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An evaluation of DNA damage in human lymphocytes and sperm exposed to methyl methanesulfonate involving the regulation pathways associated with apoptosis



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1 An evaluation of DNA damage in human lymphocytes and sperm exposed to methyl
2 methanesulfonate involving the regulation pathways associated with apoptosis

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19 Keywords: DNA damage, methyl methanesulfonate, genotoxicity, apoptotic
20 pathways.

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29 Highlights

- 30 • Human Lymphocytes and sperm cells exposure to MMS produced significant
31 DNA damage and apoptosis.
- 32 • DNA damage following MMS exposure *in vitro* was evaluated in the Comet
33 assay in both cell types.
- 34 • DNA damage and apoptosis resulted in increased P53 and decreased
35 CDKN1A and BCL-2.
- 36 • Human sperm were more susceptible to DNA damage than lymphocytes.
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100 Exposure to DNA-damaging agents produces a range of stress-related responses.

101 These change the expression of genes leading to mutations that cause cell cycle
102 arrest, induction of apoptosis and cancer. We have examined the contribution of
103 haploid and diploid DNA damage and genes involved in the regulation of the
104 apoptotic process associated with exposure, The Comet assay was used to detect
105 DNA damage and quantitative RT-PCR analysis (qPCR) to detect gene expression
106 changes in lymphocytes and sperm in response to methyl methanesulfonate. In the
107 Comet assay, cells were administered 0-1.2 mM of MMS at 37°C for 30 min for
108 lymphocytes and 32°C for 60 min for sperm to obtain optimal survival for both cell
109 types. In the Comet assay a significant increase in Olive tail moment (OTM) and %
110 tail DNA indicated DNA damage at increasing concentrations compared to the
111 control group. In the qPCR study, cells were treated for 4 hr, and RNA was isolated
112 at the end of the treatment. QPCR analysis of genes associated with DNA stress
113 responses showed that TP53 and CDKN1A are upregulated, while BCL-2 is
114 downregulated compared with the control. Thus, MMS caused DNA damage in
115 lymphocytes at increasing concentrations, but appeared not to have the same effect
116 in sperm at the low concentrations. These results indicate that exposure to MMS
117 increased DNA damage and triggered the apoptotic response by activating TP53,
118 CDKN1A and BCL-2. These findings of the processing of DNA damage in human
119 lymphocytes and sperm should be taken into account when genotoxic alterations in
120 both cell types are produced when monitoring human exposure.

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124 1. Introduction

125 The alkaline Comet assay is widely used for human biomonitoring, ecotoxicology
126 and routine genotoxicity assessment of chemicals. It has been used extensively to
127 assess DNA damage as single and double strand breaks and alkali-labile sites in the
128 whole genome of the individual cells (Anderson and Plewa, 1998; Tice et al.,
129 2000a). The connections between cell cycle and cell death have been studied and it
130 has been commonly found that cycling cells are more vulnerable to apoptosis, while
131 inactive cells are comparatively more resistant to killing (Pucci et al., 2000). It is
132 known that cancer treatments recruit additional cells into the commonly small growth
133 fraction of the tumour, so that cells could be vulnerable to chemotherapeutic drugs
134 (Hardwick and Soane, 2013). Cells treated with the methylating agent methyl
135 methanesulfonate (MMS) results in alkylated DNA that is badly replicated via DNA
136 polymerases *in vitro* and *in vivo* (Tercero and Diffley, 2001). This DNA damage
137 induced via genotoxic stress leads to changes in the expression of several critical
138 genes. The TP53 gene is the most relevant of these genes, also known as tumour
139 protein 53, which encodes for a 393 amino acid nuclear protein that functions as a
140 transcription factor p53 (Soussi et al., 1990). The p53 tumour suppressor gene is
141 important and included in cell cycle regulation, detection and repair of DNA damage,
142 apoptosis and senescence (Hamzehloie et al., 2012). The ability of p53 to induce
143 senescence or apoptosis of cells exposed to oncogenic stress establishes a main
144 pathway by which p53 functions as a tumour suppressor (Pietsch et al., 2008). Over
145 the past several decades, research has revealed that the p53 protein is superfluous
146 for normal progress but is essential for cellular response to DNA damage (Liu and
147 Kulesz-Martin, 2001; Liu et al., 2010a). The activity of p53 is firmly controlled at
148 insignificant levels in normal cells. p53 protein is rapidly induced by DNA damaging

149 stimuli such as UV light, chemical carcinogens and chemotherapeutic agents (Liu
150 and Kulesz-Martin, 2001; Purvis et al., 2012). The induction of p53 is attained during
151 a post-translational mechanism which decreases the p53 turnover. This p53
152 induction plays a crucial role in transcriptional activation of the cell cycle inhibitor p21
153 and cell cycle arrest (Wulf et al., 2002). The cyclin-dependent kinase (CDK) inhibitor
154 p21^{CDKN1A} is mostly controlled at the transcriptional level, while induction of p21
155 mainly leads to cell cycle arrest (Gartel and Radhakrishnan, 2005). In addition, p21
156 plays an important role in the inhibition of DNA replication during the proliferation of
157 the cell nuclear antigen PCNA (Perucca et al., 2006). The level of expression of p21
158 is up-regulated via the p53 tumour suppressor gene *in vitro*, in response to DNA-
159 damaging agents (Macleod et al., 1995; Benson et al., 2014). p21 mediates growth
160 arrest when cells are exposed to DNA damaging agents such as chemotherapy
161 drugs (Gartel and Radhakrishnan, 2005). Furthermore, p21 expression can be
162 regulated p53 independently in several situations involving cellular differentiation and
163 normal tissue development (Liu et al., 2010b). The members of the Bcl-2 family of
164 proteins are included in the regulation of apoptosis pathways as inducers and
165 inhibitors in many cell types (Hardwick and Soane, 2013). They are regulated and
166 mediate the process by which mitochondria contribute to cell death. This pathway is
167 required for normal embryonic development and for preventing cancer (Hardwick
168 and Soane, 2013). The Bcl2 protein also has important roles in normal cell
169 physiology associated with mitochondrial dynamics and other processes of normal
170 healthy cells (Hardwick and Soane, 2013).

