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Sex-dependent expression of metallothioneins MT1 and MT2 and concentrations of trace elements in rat liver and kidney tissues: Effect of gonadectomy☆

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ABSTRACT

Metallothioneins (MTs) exhibit binding affinity for several essential and toxic trace elements. Previous studies in rodents indicated sex differences in the hepatic and renal expression of MTs and concentrations of various elements. The mechanism responsible for these differences has not been resolved. Here, in the liver and kidney tissues of sham-operated and gonadectomized male and female rats we determined the expression of *MT1* and *MT2 (MT1&2)* mRNA by RT-PCR, abundance of MT1&2 proteins by Western blotting and immunocytochemistry, concentrations of essential (Fe, Zn, Cu, Co) and toxic (Cd, Hg, Pb) elements by ICP-MS, and oxidative status parameters (SOD, GPx, MDA, GSH) by biochemical methods. In both organs, the expression of *MT1&2* mRNA and MT1&2 proteins was female-dominant, upregulated by castration, and downregulated by ovariectomy. Concentrations of Fe in the liver and Co in the kidneys followed the same pattern. Most other elements (Zn, Cu, Cd, Hg) exhibited female- or male-dominant sex differences, affected by gonadectomy in one or both organs. Pb was sex- and gonadectomy-unaffected. GPx and MDA were elevated and associated with the highest concentrations of Fe only in the female liver. We conclude that the sex-dependent expression of *MT1&2* mRNA and proteins in the rat liver and kidneys may include different mechanisms. In the liver, the female-dominant tissue concentrations of Fe may generate oxidative stress which is a potent enhancer of MTs production, whereas in kidneys, the female-dominant expression of MTs may be unrelated to Fe-mediated oxidative stress.

1. Introduction

Metallothioneins (MTs) are small (6–7 kDa), cysteine-rich proteins that bind several trace elements with different affinities. In mammals, functional MTs exist in four forms, and, depending on the species, some forms exist in two or more isoforms. MT1 and MT2 (MT1&2) are expressed in various organs [1–3], MT3, alias growth inhibitory factor (GIF), is found in the brain and kidneys [3–5], whereas MT4 is located

Abbreviations: ARE, androgen response element; BSA, bovine serum albumin; C, castrated males; ERE, estrogen response element; GPx, glutathione peroxidase; GRE, glucocorticoid response element; GSH, glutathione; MDA, malondialdehyde; MT-ab, anti-metallothionein antibody; MTs, metallothioneins; MT1, MT2 MT3 and MT4 metallothionein 1 2 3 and 4, respectively; O, ovariectomized females; PBS, phosphate-buffered saline; PFA, *p*-formaldehyde; RNS, reactive nitrogen species; ROS, reactive oxygen species; SO, sham operated; SOD, superoxide dismutase

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in stratified squamous epithelia [6]. As mainly studied in the liver and kidneys of experimental animals, the expression of MT1&2 can be induced by a wide range of stimuli, such as treatment with essential and non-essential trace elements, physical stress, glucocorticoids, inflammation, irradiation, caloric restriction, and chemicals that produce reactive oxygen (ROS) and nitrogen (RNS) species, while MT3 and MT4 are poorly or not at all affected by these stimuli [3,4,7–10]. Based on these studies, the proposed functions of MT1&2 include intracellular storage and homeostatic control of essential metals (Zn, Cu, Fe, Co), absorption and/or excretion of some essential and toxic metals, protection from cytotoxicity of toxic trace elements (Cd, Hg, Pb, Ag, Pt) by scavenging and sequestration, scavenging free radicals (ROS and RNS) generated in physiological and pathophysiological metabolic processes, protection against alkylating agents, and resistance to and/or protection from anticancer drugs [1–3,9–15]. A potential role of MTs in the development and progression of various human tumors may be an important, but still unclear phenomenon [16].

In mice and rats, the highest abundance of *MT1&2* mRNA and protein, and the related tissue concentration of Zn occur perinatally in the liver, less in the kidneys, and thereafter both decrease to the basal levels in adults [14,17–19]. Sex-related differences in the expression of hepatic and renal MT1&2 proteins and mRNAs have been studied in the same species with variable results. In *MT1*-transgenic mice, females exhibited 4-5-fold higher levels of the hepatic MT1 protein as compared to males [20], while Shaikh et al. [21] reported that both hepatic and renal levels of MTs in adult mice were strain-related; in some strains the renal (but not hepatic) MT protein levels in females were about 2-fold higher than in males. Sogawa et al. [22] showed that the hepatic *MT1* mRNA expression in adult female mice was also about 2-fold higher than in males, and significantly diminished by ovariectomy. This sex difference in gene expression was present in 46 week-old, but not in prepubertal, 4 week-old mice. In rats, neonatal animals exhibited the male-dominant abundance of the MT protein in their liver, whereas adult animals showed just the opposite, the estrogen-driven female-dominant tissue abundance of this protein. These sex differences in hepatic MT abundance correlated well with the tissue accumulation of Cd following treatment of animals with $CdCl_2$ [23]. In the kidneys of untreated adult rats, however, the tissue MT concentration was also higher in females compared to males, but the corresponding differences in Cd accumulation were not observed [18,23]. These studies thus indicated estrogens as the physiological regulator of MT1&2 protein abundance in the rat liver and kidneys in rodents. However, the promoters of *MT1&2* genes do not contain either androgen (ARE) or estrogen (ERE) response elements, which usually mediate transcriptional regulation by the respective sex hormones (reviewed in [10]), and the question remains whether some other element and/or mechanism contributes to the phenomenon of the sex-dependent expression of MTs.

We recently studied sex-related differences in the tissue content of various trace elements in the liver and kidneys of adult rats in the presence and absence of circulating blood [24]. In a limited number of animals (5 in each experimental group), some elements supposed to have affinity for MTs were more abundant in the female liver (Fe, Cu, Cd), whereas in kidneys, Fe, Co, Zn, and Cd were more abundant in the females, and Hg and Pb were more abundant in the males. A possible correlation of these elements concentration with the tissue expression of *MT1&2* mRNA and protein, and sex hormones responsible for the observed phenomena represent the topic of the present study.

2. Material and methods

2.1. Animals and treatment

Experiments were performed using 10–12 week-old Wistar strain male and female rats from the breeding colony at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Animals were bred and maintained according to Directive 2010/63/EU on the protection of animals used for scientific purposes. Before and during experiments, animals had free access to standard pelleted food (4RF21 from Mucedola, Settimo Milanese, Italy) and tap water. Two weeks before the sacrifice, males were castrated *via* scrotal route, whereas females were ovariectomized *via* dorsal (lumbal) approach. The sham-operated animals underwent the same operation procedure, but without removing the respective organs. The operations were performed under general anesthesia induced by Narketan (80 mg/kg body mass (b.m.)) and Xylapan (12 mg/kg b.m., i.p.), both purchased from Chassot AG (Bern, Switzerland). The studies were approved by the Institutional Ethics Committee and by the Croatian Ministry of Agriculture.

