Original Article

Spermatozoa HSP90β expression correlates with ROS generation and altered motility in response to methyl parathion treatment *in vitro*

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ABSTRACT

Objectives: Widely used organophosphorus pesticides, methyl parathion (MePa), alter the reproductive functions in various animals and humans by induction of oxidative stress on augmented release of reactive oxygen species (ROS). MePa affects semen quality by inducing DNA damage through spermatogenic stages. Several heat shock proteins (HSPs) are expressed in response to environmental stressors particularly the redox-active ones for regulation of protein turnover. Since oxidative stress and sperm motility are implicated in MePa toxicity, studying the expression of HSP90β will unravel the mechanism behind its noxiousness.

Material and Methods: Spermatozoa isolated from healthy donors were subjected to various concentrations of MePa (50, 250, 500, and 750 μM) for studying its effect on sperm motility, ROS generation, sperm chromatin integrity, and expression of stress responsive molecular chaperone HSP90β.

Results: *In vitro* exposure of MePa at concentrations ≥500 μM results in a decline in sperm motility and an increased generation of ROS, DNA damage, and HSP90β expression.

Conclusion: ROS-mediated modulation of HSP90β expression may affect the structural integrity of client proteins and oxidative injury to membrane lipid, along with DNA integrity resulting in declined sperm motility in response to MePa.

Keywords: Methyl parathion, Oxidative stress, HSP90β, Spermatozoa, Reactive oxygen species, Motility

INTRODUCTION

Agrochemicals like organophosphorus pesticides (OPs) are used worldwide as pesticides and are hazardous to human health. However, they are mainly known for their neurotoxic effects on non-target species through inhibition of acetylcholinesterase (AChE) activity together with the potential to generate reactive oxygen species (ROS) inducing oxidative stress during their metabolism.[1] Based on this, majority of studies are focused on neurotoxicity of OPs in both non-target and model organisms. The previous studies on methyl parathion (MePa) revealed that it has adverse effects on semen quality in humans and animals.[2,3] Reproductive potential principally in terms of semen quality and hormonal level are reported to be adversely impacted in response to OPs.[4,5] Furthermore, epidemiological studies reveal multifarious reproductive disorders such as infertility and adverse pregnancy outcomes including perinatal mortality and congenital defect

as a consequence of OP exposure.[6] The OP pesticides alter reproductive functions primarily through reduction in the activity of brain AChE and, in turn, influence the gonads. Several studies on human exposure to OP pesticide have reported reduction in sperm count and motility, hormonal imbalances such as low testosterone and high luteinizing hormone levels, and increased sex chromosome aneuploidy in sperm.[7] Decline in sperm chromatin integrity is observed in agricultural workers in response to OP exposure particularly MePa, methamidophos, and DZN.[5]

MePa, a toxic organophosphate insecticide has great demand for its broad-spectrum activity. It plays a significant role in impairing sperm function. Changes in the histoarchitecture of testis of adult white throated munias (*Lonchura malabarica*) are observed on exposure to MePa (5–20 g/100 g body weight/day), where the higher doses reduced the testicular weight after 10 days. On the other hand, a marked decline in seminiferous tubules was evidenced after single administration of lower dose, thus implying impairment of the cholinergic functions of the testes by MePa.[8] A recent study on HepG2 cells exposed to malathion toxicity reported DNA damage and oxidative stress by increase in the level of malondialdehyde (MDA) level, a by-product of lipid peroxidation.[9] Although, Salazar-Arredondo *et al.* (2008) have reported MePa to decline the motility of human spermatozoa at a concentration of 750 μM, its mechanism of induction is largely unknown.[10] Since oxidative stress and sperm motility are implicated in MePa toxicity, it will not be out of context to hypothesize the heat shock proteins (HSPs) involvement in this process. In fact, the previous proteomic studies by our group have reported the down regulation of different HSPs in human spermatozoa in various male infertility conditions.[11,12] Sagare-Patil *et al.* (2017), have also reported a decline in HSP90 in oligoasthenozoospermic men and found a positive correlation with the number of motile spermatozoa. The authors also reported differential expression of HSPs. HSP90α was typically localized in residual nuclear envelope of capacitated human spermatozoa, whereas HSP90β isoform was predominantly flagellar. They also reported that HSP90 inhibition by geldanamycin or 17-AAG was able to suppress progesterone-mediated forward progressive motility, hyperactivation, and acrosome reaction.[13] Under this scenario, the present study is designed to find out the effect of MePa on ROS generation, DNA damage vis-à-vis HSP90β expression, and its correlation with sperm motility *in vitro*.

