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Shivani Chhillar
ICAR-NDRI
VIPUL BATRA
National Dairy Research Institute
Arumugam Kumaresan
SRS of National Dairy Research Institute
Rakesh Kumar
ICAR-NDRI
Ankit Pal
ICAR-NDRI
TIRTHA DATTA (tirtha.datta@icar.gov.in)
ICAR-Central Institute for Research on Buffaloes

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Acute Exposure to Organophosphosphorus Pesticide Metabolites compromises mammalian sperm survival, functional and fertility

Shivani Chhillar[1#], Vipul Batra[1, 2#], Arumugam Kumaresan[3], Rakesh Kumar[1], Ankit Pal[1] and Tirtha Kumar Datta[1, 4*]

[1]Animal Genomics Lab., Animal Biotechnology Centre, ICAR-NDRI, Karnal, India
[2]School of Medicine, Division of Child Health, Obstetrics and Gynecology, University of Nottingham, Nottingham, England
[3]Theriogenelogy Lab., SRS of National Dairy Research Institute, Bengaluru, India
[4] ICAR-Central Institute for Research on Buffaloes, Hisar, India
[#]These authors contributed equally to the work
[*]Animal Genomics Lab., Animal Biotechnology Centre, National Dairy Research Institute, Karnal, India. email-tirthadatta@gmail.com

Abstract

Agrichemicals such as metabolites (OPPMs) of the commonly-used organophosphorus pesticides (OPPs) are considered more hazardous and pervasive than their parent pesticides which. This is because parental germline exposure to such xenobiotics has been demonstrated to cause an elevated susceptibility towards various reproductive failures e.g. sub- or in-fertility

Buffalo is a vital livestock resource, however, has numerous reproductive restraints e.g. male subfertility (low conception) that negatively impact its lifetime productivity. This study sought to examine the effects of low-dose, acute OPPM exposure on the mammalian sperm survival and its fertilizing ability. The buffalo spermatozoa were briefly exposed to three most prevalent organophosphorus pesticides metabolites viz. Omethoate (Dimethoate), paraoxon-methyl (methyl parathion) and 3, 5, 6-trichloro-2-pyridinol, (chlorpyrifos). The structural and functional integrity of the OPPM-exposed and control sperm were evaluated by various Cytomics and Biomimetics experiments including assessment of capacitation, lipid-peroxidation, mitochondrial activity and kinematic parameters (CASA), and in vitro fertilization (IVF) apart from their gene-expression profiling (RT-qPCR).

Preliminary data indicated that a perturbed phosphoproteomic endowment (PTP) and a dysregulated ROS-homeostasis in the OPPM-exposed spermatozoa ($P<0.05$) negatively affected their fertilizing ability ($P<0.01$) vis-à-vis control sperm. This is the first study demonstrating the spermatotoxic effects of OPPMs on gamete survival and function.

Keywords: Organophosphorus pesticides, oxons, metabolites, paternal programming, DoHaD, Male fertility

61 **Graphical abstract**

62

63

1. INTRODUCTION

Organophosphorus pesticides (OPPs) are amongst the most widely used synthetic pesticides around the globe. Despite being banned or restricted in many developed nations their exposure is still prevalent, particularly in developing economies, rendering the living organisms vulnerable to pesticide-induced reproductive hazards (**1-3**). Consequently, a large fraction of the human and animal population is exposed to the OPPs (**4-7**). The situation is further exacerbated by a lack of regulation, legislation and education which thus affects human, and animal health and the environment (**8-9**). Most pesticides contain at least one agent/metabolite that can affect any of the reproductive or developmental endpoints in humans and animals (**10**). The organophosphorus pesticides (OPPs) and their metabolites (OPPMs) are well-known mammalian male reproductive toxicants that damage the spermatozoa, alter testicular-somatic cells' function and adversely affect the semen quality (genotoxic and teratogenic). As a result, concerns regarding their safety are growing among environmental scientists (**11-13**).

The adverse effects of these pollutants on male fertility have also become an anthropogenic issue of global relevance among reproductive biologists owing to an increase in occupational and household chemical contamination. Moreover, the biotransformation of the OPPs often leads to the production of more hazardous chemical compounds, and their metabolites (OPPMs) e.g., oxons. These OPP metabolites persist and pervade the environment and interact with various organisms for decades leading to widespread body burdens (**14-17**). For example, the OPP, Dimethoate, *O*,*O*-Dimethyl *S*-[2-(methylamino)-2-oxoethyl] phosphorodithioate, a systemic and contact organophosphorus insecticide and its metabolite, Omethoate, O,O-Dimethyl S-(2-(methylamino)-2-oxoethyl) phosphorothioate reportedly persist in the reproductive organs of mice and rats for weeks after exposure(**18-20**). Omethoate is a toxic organophosphate insecticide metabolite that has been implicated in dimethoate toxicity in insects and mammals (**21-22**). Epidemiological data and experimental studies indicate a correlation between exposure to pesticides and perturbed sperm functional parameters and a decline in fertility (**23-25**). Spermatozoa are particularly vulnerable to the adverse effects of various agrichemical exposures and insults. The near lack of apoptotic mechanisms; repair, proofreading and protective enzymes; exceptionally high surface area/volume ratio (>50:1) and a considerable proportion of unsaturated fatty acids (PUFAs) in membrane render them comparatively more susceptible to

environmental insults vis-à-vis the female gamete and other somatic cells (**26-29**). Besides, the male-specific traits e.g., the higher abundance of androgen receptors which can interact with several pesticides, may result in depletion of testicular cell populations, altered testicular pathology, a decline in sperm count and reduction in sperm motility are also implicated in the elevated severity observed in male reproductive function **(30-34).**

Both chronic and acute exposure to these agrichemicals (OPPs) and their metabolites (OPPMs) have been reported to affect fertility and disrupt endocrinal homeostasis which may also cause undesirable effects on embryonic/offspring development, metabolic health and reproductive function (**35-38**). Many of these OPPs e.g., chlorpyrifos, *O*,*O*-Diethyl *O*-(3,5,6-trichloropyridin-2-yl) phosphorothioate, dimethoate and parathion have been classified as endocrine-disrupting chemicals (EDCs) which are known to perturb the cellular and endocrinal system which is crucial for normal development and reproduction homeostasis (**39-41**). However, the OPPs are known to primarily diminish the brain AChE activity (mostly at higher doses) which may consequently impact the sperm and the gonads through altered hypothalamic function leading to perturbations in endocrinal homeostasis via the HPT-axis (**42-44**). Interestingly, such programmed infertility is also known to be inherited in a trans-generational manner through environmentally induced changes in the epigenome, as posited in the DoHaD (Development Origins of Health and Disease) paradigm (**45-47**). For instance, the exposure to pesticide methoxychlor and fungicide vinclozolin leads to oligozoospermia which is observed in four successive generations indicating transgenerational transmission of parental programming effects (**48-50**).

Notably, under the Indian grazing systems animals are reared as per the semi- intensive farming paradigm. Thus, most of the livestock animals (e.g. buffaloes) are let out for grazing in open fields rather than being stall-fed which greatly increases the chances of exposure to agrichemicals such as OPPs because of their rapidly increasing consumption, particularly in emergent nations like India (**51-55**). We chose buffalo as a model for this study because it is a primary dairy animal in south south-east and Middle Eastern Asian countries. A crucial milch and meat species, more people bank on buffalo for their livelihood than on any other livestock animal (**56**). Nevertheless, it suffers from numerous reproductive constraints e.g., low conception rates (sub-fecundity) despite producing morphologically normal spermatozoa (idiopathic causes).

