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Detection of Sperm DNAAlterations and Heat Shock Protein -70 Levels in Albino Rats Exposed to Methoxychlor

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Abstract: Methoxycholor ($C_{16}H_{15}Cl_3O_2$) is an organochorine insecticide used for the control of parasites in livestock as well as a variety of pests on ornamentals, fruits and vegetables. The current investigation threw the light on the possible effect of MXC on the DNA of rat's spermatozoa using RAPD-PCR technique and the level of HSP-70 of seminal plasma. To achieve this task, 20 adult male rats were used and classified into 4 equal groups. 1st group of rats was gavaged with sesame oil (vehicle) and the remaining 3 groups received methoxychlor. The second group received 5mg/kg/d for 10 days, the 3rd group treated with 50mg/kg/d and the 4th group gavaged with 150 mg/kg/d for 10days. To capture spermatozoa first exposed to methoxychlor during spermiogenesis, animals were sacrificed by decapitation on 21 days after the initiation of treatment. Methoxychlor induced DNA alterations in dose dependent pattern as elucidated by using RAPD-PCR and denrogarmatic analysis. On the similar ground, methoxychlor provoked significant increases in HSP-70 of seminal plasma in dose parallel behavior. It was concluded that methoxychlor induced oxidative stress in epididymal sperm of rats via elevation of HSP 70 of seminal plasma and alterations in DNA fingerprinting.

Key words: RAPD-PCR · Oxidative stress · DNA damage

INTRODUCTION

Methoxychlor (MXC) is a synthetic organochlorine which is used as an insecticide. The use of MXC has been banned in the United States and the European Union. MXC is used to protect crops, ornamentals, livestock and pets against fleas, mosquitoes, cockroaches and other insects. It has been used to some degree as a replacement for DDT, as it is metabolized faster and does not lead to bioaccumulation [1]. Human exposure to MXC occurs via air, soil and water, primarily in people who work with the substance or who are exposed to air, soil, or water that has been contaminated. It is unknown how quickly and efficiently the substance is absorbed by humans who have been exposed to contaminated air or via skin contact. In high doses the agent can lead to neurotoxicity as observed in animal experiments. Some of the agent's metabolites have estrogenic effect as shown in adult and developing animals before and after birth [2].

One studied metabolite of MXC is 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) which is considered to have reproductive toxicity in the animal model by reducing testosterone biosynthesis. Such effects adversely affect both male and female reproductive systems [3]. MXC has a proliferative effect on the ovarian epithelial cells, which is mediated through stimulation of cell cycle regulators and inhibition of apoptosis [4]. MXC has been shown to induce oxidative stress in the mouse ovarian follicle. The mechanism by which the response to MXC-induced oxidative stress relates to our previous demonstration of MXC-induced ovarian epithelial cells proliferation and decreased apoptosis at present is unclear [5]. MXC produce oxidative DNA damage in the ovarian epithelial cells and reduces fertility in female rodents, decreases follicle numbers and increases atresia through oxidative stress pathways [6]. MXC-induced oxidative stress may decrease the levels of cell cycle regulators, which in turn, results in inhibition of the growth of follicles [7].

The 70 kilodalton heat shock proteins (Hsp70s) are a family of ubiquitously expressed heat shock proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are an important part of the cell's machinery for protein folding and help to protect cells from stress [8].

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Oxidative stress-related parameter, HSP70, was estimated in the testis of adult male rats that exposed to a single dose of MXC (50 mg/kg body weight) orally. MXC elicited increase in the levels of inducible HSP70 which could be a part of protective mechanism mounted to reduce cellular oxidative damage [9].

The present investigation was carried out to cast the light on the possible effect of MXC on the DNA alterations of rat's spermatozoa using RAPD-PCR and the level of HSP-70 in the seminal plasma.

MATERIALS AND METHODS

Animal Treatments: Twenty albino male rats (250-300 g) were purchased from the Farm of the Egyptian Organization for Vaccine and Biological Preparations at Helwan, Cairo. Rats were provided with food and water *ad libitum* and were divided into 4 equal groups , 1st group of rats was gavaged with sesame oil (vehicle) and the remaining 3 groups received MXC (Sigma Chemical Co., St. Louis, MO).) . The second group received 5mg/kg/d, the 3rd group treated with 50mg/kg/d and the 4th group gavaged with 150 mg/kg/d for 10days. To capture spermatozoa first exposed to MXC during spermiogenesis, animals were sacrificed by decapitation on 21 days after the initiation of treatment [10].