MATERIAL AND METHODS

Ethics statement and study population

The proven fertile donors were recruited for the study after the approval by the Institutional Ethics Committee of Kar Clinic and Hospital Pvt. Ltd., Bhubaneswar, Odisha, India. All donors within reproductive age group 20–35 years having no known medical conditions were included in this study signed an informed written consent.

Semen analysis

The semen samples collected after 3–5 days of sexual abstinence were analyzed following liquefaction for 20–30 min at 37°C according to the World Health Organization (WHO) 2020 guidelines.[14] Motile spermatozoa fraction was obtained by layering the sample on a double-layer density gradient media (90% and 45% from AllGrad 100%, LifeGlobal) and centrifuging at 2500 rpm for 20 min at 37°C. The pellet containing motile spermatozoa was resuspended in sperm preparation medium (SpermWash, VITROMedia) and subjected to further experimentation.

Spermatozoa treatment with MePa

About 10^6 motile spermatozoa with approximately 65% motility was incubated with sperm media (1 ml final volume) having different concentrations of MePa (i.e., 50, 250, 500, and 750 μM). Stock solution of MePa was prepared in DMSO. Incubation tube contains spermatozoa and sperm media along with DMSO devoid of MePa served as control. All experiments were replicated 5 times. Sperm motility was reassessed after the completion of incubation period. Since the motility declined significantly at 500 μM MePa, the study of effect of time (0, 15, 30, 45, and 60 min) was carried out by exposing the spermatozoa to 500 μM MePa.

ROS quantification by 2',7'-dichlorodihydrofluorescein diacetate

The intracellular production of ROS was assessed by oxidation of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich). The non-fluorescent probe, H_2DCF-DA on deacetylation by intracellular esterases in presence of ROS, produces highly fluorescent 2,7-dichlorofluorescin. Briefly, spermatozoa (1×10^6/ml) treated with 10 μM H_2DCF-DA for 30 min in dark was washed thrice in phosphate buffer saline. Then, the fluorescent signal was detected at 488 nm excitation and 525 nm emission by spectrofluorimetry.

Sperm DNA fragmentation assay

Based on the results of MePa exposure on sperm motility and ROS generation, sperm DNA integrity was assessed after exposure of spermatozoa to 500 μM MePa by Acridine orange (AO; Sigma-Aldrich).[15] AO, a metachromatic fluorescence probe, on binding to double-stranded DNA emit green fluorescence (515–530 nm) while that with single stranded or denatured DNA emits red fluorescence (>630 nm). DNA fragmentation index (DFI) was calculated

for quantification of DNA denaturation by the ratio of red to red + green fluorescence and is denoted by proportion of sperm with DFI (DFI%). In brief, smears of the spermatozoa from both control and experimental tubes were air dried, then fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) for a minimum of 2 h at 4°C and stained with freshly prepared AO (0.19% w/v in McIlvain phosphate-citrate buffer, pH=4) for 10 min and visualized immediately under fluorescence microscope (Leica, USA) with a 460-nm filter.

Immunocytochemistry (ICC) of expression profile of HSP90β

For ICC, 500 µM MePa (based on results of motility and ROS generation) treated and control spermatozoa were fixed with 4% paraformaldehyde, permeabilized with Triton X-100 (0.1% v/v in PBS) for 5 min and blocked with 1% bovine serum albumin (BSA) (w/v) in phosphate-buffered saline tween (PBST) (0.1% Tween-20) for 2 h. Then, the cells were exposed to primary antibody (HSP90β anti-human goat immunoglobulin [Ig] G, 1:200 dilution; Abcam) in 1% BSA in PBST overnight at 4°C. After washing with PBST, the cells were incubated in FITC conjugated secondary antibody (anti-goat IgG, 1:5000 dilution; Abcam) diluted in blocking solution for 1 h at room temperature in dark. After washing the cells 3 times in PBST, imaging was done on Leica fluorescence microscope (Leica, USA) and the expression of HSP90β was analyzed by measuring the fluorescence intensity using IMAGE J Software.