171 In the present study, DNA damage was assessed using the Comet assay. The
172 expression of the apoptosis regulatory genes, *TP53*, *CDKN1A* and *BCL2* were

173 determined using qPCR methods in somatic and germ cells after MMS treatment of
174 human lymphocytes and sperm to determine effects in diploid and haploid cells.

175 2. Materials and Methods

176 2.1. Collection of semen and blood samples

177 Ethical approval for the collection of semen and blood samples has been provided by
178 the University of Bradford's Research Ethics Subcommittee involving human
179 subjects (reference number: 0405/8). After informed consent, peripheral blood from
180 four healthy, non-smoking volunteers (average age of 38 ± 6.7 years) was obtained
181 in heparinised vacutainers (Greiner-Bio-One, Germany) by venepuncture. Also, four
182 semen samples were provided and consented and each sample was analysed within
183 2 h after ejaculation according to the WHO criteria (World Health Organization, 1999)
184 for general appearance, viscosity, volume, pH, sperm concentration, motility and
185 morphology. After aliquoting, semen samples were snap-frozen in liquid nitrogen and
186 subsequently stored at $-80\text{ }^{\circ}\text{C}$ until analysis.

187 2.2. Lymphocyte isolation for the Comet assay

188 Whole blood was diluted 1:1 with saline and lymphocytes were isolated using of
189 Lymphoprep (Axis-Shield, Norway) according to the manufacturer's instructions. The
190 lymphocyte pellet was then resuspended in foetal bovine serum (FBS; Invitrogen,
191 UK) and transferred to a cryovials containing FBS/DMSO (9:1). This cell suspension
192 was frozen at $-20\text{ }^{\circ}\text{C}$ overnight and then transferred to $-80\text{ }^{\circ}\text{C}$ for storage before
193 use.

194

195 2.3. Cell treatment

196 Cell suspensions (1 ml, 10^6 cells/ml) were mixed with fresh Roswell Park Memorial
197 Institute (RPMI) 1640 Medium (total volume 1000 μ l). One hundred μ l of cell
198 suspension were then added to each treatment tube with, 890 μ l RPMI medium, plus
199 10 μ l of MMS or RPMI for the negative control). Cells were treated with different
200 concentrations (0, 0.3, 0.6, 0.8 and 1 mM) of MMS for 30 min at 37 °C (lymphocytes)
201 or for 60 min at 32 °C (sperm). The treated and untreated cells were used for the
202 Comet assay and quantitative reverse transcription PCR (RT-qPCR).

203 2.4. Cell viabilities

204 To prevent the effect of DNA degradation related to cytotoxicity, viability staining of
205 lymphocytes was performed prior to the experiments (Tice et al., 2000b). For both
206 lymphocytes and sperm, cell viability was measured by use of the Trypan blue
207 exclusion test (10 μ l of 0.05% Trypan blue added to 10 μ l of cell suspension (Pool-
208 Zobel et al., 1992). Viability was generally >90%, but always >75% (Henderson et
209 al., 1998).

210 2.5. Comet assay on sperm and lymphocytes

211 DNA damage was measured with the alkaline version of the Comet assay. In brief,
212 after treatment, cell samples were centrifuged and the supernatant was discarded.
213 To the cell pellet 100 μ l of 0.5% low melting agarose (LMP) (Invitrogen, Paisley, UK:
214 15517-022) was added. This cell suspension was transferred to slides pre-coated
215 with 1% normal melting point (NMP) agarose. For sperm, 2% LMP agarose was
216 used. The slides were placed on an ice block for 5 min, after which 100 μ l of 0.5%
217 LMP was added on top and slides were placed on ice for another 5 min. When using

218 lymphocytes, slides were placed in freshly prepared, cold lysing buffer (2.5 M NaCl,
219 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 and 10% DMSO added just
220 before use) and kept overnight at 4°C. For sperm, the lysis solution was
221 supplemented with 10 mM dithiothreitol (Sigma, UK) and 0.05 g/ml proteinase K
222 (Sigma, UK), respectively, and incubation took place in each solution for 1 h at 4 °C.
223 The slides were placed on a horizontal gel electrophoresis platform and covered with
224 an alkaline solution of 300 mM NaOH and 1 mM Na₂EDTA, pH ~13.5) for a pre-
225 incubation prior to electrophoresis. Electrophoresis was carried out for 30 min
226 (lymphocytes) or 20 min (spermatozoa) at 4 °C at ~0.75 V/cm (20-25V, ~300 mA).
227 The DNA was electrophoresed for 20 min and the slides rinsed gently 3 times with
228 400 mM Tris (pH 7.5) to neutralize the excess alkali. Each slide was stained with 60
229 µl of 20 µg/ml ethidium bromide (Sigma) and covered with a coverslip. Slides were
230 analyzed by a computerized image analysis system (Comet 6.0; Andor Technology,
231 Belfast, UK). In the Comet assay, Olive tail moment and % tail DNA were measured
232 as DNA damage parameters for sperm and lymphocytes. All of these steps were
233 conducted under dimmed light to prevent the occurrence of additional DNA damage.

