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Immunohistochemical and qReal time – PCR Expression of Matrix Metalloproteinase (MMP-2) in Testes of Male Wister Rats after Cadmium Chloride (CdCl2) exposure

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ABSTRACT

Matrix metalloproteinases (MMPs) are a family of structurally related proteins that degrade most if not all components of the extracellular matrix and basement membranes in a zinc dependent manner at a physiological PH. Cadmium is one of environmental pollutants arising from electroplating, fertilizers, pigment and plastic manufactures, most animals with scrotal testes are susceptible to cadmium–induced testicular toxicity. In this sense, This study conducted to investigate the effect of cadmium chloride (CdCl2) injection with different dose on the quantitative expression of MMP-2 and immunohistochemistry of male rats testes. 30 male Wister rats were conducted in this study, control group received saline IP (n = 10), CdCl2 treated group (n=10) were injected IP with of 5 mg/kg B.W., the third group (n= 10) which injected IP of CdCl2 in dose 10 mg / kg BW. The injections were twice per week continued for 3 weeks. The animals were euthanized and testicular tissues were collected and used for RNA extraction and real time PCR for quantitative expression of MMP-2 and immunohistochemical demonstration. We have found MMP-2 in control groups expressed in the leydig cells and sertoli cells and different stages of spermatogenesis. After injection of CdCl2 (5mg) of male rats showed decrease the quantity and expression of MMP-2 in the testicular tissue $P<0.05$ compare with control group, the group injected with (10mg) CdCl2 recorded more decrease of MMP-2 expression ($P<0.01$). In conclusion, our results highlight that cadmium directly cause reduction the quantity of MMP-2 in the testes without induced any inflammation in testicular tissue, the reduction of MMP-2 interfere with down regulation of reproductive activity that may lead to infertility due to environmental exposure to toxic metals of male.

Introduction

Matrix metalloproteinases (MMPs) are a family of structurally related proteins that degrade most if not all components of the extracellular matrix and basement membranes in a zinc dependent manner at a physiological pH. They have been implicated in extracellular matrix remodeling in relation to embryonic developmental processes, inflammation and tumor invasion and metastasis. (1,2).

These enzymes are produced as latent precursors, named zymogens or pre-MMPs, which require prior activation to be able to break the ECM components (3-4), Among them, MMP-2 is a pivotal MMP in remodeling of basement membrane, pericellular, and cell attachment proteins. It is secreted from cells as an inactive zymogen, proMMP-2. Thus, controlling its activity. (5)

The expression/activity of MMP-2 and MMP-9 is directly correlated with ECM remodeling, in which an unbalanced activity may contribute to several diseases in different tissues such as rheumatoid arthritis, osteoarthritis, cardiovascular dysfunction, and cancer (6,7). Therefore, any substance that has the ability to deregulate the homeostasis of the MMP activity may be potentially harmful. (8)

Remodeling of ECM may plate an important role in fetal gonadal development including cell integral, organization, differentiation and function. Regulation of ECM remodelling by MMP and TIMP is vital provide an environment that support initiation of growth, migration and differentiation by range of mechanisms (9). Cadmium is one of environmental pollutants arising from electroplating, fertilizers, pigment and plastic manufactures. Therefore it easily contaminates the soil, plants, air and water (10). Humans and animals can easily be exposed to cadmium toxicity by consuming plants, water and air. Cadmium is absorbed and accumulates in various tissues (11) even red blood cells (12), the heart (13) and the skeletal muscle of rats (14). Most animals with scrotal testes are

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susceptible to cadmium–induced testicular toxicity (15). Although, only about 1–2 % of acute cadmium dose is usually taken up by the testes, testicular toxicity is almost invariably evident. It has been reported that as low as 1-2 mg Cd/kg body weight can cause testicular damage without pathological changes to other organs (16). Exposure to cadmium has been reported to reduce male fertility in both humans and rodents (17), but the mechanism is still unknown. This study conducted to investigate the effect of cadmium chloride (CdCl2) injection with different dose on the quantitative expression of MMP-2 and immunohistochemistry of male rats testes.