Statistical analysis

All statistical analysis was done using version 17.8 of MedCalc software (Ostend, Belgium). The data were represented as mean ± Standard deviation. As the data do not follow normal distribution, non-parametric tests were executed for analyses of data on sperm motility and ROS generation (Kruskal–Wallis test), and DFI and time kinetics of sperm motility (Wilcoxon – Mann–Whitney U-test). The expression of HSP90β (fluorescence image intensity) from control and experimental spermatozoa was compared using two-tailed Student's *t*-test. Statistical significance was considered if $P < 0.05$.

RESULTS

Sperm motility, ROS generation, sperm chromatin integrity, and expression of stress responsive molecular chaperone HSP90β were studied after exposure of motile human spermatozoa to different concentrations of MePa.

MePa exposure impaired sperm motility

Lower concentrations of MePa (i.e., 50 and 250 µM) for 60 min at 37°C did not show any effect on motility. However,

a significant decrease in motility was observed at 500 and 750 µM MePa [Figure 1]. Since significant decrease in sperm motility was observed at 500 µM, time kinetics of effect of MePa on spermatozoa were studied at this concentration of MePa. When spermatozoa were exposed to 500 µM MePa for different time, their motility significantly decreased at 60 min [Figure 2].

Effect of MePa on ROS generation by spermatozoa

MePa induces increased generation of ROS in spermatozoa in a dose-dependent manner up to 750 µM [Figure 3] when incubated at 37°C for 60 min. A significant increase in the level of ROS was observed in higher treatment group (i.e., 500 and 750 µM) as compared to control, though no statistical significance was observed between the groups.
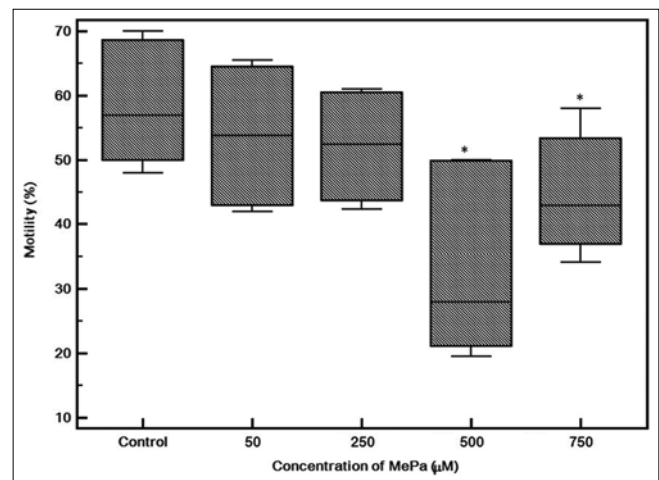
Figure 1: Effect of different concentrations of methyl parathion (i.e., 50, 250, 500, and 750 µM) at 37°C for 60 min on motility of human spermatozoa. *$P < 0.05$ with respect to control.
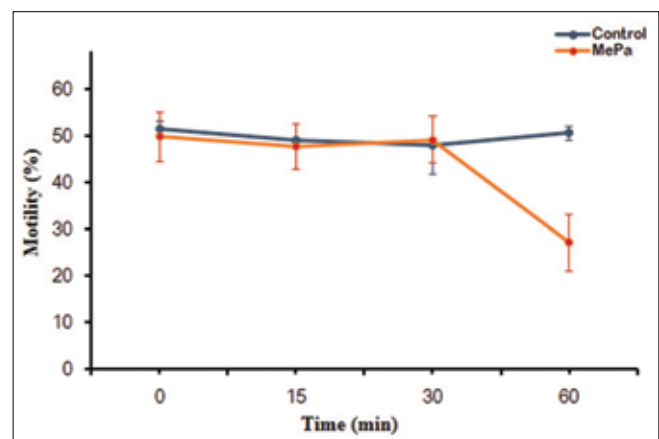

Figure 2: Effect of exposure time (0, 15, 30, 45 and 60 min) of 500 µM methyl parathion at 37°C for 60 min on motility (%) of human spermatozoa. *$P < 0.05$ with respect to control.