234 2.6. Isolation of total RNA and cDNA synthesis

235 Total RNA from cells (lymphocytes and sperm) was isolated using TRIzol® following
236 the manufacturer's (Invitrogen) manual and RNA quantity and quality were checked
237 by OD_{260/280} measurements. To remove any genomic DNA, the RNA was treated
238 with DNase I (Sigma–Aldrich) according to the manufacturer's instructions. Random
239 hexamer primed reverse transcription reactions were performed for 400 ng of total
240 RNA in a 20 µl setup using ImProm-II™ Reverse Transcription System reaction
241 following the manufacturer's instructions (Promega). The synthesised cDNA samples
242 were diluted 1:10 in nuclease free water and stored at -20 °C.

243 2.7. Quantitative real-time PCR assay

244 Reactions were carried out using the StepOnePlus™ real-time PCR instrument
245 (Applied Biosystems). Quantitative real-time PCR was used to quantify the mRNA
246 expression of *TP53*, *CDKN1A* and *BCL2* in lymphocytes and sperm. QPCR was
247 prepared in triplicates of 20 µl reaction mixture in MicroAmp optical 96-well reaction
248 plates and sealed with optical adhesive covers (Applied Biosystems). Each reaction
249 well contained 2 µl of template DNA, 2 µl of 10 × SYBR® Green PCR Master Mix
250 (Applied Biosystems), and 12.5 pmol each of forward and reverse primers. Real-time
251 qPCR was conducted with the following cycling conditions: 50 °C for 2 min, 95 °C for
252 20 s, followed by 50 cycles of 95 °C for 15 s and 60 °C for 30 s each. The data
253 obtained from each reaction was analysed by StepOne™ Software v 2.2.2. Relative
254 quantification representing the change in gene expression from real-time quantitative
255 polymerase chain reaction between experimental groups was calculated by the
256 comparative C_T method. The data were analysed by calculating the relative
257 quantification (RQ) using the equation: $RQ = 2^{-\Delta C_T} \times 100$, where $\Delta C_T = C_T$ of target
258 gene- C_T of an endogenous housekeeping gene. Evaluation of $2^{-\Delta C_T}$ indicates the fold
259 change in gene expression, normalized to the internal control (β -actin) which enables
260 the comparison between differently treated cells.

261 3. Results

262 The responses of human lymphocytes to MMS for the Comet assay parameters
263 Olive tail moment (OTM) and percent DNA in tail (% tail DNA) are shown in Table 1,
264 Fig.1 and 2. A significant increase was seen in tail moment and % tail DNA in the
265 lymphocytes from 5.70 (OTM) and 22.42% (% tail DNA) compared to the untreated
266 control groups to 1.49 (OTM) 7.65% (% tail DNA), respectively, when cells were

267 treated with 0.6 mM MMS. Further increases to 6.97 in (OTM) and 27.57% in (% tail
268 DNA) were observed when cells were treated with 0.8 mM MMS. At 1.2 mM, in the
269 OTM and % tail DNA further increased to 11.00 and 36.71% respectively. For sperm,
270 the corresponding mean tail moments increased from 4.93 in control to 6.28 at 0.3
271 mM and 8.44 at 0.6 mM. After treatment, significant increases in tail moment of the
272 nuclei were seen (Table 1, Fig. 1 and 2). This significant increase remained at
273 approximately the same level in OTM to a final concentration of 1.2 mM MMS. The
274 same significant MMS induction of DNA damage could also be seen when the % tail
275 DNA was considered, as increases from 27.98 % in control to 34.68% (at 0.3 mM)
276 and 39.60% (at 0.6 mM) were observed. Following exposure to 0.8 mM, cells treated
277 with 0.8 mM MMS showed statistically significant increased % tail DNA damage to
278 46.61%, when compared with the control. A further increase to 51.15% in % tail DNA
279 was observed when cells were treated with 1 mM MMS.

280 For the qPCR assay, different levels of expression of TP53, CDKN1A and BCL2
281 mRNA in lymphocytes were seen after treatment with different concentrations of
282 MMS. The samples were taken at 4 h following MMS treatment for both treated and
283 untreated control cultures, and the expression levels of TP53, CDKN1A and BCL2
284 were normalised against those of β -actin and compared with the equivalent control
285 value.

286 Fig. 3 and 4 shows RT-PCR results of different apoptotic genes after lymphocytes
287 and sperm cells were treated with MMS. When the MMS concentration was
288 increased from 0 to 1.2 mM, the band intensities for TP53 and CDKN1A were found
289 to be increased while the intensities for bands of BCL2 were found to be decreased
290 with the increased MMS concentration. To ensure even loading of the total proteins,

291 the β -actin was used. Fig. 3B and 4B show mRNA expression of TP53 and
292 CDKN1A, and BCL2 in human lymphocytes. The expression levels of these genes
293 were evaluated by the qPCR.

294 There were statistically significant differences in the levels of TP53 and CDKN1A
295 after 4h of treatment with 0.3, 0.6, 0.8 mM and 1.2 mM MMS in both lymphocytes
296 and sperm. However, a significant decrease in the level of expression of BCL2 in
297 both cell types treated with 0.3, 0.6, 0.8 mM and 1.2 mM MMS (* p 0.05, ** p 0.01 and
298 *** p 0.001) and respectively as shown in Fig. 3 A and B and 4 A and B.