2. Material and methods

Animals and treatments

The study was carried out in Lanzhou Institute of Husbandry and Pharmaceutical Sciences, CAAS, China, from the period of February – may 2014. 30 male Wister rats were conducted in this study purchased from animal house of Lanzhou military hospital. and maintained in cages (3 rats/cage) under controlled temperature (20 – 22°C) and lighting conditions. The animals were fed a standard pellet diet and water ad libitum. Experimental protocols met the "Guidelines of Animal Experimentation, After 7 days of acclimatization, the animals were randomized into three experimental groups, The control group received saline IP (n = 10), and the CdCl2 treated group were injected IP of CdCl2 (Sigma) in dose of 5 mg/kg B.W., the third group (n= 10) which injected IP of CdCl2 in dose 10 mg / kg BW. The injections were twice per week continued for 3 weeks then the animals were euthanized and testes were collected for RNA extraction and immunohistochemistry of MMP-2 detection.

Total RNA extraction and Real-time PCR

Total RNA was extracted from testicular tissue with a total RNA extraction kit (Omega Biotech, USA) according to the manufacturer's instructions. Testes (100 mg) was homogenized briefly in liquid N2, 1 mL of RNA-Solv reagent was added, incubation them adding 0.2 mL of chloroform, after centrifugation, 80% of the aqueous part was transferred into a clean tube, adding 1/3 of the volume absolute ethanol was added., 700 µL of mixture was transferred into a HiBind RNA column, adding 300 µL of RNA washing buffer I. This solution was centrifuged, followed by adding 500 µL of RNA washing buffer II diluted in ethanol, after centrifugation the column was transferred to a new tube, 50 µL DEPC water was added and then centrifugation occurred at full speed for 1 min. The amount of total RNA was determined using a *Nano Drop spectrophotometer* (Thermo Scientific, USA)at 260 nm. The RNA was reverse-transcribed into cDNA with random primers (PrimeScript RT Reagent Kit, TAKARA Biotechnology Co., China). The quantity of MMP-2 gene expression was measured by Real-Time RCR using a SYBR green assay (TAKARA Biotechnology Co., China) used BIO-RAD Multicolor Real-Time PCR machine (USA). Primer pairs for MMP-2 were designed using Primer Premier 5.0software based on the rat sequences of MMP-2 (forward: GTGCCCCATGTGTCTTCCCCTTCA. Reverse: GCCCCACTTCCGGTCATCATCGTA). The PCR was conducted in a

2.3. Immunohistochemistry

MMP-2 primary antibodies were purchased from Lifespan Bio-sciences (USA). Streptavidin-biotin-peroxidase compound (SABC), 3,3_-diaminobenzidine (DAB), biotinylated goatanti rabbit IgG and bovine serum albumin (BSA) werepurchased from (Boster Company, Wuhan, China). Testes were fixed in 10% formalin, dehydrated in ethanol, embedded in paraffin wax and sectioned at 5 _m thick. after dewaxing, rehydration, inactivation of endogenous enzymes with 3% H_2O_2 for 30 min, washing in D.W. then we added 5% BSA (confining liquid) and incubated at room temperature for 20 min. The sections were incubated in primary antibodies against MMP-2 for 17 h at 4◦C and washed in PBS. Biotinylated goat anti-rabbit IgG (secondary antibody) incubation for 20 min at 20–37◦C, after that washing and incubated in SABC 37◦C for 20 min. After a final wash, tissues were stained with DAB for 20 min, counter-stained with Harris hematoxylin, and mounting.

2.4. Statistical analysis

Real-time PCR was performed in triplicate. Quantitative values are expressed as the mean ± SD. An independent *t*-test was used to compare the differences between normal and experimental groups. $P < 0.05$ was considered significant.