124 The anti-androgenic effects and the alterations in the reproductive enzyme pathways as a result
125 of acute or chronic OPP exposure are known to negatively impact the spermatozoa quality and
126 function apart from causing pernicious effects in the offspring (**54, 57-59**). Despite a burgeoning
127 rise in the studies on DoHaD and environmental (Agri)chemical exposure, the effects of the
128 prevalent pesticide metabolites (OPPMs) and their acute exposure on sperm quality parameters,
129 and fertility haven't been studied in detail. Only a limited number of in vitro reproductive,
130 environmental or toxicological studies have investigated the effect of pesticide metabolites on
131 livestock sperm function and fertility. Moreover, interest in defined, low-dose acute or chronic
132 pesticide exposure on the male reproductive system has recently increased apparently because of
133 the lack of a consented definition of the chronic or low-level exposures in many nations which
134 makes it difficult to decide the class of the exposure **(23, 60-62).**

135 Against this background, this work aimed to investigate the effects of low-dose, acute pesticide
136 (metabolites) exposure viz. Dimethoate (omethoate), methyl parathion (paraoxon-methyl) and
137 chlorpyrifos (3,5,6-trichloro-2-pyridinol) on mammalian sperm function following exposure for
138 2 h at 38˚C. We used the buffalo as a model and tested a continuum of doses ranging from 0.5-20
139 µM, based on previous OPP toxicology studies. Besides, in line with the 3R principles, the
140 mammalian spermatozoa from larger animals can serve as the best potential alternative to the use
141 of mammalian model organisms for in vitro reproductive toxicology studies **(63-65)**.

142 2. **RESULTS**

143 Ejaculates milky or creamy in appearance, with consistent homogeneity i.e. absence of
144 flakes/clumps and a minimum concentration of 600×10^6 spermatozoa/mL were considered for
145 downstream processing of semen. The mean motility and viability of representative (control)
146 sample ejaculates (after semen processing) were $86.35 \pm 1.21\%$ and $88.86 \pm 1.33\%$, respectively
147 (**Supplementary Fig.1**). The processed samples were diluted, wherever required. The motile
148 fraction of the spermatozoa was then assessed for morphological abnormalities and motility. No
149 changes in the mass motility of the buffalo spermatozoa were observed upon exposure to either
150 DMSO.

151 **2.1 Sperm Functional Parameters:**

152 **2.1.1 Membrane integrity assessment**

153 Membrane integrity is a crucial morphological/structural criterion for a spermatozoon to traverse
154 the FRT and fertilize the oocyte. The CFDA-PI dual staining discerned three spermatozoa
155 populations viz. live, dead and moribund (dying sperm, ROS producers.) spermatozoa based on
156 their fluorescence patterns indicative of the functional or compromised membrane integrity. The
157 integrity of the buffalo spermatozoa plasma membrane appeared to be compromised upon
158 incubation with three pesticide metabolites in a dose-dependent manner, especially at higher
159 concentrations (**Fig.1A-C and Supplementary Fig.2**).

160 **2.1.2 Capacitation status**

161 Capacitation is an imperative process that a spermatozoon must go through before attaining the
162 ability to fertilize an egg. Since the membrane dynamics undergo dramatic changes during
163 capacitation, the spermatozoa have a limited shelf life thereafter and as expected, premature
164 capacitation would negatively affect fertility. Chlortetracycline (CTC), which is used to detect
165 Ca^{2+}-related changes in the intracellular calcium re-distribution in the sperm head, particularly
166 during capacitation, discerned three distinct fluorescent patterns. These were classified as non-
167 capacitated (NC), capacitated (C) and acrosome-reacted (AR) spermatozoa upon exposure to
168 either of the three OPPMs (**Fig.1D-F and Supplementary Fig.2**). This induced precocious
169 capacitation in buffalo spermatozoa which increased linearly with concentration (**Fig.1D-F**).

170 **2.1.3 Mitochondria membrane potential**

171 The mitochondria membrane potential (MMP) is an indicator of health since the mitochondrion
172 is the single source of ATP and drives the self-destruct mechanisms (apoptosis) in the
173 mammalian sperm The JC-1 dye was used for the assessment of buffalo spermatozoal MMP
174 post-exposure to OPPMs. It renders the mitochondria with high MMP as fluorescing bright green
175 whereas the mitochondria with low membrane potential produced a dull fluorescence after
176 labelling (**Supplementary Fig.2**). The mitochondria membrane potential (MMP) of buffalo
177 spermatozoa diminished significantly upon incubation with OPPMs. This led to a decline in the
178 percentage of spermatozoa with a high mitochondrial membrane potential (**Fig.1G-I and**
179 **Supplementary Fig.2**).

180 **2.1.4 Lipid peroxidation**

181 Lipid peroxidation is implicated in the aetiology of defective spermatozoa function in multiple
182 mammalian species. BODIPY can incorporate into the spermatozoa and undergo a spectral
183 emission shift upon interacting with reactive oxygen metabolites, indicating oxidative stress. The
184 incubation of buffalo spermatozoa with the three OPPMs induced lipid peroxidation, as observed
185 by BODIPY staining, especially in the mid-piece (**Fig.1J-L and Supplementary Fig.2**).
186 However, it was less evident in the sperm head or the rest of the sperm tail (**Supplementary**
187 **Fig.2**).

188 **2.2 Protein tyrosine phosphorylation**

189 The OPPs/OPPMs may cause multiple asymptomatic effects at comparatively lower exposures
190 rather than overt signs and symptoms (**66**). We report similar subclinical toxic effects,
191 particularly at doses ≤ 5 μM. These doses didn't affect the sperm morphology, mass motility or
192 any functional parameters (except capacitation), nonetheless, appeared to negatively affect the
193 phosphorylation status of the protein tyrosine residues present on the cell surface
194 (**Supplementary Fig. 3**). Therefore, only OPPM concentrations ≤ 5μM were used in subsequent
195 experiments. A higher abundance of non-phosphorylated (NP) spermatozoa vis-à-vis the
196 phosphorylated spermatozoa (EM and AEM patterns) was observed in the control and the treated
197 samples (**Fig.2 and supplementary Fig.3**). Notably, the localization of phosphorylated proteins
198 on the sperm mid-piece always coincided with that of the equatorial region. The incubation with
199 OPPMs appeared to induce the phosphorylation of tyrosine residues (a conserved feature of
200 mammalian sperm capacitation) of sperm proteins (**67**) thus causing a reduction in the fraction of
201 NP spermatozoa (**Fig.2**).

202 **2.3 Expression dynamics of metabolic genes and oxidative stress-related genes**

203 The relative expression profiles of the selected panel of metabolic genes (ATP 6, ATP 8, COX 2,
204 CYT B, ND1 and ND2) and the antioxidant defence system (GPx and GST) were generated
205 using RT-qPCR, in the spermatozoa treated with different concentrations of OPPMs. A notable
206 rise in the expression of metabolic genes and an appreciable decline in genes of the antioxidant
207 defence system (AODS) was observed upon exposure to OPPMs (**Fig. 3**).

208 **2.4 Computer-Assisted Sperm Analysis (CASA)**

209 The buffalo sperm kinematic parameters were found to be remarkably perturbed upon exposure
210 to Organophosphorus pesticide metabolites (**Fig. 4**). It is worth mentioning that although the
211 lower doses of pesticide metabolites didn't affect motility per se, the motion and velocity
212 characteristics of buffalo spermatozoa were, however, altered upon exposure to OPPMs (**Fig. 4**).

213 **2.5 In vitro fertilization (IVF)**

214 The incubation of the semen sample with pesticide metabolites hindered the fertilization and
215 impeded the subsequent embryonic development in a dose-dependent manner (**Fig.5**). The rate of
216 oocyte cleavage and formation of blastocyst declined significantly upon exposure to OPPMs
217 indicating detrimental effects of OPPM exposure on the fertilizing ability of the buffalo
218 spermatozoa (**Fig.5**).

219 **3. DISCUSSION**

220 This study was undertaken to assess the effects of acute exposure to three organophosphorus
221 pesticides' metabolites (OPPMs) viz. paraoxon-methyl (methyl and ethyl parathion), omethoate
222 (dimethoate), 3, 5, 6-trichloro-2-pyridinol (chlorpyrifos, chlorpyrifos-methyl) on the viability
223 and fertilizing ability of the bubaline spermatozoa. The OPPs and some of their metabolistes
224 (e.g. oxons) are known to persist in the environment and interact with various organisms
225 (pervading for decades after exposure) resulting in widespread body burdens. Consequently the
226 exposure to these metabolites not only affects the metabolic, physiologic and reproductive health
227 of the parent but also causes pernicious effects in the forthcoming generations. Mammals are
228 exposed to a variety of (organophosphorus) pesticides during their lifetime, however, most of the
229 OPP toxicological studies have focused on either their roles as EDCs or the epigenetic changes
230 induced upon exposure to OPPs/OPPMs. The effects of acute exposure to OPPMs on
231 mammalian male gamete at low doses or from low environmental exposures have not been
232 completely elucidated. We employed the major pesticide metabolite for the three widely used
233 organophosphorus pesticides (OPPs) for assessing the functional ramifications of their acute
234 exposure on buffalo spermatozoa. Our results indicated that all three OPPMs used in this study
235 induced toxic effects on bubaline spermatozoa in a dose-dependent manner including impairment
236 of sperm function, perturbation of the kinematic parameters, and decreased fertilizing ability.

