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RESEARCH ARTICLE

CONSECUTIVE CHANGES OF HEAT SHOCK PROTEIN 70 DURING TCDD(2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN) TOXICITY IN RAT TESTIS

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ABSTRACT

Purpose: To investigate how heat shock protein (HSP) expression, especially inducible HSP 70, is related to the infertility of dioxin-induced testicular toxicity by animal study

Materials and Methods: Four week old 30 male Sprague-Dawley rats were divided into 10 groups. Group I, II, III received TCDD(2,3,7,8-tetrachlorodibenzo-p-dioxin) 40μg intraperitoneally and were sacrificed 12hr, 24hr, 48hr later respectively. Group IV, V, VI received vehicle as control group and were sacrificed at the same time. Group VII, VIII received TCDD at 5 weeks of age and were sacrificed 1 week, 4 week later respectively. Group IX, X received vehicle as control group and were sacrificed at the same time. All extracted testes were examined with light microscopy, western blot and immunofluorescence for HSP 70, HSP 90 and inducible HSP 70 and apoptosis stain with TUNEL method.

Results: On light microscopy, only group VIII showed significant difference between study and control group. Group VIII and group X showed body weight 139.7gm, 275gm, testis weight 2.17gm, 3.67gm, seminiferous tubule diameter 252μm, 281μm, Johnson score 8.8, 10.0 points, injured tubule ratio 0.131, 0.024 respectively ($p<0.01$). In western blot and immunofluorescence, inducible HSP 70 was expressed in the group I, II, III, VII, VIII and the intensity increased with time, but in the control group, no expression for inducible HSP 70 was seen. Total HSP 70 was expressed in all study and control groups. On apoptosis stain, intensity peak was noticed at 12hr and decreased after then. HSP 90 was expressed in all study and control groups as well

Conclusions: The expression of inducible HSP 70 in the TCDD injured rat testis increased with time and on immunofluorescence stain, the expression site was spermatocytes. Apoptosis appeared initially but decreased after 12hrs. HSP90 and total HSP 70 expression was not changed by TCDD.

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INTRODUCTION

During the past 50 years, the qualitative and quantitative deterioration was noted in the sperms of men in general population (Sengupta *et al*., 2017; Auger *et al*., 1995). This is related with environmental hormones called endocrine disruptors which could harm to male reproductive system (Sidorkiewicz *et al*., 2017). These environmental hormones are chemically stable and accumulated upstream food chain without degradation, finally to human. These substances could transmit to fetus, infant and baby through placenta, breastfeeding and could evoke teratogenesis and insult to reproductive systems (Feng *et al*, 2016). Solving these problem theoretically would be non-production of these harmful substances but it is impossible. So, preventive measures to less production of these substances and treatment with more knowledge of the mechanisms would be second best options.Of the many endocrine disruptors, dioxin is one of the most well-known, harmful andimportant substances. When testes are exposed to dioxin, the seminiferous tubules can undergo spermatocytic arrest and eventually oligospermia comes (Choi *et al*., 2008). Heat shock protein (HSP) is known as stress gene product and has an important role in the development and cell division as well as responing to various stress situations as like heat, toxinsand oxidative stress (Barnes *et al*., 2002). HSP would have a role in responding to dioxin toxicity to testes. Of the HSP family, HSP 70 is known to be related with key process during various stressful situations or normal development. Gene disruption of HSP 70 resulted in germ cell apoptosis (Dix *et al*., 1996) and HSP 70 expression is closely related with male infertility (Feng *et al*., 2001; Bae *et al*., 2014). HSP 70 was reported to increase to dioxin induced liver toxicity in rats (Kim *et al*., 2012) and it means HSP is related with early recovering efforts of the cells to external stresses. We investigated how HSP expression, especially

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inducible HSP 70, is related to the dioxin-induced testicular toxicity using animal study.

