperature for 15 minutes, and then pretreated with 1% (w/v) SDS for 4 min, an antigen retrieval technique as previously described [32]. After three wash in PBS of 5 min for each, slides were incubated in 1% (w/v) BSA in PBS/0.02% sodium azide for 15 minutes to block nonspecific staining, and once in PBS, after which they were incubated in MT-1 (diluted 1:400 in PBS) or V-ATPase antibodies (diluted 1:200 in PBS) for 60 min at room temperature. Sections were washed twice for 5 min in high-salt PBS (2.7% [w/v] NaCl) to reduce nonspecific staining, and once in normal PBS. They were then incubated for 60 min at room temperature with secondary antibodies, a goat anti-mouse immunoglobulin (Ig) G coupled to fluorescent dye, FITC (Jackson Immunologicals, West Grove, PA) or donkey anti-chicken immunoglobulin (Ig) G conjugated with fluorescent cyanine dye, CY3. The sections were again washed as described above. Topro 1:1500 were co-incubated with secondary antibody for nucleus staining. The slides were mounted with VectaShield mounting media (Vector Labs, Burlingame, CA) and examined using Leica SL confocal microscope after drying.

2.3.4 Fluorescent microscope

Two IF experiments were carried out for each animal of control and Cd treated groups. All incubations were performanced under same conditions modified from PI's lab protocol [23]. Sections were examined using a Leica SL confocal microscope. All the images were obtained with identical exposure time and gains on the same day.

2.4 WESTERN BLOTTING

Adult rats were anesthetized and perfused through the left ventricle with PBS pH 7.4 containing protease inhibitor. One kidney/epididymis from each animal was removed and snap-frozen using liquid nitrogen before storing at -80^{o}C before use. The other tissue (kidney or epididymis) was kept in situ and fixed via left ventricle perfusion as indicated above, and used for immunocytochemistry.

Each epididymis (kidney) tissue was thawed on ice, cut into smaller pieces with a razor blade, and quickly washed in 1 ml of ice-cold PBS containing complete protease inhibitor (Roche, Indianapolis, IN). Protein was extracted by the addition of 200 µl cold extraction buffer per Dr. Pastor-Soler's lab protocol [23]. Extra DTT (350mM) was added to the buffer to reduce disulfide bonds formation, therefore limit large polymerization of MT protein. Sample buffer was added to protein after the concentration was assayed. The samples were solubilized at 100 ^{o}C for 15 min) with a final concentration of 10% NuPAGE reducing agent. The equal amount of samples were loaded with 4-15% Gel, running at 100 v for 1 hour, and then transferred at 4^{0}C under 100 v for 1 hour. The transferred membranes were blocked with 5% milk/TBS, 50mM Tris-HCl, and 150 mM NaCl. The membranes were washed in TBS containing 0.05% Tween-20 (Sigma). Primary antibody (1:2000 dilutions) was incubated for 1 hour, then secondary antibody for 1hr. Films were developed after washing 3 times with 20 min for each time. The sample membranes were blotted with β-actin as an internal control to confirm samples equal loading.

3.0 RESULTS

3.1 METALLOTHIONEIN IN THE KIDNEYS

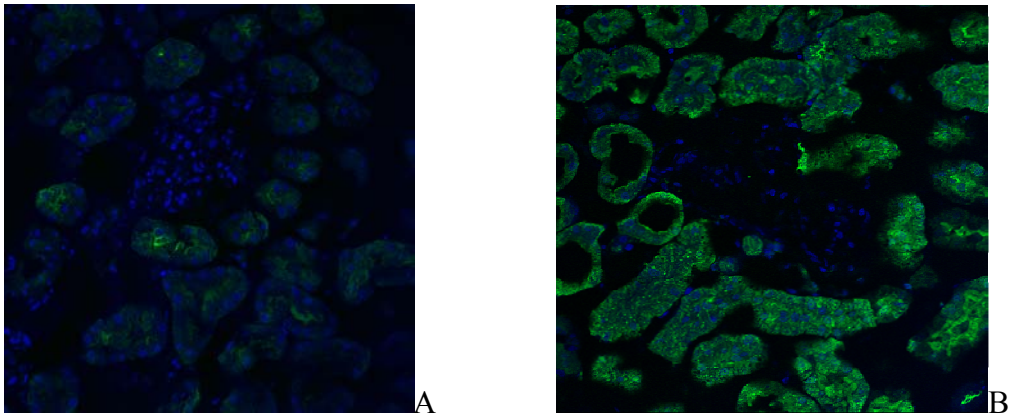
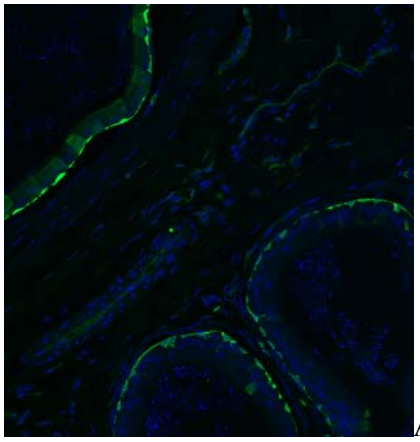