299 4. Discussion

300 Methyl methanesulfonate was the chemical of choice for the induction of DNA
301 damage in human lymphocytes and sperm as a well-known genotoxic compound
302 that can directly react with guanine and adenine bases of DNA to generate
303 interstrand and intrastrand cross-links (Hosseinimehr et al., 2011). During cell
304 division, however, the replication fork could be stalled and collapses at the sites of
305 DNA cross-links, leading to formation and subsequent processing of DNA double
306 strand-breaks (DSB), which are considered the most deleterious form of DNA
307 damage (Yu et al., 2006). Through obstructing the structural and functional
308 properties of DNA, DSBs can have deleterious effects on many aspects of DNA
309 metabolism, including DNA replication and transcription, and because they can
310 eventually cause mutations and chromosomal aberrations (Shanbhag et al., 2010;
311 Polo and Jackson, 2011). Double strand-breaks can also create various signal
312 transduction pathways that can ultimately result in cell tumorigenesis, to
313 programmed cell death (Suwaki et al., 2011). These DNA strand breaks inducing
314 programmed cell death is a crucial event for numerous regular chemotherapeutic

315 agent applications (Waxman and Schwartz, 2003). Programmed DNA lesions also
316 form as intermediates through developmentally regulated genome rearrangements in
317 germ cells and somatic cells (Longhese et al., 2009; Tsai and Lieber, 2010). The
318 induction of DNA breaks and the changed expression of the apoptosis regulatory
319 genes, *TP53*, *CDKN1A* and *BCL2* by MMS were assessed using the comet and
320 qPCR assays on human lymphocytes and sperm. For the Comet assay, DNA
321 damage response patterns for the OTM and % tail DNA Comet-assay parameters
322 were observed for both cell types (Tables 1 and 2); however, sperm additionally
323 showed a significant increase in OTM and % tail DNA after being exposed to lower
324 concentrations of 0.3 mM for both OTM and % tail DNA (Fig.1). MMS genotoxicity on
325 germ cells has been well studied and described in numerous *in vivo* studies reporting
326 the induction of chromatin alterations also dominant lethal mutations and heritable
327 translocations in sperm (Ehling and Neuhauser-Klaus, 1990; Russell et al., 1992;
328 Cordelli et al., 2007). This suggests that the damage to the spermatozoa DNA was
329 potentially introduced by inhibiting replication, causing formation of replication-
330 related to DNA lesions, and potentially DSBs. Late spermatids and immature
331 spermatozoa are most sensitive to MMS due to the absence of DNA repair during
332 postmeiotic stages (Inoue et al., 1993). MMS also showed significantly increased
333 concentration-dependent responses in lymphocytes for the Comet assay
334 parameters. OTM values significantly increased with the MMS concentration of 0.6
335 mM. This significant increase continued to stay at approximately the same level up to
336 concentrations of 0.8 mM and 1.2 mM MMS (Table 1). This positive result is similar
337 to results of Baohong et al. (2005), where earlier significantly increased incidences of
338 DNA damage were observed in human lymphocytes after *in vitro* treatment with
339 MMS using the Comet assay (Baohong et al., 2005). Our results show that sperm

340 reach significance at a lower threshold of sensitivity with lower concentrations of
341 MMS. This may be due to the fact that they are unable to repair damaged DNA and
342 they are structurally different. This has been previously shown for other chemicals
343 (Baumgartner et al., 2012). In another study, it has been reported that DNA damage
344 was evaluated in human lymphocytes and sperm, highly increased DNA damage in
345 sperm was observed when compared with the response in lymphocytes using the
346 alkaline comet assay *in vitro* (Anderson et al., 2003; Migliore et al., 2006; Pandir,
347 2015). In contrast to somatic cells, sperm protamines contain a significant number of
348 cysteine residues which are essential in the last stage of sperm nuclear maturation
349 as they form protamine disulfide cross bonds (Loir and Lanneau, 1984). This S-
350 methyl-L-cysteine group is the major reaction product after exposure to MMS (Sega
351 and Owens, 1983). Alkylation of cysteine sulfhydryl groups contained in sperm
352 protamine blocks normal disulfide bond formation, preventing proper chromatin
353 condensation in the sperm nucleus. Subsequent stresses produced in the chromatin
354 structure eventually lead to chromosome breakage, with resultant dominant lethality
355 (Sega and Owens, 1983). The results also showed that the defective spermatid
356 protamination and disulphide bridge formation could be attributable to insufficient
357 oxidation of alkylation groups. This destructively affects sperm chromatin packaging
358 and creates sperm cells more susceptible to reactive oxygen species (ROS) while
359 subsequently inducing DNA fragmentation. Lymphocytes, however, seem to be less
360 susceptible to MMS during the cell cycle. This implies that less damage to the DNA
361 from lymphocyte was seen due to repair of DNA damage before replication start.
362 Fast repair of DNA damage was observed in human lymphocytes during the first
363 hours of cultivation after treatment with MMS using the comet assay (Bausinger and
364 Speit, 2015). Mammalian cell responses to several stresses fluctuate importantly;

365 reliant on the type of cells exposed to stress and time and type of toxicant exposure.
366 MMS induces apoptosis during the activation of p53-dependent and independent
367 pathways (Lackinger et al., 2001; Ryu et al., 2001). In agreement with these studies,
368 our data showed that for both cell types, after 4h treatment with MMS (0.3, 0.6, 0.8
369 and 1.2 mM), *TP53* and *CDKN1A* were induced and *BCL2* expression was
370 downregulated in a dose-dependent manner. The p53 plays a key role in the
371 regulation of cell cycle events (Sionov et al., 2000). In response to DNA damage,
372 p53 is activated and turns on the transcription of one of its important downstream
373 genes, p21 (el-Deiry et al., 1993). p21 subsequently binds and inhibits, preventing
374 phosphorylation of important CDK substrates and blocking cell cycle development, so
375 allowing further time for the cell to repair DNA damage (Ouhtit et al., 2000). Our
376 findings that MMS induction of *TP53* led to the induction of the *CDKN1A* gene (Fig. 3
377 and 4) implies that MMS induces *TP53*, which, in turn, activates *CDKN1A* and
378 results in cell cycle arrest to allow the repair of induced DNA damage. These results,
379 also combined with the disruption of mitochondrial membrane permeabilization,
380 release of cytochrome c from mitochondria, and downregulation of *BCL2*, indicate
381 that the accumulation of DSB contributes to the induction of mitochondria-dependent
382 cell apoptosis under these experimental conditions.