2.2. Determination of body and kidney parameters, and of sex hormones in blood plasma

Prior to sacrifice of anesthetized rats by exsanguination, the mass of each animal was measured, and 3–4 ml of blood was collected by percutaneous puncture of the left heart ventricle. The blood was centrifuged at 1000 *g* for 10 min to pellet blood cells; the supernatant contained blood plasma for determination of sex hormones. The commercial Chemiluminescent Microparticle Immunoassay kits were used for quantitative determination of testosterone (7K73) and progesterone (7K77) (both from Abbott Laboratories, Abbott Park, IL, USA). Estradiol was determined by the immunoassay reagent kit from Ortho Clinical Diagnostics (8552630 ECI; Raritan, NJ, USA). Following sacrifice, the kidneys were removed, decapsulated, and their mass was measured to calculate the renal index.

2.3. Isolation of RNA and synthesis of first strand cDNA

The liver tissue and decapsulated right kidney were removed and briefly rinsed in cold phosphate-buffered saline (PBS). From each organ, ~1-mm thick slices (80–100 mg wet mass (w.m.)) of the liver (largest lobe) and kidney (transversal middle slice) tissues were cut off and immediately submerged into the RNAlater (Sigma, St. Louis, MO, USA). The slices were used *in toto* for total cellular RNA extraction with Trizol(Invitrogen) and cleaning with RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The RNA concentration and purity were determined spectrophotometrically in BioSpec-nano (Shimadzu, Japan). The quality and integrity of isolated RNA were checked by agarose gel electrophoresis, stained with StarGel (Lonza, Rockland Inc., ME, USA), and stored at −70 °C until further use.

First strand cDNA was made using the High Capacity cDNA RT Kit (Cat #4374966, Applied Biosystem, Foster City, CA, USA) following the manufacturer's instructions. Total cellular RNA (1 μg) was incubated at 25 °C for 10 min in the reaction mixture containing random primers and reverse transcribed in a total volume of 50 μL containing 1× reverse transcription buffer, 20 units of ribonuclease inhibitor, 1 mM of dNTP mix, and 40 units of Multiscribe reverse transcriptase, by incubation at 37 °C for 120 min and final denaturation at 85 °C for 10 min. cDNAs were stored at −20 °C until further use.

2.4. End-point RT-PCR

PCR was performed in a total volume of 20 μL using 1 μL of 5× diluted first strand cDNA, 0.4 μM specific primers, and ready to use PCR Master Mix (Applied Biosystem) following instructions by the manufacturer. To avoid amplification of genomic DNA, intron over-spanning primers were used. Custom primers for rat *MT1*, *MT2*, and *GAPDH* genes were purchased from Invitrogen (*online*). The sequences of specific primers used for RT-PCR reactions and predicted RT-PCR product sizes are listed in Table 1. Reaction conditions used for PCR included initial denaturation for 3 min at 94 °C, denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C, and elongation for 45 s at 72 °C. The non-template control reactions, where the cDNA was replaced with DNase/RNase-free water, gave no PCR products, indicating an absence of relevant contamination in our tests (data not shown). RT-PCR products

Table 1
Primer sequences used in end-point RT-PCR studies of rat genes.

Gene	Forward (F)/Reverse (R) Primers (5'-3')	Accession No. Gene Bank	Location	RT-PCR product (bp)
MT1 A	F:CACCAGATCTCGGAATGGAC	NM_138826.4	56-75	220
	R:CAGCAGCACTGTTCGTCACT		275-256	
MT2 A	F:CACAGATGGATCCTGCTCCT	NM_001137564.1	86-105	249
	R:AAGTGTGGAGAACCGGTCAG		334-315	
GAPDH	F: GGTGATGCTGGTGCTGAGTA	NM_017008.2	1105-1125	369
	R:GGATGCAGGGATGATGTTCT		1453-1473	

were resolved by electrophoresis in 1.5% agarose gel stained with 1 × GelStar (Lonza, Rockland Inc., ME, USA), visualized and recorded under UV light. The housekeeping gene *GAPDH* was used as a control for variations in RNA input. In preliminary experiments, the optimal number of PCR cycles within the exponential phase of the reactions was found to be 20 for *MT1* and *MT2* mRNA, and 24 for *GAPDH* mRNA. The bands of PCR products were evaluated by densitometry using the noncommercial Image-J program (http://imagej.nih.gov/ij/). The density of each *MT1* or *MT2* mRNA band (same area) was measured and divided by the density of the corresponding *GAPDH* mRNA band in order to obtain numerical, relative values of the gene expression.

2.5. Antibodies and other material

Monoclonal antibody against the horse MT1&2 (clone E9) (further, MT-ab), which recognizes a highly conserved domain common to both MT1 and MT2 proteins was purchased from DAKO (Carpinteria, CA, USA; Cat. No. M0639). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA); the CY3-labeled donkey anti-mouse IgG (DAMCY3; Cat. No. 715-165-151) was used for immunofluorescence studies, whereas the alkaline phosphatase-labeled goat anti-mouse IgG (GAMAP; Cat. No. 115-056-068) was used in immunoblotting studies. Various other chemicals in the study were of the highest purity available and were purchased from either Sigma or Fisher Scientific (New Jersey, NJ, USA).

2.6. SDS-PAGE and Western blotting

The liver (largest lobe) and kidneys were removed and dissected manually to prepare 10% tissue homogenates in a chilled buffer (in mM; 300 mannitol, 5 EGTA, 12 Tris/HCl, pH 7.4, protease inhibitors (Sigmafast) using a Powergen 125 homogenizer (Fisher Scientific, New Jersey, NJ, USA). The homogenates were centrifuged at 5000 *g* for 10 min, the pellet was discarded and the supernatant (further denominated as tissue sample) was used in further studies for detection of MT1&2 by immunoblotting. Proteins were measured by dye-binding assay [25] using bovine serum albumin (BSA) as standard, and the tissue samples were stored frozen at −20 °C until further use.

Before PAGE, the tissue samples were mixed with sample buffer (4% SDS, 18% v/v glycerol, 167 mM Tris/HCl, 4% bromphenol blue, 5% 2-mercaptoethanol, pH 6.8), and denatured at 95 °C for 5 min. Proteins (60 µg per lane) were separated through 18% SDS-PAGE mini gels using the Vertical Gel Electrophoresis System and then electrophoretically wet-transferred using Mini Trans-Blot Electrophoretic Transfer Cell (both from Bio-Rad Laboratories, Hercules, CA, USA) to Immobilon membrane (Millipore, Bedford, MA, USA). Following transfer, the Immobilon membrane was incubated in 1% glutaraldehyde for 30 min to enhance MT1/2 retention in the membrane [26], washed several times with water and blocked in the blotting buffer (5% nonfat dry milk, 0.15 M NaCl, 1% Triton-X-100, 20 mM Tris/HCl, pH 7.4) at room temperature for 30 min, followed by an incubation in the same buffer that contained MT-ab (1:500) at 4 °C overnight. The membrane was then washed with several changes of blotting buffer, incubated for 60 min in the same buffer that contained 0.1 µg/mL GAMAP, washed again, and stained for alkaline phosphatase activity using the 5-bromo-4chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) method as indicator [27]. The protein bands were evaluated by densitometry using the above-mentioned Image-J program. The density of each band (same area) was determined and expressed relatively to the density of the strongest band (= 1 arbitrary unit) in control samples.