MATERIALS AND METHODS

Animals

30four-week-old male Sprague Dawley rats were used.The rats were divided into 10 groups (I, II, III, IV, V, VI, VII, VIII, IX and X) with each group of 3 rats. Group I, II and III received TCDD(2,3,7,8-tetrachlorodibenzo-p-dioxin) 40μg intraperitoneally and were sacrificed 12hr, 24hr and 48hr later respectively. Group IV, V, VI received the same volume of vehicle (acetone:corn oil=1:19) as control groups and were sacrificed at the same time with the groups I, II and III. After sacrificing the animals, testes were extracted and frozen for western blot and immunofluorescence. To see the later change, group VII and VIII received TCDD at 5 weeks of age and were sacrificed 1 week and 4 week later respectively. Group IX, X received vehicle (acetone:corn oil=1:19) as control groups and were sacrificed at the same time. The rats of group VII, VIII, IX and X were injected TCDD at 5 weeks rather than 4 weeks because four-week-old rats could not survive 1 or 4 weeks after TCDD injection. In the rats of group VII, VIII, IX and X group, testes were also extracted for western blot and immunofluorescence and additionally for light microscopic examination after sacrificing the animals.

Histologic examination

Testes tissues were fixed in Bouin's solution and paraffin block was made using routine procedures and sliced with thickness of 3μm and Hematoxylin and Eosin staining was performed for light microscopic examination. The testes of group VIII and X were examined for histologic changes using the diameter of seminiferous tubules and Johnson's score and the number of injured seminiferous tubules were assessed. But, in the testes of group VII and IX, the immature testes tissue of less than 8 weeks made it meaningless to measure the diameter of seminiferous tubules and Johnson's score, so, simple histologic findings were compared.

Western blot analysis

testes tissues were washed in phosphate buffer solution two times and lysed in lysis buffer (50mM Tris-HCl, pH7.4, 1% NP-40, 150mM NaCl, 1mM EDTA, 1mM PMSF, protenase inhibitor cocktail solution) and after centrifugation, the upper layer contents were separated. 10% SDS-PAGE(SDS-polyacrylamide gel electrophoresis) gel was made and cell lysates were added for gel electrophoresis under denaturing conditions. And then protein samples were fractionated on PVDF(Polyvinylidene Fluoride, Amersham, Pharmacia Biotech Inc., Piscataway, NJ, USA) with 150mA for 90minutes. PVDF was stained in Ponceaus solution and protein band was confirmed and washed with distilled water to remove Ponceaus solution and blocking buffer (TBS+ 5% Skim milk) was treated for 1hour and then primary antibodies HSP 70 (NeoMarkers, Fremont, CA, USA), inducible HSP 70 (Stressgen, Canada) and HSP 90 (Stressgen, Canada) was treated to blocking buffer with dilation of 1:1000 and secondary antibodies of Anti-rabbit IgG, HRP linked donkey Ab (Amersham, Pharmacia Biotech Inc., Piscataway, NJ, USA), anti-mouse IgG HRP linked rabbit Ab were treated respectively with 1:3000 dilution. PVDF membrane was mixed with same amount of ECL kit A, B solution (Amersham, Pharmacia Biotech Inc., Piscataway, NJ, USA) and exposed to Hyperfilm ECL (Amersham, Pharmacia Biotech Inc., Piscataway, NJ, USA) and then photosensitized for 1 minute. Quantitative analysis was perfomed using Alpha Ease Version 5.1 (Alpha Innotech Comp. San Leandro, CA, USA). All measurements were repeated 3 times and the mean of the results were used.

Immunohistochemistry

Frozen testes tissues were sliced with a thickness of 6-μm and exposed to air for 30 minutes and fixed in cold acetone for 2 minutes and then dried to air for 1 or 2 minutes. The specimens were washed 3 times and primary antibody of anti-inducible HSP antibody (Stress gene, Canada) was treated for 1 hour and then washed 3 times in PBS and then FITC attached secondary antibody (anti-mouse IgG FITC conjugated Ab, Zymed, Ca, USA) was treated for 1 hour. After washing again, fixation was perfomed using glycerine jelly. Light microscpy with cooled CCD was used and images were captured for analysis.

TUNEL stain

To examine whether cell death occurs in seminferous tubules after TCDD injection, TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining was perfomed.