383 5. Conclusions

384 The present study reveals the effects of MMS on human somatic cells and germ
385 cells and provides significant insight into potential mechanisms through which MMS
386 exerts its genotoxic effects on these cells. In addition to the Comet assay data
387 evaluation of DNA damage via qPCR data using differential expression analysis of
388 *TP53*, *CDKN1A* and *BCL-2* genes have provided the evidence for the genotoxic

389 effects of MMS in healthy human lymphocytes and sperm. Thus, the sperm appear
390 to be more sensitive to MMS. Despite the differences in cell packaging of the two cell
391 types, they were examined at optimal conditions of survival for both types, so can
392 more readily be made.

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397 Conflict of Interest Statement

398 The authors have no conflicts of interest with regard to the funding of this research.

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547 Figure legends

548 Fig1. Comet assay results obtained from exposure of 0, 0.3, 0.6, 0.8 and 1.2 mM
549 concentrations of MMS to lymphocytes and sperm cells. Comet parameters, % tail
550 DNA were taken into account to measure DNA damage showing a clear
551 concentrations related increase in DNA damage. All experiments were performed at
552 least three times. Mean values \pm SE. * = comparison with negative control. * P <
553 0.05, ** P < 0.01, *** P < 0.001.

554 Fig 2. Comet assay results obtained from exposure of 0, 0.3, 0.6, 0.8 and 1.2 mM
555 concentrations of MMS to lymphocytes and sperm cells. Comet parameters, OTM
556 were taken into account to measure DNA damage showing a clear concentrations
557 related increase in DNA damage. All experiments were performed at least three
558 times. Mean values \pm SE. * = comparison with negative control. * P < 0.05, ** P <
559 0.01, *** P < 0.001.

560 Fig 3. Concentration-dependent effects of MMS on *TP53*, *CDKN1A*, and *BCL-2*
561 mRNA expression levels in lymphocyte cells, treated with different concentrations of
562 MMS (0, 0.3, 0.6 and 1.2 mM) for 4 h. mRNA expression levels were determined by
563 qPCR. β -actin mRNA was used as an internal control. (A) The relative gene
564 expression level of *TP53*, *CDKN1A*, and *BCL-2*, analyzed from the qPCR results. (B)
565 The mRNA of lysed cells was extracted and was converted to cDNA. The gene
566 expression levels of *TP53*, *CDKN1A*, and *BCL-2* were evaluated by reverse-
567 transcription PCR. β -actin mRNA was used as the internal control. The data shown
568 are representative of three independent experiments. The significant differences
569 from control are indicated by * P < 0.05, ** P < 0.01, *** P < 0.001.

570 Fig 4. Concentration-dependent effects of MMS on *TP53*, *CDKN1A*, and *BCL-2*
571 mRNA expression levels in sperm cells, treated with different concentrations of MMS
572 (0, 0.3, 0.6 and 1.2 mM) for 4 h. mRNA expression levels were determined by qPCR.
573 β -actin mRNA was used as an internal control. (A) The relative gene expression
574 level of *TP53*, *CDKN1A*, and *BCL-2*, analyzed from the qPCR results. (B) The mRNA
575 of lysed cells was extracted and was converted to cDNA. The gene expression levels
576 of *TP53*, *CDKN1A*, and *BCL-2* were evaluated by reverse-transcription PCR. β -actin
577 mRNA was used as the internal control. The data shown are representative of three
578 independent experiments. The significant differences from control are indicated by
579 * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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598 Table 1. Concentrations-response of MMS in human lymphocytes and sperm was measured
599 using the alkaline Comet assay with the parameters Olive tail moment (OTM) and % tail
600 DNA. Data shown represents group values (mean \pm SE) of three experiments (100 cells per
601 experiment). Ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control.

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Exposure concentrations for lymphocytes	Olive Tail moment	%Tail DNA Mean \pm
	Mean \pm SE	SE
Control	1.49 \pm 0.14	7.65 \pm 0.82
0.3 mM	3.14 \pm 0.46	14.97 \pm 1.65
0.6 mM	5.71 \pm 0.84 *	22.42 \pm 2.14 *
0.8 mM	6.97 \pm 1.21 **	27.57 \pm 2.36 *
1.2 mM	11.00 \pm 1.34 **	36.71 \pm 3.73 **

Exposure concentrations for sperm	Olive Tail moment	%Tail DNA Mean \pm
	Mean \pm SE	SE
Control	4.93 \pm 0.26	27.98 \pm 1.69
0.3 mM	6.28 \pm 0.44 *	34.68 \pm 0.54 *
0.6 mM	8.44 \pm 0.58 *	39.66 \pm 2.85 **
0.8 mM	10.11 \pm 0.43 **	46.61 \pm 2.13 **
1.2 mM	11.58 \pm 0.14 ***	51.15 \pm 3.62 ***