Sperm Collection: To isolate spermatozoa from the cauda epididymides, the epididymides were first removed, trimmed free of fat and washed in 2 ml of prewarmed (37°C) 10 mM Tris-HCl buffer containing 50 mM NaCl and 50 mM EDTA, pH 8.2-8.4. This medium maintains the genetic integrity of frozen spermatozoa [11]. The corpus-cauda epididymal junction was then clamped with a hemostat and an incision was made in the distal cauda epididymidis. Spermatozoa released from the site of incision were collected in 2 ml of media at 37°C, incubated for 5 minutes to allow the spermatozoa to disperse, centrifuged at 5000 rpm for 20 min. to separate sperms from seminal plasma and then pellet of sperms were diluted 1:10 in fresh media. Aliquots of 100 µL were stored at -80°C until RAPD-PCR analysis was performed.

DNA Extraction and RAPD Reaction: Caudal epididymal spermatozoal DNA was isolated by proteinase K digestion (Boehringer Mannheim). Followed by phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation as previously described by Cummings and Thorgaard [12]. The quantity and quality of the DNA was assessed by ultra-violet .The RAPD protocol as previously described by Ferrero *et al*., [13]. Briefly, an initial heating at 96°C for 5 min. followed by 45 cycle of denaturation at 94°C for 1 min, annealing at 36°C for 90 seconds and extension at 72°C for 5 min in the thermal cycler (Perkin_Elmer model 2400).The primer used was AA-41 (5′-GATTCACTGC-3′; Biopolymers Department, CNBCR, I.S. Carlos III, Madrid, Spain). This Primer was selected because with rat's sperm cells it generates different amplification products within a wide range of molecular weights.

Amplification products (22µl) were resolved electrophoretically (150 V for 3 h) on 2.1% agrose gels in 1X TBE and stained with ethidium bromide. The image was recorded by the Grab_it program (UVP. Uplad, CA).

Optimization of Primer Concentration: In order to avoid the presence of artifacts, 20 amplifications were carried out on different days, without DNA template and using 3 different primer concentrations (2.5, 4 and 5 pM) following the above described amplification conditions. The optimization trails revealed that 5 PM was the suitable primer concentration in the experiment.

Amplification of PCR Product: Amplification reaction was preformed in a total volume containing 26.05µl (8.33µl distilled water ,and 3µl of primer (30 pM /ml), 3µl genomic DNA (10 ng/µl), 2.5µl of PCR buffer(10 X), 5 µl stock solution of $MgCl_2$ (25 mM), 4µl DNTP mix(25mM), 0.22µl Tag polymerase enzyme (5units/ µl).

Analysis of the Band Pattern of Control and Exposed Cells: The genomic DNA extract in the different flasks was obtained and amplified at least twice on different days. The control and exposed cells for each experiment were individually amplified, but developed together in the same gels. Using the molecular weight marker TriDye™, 1oo bp ladder (BioLabs Inc., USA) the agrose gels were analyzed by densitometry (Gel works ID: UVP).

Qualitative analysis was performed by comparing the percentage of each peak of the control and exposed cells. After eliminating the background, quantitative differences were studied using molecular weight and percentage of the amplified band.

Cluster Analysis Methods for the Electrophoretic Profile of the RAPD Products: The pattern of the polymorphism conferred by the primer used was analyzed using the cluster analysis system (AAB- Clustering analysis software, USA). Genetic relatedness and divergence between the control and treated samples from various

groups were calculated on the bases of similarity coefficient values and average linkage which displayed the homogenisity and heterogenesity as determined according to the simple band match (SBM) and amount of damage in percentage in dendrogram construction.

Estimation of Heat Shock Protein 70 (HSP70) in Seminal Plasma: To achieve this task, Rat Total HSP70 ELISA kit (Surveyor™ IC, Catalog Number SUV1663) was used [14]

Statistical Analysis: Data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL).

ANOVA with Tukey's post hoc test was used for multiple comparisons between treatment groups. Data are presented as means ± standard error of the mean. p value ≤ 0.05 was considered significant.

RESULTS

The obtained results reveal that Methoxychlor exert DNA changes in different regime of applications . Briefly, Fig. 1 and Table 1 represent DNA finger printing pattern obtained by RAPD-PCR using the selective primer of rat's spermatozoa. The result display that the 1st group shows 7 DNA bands ranging from 1019 to 127 bp. The 2nd group shows 6 DNA bands ranging from 782 to 197 bp while the 3rd group displays 5 DNA bands ranging from 782 to 144 bp. Meanwhile, 4th group shows 7 DNA bands ranging from 782 to 167 bp.