Statistics

Statistical analysis was made using SPSS 12.0 for Windows and Mann-Whitney test was used for analysis and p-value of 0.05 was used.

RESULTS

Body weight and histologic changes

Group I, II, III and VII (12, 24, 72 hours and 1 week after TCDD injection, respectively) was not different from control group IV, V, VI and VII respectively in terms of body weight, testis weight and histologic findings. On the light microscopy, only group VIII (4 weeks after TCDD injection) made significant difference from control group X. The body weights of group VIII and group Xwere 139.7 and 275gm, testis weights were 2.17 and 3.67gm, seminiferous tubule diameters were 252 and 281μm, Johnson scoreswere 8.8 and 10.0, the injured tubule ratios were 0.131 and 0.024 respectively ($p<0.01$) (Table 1).

Table 1. Pathologic changes of TCDD-treated rats after 4 weeks later

	TCDD group	control group
Body weight (gm)	139.7*	275.0
Testis weight (gm)	2.17*	3.67
Diameter of seminiferous tubule (μm)	252**	281
Johnson score	8.84*	10
Injured tubule/total tubule	0.131*	0.024

* $p<0.01$, ** $p<0.05$
TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

The changes of spermatids, formation of giant cells and arrested maturation were frequently observed in seminiferous tubules of group VIII compared with normal histologic findins of group X (Fig 1).

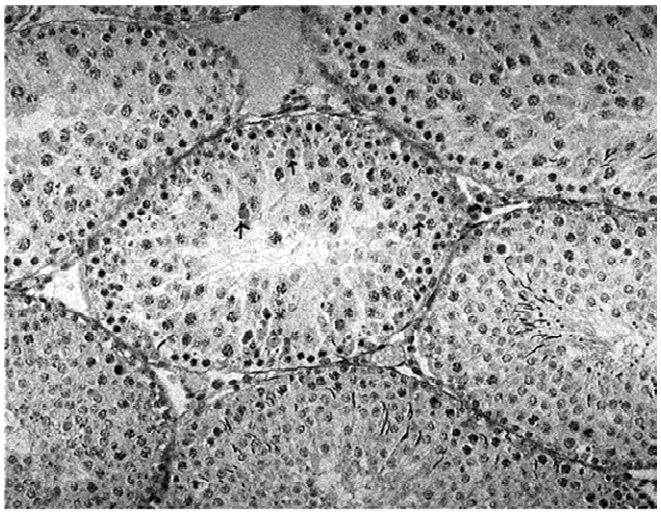
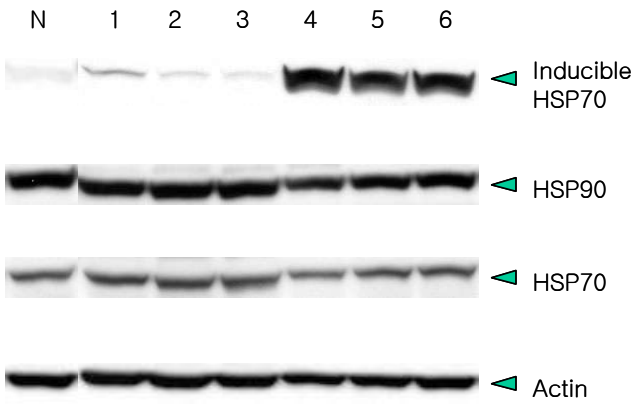
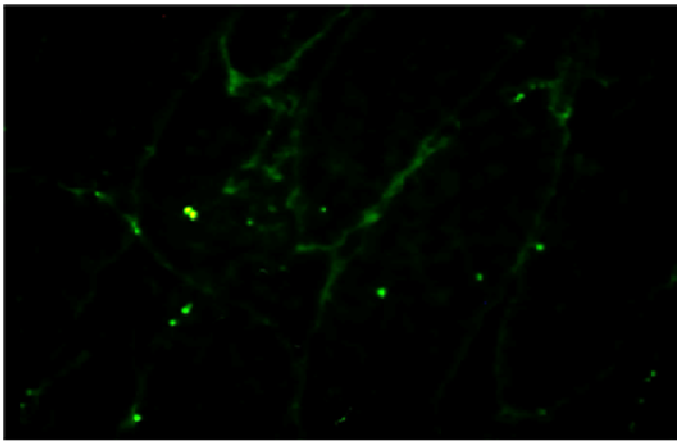