Alteration in the sperm chromatin integrity by MePa

As significant reduction in motility and augmented generation of ROS was observed in 500 µM treated group, so the effect of MePa on the sperm chromatin was evaluated by DFI at this concentration. It has been observed that MePa-treated spermatozoa have three-fold increase in DFI as compared to control [Figure 4].

Expression of HSP90β in MePa-treated spermatozoa

HSP90β is found to be primarily expressed in the head and neck region of the spermatozoa and its expression increased significantly when exposed to higher concentration of MePa, that is, 500 µM as evidenced by the difference in intensity of fluorescence measured by fluorescence microscope (Leica, Microscope) [Figure 5].
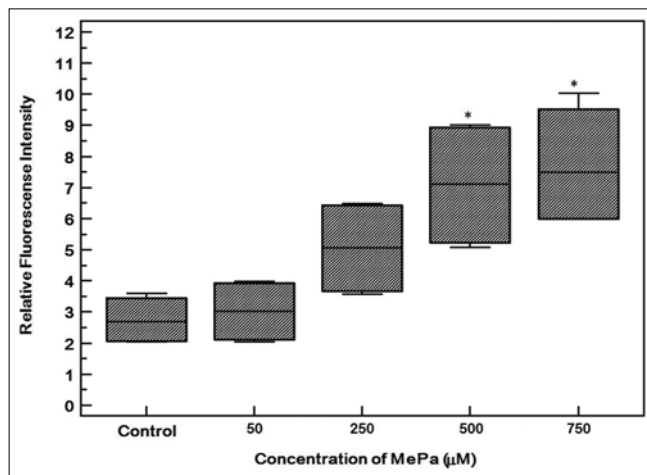

Figure 3: Effect of different concentrations of methyl parathion (i.e., 50, 250, 500, and 750 µM) at 37°C for 60 min on reactive oxygen species generation (H2DCF-DA fluorescence). *$P < 0.05$ with respect to control.
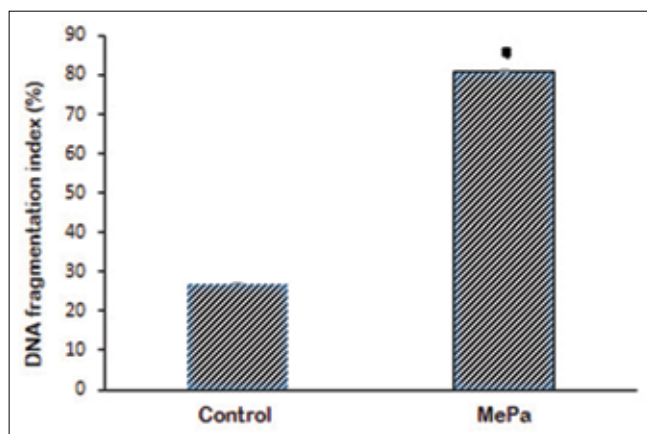
Figure 4: Comparison of DNA fragmentation index between control and 500 µM methyl parathion treated group (Acridine Orange Test). *$P < 0.05$ with respect to control.