237 This dose-response effect indicated the toxicological implications of acute pesticide exposure on
238 mammalian spermatozoa.

239 Exposure to OPPs and their metabolites (OPPMs)are known to induce deleterious (cytotoxic)
240 effects on the reproductive system of animals through distinct physiological mechanisms,
241 components and pathways and are thus associated with deteriorated sperm quality (**40, 54, 68-**
242 **70**). For example, acute exposure (1-3h) to Roundup (and its major component Glyphosate) has
243 recently been reported to cause a reduction in sperm viability, acrosome integrity, mitochondrial
244 activity and motility in a dose-dependent manner thus exerting negative impacts on male
245 gametes (**71**). Notably, most of the studies on pesticide exposure have reported adverse impacts
246 on one or more sperm functional parameters (SFPs) e.g., membrane integrity, MMP, capacitation
247 and motility (**72-74**). Many of these parameters are correlated with fertility and have been
248 proposed as fertility biomarkers in addition to their reported associations with the life expectancy
249 of the offspring (**75-78**). We also observed a rise in the percentage of spermatozoa with impaired
250 sperm function parameters (SFPs) upon exposure to OPPMs (**Fig.1**), particularly at higher doses
251 (≥5μM). The presence of pesticide residues or their metabolites in the fluids surrounding
252 spermatozoa negatively influences the spermatozoa function (impairing survival and fertility)
253 and is also implicated in developmental malformations or defects in the fetus/offspring (**41, 55,**
254 **70, 79**). Several studies across multiple mammalian species have focused on the effects and
255 putative mechanisms of action of (organophosphorus) pesticides on the male reproductive
256 system (**7, 57, 73, 80**). For instance, both in vivo (via oral gavage) and in vitro exposure to
257 chlorpyrifos have been shown to interfere with male reproductive functions leading to reduced
258 fertility in mammals (**3, 81**). Our results agree with the aforementioned studies that exposure to
259 these xenobiotics potentiates the perturbation of functional parameters of mammalian
260 spermatozoa e.g., mitochondrial function and activity (**Fig.1G-I**). The oxidative stress associated
261 with pesticide exposure is known to trigger mitochondrial deficiency among other cytotoxic and
262 genotoxic effects (**82, 83).** The deficiency in mitochondrial activity and function results in the
263 manifestation of oxidative stress thus creating a vicious cycle and is itself entwined in it
264 (discussed below). The buffalo spermatozoa have been reported to contain an elevated amount of
265 unsaturated fatty acids (PUFA) vis-à-vis other bovids which render them vulnerable to oxidative
266 stress-induced damage **(84)**. We observed a reduced MMP along with a concomitant rise in lipid
267 peroxidation (LPO) of bubaline spermatozoa (**Fig.1J-L**) which has been demonstrated to be

268 highly associated with Murrah buffalo bull fertility (**85**). The exposure of mammalian
269 spermatozoa to OPPs/OPPMs e.g., methyl parathion or its metabolite, methyl paraoxon (one of
270 the most potent insecticides) reportedly caused oxidative stress through elevated LPO, as
271 assessed by malondialdehyde production at 7 and 28 days post-treatment (dpt) in male mice (**31**).
272 A different OPP, Dimethoate (along with Chlorpyrifos) is amongst the most frequently used
273 OPPs, especially in developing economies, as mentioned previously (**3, 38**). As expected, the
274 exposure to another OPP, Dimethoate through oral gavage has also been reported to affect the
275 reproductive performance of male mice leading to a decline in sperm motility, fraction of live
276 spermatozoa, hormone levels and alterations (degenerative changes) in testicular histology (**19,**
277 **20**). Akin to chlorpyrifos metabolite TCPy, Omethoate is considered more toxic than its parent
278 pesticide, Dimethoate (**13, 18, 21, 22**). Overall, acute exposure to OPPMs negatively impacted
279 the sperm functional parameters which are considered biomarkers of mammalian sperm fertility.

280 It is worth mentioning that capacitation was the only functional parameter which was affected at
281 low OPPM exposure (≥1μM) to buffalo sperm (**Fig.1D-F**). We didn't observe any significant
282 effect on sperm mass motility at these doses (**Supplementary Fig.1**), nonetheless, it has often
283 been observed that such subclinical effects may appear to be subtle and can cause chronic illness
284 through functional alterations in diverse biological processes (**66**). The observed rise in
285 precociously capacitated sperm at low OPPM exposure (1μM) intrigued us to assess similar,
286 capacitation-associated functional alterations e.g., PTP status and buffalo sperm kinematics. We
287 also observed a rise in buffalo spermatozoa protein tyrosine phosphorylation (PTP) post the low,
288 acute exposure of OPPMs. PTP is an important intracellular mechanism that is implicated in
289 many cellular processes e.g., regulating sperm functions. A rise in the tyrosine protein
290 phosphorylation is considered a robust indicator of capacitation, in several species including
291 humans (**86**), rodents (**87**), pigs (**88**), and bovids including buffalo **(89, 90**). Most OPPs are well-
292 known phosphorylating agents and our results indicate that the same is true for their metabolites
293 that induced dose-dependent tyrosine phosphorylation in buffalo spermatozoa (**Fig.2**). Our
294 observations corroborate with these studies and were also underpinned by the observed rise in
295 the number of precociously capacitated spermatozoa and tyrosine phosphorylation (**67**).
296 Nevertheless, whether this rise in phosphorylation was genotoxic or induced any epigenetic
297 changes were not assessed in this study. Most of the sperm kinematic parameters are not
298 perceptible by the naked eye, however, they exhibit sensitivity to various reproductive toxicants

299 such as OPPs (**91**). Surprisingly, many of the velocity and motion characteristics of buffalo
300 sperm were found to be altered upon OPPM exposure (**Fig.4**). These characteristics are crucial to
301 the fertilizing ability of the mammalian spermatozoa, for instance, the straight line velocity
302 (STR) has been proposed to play crucial roles in sperm transport through the FRT and oocyte-
303 penetration (**92**). Exposure of mammalian spermatozoa to the OPPs has been demonstrated to
304 perturb many of the sperm kinematic parameters e.g., Chlorpyrifos exposure to buffalo
305 spermatozoa reportedly results in a reduction in straight line velocity (STR) and average path
306 velocity (VAP) (**53**). Overall, the results of the CASA experiments indicated perturbed motility
307 and velocity parameters upon OPPM (akin to OPP) exposure that can potentially affect their
308 reproductive function (**51, 54**).