Fig.1. Apoptotic spermatocytes (arrows) and decreased sperm count is seen definitely in centrally located seminiferous tubule at 4 weeks after TCDD injection. H-E stain (x400)

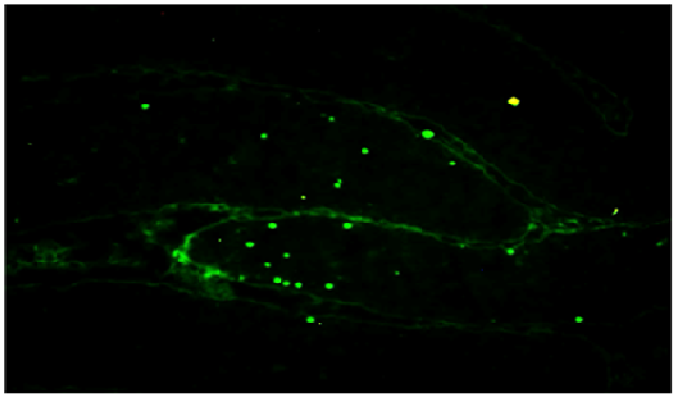


N : Normal (control)
1,2,3 : 1 weeks later sacrificed after TCDD 40ug injection, Group VII in text.
4,5,6 : 4 weeks later sacrificed after TCDD 40ug injection, Group VIII in text.

Fig.2. Western blot for HSP 70, 90 and inducible HSP 70. Inducible HSP 70 was expressed weakly at 1 week later (1, 2, 3), and strongly at 4 weeks later (4, 5, 6) after TCDD injection. HSP 90 and total HSP 70 expression was not different from control



A



B

Fig. 3. Inducible HSP 70 is detected as bright spot in immunofluorescence 12 hours after TCDD injection (A). After 48 hours after TCDD injection, the expression of inducible HSP 70 was intensified. Viewing the location of expression within the tubule, the site seems to be spermatocytes (B)

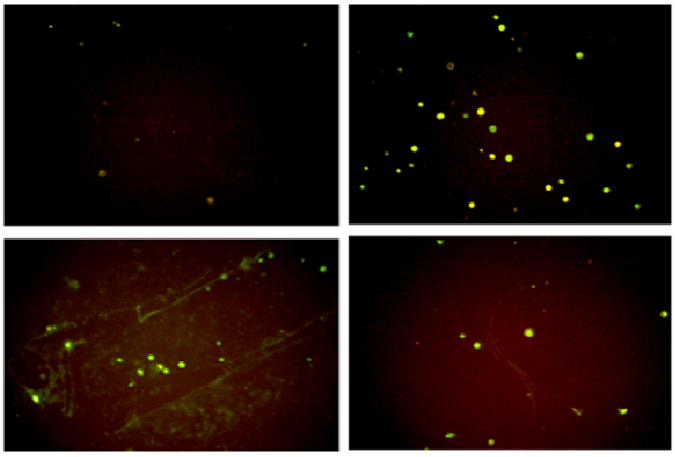


Fig. 4. Effect of dioxin on apoptosis of the seminiferous tubular cells in the testis. The TUNEL reaction is markedly increased at 12 hr, after then is gradually diminished at 24 hrs and 48hrs after TCDD injection. Even at 48hr, TUNEL reaction is more frequent than normal. Top left control, top right 12hrs, bottom left 24hrs, bottom right 48hrs after TCDD injection

Western blot for inducible HSP 70, HSP 70 and HSP 90

Inducible HSP 70 was expressed in group I, II and III (12, 24 and 48 hours later, respectively) but the expression was not found in control groups (IV, V, VI). In the group VII and VIII (1 and 4 weeks later, respectively) the intensity of expression of inducible HSP 70 increased with time but the expression was not found in control groups (IX and X). Total HSP 70 (Neomarker) including both constitutive and inducible forms was expressed in all testes tissues including exprimental and control groups. HSP 90 was expressed in all testes tissues including exprimental and control groups (Fig. 2).