Figure 4. MT-1 expression in kidney (immunostaining)

The metallothionein expression assessed by IF in the kidney proximal tubules is shown in Figure 4. Low level staining was observed over the cytoplasm of the proximal tubule epithelium in control animals (A). MT staining was distinctively increased in both the areas of the epithelium and the intensity of the reaction with $CdCl_2$ treatment (B) for 10 days (2 mg /kg/day) vs. control rats (A). WB of kidney also shows overall MT expression upregulated in the $CdCl_2$-treated group (Figure 7).

3.2 MT EXPRESSION ON THE EPIDIDYMAL DUCT EPITHELIUM


A

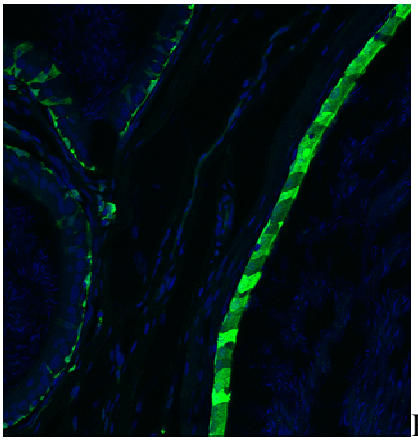

B

Figure 5. MT staining at cauda epididymal epithelium

MT staining shows that most basal cells and some clear cells were positive at the distal duct in control animals. In the caudal epididymal epithelium, significantly increased number of clear cells was reactive to the staining in the $CdCl_2$ treated rats (B) compared to the control rats (A). These results presented in Fig 4-7 are representatives of all 6 animals.

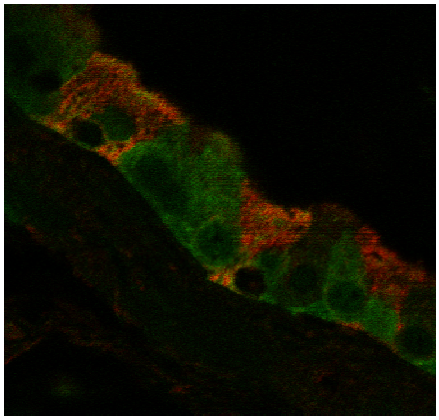


Figure 6. MT and V-ATPase co-staining at the distal epithelium

Co- staining on the distal epithelium shows that most MT positive stained clear cells (green) was also reactive to V-ATPase staining (red). The overlapped staining shows yellow.