309 Interestingly, this alteration in motility-related spermatozoal kinematics parameters has
310 reportedly been ascribed to the elevated mitochondrial gene activity affecting ATP production
311 which results in precocious capacitation (**91, 92**). Therefore, we selected a panel of
312 mitochondrial metabolic (and antioxidant) genes to assess if the same was also true for the
313 OPPMs. The motility patterns which are very crucial for the fertilizing ability of mammalian
314 spermatozoa are dependent on energy sources. We observed an elevated expression of genes
315 involved in mitochondrial bioenergetics upon OPPM exposure (**Fig.3).** This was expected since
316 mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis are the two main pathways
317 to generate ATP (**95, 96**). Notably, induction of the OXPHOS pathway produces a large amount
318 of ATP in sperm which in turn is a potent inducer of sperm capacitation and acrosome reaction.
319 The genes such as ATP6, ATP8 and the NADPH dehydrogenase subunits (ND1, ND2, and ND4)
320 are involved in ATP formation (**95, 96**) while GST and GPxare the representative enzymes of the
321 antioxidant defense system (AODS) (**74, 97**). The activity of cellular antioxidant enzymes is
322 considered a marker of ROS homeostasis which is reportedly altered upon exposure to
323 xenobiotics, such as OPPs and OPPMs (**98, 99**). We observed a reduction in the expression of
324 antioxidant enzymes (GPx and GST) in buffalo sperm upon exposure to OPPMs (**Fig.3**) which is
325 indicative of the potential manifestation of an imbalance in the redox state/ROS homeostasis
326 (**100**). A similar decline in AODS has been implicated in enhanced ROS production further
327 aggravating the oxidative stress e.g., dose-dependent rise in LPO, as observed in this study (27,
328 28, **30, 31, 101**). A compromised structural or functional integrity of the sperm membrane and
329 sperm mitochondria may lead to a reduction in sperm fertility (**102**). Besides, as mentioned

330 earlier altered functional and motility parameters are expected to cause a decline in the fertilizing
331 ability of the spermatozoa used in IVF (**39**). As anticipated, low OPPM exposure (e.g., 0.5µM,
332 TCPy) led to a significant reduction in the fertilizing ability of spermatozoa (**Fig.5**). As reported
333 previously, precocious capacitation could also be implicated in their impaired fertilizing ability,
334 as observed in our study (**96**), Furthermore, the paternal seminal fluid is capable of influencing
335 the developmental programming effects in the progeny and dictating changes in the uterine
336 luminal components (**103**). Such exposures can lead to the impairment of various biochemical
337 pathways and high ROS production resulting in low-quality embryos which consequently leads
338 to poor clinical outcomes in IVF programs (**39, 53, 104, 105**). A growing body of evidence
339 indicates that prenatal exposure to environmental xenobiotics e.g., OPPMs can adversely affect
340 fertility, in *utero* and post-natal development and may have multigenerational effects as
341 addressed under the novel, E-DOHaD (Environmentally-induced DOHaD) model of trans- or
342 inter-generational inheritance (**17, 54, 106, 107**). For example, men exposed to pesticide residues
343 had children with an elevated risk of male reproductive developmental disorders including birth
344 defects such as cryptorchidism, hypospadias, reduced fertility, stunted growth and development
345 leading to clinical manifestations (**61, 108-110**). It would be interesting to assess the effects of
346 OPPM exposure at different embryonic cell stages and post-natal stages in the offspring through
347 adulthood.

348 There are certain caveats and limitations of this study that are worth discussing. For example, the
349 epigenetic changes and genotoxicity of the OPPM exposure were not ascertained. The impact of
350 these OPPMs on male fertility cannot be ruled out due to the use of recommended OPP doses
351 and the existence of regulatory frameworks. Hence, it would also be interesting to study the
352 effects of cumulative, minimal dosage, chronic exposure on male reproductive physiology. This
353 is because the mechanisms underlying chronic and acute exposure have been proposed to be
354 distinct. For example, in rodents, the mechanisms behind the reduction of fertilizing ability of
355 spermatozoa at 7- and 28-days post-treatment (dpt) of methyl parathion have been ascribed to the
356 sperm surface remodelling events and acrosomal defects, respectively (**31, 39**). Moreover, we
357 chose the OPPM concentrations based on previous reproductive toxicology studies wherein a
358 huge variation in the selected concentrations (0.005µg/ml to 750µM) was observed (**53, 54, 57**).
359 Importantly, the assessment of pesticide concentration in representative semen samples (as
360 reported in food products and air samples) would additionally help employ the bio-available

361 equivalent doses for similar studies (**111-117**) Overall, the spermatotoxic effects of three OPPMs
362 used in this study appear to share the mechanisms of toxicity viz. oxidative stress and protein
363 phosphorylation, as reported for various OPPs (**118-119**). The dose-dependent rise in PTP, LPO,
364 and OXPHOS gene expression indicated the involvement of regulatory protein modifications
365 (phosphorylation) and oxidative stress in mediating the reproductive toxicity of OPP metabolites.

366 **CONCLUSION**

367 Exposure to environmental pollutants such as OPPs appears to be one of the preponderant,
368 previously unidentified pathological factors to be associated with idiopathic male infertility (**120,**
369 **121**). By using the mammalian (Bubaline) spermatozoa as a model, our results revealed transient
370 exposure to OPPMs viz. Omethoate, MePa and TCPy at low concentrations (0.5-2 μM)
371 detrimentally affect the function and survival of the male gamete. It appears that transient
372 exposure to OPPMs led to the induction of oxidative stress that caused sperm damage and also
373 increased phosphorylation leading to precocious capacitation thus affecting the fertilizing ability
374 and subsequent embryo development. Nevertheless, caution should be exercised while
375 extrapolating the results of these in vitro models to in vivo studies. Moreover, since both parents
376 contribute equally to the genetic material of their offspring and a male exposed to pesticide
377 (bodily fluids) renders a woman's uterine environment susceptible to exposure during coitus
378 (**122**), both parents should be considered particularly in inter- and trans-generational studies.
379 Furthermore, most couples often share lifestyle habits, and diet choices and occupy the same
380 niche and together transmit the molecular memory of their past environmental experience to their
381 offspring (**123**).

382 **4. METHODS**

383 **Reagents** All chemicals, media and reagents including the commercial formulation of OPPMs
384 used in this study were procured from Sigma-Aldrich Chemical Co. Ltd, (USA) unless stated
385 otherwise. All plasticware was procured from Nunc Inc. (ThermoScientific, USA). The OPPM
386 formulations were diluted with dimethyl sulfoxide (DMSO).

387 **4.1 Sample collection and processing**

388 Frozen semen straws of Murrah buffalo bulls (N =9) were procured from the Artificial Breeding
389 Research Centre (ABRC), ICAR-NDRI, India. The straws were thawed by immersing them in a
390 water bath at 38˚C for the 30s. The contents were collected into 15mL centrifugation tubes
391 containing 2mL working Sp-TALP medium (1 mM 60% Na-lactate and 0.98 mM Na-Pyruvate in
392 2X filtered stock mixed with an equal amount of Mili-Q water) (**Supplementary sheet-**
393 **Methods: Table 1**). The spermatozoa were separated from the seminal plasma and extender
394 components by centrifuging at 280 x g for 6min (thrice) in the working Sp-TALP medium. The
395 supernatant was discarded each time and the sperm pellet obtained after the final wash was
396 subject to the swim-up technique. The obtained fraction containing the motile spermatozoa was
397 suspended in 250µL working-NCM (non-capacitating, Sp-TALP medium). Since the semen
398 straws were procured from a commercial government-funded farm that operates under standard
399 conditions, specific authorization from the Ethics Committee was not required to conduct this
400 study.

401 **4.2 Experimental Design**

402 The motile spermatozoa (10 x 10^6) obtained from the previous step were incubated with
403 different concentrations (0.5µM, 1µM, 2µM, 5µM, 10 µM, 20 µM) of three pesticide metabolites
404 viz. Omethoate (from Dimethoate), Paraoxon-methyl (from Methyl parathion), and 3,5,6-
405 Trichloropyridinol (from Chlorpyrifos), the three most consumed OPPs (**124**) for 2 hours at 37˚C
406 in a CO_2 incubator along with negative control (no pesticide metabolites) and vehicle control
407 (DMSO only). We chose a continuum of doses based on values reported previously in various in
408 vitro and in vivo reproductive toxicological studies on mammalian spermatozoa survival and
409 function (**53, 55, 57, 125**). Likewise, the time of incubation was decided based on previously
410 published literature on acute toxicity, most of which have reported an incubation time between 1-
411 3h (**53, 57, 71**).