DISCUSSION

OPs, including MePa, were found to be associated with reproductive failures with trans-generational effects. Their genotoxic effects on sperm are opined to be through oxidative damage.[13,16] Pina-Guzman *et al.* studied on the exposure of MePa on male mice affecting spermatozoa functions decreasing its fertilization ability.[17] Verma *et al.* (2009), reported that chronic oral toxicity of MePa causes lipid peroxidation in different parts of rat brain (i.e., fore-, mid-, and hind-brain) as evidenced by formation of MDA and 4-hydroxynonanal.[18] In another study, very low doses of MePa (i.e., 0.5–1.5 mg/kg) on rats reported decreased sperm count, along with the formation of abnormal sperms.[4] Similarly, *in vitro* exposure to MePa is reported to affect DNA integrity in human ejaculated spermatozoa without substantially affecting the motility until 750 µM concentration.[10] Our results corroborated the findings of Salazar-Arredondo *et al.*, with regard to sperm DNA integrity and induction of oxidative stress as revealed by augmented ROS generation. Furthermore, there is a significant decline in sperm motility at 500 and 750 µM MePa exposures with respect to control; although, no marked change was observed between the doses. The generation of ROS is a function of the source of production, for example, number of mitochondria present or number of moles of sperm NADH oxidase. Therefore, maximum rate of generation might have been achieved at 500 µM which did not increase further by augmenting the exposure level to 750 µM. It has been reported that in response to augmented ROS production, transcription and ATP production in mitochondria of boar spermatozoa are severely affected with a damage inflicted to electron transport chain proteins.[19] Therefore, the observed decline in motility might be due to an energy deprived state along with a change in membrane fluidity due to ROS-mediated oxidative damage to the membrane lipids. Interestingly, we observed a sharp increase in HSP90β expression in response to 500 µM MePa exposure. HSPs are constitutively produced molecular chaperones which act in response to several environmental stressors.[20] HSP90β is principally localized in the head (equatorial region). In a recent report, Profumo *et al.*[21] have shown that induction of oxidative stress by H_2O_2 upregulated HSP90 on the surface of primary human endothelial cells. Therefore, it is natural to expect an upregulated expression of the chaperone in MePa-induced oxidative stress condition. Furthermore, oxidative damage to HSP90 is shown to cleave the protein into Hsp90cl resulting in degradation of its client proteins.[22] On cleavage, Hsp90cl gains a new function that leads to the accumulation of actin aggregates.[23] Actin polymerization-depolymerization is suggested to regulate sperm motility including acrosome reaction and capacitation.[24] Therefore, this may also contribute to the decline in sperm motility.
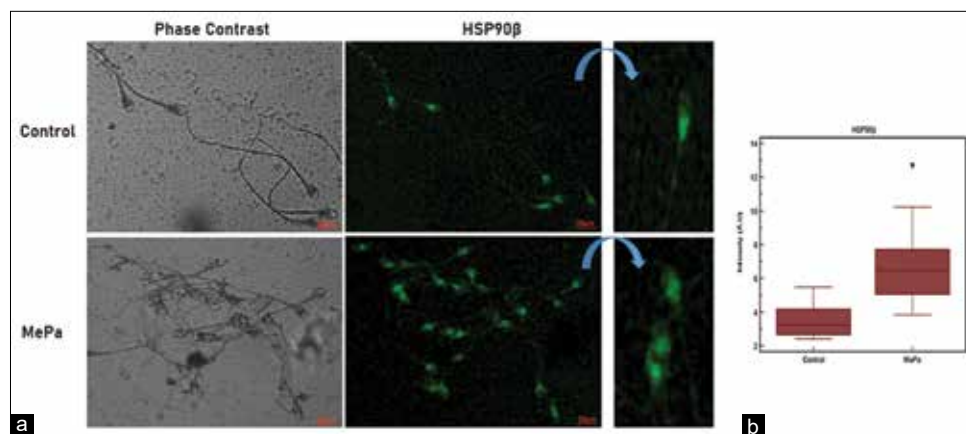
Figure 5: Expression profile of heat shock protein HSP90? In control and methyl parathion treated spermatozoa (500 µM) (a); corresponding box-whisker plot of fluorescence intensity of Immunocytochemistry (in arbitrary units) (b). *$P < 0.05$ with respect to control.

Overall, our study demonstrated that MePa is capable of generating ROS *in vitro* inflicting damage to the spermatozoa DNA and upregulates HSP90β expression resulting in impaired sperm motility.

CONCLUSION

The organophosphorus pesticides such as methylparathion alter the reproductive functions of animals and humans by inducing oxidative stress through the production of ROS. This ROS mediates the modulation of HSP90β expression which may affect the structural integrity of client proteins and causes oxidative injury to membrane lipids along with DNA integrity resulting in declined sperm motility in response to MePa.

Ethics statement

Institutional Ethics Committee, Kar Clinic and Hospital Pvt. Ltd, Bhubaneswar, Odisha approved the study and all participants gave written informed consent.

Author contributions

LS: Conceptualization. JN and SRJ: Writing-original draft. Methodology: JN, SRJ, BP. LS and JN: Writing-review and editing. The final version of the manuscript is approved by all authors.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent.

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Conflicts of interest

There are no conflicts of interest.

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