3. Results

Animals that were injected to Cadmium Chloride IP presented alterations in the activity of MMP2 and MMP9 within their tissue extracts. Testes extracts animals groups injected with CdCl2 exhibited a significant reduction of quantity expression of MMP-2 measured by real time PCR, when compared to untreated animals shown in figure (1). MMP-2 expression of testicular extract recorded 1.07 ± 0.06 (mean ± SE) in the normal rats, while the PCR results of MMP-2 expression of injected animals with (5 mg) CdCl2 decreased markedly record 0.74 ± 0.03. this difference was significant by student *t*-test (P<0.05). The reduction of MMP-2 expression was relative with increase the dose of CdCl2 injection to (10 mg), the results was 0.55 ± 0.04, this decrease record a highly significant (P<0.01) compared with normal animals.

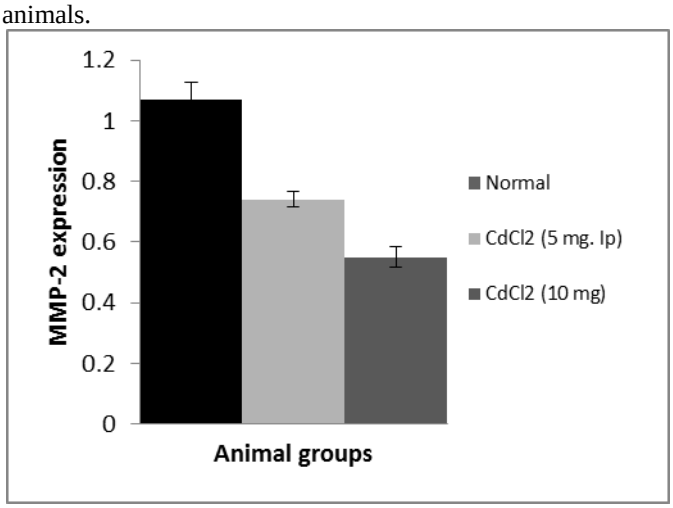

Figure (1). Expression of MMP-2 genes in the testes of normal rats and CdCl2 injected rats measured by real-time PCR. Gene expression decreased in testicular extracts of injected groups compared normal group.

These results approved by immunohistochemistry expression of MMP-2 in testes tissue samples of different groups that revealed a specific gene expression pattern of MMP-2 in the testicular structures (shown in figure 2). In normal rats , MMP-2 expressed in the cytoplasm of sertoli cells and interstitial cells of leydig as well as the different stages of spermatogenic cells. This expression were clearly decreased in the tissue of the injected rats especially those were injected with (10 mg) CdCl2.

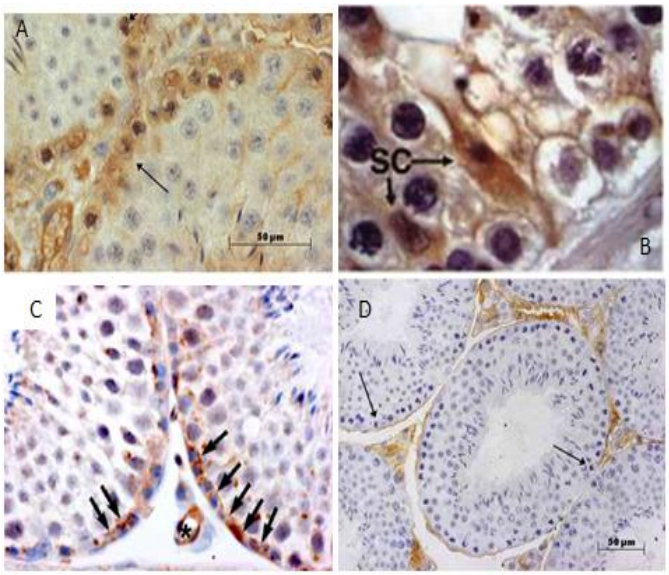

Figure (2)