2.7. Tissue fixation and immunocytochemistry

In anesthetized rats, the organs were perfused *in vivo via* the heart left ventricle (the perfusate was released through the incised abdominal large vein), first with aerated (95% O_2/5% CO_2) and temperature-equilibrated (37 °C) PBS for 2–3 min, and then with 4% paraformaldehyde for 5 min. Liver (largest lobe) and kidneys were removed, sliced in 1–2 mm-thick slices, and kept in the same fixative at 4 °C overnight, followed by extensive washing in PBS, and storage refrigerated in PBS containing 0.02% NaN_3 until used.

To cut 4-µm thick frozen sections, tissues were infiltrated with 30% sucrose (in PBS) overnight, embedded in OCT medium (Tissue-Tek, Sakura, Japan), frozen at -25 °C, and sectioned in a Leica CM 1850 cryostat (Leica instruments GmbH, Nussloch, Germany). Cryosections were collected on Superfrost/Plus microscope slides (Fischer Scientific, Pittsburgh, PA, USA) and rehydrated in PBS for 10 min. Before immunostaining, the sections underwent optimal antigen retrieval steps, as described in detail previously [27,28]. Nonspecific binding of the antibodies was then prevented by incubating the sections in 1% BSA (in PBS) for 30 min, followed by MT-ab (1:200 in PBS) at 4 °C overnight. The consecutive steps included two washings in 0.1% Triton-X-100 (in PBS) plus two washings in regular PBS (5 min each), incubation in DAMCY3 (1.6 µg/mL) at room temperature for 60 min, two washings in 0.1% Triton-X-100 and twice with regular PBS (5 min each), and mounting a fluorescence fading retardant (Vectashield; Vector Laboratories Inc., Burlingame, CA, USA).

The staining was examined with an Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany). The images were captured by a computer guided Spot RT Slider camera and software (Diagnostic Instruments, Sterling Heights, MI, USA), and imported into Adobe Photoshop for processing and labeling. To obtain better contrast, the CY3-related red color in immunofluorescence images was converted into black and white mode using Photoshop.

2.8. Determination of chemical elements in the liver and kidney tissues

In animals sacrificed by exsanguination, the abdominal organs were rinsed of blood with a stream of chilled ultra-pure water. With this approach, a limited number of blood-associated elements represent a small, but similar contribution in the tissues from all animal groups. A piece of the liver largest lobe (~1 g wet weight) and the whole decapsulated left kidney from each animal were removed, rinsed with ultra-pure water, briefly blotted onto filter paper, and further processed as described in detail recently [24]. The cutting of the tissues for these studies was performed using the ceramic knife and Teflon-based support. The chemical elements were measured by ICP-MS in Agilent 7500cx (Agilent Technologies, Tokyo, Japan) under conditions described in detail previously [24]. The instrument detection limits for the studied elements, calculated as mean plus three standard deviations of blanks (1% HNO_3), were (in µg/L) 2 for Fe, 0.4 for Zn, 0.01 for Cu, 0.0009 for Co, 0.0007 for Cd, 0.004 for Hg, and 0.001 for Pb.

2.9. Determination of SOD and GPx activities

To determine the activity of antioxidant enzyme superoxide dismutase (SOD; EC 1.15.1.1), the liver (largest lobe) and kidney (central transverzal slice) tissue pieces (~100 mg w.m.) were rinsed with cold PBS and homogenized on ice in 0.5 mL of cold buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 20 mM HEPES/Tris, pH 7.2). Tissue homogenates were centrifuged at 1500 *g* for 5 min at 4 °C (Eppendorf Microcentrifuge 5417R). The supernatant was separated and kept frozen at −80 °C until enzyme analysis. The total SOD activity was measured on a TECAN Infinite M2000 Pro microplate reader (TECAN, Grödig, Austria) using the commercial Superoxide Dismutase Assay Kit (Item No. 706002; Cayman Chemical Company, Ann Abor, MI, USA). As indicated in the product datasheet, this assay measures all three types of SOD (Cu/Zn-, Mn-, and Fe-) and utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. SOD activity is expressed in U/mg protein.

To determine the activity of cytoplasmic enzyme glutathione peroxidase (GPx; EC 1.11.1.9), the liver (largest lobe) and kidney (central transverzal slice) tissue pieces (~100 mg w.m.) were rinsed with cold PBS and homogenized on ice in 0.5 mL of cold buffer (5 mM EDTA, 1 mM DTT, 50 mM Tris-HCl, pH 7.5). The homogenates were centrifuged at 10,000 *g* for 15 min at 40 °C (Eppendorf Microcentrifuge 5417 R). The supernatant was separated and kept frozen at −80 °C until enzyme analysis. The activity of GPx was measured on a TECAN Infinite M2000 Pro microplate reader using the commercial Glutathione Peroxidase Assay Kit (Item No. 703102; Cayman Chemical Company, USA). The kit measures the GPx activity indirectly, in a coupled reaction with Cumene hydroperoxide as a substrate and glutathione reductase, and was assessed from the decrease in absorption at 340 nm due to oxidation of NADPH to $NADP^+$. The enzyme activity was expressed in U/mg protein. Proteins in tissue samples aimed to be used for enzyme determinations were measured by the dye-binding assay [25].

2.10. Determination of tissue concentrations of GSH and MDA

Glutathione (GSH), an antioxidant peptide, was analysed according to Ellman's method [29]. Briefly, 10% liver and kidney (central transverzal slice) tissue homogenates were prepared, 100 µL of 5% trichloroacetic acid was added, mixed, and centrifuged at 3000 *g* for 10 min. To 100 µL H_2O (blank solution), standards, or samples (supernatant following centrifugation of the tissue homogenates), added were 850 µL 0.3 M Na-phosphate buffer (pH 7.4), and 50 µL 1 mM 5,5-dithio-bis-2-nitrobenzoic acid. Absorbance of blank, standards, and tissue samples were measured spectrophotometrically at 412 nm (Cecil 9000, Cambridge, UK). GSH concentration was calculated from the calibration curve.

Malondialdehyde (MDA), a lipid peroxidation marker and a measure of free radical activity in the tissue, was analyzed according to Drury et al. [30]. 10% liver and kidney tissue homogenates were prepared in 0.3 M Na-phosphate buffer (pH 7.4). To 50 µL sample or standard (2.5 µM 1,1,3,3-tetraethoxy propane) added were 5 µL butylated hydroxytoluene (0.2%, w/v), 750 µL phosphoric acid (1%, v/v), 250 µL thiobarbituric acid (0.6%, w/v) and 445 µL water. Samples were mixed and incubated in a boiling water bath for 30 min. MDA was determined by HPLC by applying the following conditions: the injection volume 20 µL, flow-rate 1 mL min^{-1} (λ_{ex} = 527 nm, λ_{em} = 551 nm), fluorescence detector at 532 nm, and temperature in the column oven 32 °C. The mobile phase consisting of 50 mM KH_2PO_4 and methanol (60:40, v/v, pH 6.8) was degassed before use in an ultrasonic bath for 15 min. The retention time of MDA was about 2.5 min. The MDA concentration was calculated by software from the calibration curve.