Immunohistochemistry

Inducible HSP 70 was expressed in group I, II and III (12, 24 and 48 hours later, respectively) but the expression was not found in control groups (IV, V, VI). The expression site in seminiferous tubule was dominantly spermatocyte location. In the group VII and VIII (1 and 4 weeks later, respectively) the intensity of expression of inducible HSP 70 increased with

time but the expression was not found in control groups (IX and X) (Fig. 3).

TUNEL-FITC stain for apoptosis

The intensity of TUNEL staining was strongest after 12 hours and then the intensity decreased with time but defintely increased than that of control groups (Fig. 4).

DISCUSSION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) is the most well known toxic chemical in dioxin family and the toxicities of other dioxins or dioxin-like acting substances are calculated as relative toxicity compared to standard TCDD toxicity(Finley *et al*., 2003). TCDD evokes various cancers and world health organization (WHO) reported TCDD as class I carcinogen in 1997 (McGregor *et al*., 1998). Besides carcinogenesis, TCDD is known to evoke teratogenesis via placenta or breastfeeding and its long term sequale was debated after the use in Vietnam war asa name of Agent orange (Hays *et al*., 2003). TCDD also makes a harmful events in reproductive systems and is regarded as an endocrine disruptor. In the TCDD exposed testes, no production of spermatid or sperm is observed due to spermatocytic arrest in pachytene stage and the fertility of already made sperms also decrease. TCDD is known to bind to Aryl hydrocarbon receptor (AhR) in cytosol and moves to nucleus. In the nucleus, AhR makes a complex with aryl hydrocarbon receptor nuclear translocator (ARNT) to bind to dioxin response elements (DREs) to activate dioxin-responsive genes (Chan *et al*., 1994). On the other hand, HSP 70 is a member of chaperone proteins to assist folding, transport and assembly of other proteins (Rodons, 2016). As the name indicates, heat shock can increase HSP 70 expression but other stresses as like oxidants or toxicants have the same effect on HSP 70. And in responding to these various stresses, HSP 70 has a role to protect and recover the cells from the harmful damage made by external stresses. In this experiment, inducible HSP 70 expression increased after 12, 24, 48 hours and 1, 4 weeks after TCDD injection proved by western blotting and immunfluorescence methods. The intensity of expression increased as time passed by up to 4 weeks. Apoptosis confirmed by TUNEL staining method was the most prominent at 12 hours later and then the intensity of TUNEL stain decreased but inducible HSP 70 expression increased steadily upto 4 weeks. It is unclear what is the meaning of this discrepancy between apoptosis marker and inducible HSP 70 expression. At 4 weeks later, the testes seem to be severely damaged by TCDD effect proved by light microscopic examination and body, testes weight and HSP 70 was strongly expressed at this time point. Therefore, we can suppose that HSP 70 is still making efforts to protect and recover cells from harmful effects of TCDD but when recalling the heat shock paradox, more complex HSP 70 effect on specific time point could be assumed (Kobba *et al*., 2011). More extensive studies could suggest the correlation or meaning of each parameters in TCDD toxicity to testes. The expression site of inducible HSP 70 was spermatocytes and this means inducible HSP 70 is related with specific steps of spermatogenic process in the seminiferous tubule. The specific arrest after TCDD toxicity is known to occur in spermatocytes and HSP 70 is expressed at the same site maybe to protect further damage of TCDD but possibility of complex effect on specific time point could not be neglected when considering the discrepancy of apoptosis results.

Conclusion

At 4weeks later after TCDD, body and testis weight decreased and seminiferous tubules were significantly injued. The expression of inducible HSP 70 in the TCDD administered rat testes increased with time and on immunofluorescence stain, the expression site in seminiferous tubule was spermatocytes. Apoptosis appeared initially but decreased after 12hrs. HSP90 and total HSP 70 expression was not changed by TCDD.

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Conflict of interest

No conflict of interest.

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