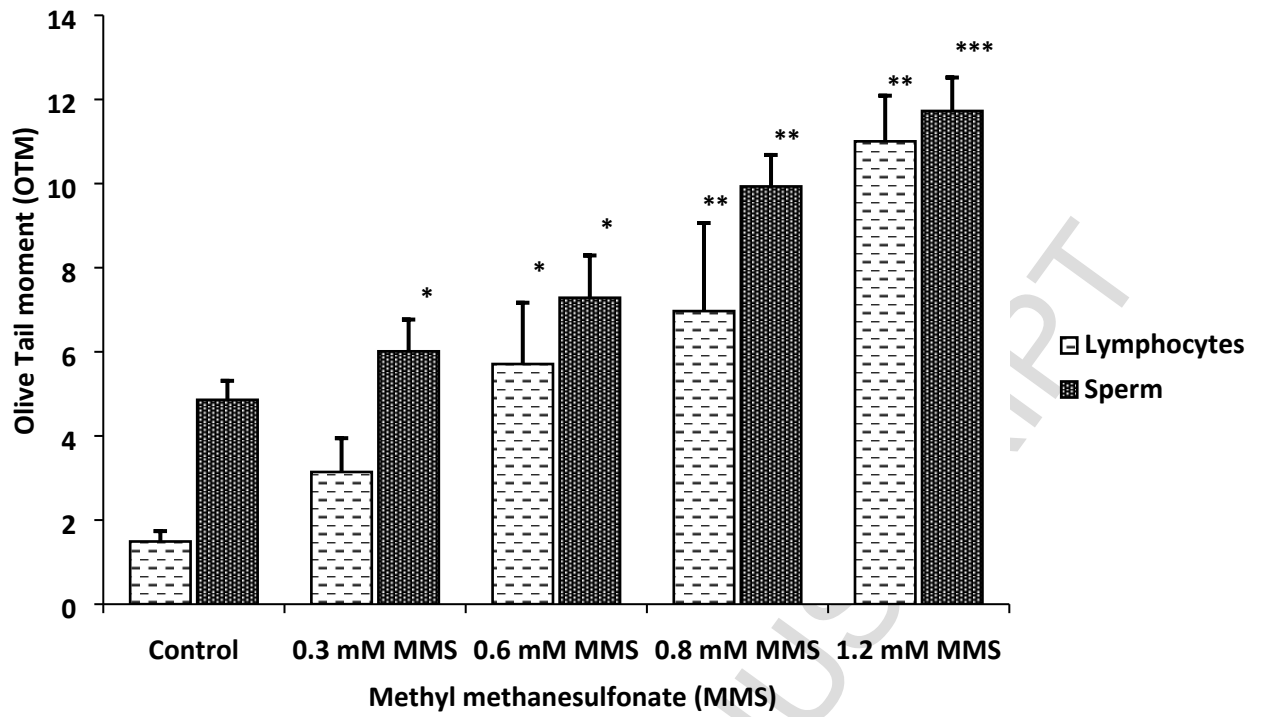
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605 Fig 1