2.11. Presentation of the data

Morphological parameters in kidneys, plasma concentrations of sex hormones, enzyme activities, oxidative stress parameters, and tissue concentrations of chemical elements were determined in 9–10 animals per experimental group. mRNA, immunoblotting and immunocytochemical data were determined in 4 animals per experimental group. The numeric data, shown as means ± SEM, were statistically evaluated by either *t*-test or ANOVA/Duncan at the 5% level of significance.

3. Results

3.1. Sex- and gonadectomy-related parameters in male and female rats

In order to prove the validity of our research data, we determined morphological data in the kidneys and plasma concentrations of sex hormones in sham-operated and gonadectomized rats. As shown in Table 2, a) the body mass (b.m.) in sham-operated male rats was ~66% higher than in sham-operated females, castrated males had ~12% lower b.m. than sham-operated males, whereas ovariectomized females had ~36% higher b.m. than sham-operated females, b) the kidney mass in sham-operated males was ~66% higher than in sham-operated females, castration decreased it by ~23%, whereas ovariectomy increased it by ~12%, and c) the renal index was the same in sham-operated males and females, but lower ~12% in castrated and ~17% in ovariectomized animals in comparison with the respective sham-operated animals. Regarding the concentration of sex hormones in blood plasma, a) testosterone was 6-fold higher in sham-operated males than in sham-operated females, gonadectomy decreased it by ~94% in males and by ~62% in females, b) estradiol in sham-operated females was 4-fold higher than in sham-operated males; it remained unaffected by castration, and decreased ~73% by ovariectomy, and c) progesterone in sham-operated animals was ~2.4-fold higher in females than in males; it remained unaffected by castration, and decreased by ~45% by ovariectomy. Therefore, all these data indicated clear sex differences in various parameters in sham-operated rats, and an efficient removal of gonads in male and female animals.

Table 2
Sex- and gonadectomy-related parameters in male and female rats.

	Males		Females	
Parameter	Sham-operated	Castrated	Sham-operated	Ovariectomized
Body and kidney parameters				
Body mass (g)	345 ± 10	303 ± 9*	208 ± 4*	282 ± 7**
Kidney mass (g)	2.40 ± 0.10	1.85 ± 0.06*	1.45 ± 0.04*	1.62 ± 0.05**
Renal index (g/kg BM)	6.98 ± 0.24	6.11 ± 0.15*	6.99 ± 0.27	5.77 ± 0.13**
Sex hormones in blood plasma				
Testosterone (nmol/L)	6.07 ± 0.62	0.34 ± 0.04*	0.98 ± 0.18*	0.37 ± 0.07**
Estradiol (pmol/L)	65.3 ± 13.7	68.3 ± 13.0	266 ± 58*	70.5 ± 9.05**
Progesterone (nmol/L)	37.8 ± 4.47	46.1 ± 4.35	89.7 ± 11.2*	49.3 ± 4.74**

Shown are means ± SEM determined from 10 rats in each experimental group. BM, body mass. Statistics (*t*-test): P < 0.05: **versus* sham-operated males; ***versus* sham-operated females.

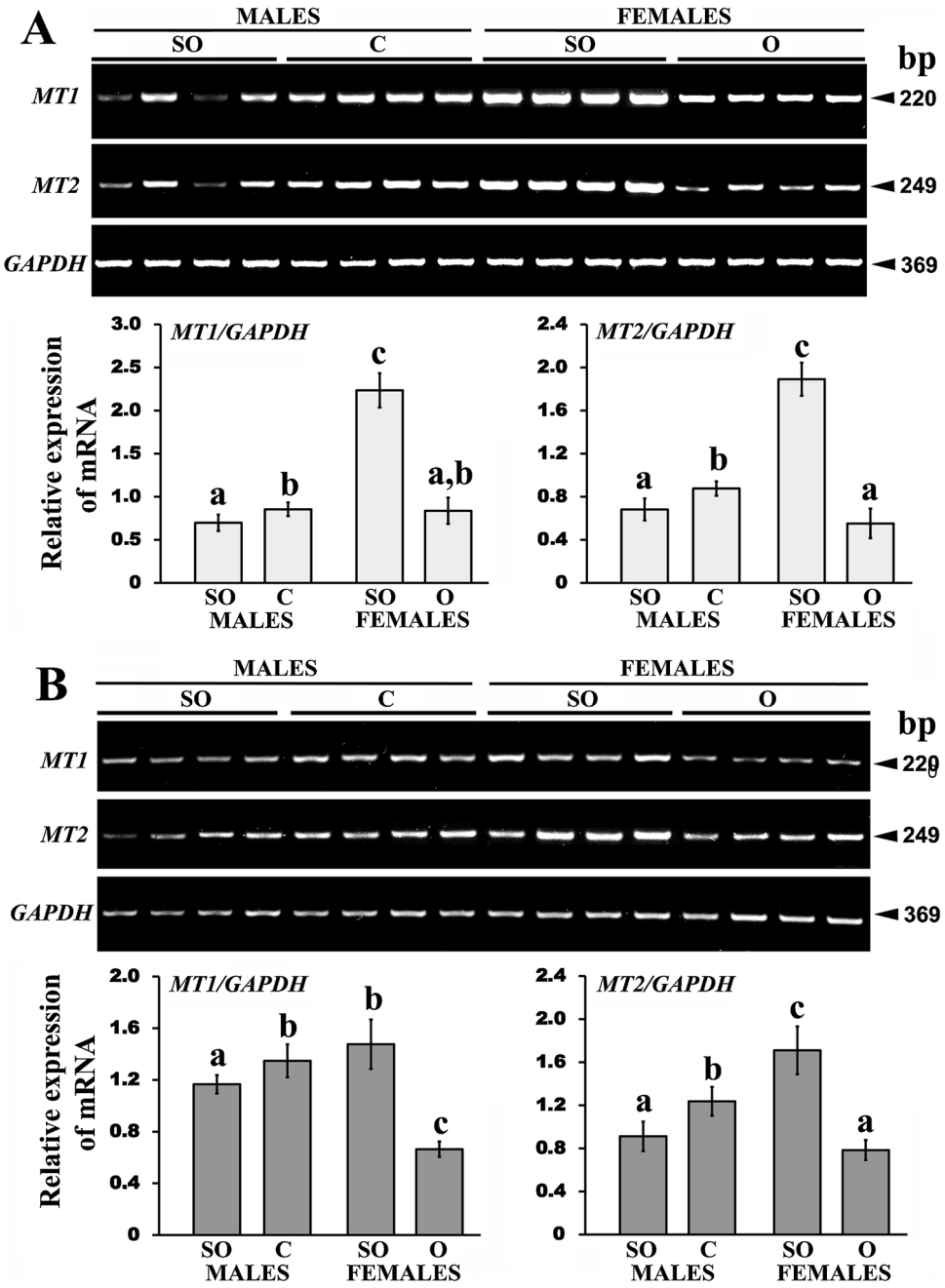

Fig. 1. Expression of *MT1*, *MT2*, and *GAPDH* mRNA in the liver (**A**) and kidney (**B**) tissues from sham-operated and gonadectomized male and female rats; end point RT-PCR data (each mRNA band represents mRNA expression in the tissue of a separate animal) and relative expression of the *MT* mRNA band *vs.* housekeeping *GAPDH* mRNA band (*MT/GAPDH*; each bar represents mean ± SEM of the data from 4 animals in the respective experimental group). SO, sham-operated; C, castrated; O, ovariectomized. bp, base pairs. Statistical differences (ANOVA/Duncan): a:b, a:c, and b:c, $P < 0.05$; a:a, and b:b, N.S.