412 **4.3 Sperm Function Parameters (SFPs)**

413 After incubation with OPPMs, the control and treated buffalo spermatozoa were evaluated for
414 intactness of structural and functional integrity. The examination of sperm-membrane integrity,
415 acrosome reaction and lipid peroxidation were done by using carboxyfluoresceindiacetate-
416 propidium iodide (CFDA-PI) and 4, 4-Difluoro-4-bora-3a, 4a-diaza-s-indacene (BODIPY) dyes,

417 respectively, as per the method described by Singh and colleagues (**85**). However, the assessment
418 of mitochondrial membrane potential (MMP) by JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-
419 tetraethylbenzimidazolyl-carbocyanine iodide) and capacitation status by Chlortetracycline
420 (CTC), a fluorescent chelate probe of Ca^{2+} was done by the methods described by Saraf and co-
421 workers (**126**). Thereafter, the excess stains were removed by washing the stain-incubated
422 spermatozoa with 200 µL of sperm-TALP by centrifugation at 800 x g for 3 min. The pelleted
423 spermatozoa were used to make a thin smear onto which a few drops of mounting medium,
424 Dabco® 33-LV were placed and was observed at 1000X magnification under an Olympus BX-
425 51 fluorescence microscope using appropriate filters. A minimum of n=200 spermatozoa (in
426 triplicates) were evaluated, in a minimum of 10 fields for observing fluorescent patterns. The
427 images of the two filters were merged to obtain the final image, wherever required.

428 **4.4 Protein tyrosine phosphorylation (PTP) status**

429 The protein tyrosine phosphorylation (PTP) status of the buffalo spermatozoa was assessed using
430 an indirect immunofluorescence assay as described by Saraf et al (**127**). The higher doses
431 ≥10µM of the OPPMs were omitted from further experiments (explained later). The control and
432 the treated spermatozoa were washed with PBS at 300 x g for 5min and a 20µL sperm
433 suspension was then smeared onto a clean glass slide and air dried. Thereafter, the spermatozoa
434 were fixed in 4% paraformaldehyde for 1h at 4˚C and washed with PBS (3X). The spermatozoa
435 were permeabilized using methanol and the slides were blocked using 5% BSA in PBS for 2h at
436 room temperature. Subsequently, the smears were incubated with monoclonal anti-
437 phosphotyrosine antibody (P1869; Sigma, 1:100) in 1% BSA for 3h at 37˚C and then washed
438 with PBS (3X). Afterwards, the smears were incubated with FITC-conjugated anti-mouse IgG
439 antibody produced in goat (F4018; Sigma, 1:100) and then washed thoroughly with PBS. After
440 the final washing step, the coverslip was mounted onto a dried glass slide. A drop of mounting
441 medium, Dabco® 33-LVwas placed on the slide and the cells were then observed under a BX-51
442 Olympus fluorescence microscope at 1000X magnification using a FITC-filter. The spermatozoa
443 were assessed for the percentage of different phosphorylation patterns and a minimum of 200
444 spermatozoa were counted (in three technical replicates) across the slide. The three patterns of
445 tyrosine phosphorylation (pattern NP – no fluorescence; pattern EM- fluorescence over the

446 equatorial region and mid-piece and pattern AEM – fluorescence over the acrosomal area,
447 equatorial region and mid-piece) were counted and expressed as percentages.

448 **4.5 Expression dynamics of the metabolic and oxidative stress-related genes**

449 Total RNA was isolated from different experimental groups and the control group of
450 spermatozoa as described previously by Batra and colleagues (**128**) using the TRI Reagent, RNA
451 isolation reagent (Sigma-Aldrich, USA). The isolated RNA was quantified using a Nanodrop
452 ND-1000 UV–Vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).
453 The cDNA synthesis and RT-qPCR optimization were done as described by Batra et al. (**128**)
454 (**Supplementary sheet-Methods**). The primer designing tool at NCBI, the Primer-BLAST was
455 used to design primers for the metabolic and oxidative stress-related genes in the buffalo
456 spermatozoa and two reference genes viz. GAPDH (glyceraldehyde-3-phosphate
457 dehydrogenase) and β-actin. Intron-spanning primers were designed, wherever possible and the
458 self-annealing sites, mismatches and secondary structures in the primers were checked using
459 OligoCalc (**129**). The specificity of each set of primers was again checked using the BLAST
460 alignment tool and in silico PCR (**130**) was run for each set of primers before commercial
461 synthesis (Sigma-Aldrich, USA). The MIQE (**131**) guidelines were followed at every step,
462 wherever possible. The relative quantification of all the genes was done on a Bio-rad CFX-96
463 Touch Deep Well Real-Time PCR system platform using the iTaqUniversal SYBR Green
464 Supermix (Bio-Rad, USA) in a 10 µL reaction mix. The thermal profile was 95˚C for 5 min, 40
465 cycles consisting of denaturation at 95˚C for 15 s, annealing at variable optimized temperatures
466 for 20s, extension at 72˚C for 20 s, followed by the melt curve protocol with 10s at 95˚C and
467 then 60s each at 0.5˚C increments between 65˚C and 95˚C. The melt curve analysis ensures a
468 specific, unique product formation and ascertains primer dimer formation. A no-template control
469 (NTC) was included in each plate to confirm the absence of nucleic acid contamination. The
470 mean sample C_q (Cycle of quantification) values for the various metabolic and oxidative stress-
471 related genes were calculated for duplicate samples and their relative expression was calculated
472 by ΔΔCt method, as described previously (**132**). The differential gene expression levels were
473 examined for normality of distribution and were analyzed by one-way ANOVA, as implemented
474 in GraphPad Prism 8.0 (**for Windows, GraphPad Software, La Jolla California USA,**
475 **www.graphpad.com**) and a p-value < 0.05 was considered to be statistically significant.

476 **4.6 Sperm kinematics using computer-assisted sperm analyzer**

477 The spermatozoa incubated with pesticide metabolites (OPPMs) were subject to computer-
478 assisted sperm analysis (CASA) for estimating the velocity and motion parameters of the buffalo
479 spermatozoa. Computer-assisted sperm analyzer (IVOS12.1, Hamilton-Thorne Biosciences,
480 Beverly, MA, USA) was used to evaluate the kinetic characteristics. The motility and movement
481 parameters like the curvilinear velocity (VCL, µm/ s), linear velocity (VSL, µm/s), average path
482 velocity (VAP, µm/s), the mean amplitude of lateral head displacement (ALH, µm), the
483 percentage of linearity i.e. the ratio between VSL and VCL (LIN, %), the straightness coefficient
484 which is the ratio between VSL and VAP (STR, %) and the frequency with which the actual
485 sperm trajectory crossed the average path trajectory (BCF, Hz) were recorded in duplicates for
486 all the experimental groups. The CASA software settings were as follows: temperature = 38˚C,
487 frame rate = 60Hz, frames acquired = 30, minimum contrast = 35, minimum cell size = 5 pixels,
488 cell size = 9 pixels, cell intensity = 110 pixels, progressive cells (VAP cut-off = 50 m/s, STR cut-
489 off = 70%), slow cells (VAP cut-off = 30 /s and VSL cut-off = 15 /s). The spermatozoa (N=500)
490 were observed in a minimum of five optical fields around the central reticulum of the chamber
491 for sperm motility analysis. The differential kinematic parameters among the various
492 experimental groups were analyzed by ANOVA and Tukey's post-hoc test, as implemented in
493 GraphPad Prism 8.0 (for Windows, GraphPad Software, La Jolla California
494 USA, www.graphpad.com) and a P-value <0.05 was considered to be statistically significant.