Immunohistochemical sections showing the expression of MMP-2 genes in the testes of normal and CdCl2 groups. (A ,B) illustrates strong gene expression in the different testicular structures of normal rats include (sertoli cells, spermatogonia and spermatocytes as well as leydig cells). (C) the expression in the group rats injected with (5mg) CdCl2 revealed a slightly expression of MMP-2 the mentioned structure, In section (D) of the rats received (10mg) CdCl2 showed restricted expression of MMP-2 in the interstitial cells of leydig and absent of MMP-2 in the seminiferous tubules structures.

4. Discussion

Infertility affects approximately 15% of all couples trying to conceive and male factor infertility is implicated in almost half of these cases(18).It has long been suggested that at least half of the cases of human male infertility of unknown etiology may be attributable to various environmental and occupational exposure to toxic metals (19) .In recent years there has been an increasing interest in the contribution of occupational and environmental exposures to toxic metals in declining sperm concentration and human male fertility (20). Cadmium has been reported to damage the testes of many mammals(21). The present study demonstrated immunohistochemical the expression of MMP-2 in the testes, illustrated normally MMP-2 found in interstitium of seminiferous tubule as well as in different stages of spermatogenesis cells that explained the important role of these peptidase in the functions of testes which require remodeling of extracellular matrix through the maturation and development of sperms also needed to maintain the sperms viability and motility in semen after ejaculation because these enzymes effect the liquefaction time of semen.the previous study approved the expression and function of MMPs in the male reproductive system. It is generally believed that the gelatinases MMP-2 and -9 are involved in remodelling processes of structural proteins (22; 23). Gelatinolytic activity coinciding with differentiation stages has been found in maturing prostate and seminal vesicles in a rat model (24).

In rat Sertoli cell cultures, MMP-2 was detected and was suspected to contribute to remodelling of the basement membrane during development of the seminiferous tubules and in the release of differentiating germ cells from the basal lamina (25).

A previous authors discussed a possible function of MMPs in liquefaction of the ejaculate, confirm the presence of the two MMPs and TIMPs in seminal plasma. In our study the CdCl2 injections showed adverse effect on the expression of MMP-2 of male rats testes through the quantitative measurement by real time – PCR that showed a decrease of MMP-2, revealed this reduction was a relative to dose of CdCl2 injection up to (10mg), these findings approved immunohistochemical technique revealed a clear decrease of these peptidase cells of seminiferous tubles with slightly expression of interstitial cells.

Animals chronically exposed to cadmium in drinking water also exhibited decreased MMP2 and MMP9 in their prostatic and testes tissues, reinforcing the relationship between cadmium consumption and accumulation in these organs. These enzymes have been reported to be potentially inhibited by Zn^{2+} and Cu^{2+}, at low concentrations of 100 µM (26) and also to higher concentrations (2 mM) of Zn^{2+} , Co^{2+}, Br^{2+}, Mn^{2+}, Mg^{2+} [24]. It is believed that these divalent ions exert their inhibitory effects by conformational changes in the catalytic domain of these enzymes (27). Furthermore, cadmium, lead, and zinc directly inhibited 25 kDa and 43 kDa enamel matrix proteinases in vitro activities when dissolved at the incubation buffer of gelatinase, even in low concentrations such as 110 mM. therefore cadmium able to directly inhibit the activities of both MMP2 . Besides the potential effects on prostate carcinogenesis, the down regulated the activity of MMPs in the testicles, which could contribute to an imbalance in reproductive success, given the important role of these peptidases on the motility of spermatozoids (28).

In conclusion, our results highlight that cadmium directly cause reduction the quantity of MMP-2 in the testes without induced any inflammation in testicular tissue, this reduction of MMP-2 interfere with down regulation of reproductive activity that may lead to infertility due to environmental exposure to toxic metals of male.

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