3.2. Expression of MT1 and MT2 mRNA and MT1&2 proteins in the liver and kidney tissue of sham-operated and gonadectomized rats

Fig. 1A shows the RT-PCR products related to the hepatic expression of *MT1* and *MT2* mRNA, which were sex- and gonadectomy-dependent, and their relation to the sex- and gonadectomy-unaffected mRNA expression of the housekeeping gene *GAPDH*. These bands, and the pattern of their density relative to the stable density of *GAPDH* band (*MT1/GAPDH* and *MT2/GAPDH*), indicate that in the liver tissue, relative mRNA expression of both *MT* genes was a) ~3-fold higher in sham-operated females than in sham-operated males, b) moderately upregulated by castration (20–30%), and strongly downregulated by ovariectomy (65–80%). In the kidney tissue (Fig. 1B), the mRNA expression of both *MT* genes was also female–dominant, being ~25% and ~80% for *MT1* and *MT2*, respectively, higher in sham-operated females than in sham-operated males, castration weakly upregulated (10–30%), whereas ovariectomy strongly downregulated (60–65%) the mRNA expression of both genes.

The immunochemical data with MT-ab are shown in Fig. 2A for hepatic, and 2B for renal tissue samples. In Western blots of all tissue samples, the 6–7 kDa band of monomeric MT1&2 forms was very weak or not observed (not shown). Rather, the MT-ab strongly labeled the protein band of ~13 kDa (dimeric form of MTs) and one or more weaker upper bands (probably polymeric forms of MTs). The densitometric evaluation of the ~13 kDa protein band and the representative immunocytochemical images show that the expression pattern of MT1&

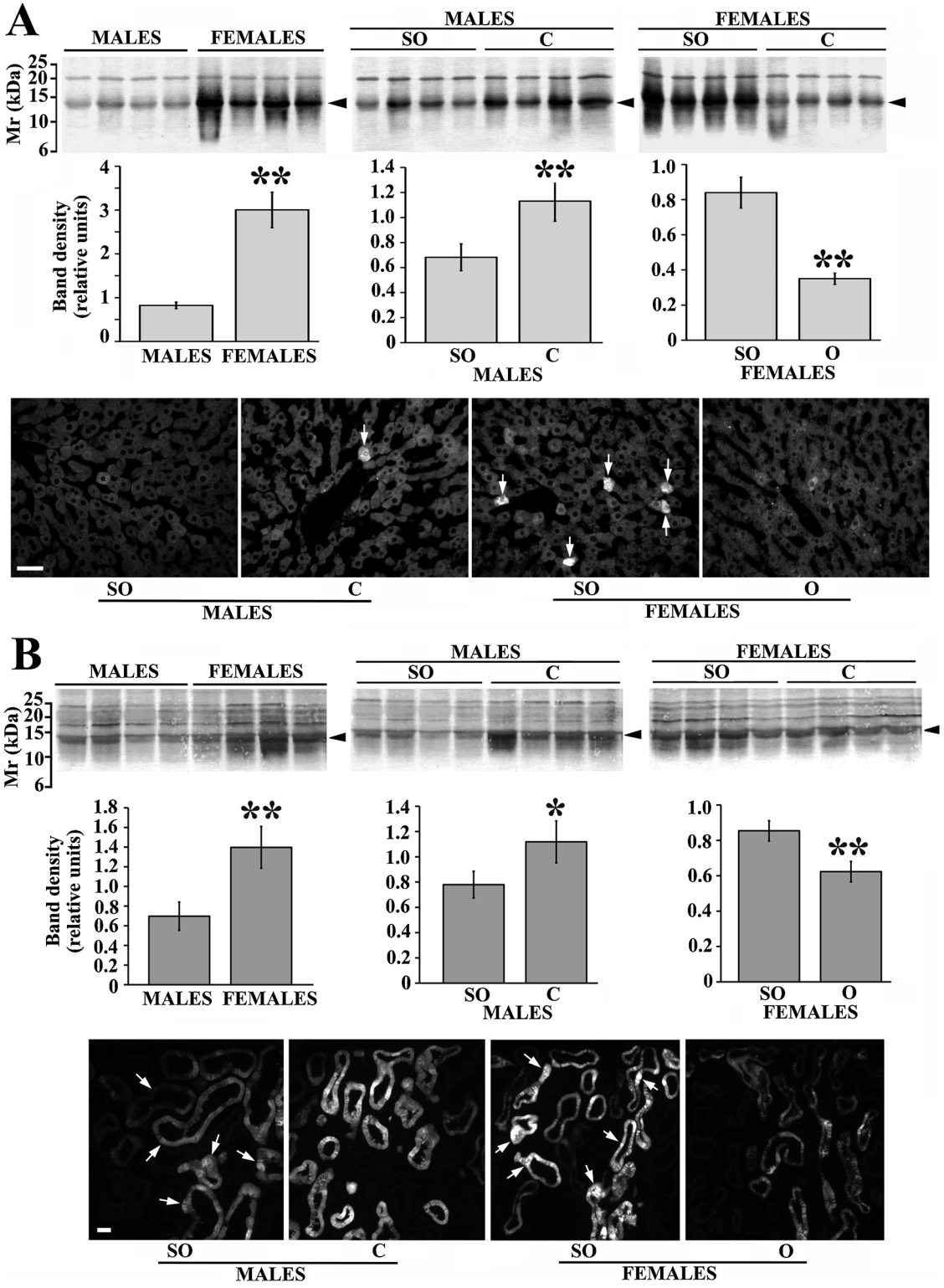

Fig. 2. Immunoblots with MT-ab and densitometric evaluation of the protein bands labeled in immunoblots of the liver (**A**) and kidney (**B**) tissue homogenates, and the MT-ab-related immunostaining in cryosections of the liver (**A**) and kidney (**B**) tissues from sham-operated male and female rats, and from sham-operated (SO), castrated (C), and ovariectomized (O) animals. In immunoblots, the strongest protein band of ~13 kDa was indicated by arrowheads and represents the MT1&2 dimer used for densitometric evaluation. Each band represents the MT1&2 protein abundance in the tissue homogenate from a separate animal. Mr, relative molecular mass. Densitometric evaluation of the protein bands is shown by bars; each bar represents the mean ± SEM of the band density from 4 animals in the respective experimental group. Statistics (*t*-test): *versus* respective males or sham-operated animals, *P < 0.05 or **P < 0.01. Immunocytochemical images of MT1 &2 in tissue cryosections represent similar findings in 4 animals per experimental group. Bars, 20 μm in all images of the respective tissue.

2 proteins in both liver and kidney tissue samples match those of mRNA. Thus, as shown by protein bands and the densitometric evaluation of the same bands (shown by bars), the expression of MT1&2 proteins in both organs was significantly higher in females than in males (both sham-operated), upregulated by castration (C) and downregulated by ovariectomy (O).