495 **4.7 IVF (in vitro fertilization) Study**

496 The IVF experiments were done as previously explained by Batra et al (**Batra et al., 2021**).
497 Briefly, buffalo ovaries from the slaughtered animals were transported to the laboratory in
498 physiological saline (0.9%, w/v NaCl) containing strepto-penicillin (50 mg/L) within 2-3h of
499 slaughter. After washing the ovaries with normal saline, the follicular fluid was aspirated using a
500 vacuum pump (Cook) in HEPES-buffered hamster embryo culture (HH) medium. After
501 extensive washing with HH medium, 15 cumulus-oocyte complexes (COCs) were placed in
502 100µL droplets of maturation medium (HEPES buffered TCM199 modified with 10% (v/v) fetal
503 bovine serum (FBS), 0.005% (w/v) streptomycin, 0.01% (w/v) sodium pyruvate and 0.005%
504 (w/v) glutamine supplemented with 5.0µg/mL FSH and 10µg/mL LH, 1 µg/mL estradiol 17-β
505 and 50 ng/mL epidermal growth factor (EGF), 64µg/mL cysteamine and 50µL ITS). The dishes

506 were cultured in duplicate for 24 h at 38.5˚C in a humidified atmosphere of 5% CO_2 in an
507 incubator. The IVF (in vitro fertilization) was carried out using 50µL of spermatozoa suspension
508 (1 x 10^6/mL) from OPPM-treated samples along with a control (vehicle). In vitro fertilization
509 (IVF) also was carried out as per the procedure described in the abovementioned study. The IVC
510 (in vitro culture) droplets of 100µL were prepared following the progressive removal of the
511 media from IVM droplets which was later replaced with IVC medium (mCR2aa —modified
512 Charles Rosenkrans medium with amino acids containing 0.8% BSA, fatty acid-free, and
513 50mg/mL gentamicin). After 12h of IVF, oocytes were denuded mechanically by brief vortexing.
514 The oocytes were subsequently washed five times in the IVC medium and transferred to IVC
515 droplets in groups of 15. A total of 50µL of IVC medium was replaced with fresh medium after
516 48 h. The replacement medium constituted of mCR2aa supplemented with 10% (v/v) FBS,
517 50mg/mL of gentamicin and 0.8% BSA (fatty acid free). The second replacement was done 48 h
518 after the first replacement. The cleavage rates were assessed at the time of 1st medium
519 replacement while blastocyst rates were determined 7 days post-IVC. The data were analyzed on
520 GraphPad prism 8.0 (for Windows, GraphPad Software, La Jolla California
521 USA, www.graphpad.com) to compare the observed differences in cleavage and blastocyst rates
522 among the control and treatment groups. A P-value <0.05 was considered to be statistically
523 significant.

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AUTHOR CONTRIBUTIONS

The study was conceptualized and designed by SC, AK, RK, and TKD. Sample collection, microscopy and expression analysis was done by SC & VB. The IVF was performed by SC & AP. RK & TKD performed statistical analyses. RK and AK implemented and conducted CASA. The manuscript was written by VB & SC. Figures were designed by VB TKD and SC. All the authors contributed to the article and approved the submitted version.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files. **Supplementary Material & Information**: Supplementary sheet-Methods and Supplementary Fig.1, 2, and 3.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS STATEMENT

Not Applicable

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994 **Fig.1 Sperm Functional Parameters (Cytomics)**The buffalo spermatozoa were exposed to 0.5,
995 1, 2, 5, 10 and 20μM of OPPM viz. Omethoate (Dimethoate), Paraoxon methyl (Methyl
996 parathion), TCPy (Chlorpyrifos) for assessment of membrane integrity (A-C), capacitation status
997 (D-F), mitochondrial membrane potential (G-I) and lipid peroxidation (J-L) using fluorescent
998 staining. CFDA with PI (see text) was used to assess the membrane integrity of the spermatozoa
999 that were categorized as live, dead or moribund. CTC (see text) was used to categorize the
1000 spermatozoa as non-capacitated (NC), capacitated (C), or acrosome reacted (AR). JC-1 (see text)
1001 was used to calculate the percentage of spermatozoa with high mitochondrial membrane
1002 potential while BODIPY (see text) for spermatozoa with high lipid peroxidation (LPO).

1003 **Fig.2 Protein Tyrosine Phosphorylation** Three major fluorescent patterns viz. non-fluorescent
1004 spermatozoa i.e. non-phosphorylated (NP), sperm bearing signal at equatorial region and mid-
1005 piece (EM) and spermatozoa with signal at acrosomal region, equatorial region and mid-piece
1006 (AEM).were observed during immunocytochemistry using monoclonal anti-phosphotyrosine
1007 antibody. The buffalo spermatozoa were exposed to 0.5, 1, and 2μM of OPPM viz. Omethoate
1008 (Dimethoate)-A, Paraoxon methyl (Methyl parathion)-B, TCPy (Chlorpyrifos)-C.

1009 **Fig.3 Pattern of expression of metabolic and oxidative stress-related genes** Relative
1010 expression profiles of the metabolic genes viz. ATP6, ATP8, COX-2, CytB, ND-1 and ND-2 and
1011 those of the antioxidant defense system viz. GPx and GST in the buffalo spermatozoa exposed to
1012 0.5, 1, and 2μM of OPPM viz. Omethoate (Dimethoate)-A-H, Paraoxon methyl (Methyl
1013 parathion)-I-P, TCPy (Chlorpyrifos)-Q-X. The expression values are normalized to GAPDH and
1014 β-actin the error bars represent the standard error of mean (SEM).

1015 **Fig.4 Kinematics of buffalo spermatozoa**. The velocity characteristics viz. curvilinear velocity
1016 (VCL), average path velocity (VAP) and (DSL) (A-C) and the motion characteristics viz. the
1017 percentage of linearity i.e. the ratio between VSL and VCL (LIN), the straightness coefficient
1018 which is the ratio between VSL and VAP (STR) and progressive STR (PGSTR) (D-F) along
1019 with the mean amplitude of lateral head displacement (G-I) and beat cross frequency (J-L) were
1020 evaluated by Computer-assisted sperm analyzer (IVOS12.1, Hamilton-Thorne Biosciences,
1021 Beverly,MA, USA).

1022 **Fig.5 In vitro fertilization (Biomimetics)** The mean ± SEM for cleavage rate (A-C), and
1023 blastocyst formation rates (D-F) in the control group and buffalo spermatozoa exposed to 0.5, 1,
1024 and 2μM of OPPM viz. Omethoate (Dimethoate)-A,D, Paraoxon methyl (Methyl parathion)-B,
1025 E, and TCPy (Chlorpyrifos)- (Fig. C, F).