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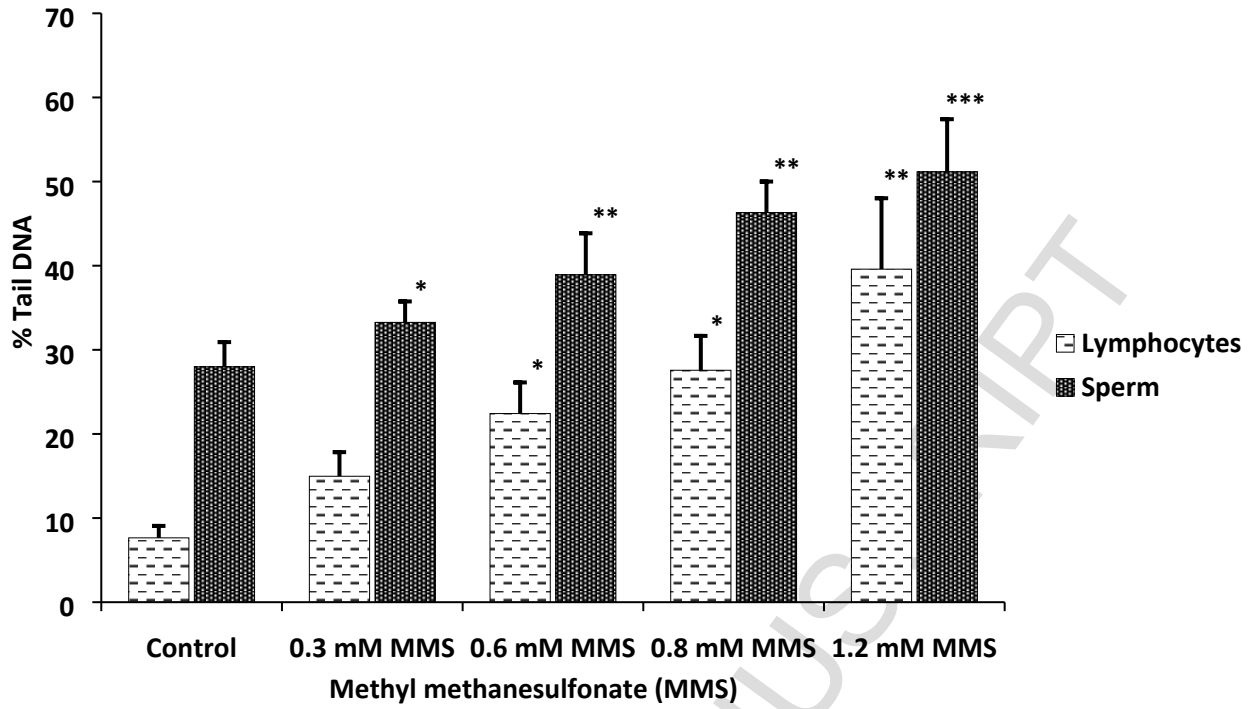
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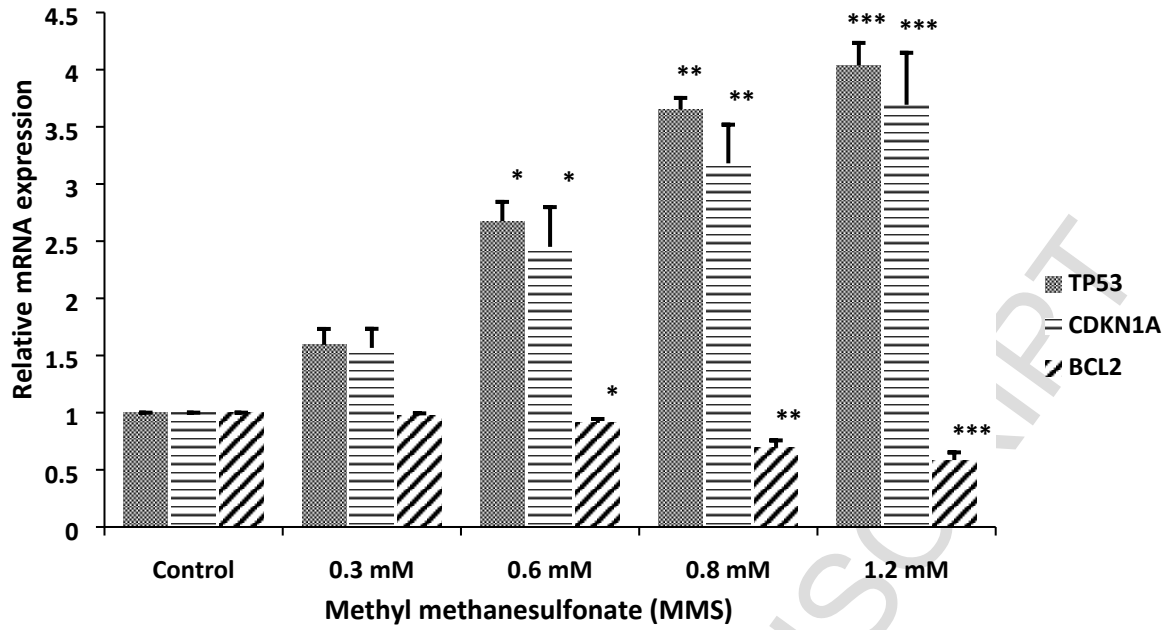
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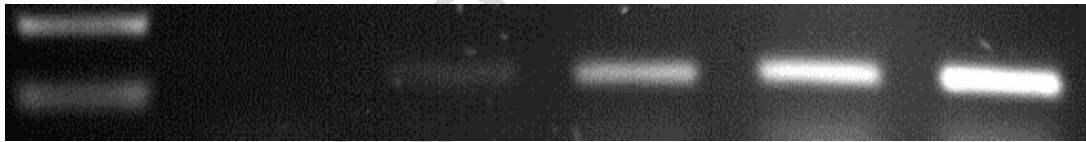
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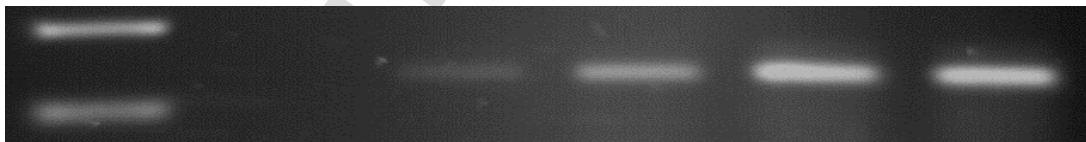
DNA Ladder 0 mM 0.3 mM 0.6 mM 0.8 mM 1.2 mM