By immunocytochemistry in cryosections of the liver tissue (Fig. 2A), in sham-operated males the heterogeneous but overall low intensity of MT-ab-related staining was evenly distributed inside the hepatocyte cytoplasm, whereas in sham-operated females, the overall staining intensity in hepatocyte cytoplasm was stronger, many cells were brightly stained in their cytoplasm, and some nuclei were also stained (arrows). In castrates, diffuse staining in most hepatocytes was generally stronger than in sham-operated males, and the strongly stained cells could be occasionally observed (arrow). In ovariectomized females, the overall immunostaining in hepatocytes was heterogeneous but much lower than that in sham-operated females. By immunocytochemistry in cryosections of the kidney tissue (Fig. 2B), in sham-operated males the MT-ab stained the cell cytoplasm and nuclei of cortical proximal tubules (arrows) with very heterogeneous intensity. The staining was much stronger and more heterogeneous in sham-operated females (arrows), thereby confirming the female-dominant expression of MT1&2 proteins in renal tubules. In castrated rats, the staining intensity and heterogeneity was enhanced as compared with that in sham-operated males, whereas in ovariectomized females, the overall staining intensity in the proximal tubule cells was strongly diminished in comparison with that in sham-operated females. Therefore, the pattern of MT-ab-related immunochemical data is in fair match with the pattern of *MT1* and *MT2* mRNA expression in both liver and kidney tissues.

3.3. Trace elements in the liver and kidney tissue of sham-operated and gonadectomized rats

Concentrations of various trace elements, known to have binding affinity for MTs [3,12], in the liver and kidney tissues from sham-operated and gonadectomized rats are summarized in Table 3.

In the liver tissue of sham-operated animals, significant female-dominant sex differences were measured for concentrations of Fe, Cu, and Cd, whereas in the kidney tissue, similar differences (females > males) were observed for Fe, Zn, Co, Cd, and Hg. Limited male-dominant sex differences were observed only for Zn and Hg in the liver tissue. Following castration, in the liver tissue the concentrations of Fe and Cd significantly increased, and that of Zn decreased, whereas in the kidney tissue, concentrations of Co and Hg were increased. Following ovariectomy, however, a significant decrease was spotted for Fe and Cu in the liver tissue, and for Fe and Co in the kidney tissue. The concentrations of Co in the liver tissue and Cu in the kidney tissue, as well as of Pb in both tissues remained unchanged in all experimental groups. Therefore, only Fe in the liver tissue and Co in the kidney tissue exhibited a pattern of concentration identical to *MT1&2* mRNA and protein expression in these tissues, while concentrations of Cu in the liver and of Fe in the kidney tissues matched this pattern only in females.

To relate the concentrations of elements in the tissues with those ingested by food and water, we have analysed the respective elements in the standard pelleted food (4RF21 from Mucedola, Italy) and tap water. The values for Fe, Zn, Cu, Co, Cd, Hg, and Pb in pelleted food were (mg/kg) 520, 99, 15, 2.1, 0.091, 0.012, and 0.18, respectively, whereas in tap water, the values were (μg/L) 3.2, 16.3, 2.3, 0.13, 0.09, 0.02, and 0.36, respectively.

3.4. Parameters of oxidative stress in the liver and kidney tissue of sham-operated and gonadectomized rats

Since amelioration of oxidative stress *via* scavenging ROS and RNS has been suggested as a possible role for MTs [1,3,31,32], in our rats we measured several oxidative stress parameters in the liver and kidney tissue. This included the activities of two enzymes that contribute to detoxification of some oxidative radicals, *i.e.*, SOD (it converts superoxide radical to H_2O_2) and GPx (a family of enzymes that reduce the lipid-damaging hydroperoxides to water), and two related markers of the oxidative stress status, *i.e.*, MDA (a highly reactive byproduct of lipid peroxidation), and GSH (thiol-containing tripeptide which neutralizes various ROS molecules by reduction).

As shown in Table 4, in the liver tissue, in comparison with sham-operated males, sham-operated females exhibited a significantly higher activity of GPx (~55%) and concentration of MDA (~45%). In castrated animals, these parameters only showed a nonsignificant tendency to increase, whereas in ovariectomized females, the same parameters exhibited a tendency to decrease in comparison with the respective controls. The activity of hepatic SOD did not exhibit significant sex differences or changes following gonadectomy in both sexes, while the concentration of GSH in the female liver was non-significantly lower (~18%) compared to that in sham-operated males, and partially increased (~40%) following ovariectomy. In the kidney tissue, however, only the GPx activity was found non-significantly lower in sham-operated females (~20% *vs.* sham-operated males), but increased significantly (~27%) in ovariectomized animals. The values of other parameters in the kidney tissue exhibited neither sex differences nor effects of gonadectomy.

4. Discussion

As a continuation of our recent paper on sex differences in trace element concentrations in several rat organs [24], the aims of the present study were: a) to test sex differences in mRNA and protein expression of MT1&2 in the rat liver and kidney tissues and detect the sex hormones responsible for these differences, b) to investigate if the related sex differences and effects of sex hormones exist in hepatic and renal tissue concentrations of trace elements known to have an affinity for MTs, and c) to detect if the former phenomena are related to oxidative status in the tested tissues. Various morphological and biochemical parameters in sham-operated and gonadectomized male and female rats, as collected in Table 2 (body and kidney mass, renal index, and the levels of sex hormones in blood plasma), showed the expected differences among sexes and following gonadectomy, thus proving these animal groups as an appropriate experimental model to resolve the abovementioned research problems.

In accordance with previous studies in rodents [18,20–23], here we confirmed in rats the female-dominant sex differences in the expression of hepatic and renal MT1&2 at the level of both mRNA and protein. In our hands, in both tissues the expression of *MT1&2* mRNA, band density of MT1&2 protein, and MT-Ab-related immunostaining intensity followed the same pattern, being female-dominant, upregulated in castrated, and downregulated in ovariectomized animals. In immunoblots, the antibody labeled the major protein band of ~13 kDa, which reflects the presence of MT dimers partially existing in the tissues *in vivo* [33], but probably mainly formed during the few-week long storage of tissue homogenates in frozen state before being used in Western blotting [34]. By immunocytochemistry in tissue cryosections, the presence of MT1&2 proteins was shown in the cell cytoplasm and some nuclei. In both hepatocytes and renal proximal tubule cells, the staining was variable in intensity, ranging from negative to strong, thus indicating a very heterogeneous abundance of the protein in cells. Similar heterogeneity of the MT1&2-related immunostaining was described previously in both organs [18,19,21,35,36].

Because in adult mice the hepatic *MT1* mRNA expression was significantly downregulated by ovariectomy [22], whereas in rats, the hepatic and renal *MT* mRNA and protein levels were upregulated by treating animals with estradiol, but not with testosterone [23] or progesterone [37–39], it has been assumed that the MT expression in rodent organs is controlled only by estrogens. In the present study we showed that the *MT1&2* mRNA and protein expression in the liver and kidneys is regulated by both sex hormones; upregulation following

Table 3
Concentrations of the essential and toxic metals in the liver and kidney tissues of sham-operated and gonadectomized male and female rats.