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Figures

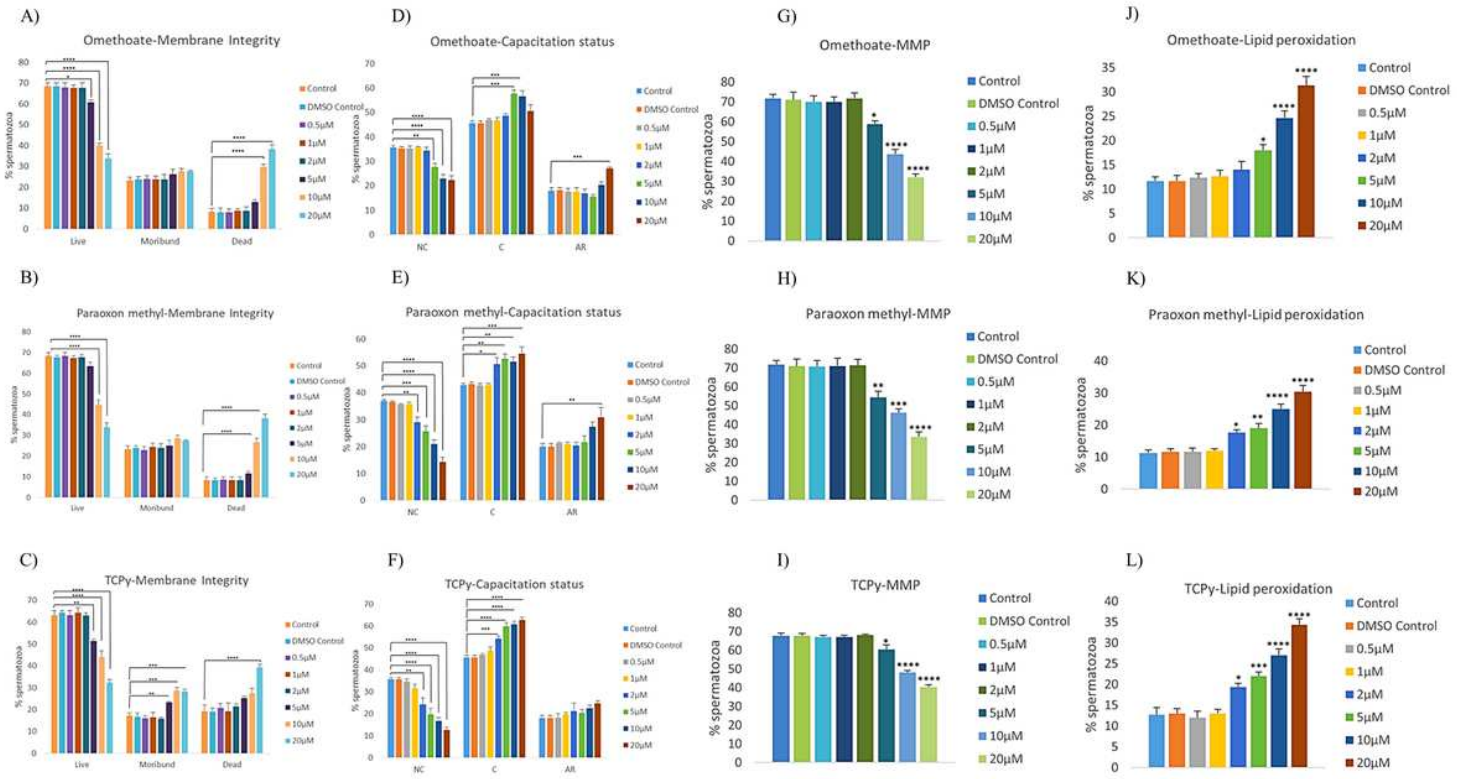

Figure 1

Sperm Functional Parameters (Cytomics)The buffalo spermatozoa were exposed to 0.5, 1, 2, 5, 10 and 20µM of OPPM viz. Omethoate (Dimethoate), Paraoxon methyl (Methyl parathion), TCPy (Chlorpyrifos) for assessment of membrane integrity (A-C), capacitation status (D-F), mitochondrial membrane potential (G-I) and lipid peroxidation (J-L) using fluorescent staining. CFDA with PI (see text) was used to assess the membrane integrity of the spermatozoa that were categorized as live, dead or moribund. CTC (see text) was used to categorize the spermatozoa as non-capacitated (NC), capacitated (C), or acrosome reacted (AR). JC-1 (see text) was used to calculate the percentage of spermatozoa with high mitochondrial membrane potential while BODIPY (see text) for spermatozoa with high lipid peroxidation (LPO).

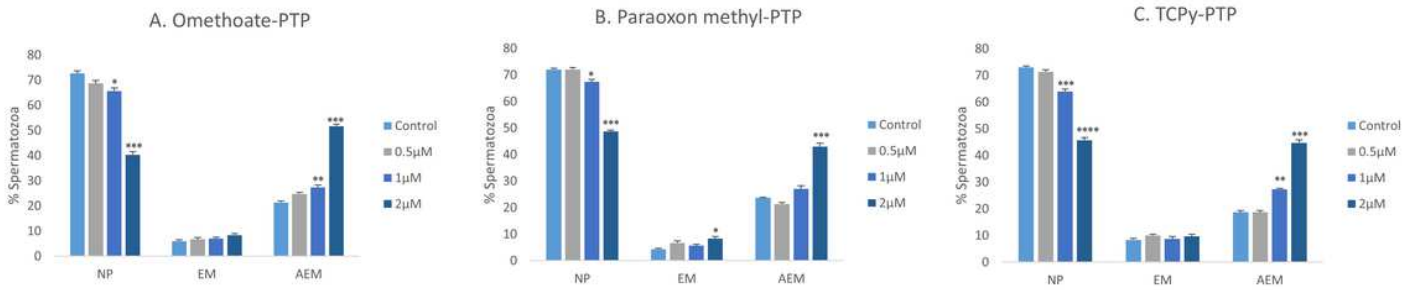

Figure 2

Protein Tyrosine Phosphorylation Three major fluorescent patterns viz. non-fluorescent spermatozoa i.e. non-phosphorylated (NP), sperm bearing signal at equatorial region and mid- piece (EM) and spermatozoa with signal at acrosomal region, equatorial region and mid-piece (AEM).were observed during immunocytochemistry using monoclonal anti-phosphotyrosine antibody. The buffalo spermatozoa were exposed to 0.5, 1, and 2μM of OPPM viz. Omethoate (Dimethoate)-A, Paraoxon methyl (Methyl parathion)-B, TCPy (Chlorpyrifos)-C.

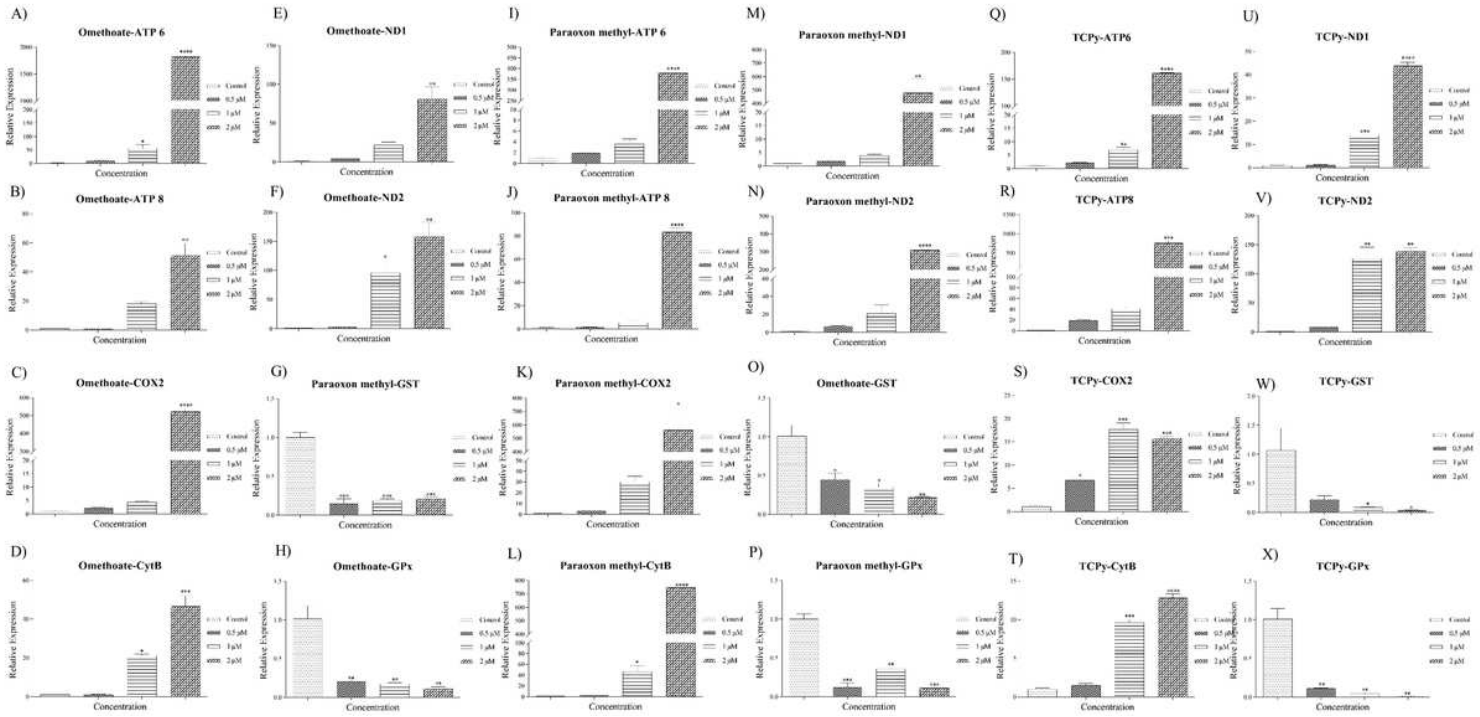

Figure 3

Pattern of expression of metabolic and oxidative stress-related genes Relative expression profiles of the metabolic genes viz. ATP6, ATP8, COX-2, CytB, ND-1 and ND-2 and those of the antioxidant defense system viz. GPx and GST in the buffalo spermatozoa exposed to 0.5, 1, and 2μM of OPPM viz. Omethoate (Dimethoate)-A-H, Paraoxon methyl (Methyl parathion)-I-P, TCPy (Chlorpyrifos)-Q-X. The expression values are normalized to GAPDH and β-actin the error bars represent the standard error of mean (SEM).