3.3 MT PROTEIN EXPRESSION ASSAY

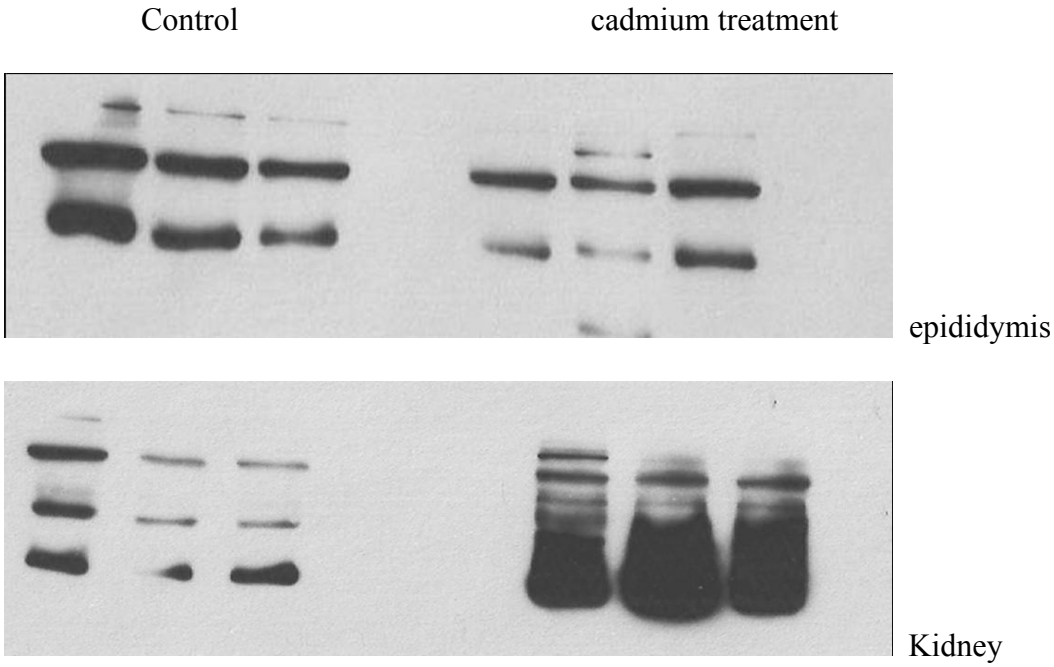


Figure 7. Western blotting of epididymis and kidneys

MT-1 western blotting was shown in Fig. 6. MT-1 protein expression decreased in the epididymis tail (upper) but increased in the kidneys (lower) after $CdCl_2$ treatment. As shown in the fig, some polymerization of MT protein formed even though up to 350 mM DTT had been added to the extraction and loading buffer. The blot membranes were blotted with monoclonal β-actin antibody (1:10,000 dilution), which indicated equal loading of all lanes (data not shown here).

4.0 DISCUSSION

4.1 CADMIUM EXPOSURE ANIMAL MODEL

For MTs, especially MT I and II, their cellular levels can be increased by heavy metals and some other agents. The sensitivity of these proteins to metals has led to their use as biomarkers of heavy metal exposure, particularly in the kidney and liver [12]. In this study, after injection of Cd (2 mg/kg/day) via the subcutaneous mini-pump for 10 days, MT expression increased according to both immunostaining and western blot in kidney. The increase of its expression in kidney indicates the viability of our Cd administration animal model.

4.2 CADMIUM EFFECTS ON THE EPIDIDYMAL EPITHELIUM MT EXPRESSION

In the present study, we successfully immunostained the rat epidicymal epithelial cells with anti-MT antibodies. We observed that immunoreactive MT was distributed in basal cells throughout the epididymis, especially in the cauda. The reaction of MT over BCs has also been reported by other groups [20].

However, few studies to date have reported MT expression on rat CCs. This study has shown that MTs are not only positive over BCs but also CCs, especially in the distal caudal area

(Fig 5 and 6). The location of MT over CCs has been further confirmed by co-staining with V-ATPase which is a CC specific marker. Our successful MT staining on CCs can be explained by our usage of different tissue fixation methods. We used PLP fixation rather than paraffin-embedded tissues as other group have used [20], suggesting that PLP fixation is favorate to MT immunostaining on the CCs study. The MT upregulations in CCs were observed in the proximal and distal area of cauda in the cadmium treated rats. This observation suggests that CCs in cauda duct may play a significant role in the protection mechanism against cadmium intoxication. Hermo et al reported that endocytic activity, the major known functions of CCs is greater in the cauda epididymis than other regions [26], suggesting that MT increase may be related to the endocytic activity.

4.3 DISCREPANCY OF THE MT EXPRESSION IN THE KIDNEY AND EPIDIDYMIS

Our study has shown the overall MT protein expression in the epididymis was down-regulated with meanwhile significant increase of MT expression in the kidney after cadmium exposure. There are some possible explanations to these phenomena.

First of all, the regulation of the MT gene by metals involves multiple signaling pathways which depend on the species of metal ion, organs and cell types [33]. Metal-responsive Transcription Factor-1 (MTF-1) is one of the most important components in the transcriptional induction of metallothionein genes [31, 34]. Wahba, et al [35] reported metallothionein decrease in rat testes by cadmium treatment. Vasconcelos also observed the discrepancy of MT gene

expression with observed MT protein levels in rats liver and kidney with Cd or Cu treatment [36]. Other studies shaw MT mRNA level changes were inconsistent in deferent epididymal regions.

On the other hand, these results may also be explained by the fact that overall epididymis weight and protein decreased in $CdCl_2$ treated animals. The proportion of epithelium, which is the main location for metallothionein, in the whole epididymis bulk may be lower in the $CdCl_2$ treated groups vs. control animals.

Furthermore, the IF, which is regional dependent, was difficult to be quantified. We can identify the rough area of the epididymal tubule, but almost impossible to locate the specific segment with our experimental method. So, there was a problem to compare between the intensity of IF of the slides from different animals.

The significance of MT expression decrease in epididymis after Cd treatment is worthy of further investigation. We do not yet know if this indicates that MT traffics away from the epididymis after scavenging cadmium.

5.0 FUTURE DIRECTION

A future study should assess Cadmium distribution in different organs and plasma to study the relevance of Cadmium effect and concentration. A possible epithelial cells apoptosis study should be conducted as well. Further greater number of MT knock-out mice model experiments should be conducted to study the effect of Cadmium to epididymal epithelium. This model could be very helpful to understand the role of MT in Cadmium toxication.

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