Element / tissue	Males		Females	
	Sham-operated	Castrated	Sham-operated	Ovariectomized
Iron				
liver (mg/kg tissue)	157 ± 9	243 ± 13*	427 ± 37*	300 ± 15**
kidney (mg/kg tissue)	47 ± 5.1	50 ± 2.3	87 ± 6.4*	66 ± 4.2**
Zinc				
liver (mg/kg tissue)	34 ± 0.5	32 ± 0.4*	32 ± 0.3*	32 ± 0.8
kidney (mg/kg tissue)	23 ± 0.4	22 ± 0.3	25 ± 0.6*	23 ± 0.7
Copper				
liver (mg/kg tissue)	4.9 ± 0.07	5.2 ± 0.11	6.0 ± 0.11*	5.4 ± 0.18**
kidney (mg/kg tissue)	11.6 ± 1.04	13.2 ± 1.23	14.2 ± 1.18	18 ± 2.61
Cobalt				
liver (μg/kg tissue)	32 ± 2.5	32 ± 1.1	31 ± 2.1	29 ± 1.7
kidney (μg/kg tissue)	192 ± 11	254 ± 10*	361 ± 13*	313 ± 10**
Cadmium				
liver (μg/kg tissue)	7.7 ± 0.65	10.7 ± 0.90*	13.0 ± 0.92*	11.9 ± 0.94
kidney (μg/kg tissue)	13.0 ± 0.77	16.6 ± 1.03	20.3 ± 1.66*	21.6 ± 1.34
Mercury				
liver (μg/kg tissue)	1.9 ± 0.22	1.6 ± 0.09	1.1 ± 0.09*	1.1 ± 0.08
kidney (μg/kg tissue)	18.1 ± 0.98	25.3 ± 0.83*	24.5 ± 1.73*	25.1 ± 1.82
Lead				
liver (μg/kg tissue)	1.0 ± 0.22	0.9 ± 0.15	0.7 ± 0.07	0.8 ± 0.13
kidney (μg/kg tissue)	4.6 ± 0.35	4.1 ± 0.33	5.0 ± 0.61	4.0 ± 0.63

Shown are means ± SEM of the data measured in the tissues from 9 (kidneys from sham-operated males and ovariectomized females) or 10 (samples from all other groups) animals. Statistical significance ($P < 0.05$): **versus* sham-operated males, and ***versus* sham-operated females.

Table 4
Parameters of oxidative stress in the liver and kidney tissues from sham-operated and gonadectomized male and female rats.

Organ/Parameter	Males		Females	
	Sham-operated	Castrated	Sham-operated	Ovariectomized
Liver				
SOD, U/mg protein	44.7 ± 2.25	44.7 ± 2.2	53.1 ± 3.8	45.7 ± 2.8
GPx, nmol/min·mg protein	587 ± 46	722 ± 52	902 ± 46*	857 ± 57
MDA, nmol/g tissue	30.7 ± 3.92	35.8 ± 2.52	44.6 ± 9.02*	40.9 ± 3.92
GSH, nmol/g tissue	7.14 ± 1.46	7.75 ± 1.28	5.83 ± 0.92	8.17 ± 1.44
Kidney				
SOD, U/mg protein	36.8 ± 2.27	30.9 ± 2.41	33.1 ± 2.37	31.7 ± 2.90
GPx, nmol/min·mg protein	272 ± 23	303 ± 21	219 ± 15	279 ± 19**
MDA, nmol/g tissue	27.5 ± 10.6	26.6 ± 3.89	33.4 ± 14.01	30.4 ± 9.33
GSH, nmol/g tissue	3.41 ± 0.34	3.29 ± 0.64	2.47 ± 0.86	4.4 ± 0.94

Shown are the data (means ± SEM) determined in 10 animals in each experimental group. Statistical significance (*t*-test; $P < 0.05$): **versus* sham-operated males; ***versus* sham-operated females.

castration indicates androgens as inhibitory, whereas downregulation following ovariectomy indicates female sex hormones as stimulatory.

The tissue concentrations and sex-dependent pattern of the tested trace elements in sham-operated rats were largely similar to those reported previously in the same organs containing residual amounts of blood [24]. The concentrations of elements in the pelleted food was much higher than in the tap water, while in the liver and kidney tissues their concentrations were comparably low, thus indicating that the tissue accumulation of elements was not directly related to the ingested load of elements, but was selectively regulated by the sex hormones-dependent mechanisms. Distinct elements exhibited the female-dominant sex differences in concentrations in the liver (Fe, Cu, Cd) and kidney (Fe, Zn, Co, Cd, Hg) tissues of sham-operated rats. After castration, Fe and Cd in the liver, and Co and Hg in the kidney significantly increased, indicating androgens as being inhibitory. Hepatic Zn and Hg showed male-dominant sex differences in concentrations. A limited drop in Zn concentration following castration indicated androgens as stimulatory, while for the very low concentrations of Hg, gonadectomy offered no clear conclusion about the hormones involved. After ovariectomy, Fe and Cu in the liver, and Fe and Co in the kidney significantly decreased, thus indicating female sex hormones as being stimulatory. Although a small but significantly higher concentration of Zn was measured in the female kidneys, gonadectomy in both sexes did not change its levels. On the other side, the female-dominant renal concentration of Hg was driven by androgen inhibition, since castration significantly increased its level, while ovariectomy had no effect. Therefore, these data show that only the hepatic concentration of Fe, and renal concentration of Co followed the whole pattern of *MT1&2* mRNA and protein expression.

Although the purified MTs from mammalian liver and kidneys contained mostly Zn and Cd, and variable amounts of Cu, Fe, and a few other elements [12,40], there is a common opinion that MTs play a major role in the intracellular handling of essential Zn, and possibly Cu, while Cd and some other elements are more or less cytotoxic [1,3,10–12,41,42]. However, Zn and Cu are also cytotoxic in higher concentrations; treatment of rodents with higher doses of Zn, Cu, as well as with Cd or other toxic elements, increases the level of oxidative stress and upregulates expression of hepatic and renal MT1&2 proteins by enhancing their synthesis at the level of gene transcription. In turn, the MT1&2 proteins bind and sequester these elements, diminishing their cytotoxicity [1–3,10–12,14,43–45].

The data in Table 3 indicate that in the liver the pattern of tissue concentration of Zn (male-dominant, lower in castrated and unchanged

in ovariectomized animals) was largely opposite to that of MTs expression, and thus, it can not be the driving force for the observed pattern of hepatic MT1&2 expression. The tissue levels of Cu, Co, Hg, and Pb were either low or the differences among experimental groups were small, and were also unlikely contributors to the observed pattern of hepatic MT1&2 expression. Although Cd is one of the strongest inducers of hepatic MT1&2 expression in rodents *via* mechanisms that include metal response elements (MREs) and antioxidant response element (ARE) in the MT1&2 gene promoters (reviewed in [10,46]), in a recent study by Madejczyk et al. [47] in rats, treatment with various doses of $CdCl_2$ for 3–7 days resulted in a significant increase of free radicals, H_2O_2, and MDA when the concentration of Cd in the liver tissue reached the level of ~4 mg/kg. In our rats, the overall hepatic concentrations of Cd (5–15 μg/kg) were too low to be able to induce any cytotoxic effect and synthesis of MTs. Therefore, Zn, Cd, and most other elements were unlikely determinators of the sex- and gonadectomy-dependent expression of the hepatic MTs. Rather, the identical pattern of MTs expression and concentration of Fe suggests a possible link between these two parameters. A similar female-dominant pattern of Fe concentration in hepatic (and also renal) tissues in rats and mice has been previously described [48–51]. It is, however, unlikely that this pattern of tissue Fe reflects the metal associated with MTs, because it is questionable whether Fe binds to MTs in significant amounts in physiological conditions *in vivo*. Previous studies in male rats fed Fe-rich or Fe-deficient diets for several weeks resulted in controversial data on MT1&2 expression [52,53], indicating that Fe unlikely affects the expression of MTs in mammalian organs directly. However, the link between Fe and MTs in hepatocytes may be indirect, *via* Fe-induced generation of oxidative stress, which may be further dependent on the action of several Fe-regulating proteins, such as hepcidin, ferroportin, and ferritin, whose expression is also affected by sex hormones [51,54].