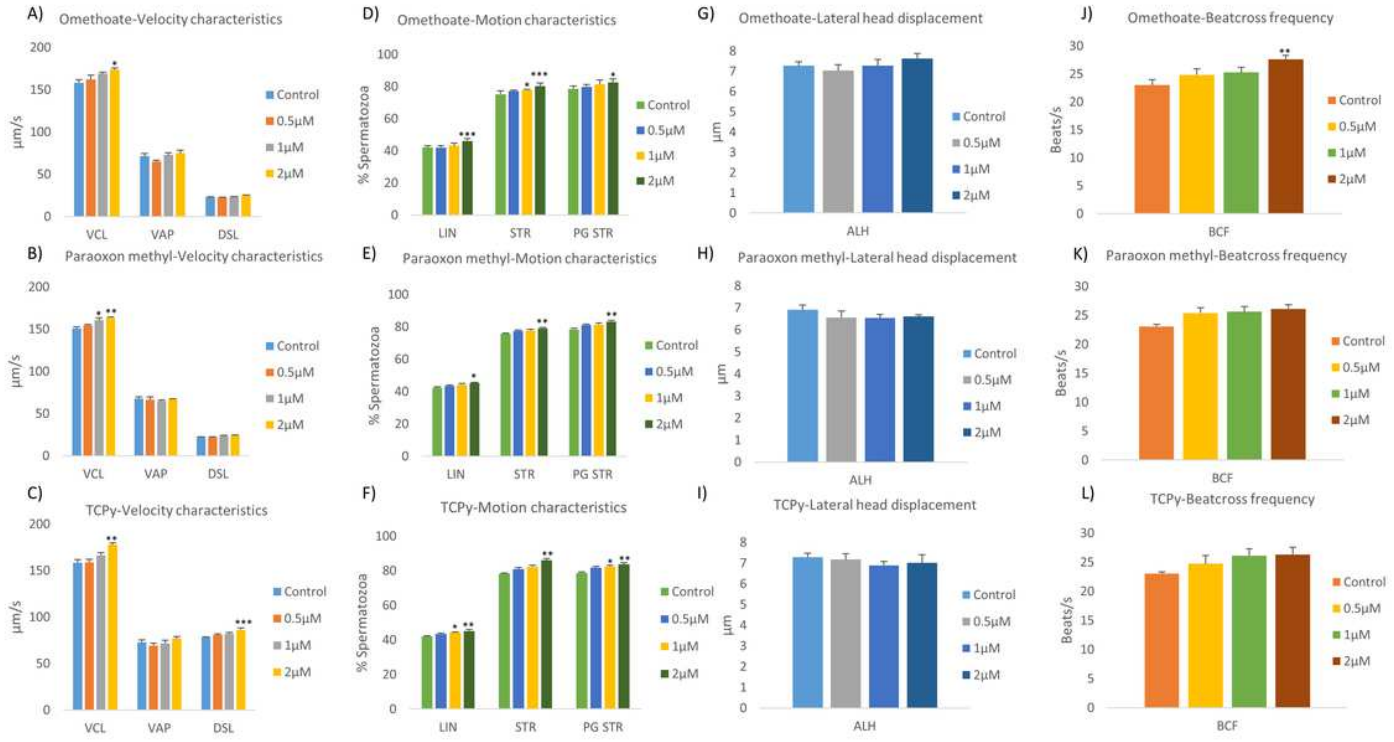

Figure 4

Kinematics of buffalo spermatozoa. The velocity characteristics viz. curvilinear velocity (VCL), average path velocity (VAP) and (DSL) (A-C) and the motion characteristics viz. the percentage of linearity i.e. the ratio between VSL and VCL (LIN), the straightness coefficient which is the ratio between VSL and VAP (STR) and progressive STR (PGSTR) (D-F) along with the mean amplitude of lateral head displacement (G-I) and beat cross frequency (J-L) were evaluated by Computer-assisted sperm analyzer (IVOS12.1, Hamilton-Thorne Biosciences, Beverly,MA, USA).

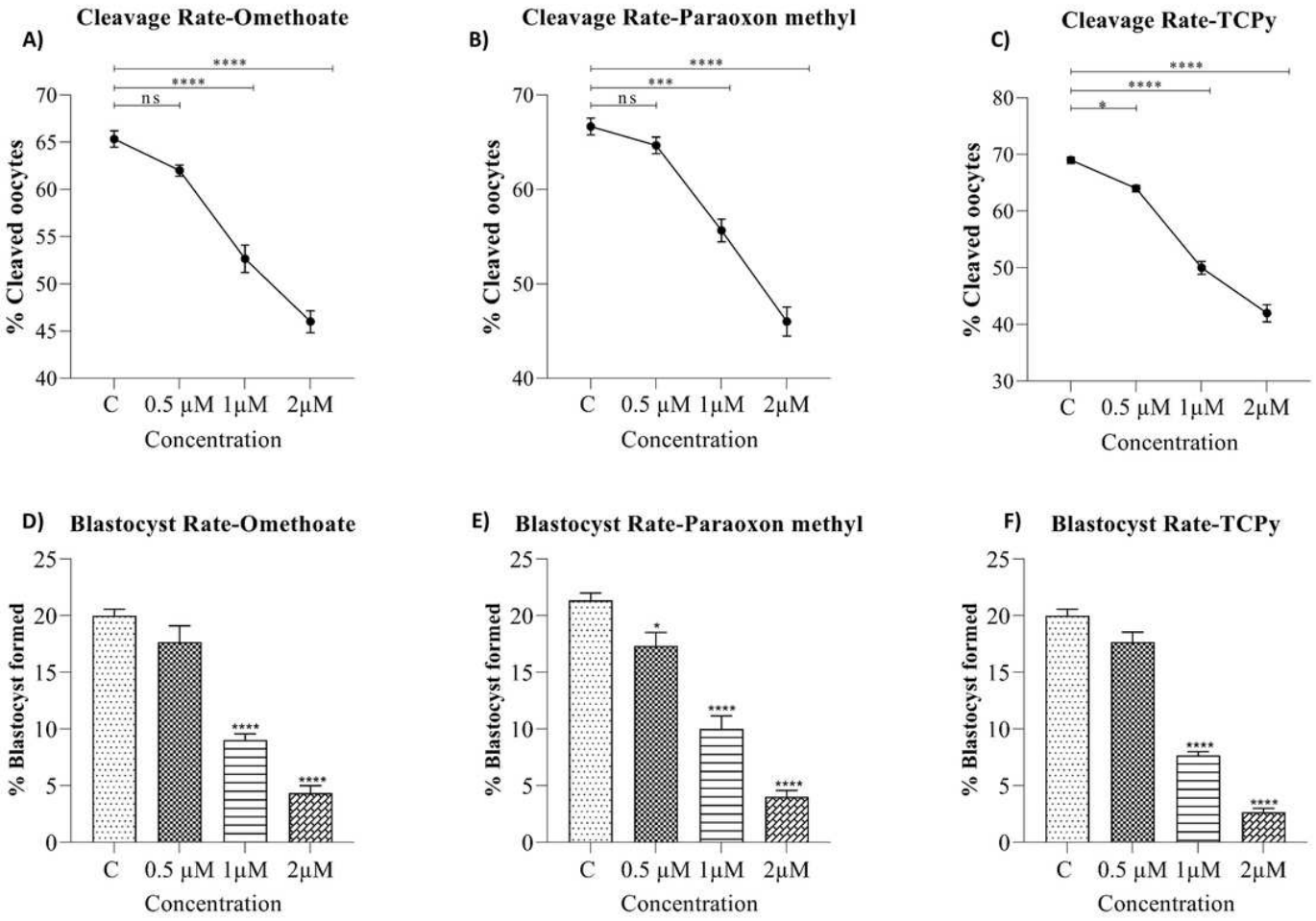

Figure 5

In vitro fertilization (Biomimetics) The mean ± SEM for cleavage rate (A-C), and blastocyst formation rates (D-F) in the control group and buffalo spermatozoa exposed to 0.5, 1, and 2µM of OPPM viz. Omethoate (Dimethoate)-A,D, Paraoxon methyl (Methyl parathion)-B, E, and TCPy (Chlorpyrifos)- (Fig. C, F).

Supplementary Files

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