The dendrogramatic analysis of RAPD profile DNAs of rat's sperms the data obtained from that analysis

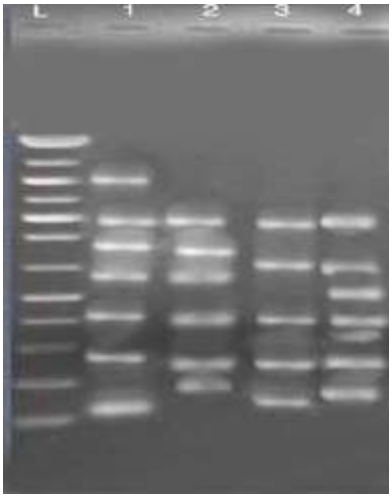

Fig. 1: Representative RAPD profiles of *Rat's sperms* .
Lane L: Marker
Lane 1: 1st group - Lane 2: 2nd group
Lane 3: 3rd group - Lane 4: 4th group

display similarility between 1st group and 2nd group with 78.57%, between the 1st and 4th group were 74.07% and 3rd and 1st group were 72.72% (Fig. 2).

The current investigation revealed that methoxychlor provoked significant (p<???) increases in HSP-70 of seminal plasma in dose dependent pattern (Fig. 3). Briefly, dose levels of 5 mg /kg b.wt./day for 10 days recorded 43.77±7.27 ng /ml; 50 mg /kg b.wt./day induced 76.08±5.16 ng/ml and the dose of 150 mg/ kg b.wt./day produce 108.64±10.82 ng/ml in comparison with control group that record 22.56±3.01 ng/ml.

Table 1: DNA fingerprinting pattern obtained by RAPD with primer due to methoxychlor treatment

	Ladder		1st group		2nd group		3rd group		4th group	
Lanes:										
Bands	bp	%	bp	%	bp	%	bp	%	bp	%
1	1517	16.49	1019	14.87	782	13.29	782	22.52	782	19.63
2	1200	6.28	782	10.54	670	24.83	608	24.04	602	11.00
3	1000	12.14	670	18.96	569	18.12	405	15.09	512	14.49
4	900	6.89	569	15.48	405	15.73	248	17.62	405	10.81
5	800	13.47	405	10.50	250	13.75	144	20.72	340	5.93
6	700	7.81	266	8.55	197	14.02			250	14.95
7	600	5.71	127	21.09					167	22.96
8	500	9.10								
9	400	5.69								
10	300	3.09								
11	200	6.03								
12	100	7.28								
Sum		100		100		100		100		100
In Lane		100		100		100		100		100

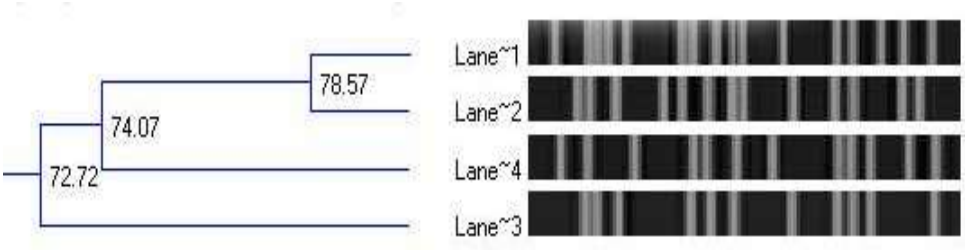

Fig. 2: Dendrogram derived from analysis of the RAPD profiles of genomic DNAs of Rat's sperms