635 TP53

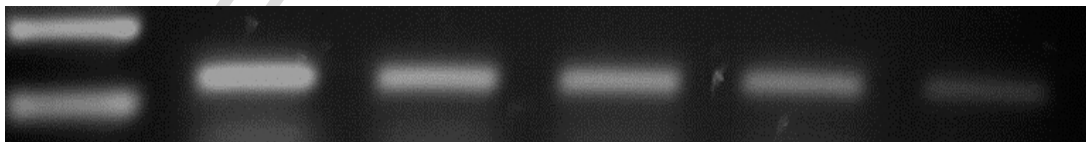


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637 CDKN1A



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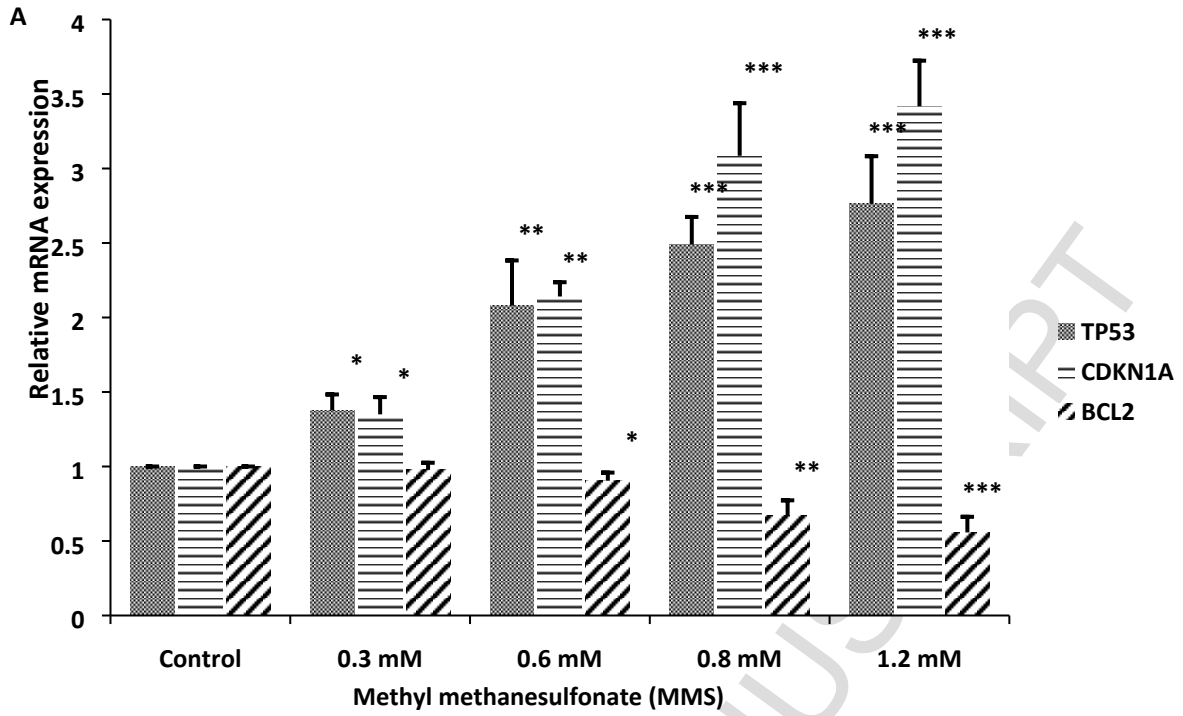
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640 β-actin



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642 Fig 4



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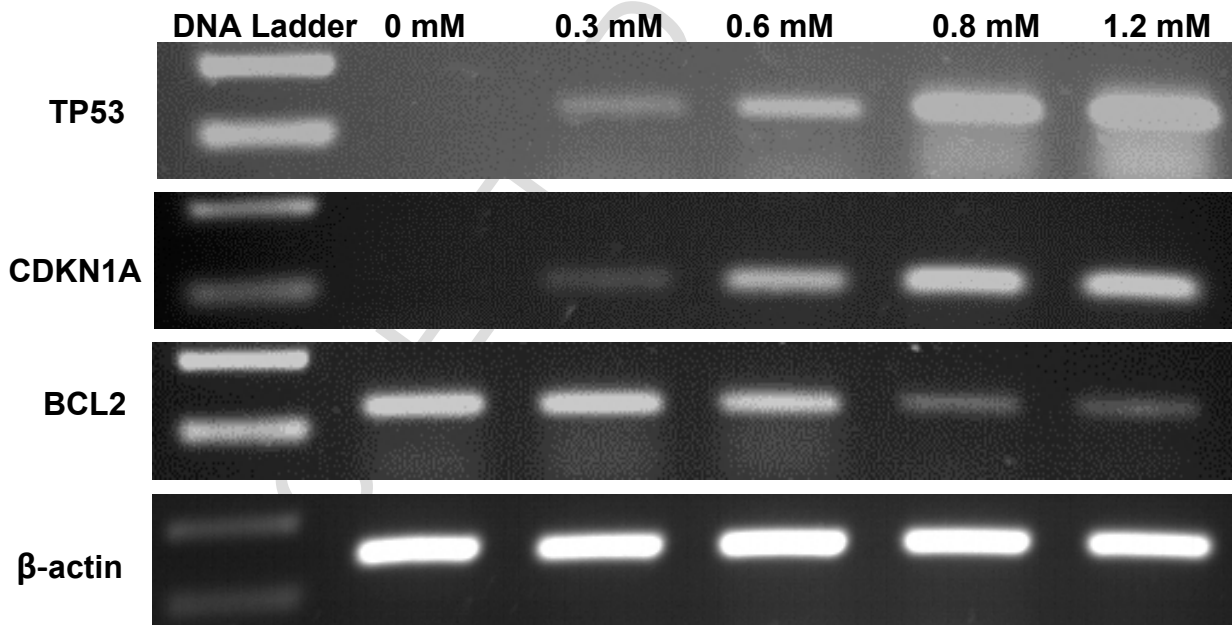
645 **B**

Table 1. Concentration-responses of MMS in human lymphocytes and sperm were measured using the alkaline Comet assay with Olive tail moment (OTM) and % tail DNA. Data shown represents group values (mean \pm SE) of three experiments (100 cells per experiment). Ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control.

Lymphocytes		
Concentrations	Olive Tail moment Mean \pm SE	%Tail DNA Mean \pm SE
Control	1.49 \pm 0.14	7.65 \pm 0.82
0.3 mM	3.14 \pm 0.46	14.97 \pm 1.65
0.6 mM	5.71 \pm 0.84 *	22.42 \pm 2.14 *
0.8 mM	6.97 \pm 1.21 **	27.57 \pm 2.36 *
1.2 mM	11.00 \pm 1.34 **	36.71 \pm 3.73 **
Sperm		
Control	4.93 \pm 0.26	27.98 \pm 1.69
0.3 mM	6.28 \pm 0.44 *	34.68 \pm 0.54 *
0.6 mM	8.44 \pm 0.58 *	39.66 \pm 2.85 **
0.8 mM	10.11 \pm 0.43 **	46.61 \pm 2.13 **
1.2 mM	11.58 \pm 0.14 ***	51.15 \pm 3.62 ***