Fe as a Fenton metal with strong pro-oxidative activity in the form of Fe^{2+} in hepatocytes is capable of stimulating the production of ROS, MDA, and peroxidation of lipids, decreasing the concentration of GSH, changing the activity of anti-oxidative enzymes, and inducing MT production [3,55–60]. The proposed mechanism includes a) release of Fe^{2+} in acidic intralysosomal medium following the breakdown of endocytosed Fe-containing proteins, 2) Fe^{2+}-induced intralysosomal production of ROS, 3) ROS-induced increased permeability of lysosomal membrane, 4) release of Fe^{2+}, ROS, and hydrolytic enzymes into the cytoplasm, and 5) further production of ROS in the cytoplasm and mitochondria. If the oxidative stress is heavily cytotoxic, the cells will die in apoptosis or necrosis, while the low-level oxidative stress will mediate release of Zn from MTs and other cytoplasmic stores, and activate a series of reactions that will upregulate the synthesis of MTs (reviewed in [10,46]). As demonstrated in recent studies in hepatic tissue and cultured cells [55,59,60], the upregulated MTs bind Zn, forming a Zn-MT complex, which scavenges and neutralizes ROS in the cytoplasm, mitochondria, and nuclei. Being internalized by autophagy into lysosomes, this complex reduces reactive Fe^{2+}, ROS, and peroxidative reactions. Accordingly, the data in Table 4 indicate the presence of a low-intensity oxidative stress in hepatic tissue, which may be related to the sex- and gonadectomy-dependent concentrations of Fe, and may cause the observed expression of *MT1&2* mRNA and proteins. In sham-operated females, we measured a significantly higher GPx activity (54%) and MDA concentration (45%) than in sham-operated males, whereas both parameters showed a tendency of increasing in castrated males and decreasing in ovariectomized females. Since sham-operated females also exhibited a ~20% lower concentration of tissue GSH compared to sham-operated males, overall these data indicate the presence of a limited oxidative stress in the female liver, which is associated with the highest tissue concentrations of Fe (c.f. Table 3) and highest expression of *MT1&2* mRNA and protein (c.f. Figs. 1A and 2 A). In other groups of animals with the gonadectomy-dependent hepatic concentrations of Fe, the oxidative stress parameters were comparably much weaker. The liver concentration of Cu, another redox active Fenton metal with the potential to induce oxidative stress and stimulate MTs [12,42,61], was also significantly higher in sham-operated, and lower in ovariectomized females (c.f. data in Table 3), but its tissue concentration was 70-fold lower than that of Fe, and its contribution to the oxidative stress in the female liver may be comparably much smaller.

In the kidney tissue, the overall pattern of *MT1&2* mRNA and protein expression in control and gonadectomized male and female rats was similar to that in the liver, but possible relations to the tissue Fe concentrations are less clear. Sex differences were observed for tissue concentrations of Fe, Zn, Co, Cd, and Hg, and these differences were either largely abolished (Fe, Co) or remained unchanged (Zn, Cd, Hg) by ovariectomy, while the concentrations of Co and Hg significantly increased by castration. Most of these elements, when used in higher doses in experimental animals, are well known inducers of the hepatic and/or renal MTs [3,7,43,62–64]. Some of them also contribute to generation of oxidative stress (Fe, Cd, Hg), whereas Zn helps to diminish it [7,57,63,65]. However, data in Table 4 showed largely unchanged parameters of oxidative stress in the renal tissue from all animal groups, thus suggesting that either the sex-related expression of renal MT1&2 was driven by an oxidative stress–independent mechanism or the levels of oxidative stress parameters were too subtle to be detected by the applied methods. In the kidney we observed that the overall tissue Fe concentrations were about one fifth of that in the liver, also exhibiting female-dominant sex differences, and its possible contribution to catalyzing oxidative stress may be minimal. Furthermore, the renal concentrations of Cd, which was overall about 2-fold higher than in the liver, and also higher in females, may have been too low to induce any cytotoxicity; previous studies in rats treated with $CdCl_2$ detected 5–7 mg Cd/kg tissue as the lowest nephrotoxic concentrations [10,66].

Contrary to other elements in the renal tissue, the concentration of Co exhibited a pattern that completely followed the pattern of *MT1&2* mRNA and proteins in control and gonadectomized rats. Co is also a Fenton metal and undergoes redox-cycling reactions; Co^{2+} reacts with H_2O_2 and induces generation of ROS and lipid peroxidation [67–69]. However, prolonged administration of Co as metal salts in adult rats resulted in either a limited upregulation of MTs in the liver, but not in kidneys [70], or in unchanged expression in both organs [71]. It is, therefore, unlikely that the concentrations of Co, measured in the kidney tissues in our animal groups, can induce MTs *via* oxidative stress. Rather, the measured concentrations of Co and other trace elements in kidneys may reflect their abundance bound to MTs and/or other intracellular proteins whose regulation may be determined by different, sex hormone-driven mechanism. One of these hormones may be progesterone, previously shown in the human cell line to upregulate the $MT2_A$ gene expression through the glucocorticoid response element (GRE) in its promoter [72].

5. Conclusion

In rats, sex differences in the expression of *MT1&2* mRNA and proteins in the liver and kidney tissues are determined by both androgen inhibition and estrogen stimulation. In both organs several trace elements with the affinity for MTs exhibited either female- or male-dominant sex differences in their tissue concentrations. In the liver, only the Fe concentration strictly followed the pattern of MTs expression; the female-dominant expression of MTs matched the tissue concentration of Fe and elevated levels of oxidative stress parameters, indicating that the expression of hepatic MTs may be determined by Fe-generated oxidative stress. In kidneys, the tissue concentrations of Fe was low and also female-dominant, but only the concentration of Co strictly followed the pattern of MTs in various experimental groups. However, the oxidative stress parameters in the kidney tissue remained unaffected by experimental procedures, or were too subtle to be detected by the applied methods, indicating that the major regulator of renal MTs may be some other sex hormone-dependent mechanism.

Author contributions

M.L., and T.O. performed experiments, prepared figures and tables, analyzed data, and drafted the manuscript; V.M., D.K., I.V.M., D.Ra., I.N.J., M.G., G.G., S.K.O., and L.N. performed experiments; J.J., D.B., M.P., D.Ro. and I.R. analyzed data. I.S. designed the concept of research, analyzed data, wrote and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise, in this study

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