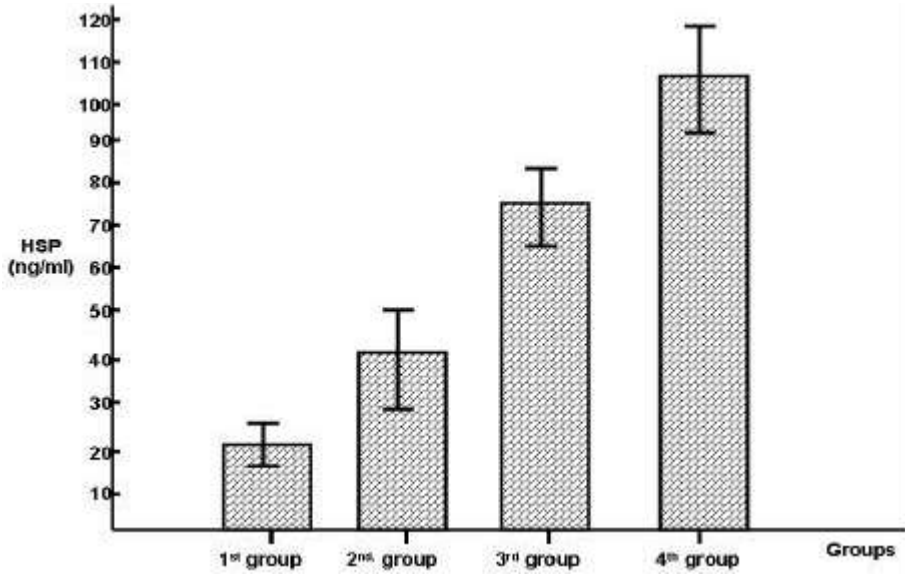

Fig. 3: Effect of methoxychlor on the HSP -70 level of the seminal plasma

DISCUSSION

An imbalance between free radical pro-oxidants and anti-oxidants has important implications for both physiological and pathological processes in the reproductive tract [15].

Methoxychlor is widely used as a pesticide in many countries and has been shown to induce reproductive abnormalities in male rats, causing reduced fertility. The mechanism of action of methoxychlor on the male reproductive system is not clear. In the present study, it was investigated whether administration of methoxychlor induces DNA alterations in epididymal sperm of adult rats [16].

Results of the current investigation displayed that RAPD technique is a useful tool for detecting methoxychlor inducing DNA damage in rat sperms. The recorded data are in same pattern of that detected by Atienzar *et al*., [17]. They evaluated the effects of a number of DNA alterations on the RAPD profiles. Genomic DNA from different species was digested with restriction enzymes, ultrasonicated, treated with benzo [a] pyrene (B[a]P) diol epoxide (BPDE) and the resulting RAPD profiles were evaluated. In comparison to the enzymatic DNA digestions, sonication caused greater changes in the RAPD patterns and induced a dose-related disappearance of the high molecular weight amplicons. A DNA sample substantially modified with BPDE, caused very similar changes but amplicons of low molecular weight were also, affected. Appearance of new bands and increase in band intensity were, also, evident in the RAPD profiles generated by the BPDE-modified DNA. Random mutations occurring in mismatch repair-deficient strains did not cause any changes in the banding patterns, whereas a single base change in 10-mer primers produced substantial differences. Also, Atienzar and Jha [18]. used the random amplified polymorphic DNA (RAPD) assay to determine DNA alterations, repair and transgenerational effects in benzo(a)pyrene [B(a)P]-exposed Daphnia magna. Qualitative and quantitative changes were observed in RAPD profiles generated not only from the B(a)P exposed Daphnia but ,also , from previously treated

organisms during the recovery experiments. On the same line, Castano and Becerril [19]. used RAPD to assess DNA damage of the RTG-2 fish cell line after short- and long-term exposure to benzo (a) pyrene. After comparing DNA fingerprints from control and exposed cells, both qualitative and quantitative analysis show an increase in the instability in the DNA fingerprint of exposed cells over a time- and concentration-dependent manner. The recoded data were in accordance with the findings of Symonds *et al*., [6]. They found that methoxychlor (MXC) induce oxidative stress damage of DNA in breast cells and mouse ovarian follicles. On the similar ground, methoxychlor demonstrate impaired male fertility with abnormal DNA methylation patterns in spermatozoa [20]. Also, Methoxychlor produce adverse effects on reproduction that correlate with altered DNA methylation patterns in the germ line of rats [21].

Recently, Fukuyama *et al*., [22] recorded that methoxychlor induced thymocyte apoptosis, resulting in thymic atrophy and predisposing the highly sensitive fetal immune system to loss of tolerance to self-antigens and subsequent increased risk for autoimmune disease and allergies. The results show that methoxychlor evoke DNA fragmentation following exposure. Furthermore, caspase-7 and -8 activities also differed between the J45.01 cells and thymocytes when treated with methoxychlor.

The obtained data were in harmony with that recorded, lately, by Vaithinathan *et al*., [9]. They found that Methoxychlor induced gonadal toxicity. Treatment with methoxychlor would alter the levels of stress proteins, heat shock proteins (HSP) and clusterin (CLU) and oxidative stress-related parameters in the testis of adult male rats. Animals were exposed to a single dose of Methoxychlor (50 mg/kg body weight) orally. The levels of HSP70 were elevated significantly . On the female level, Papaconstantinou *et al*., [23] show increases in mouse uterine heat shock protein levels are a sensitive and specific response to uterotrophic agents post methoxychlor exposure.

In conclusion, methoxychlor induced oxidative stress in epididymal sperm of rats via elevation of hsp-70 of seminal plasma and alterations in DNA fingerprinting.

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