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# Accepted Manuscript

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

## 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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66  
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.

## 1. Introduction

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

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

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

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

## 2. Materials and Methods

### 2.1. Collection of semen and blood samples

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

### 2.2. Lymphocyte isolation for the Comet assay

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



### 2.3. Cell treatment

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

### 2.4. Cell viabilities

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

### 2.5. Comet assay on sperm and lymphocytes

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

lymphocytes, slides were placed in freshly prepared, cold lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 and 10% DMSO added just before use) and kept overnight at 4°C. For sperm, the lysis solution was supplemented with 10 mM dithiothreitol (Sigma, UK) and 0.05 g/ml proteinase K (Sigma, UK), respectively, and incubation took place in each solution for 1 h at 4 °C. The slides were placed on a horizontal gel electrophoresis platform and covered with an alkaline solution of 300 mM NaOH and 1 mM Na<sub>2</sub>EDTA, pH ~13.5) for a pre-incubation prior to electrophoresis. Electrophoresis was carried out for 30 min (lymphocytes) or 20 min (spermatozoa) at 4 °C at ~0.75 V/cm (20-25V, ~300 mA). The DNA was electrophoresed for 20 min and the slides rinsed gently 3 times with 400 mM Tris (pH 7.5) to neutralize the excess alkali. Each slide was stained with 60 µl of 20 µg/ml ethidium bromide (Sigma) and covered with a coverslip. Slides were analyzed by a computerized image analysis system (Comet 6.0; Andor Technology, Belfast, UK). In the Comet assay, Olive tail moment and % tail DNA were measured as DNA damage parameters for sperm and lymphocytes. All of these steps were conducted under dimmed light to prevent the occurrence of additional DNA damage.

## 2.6. Isolation of total RNA and cDNA synthesis

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

## 2.7. Quantitative real-time PCR assay

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

## 3. Results

The responses of human lymphocytes to MMS for the Comet assay parameters Olive tail moment (OTM) and percent DNA in tail (% tail DNA) are shown in Table 1, Fig.1 and 2. A significant increase was seen in tail moment and % tail DNA in the lymphocytes from 5.70 (OTM) and 22.42% (% tail DNA) compared to the untreated 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  $\beta$ -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,

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

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

#### 4. Discussion

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

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

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

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

## 5. Conclusions

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



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

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#### Conflict of Interest Statement

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

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## Figure legends

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

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

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

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

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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 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 **

Exposure concentrations for sperm	Olive Tail moment Mean $\pm$ SE	%Tail DNA Mean $\pm$ 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 ***

Fig 1



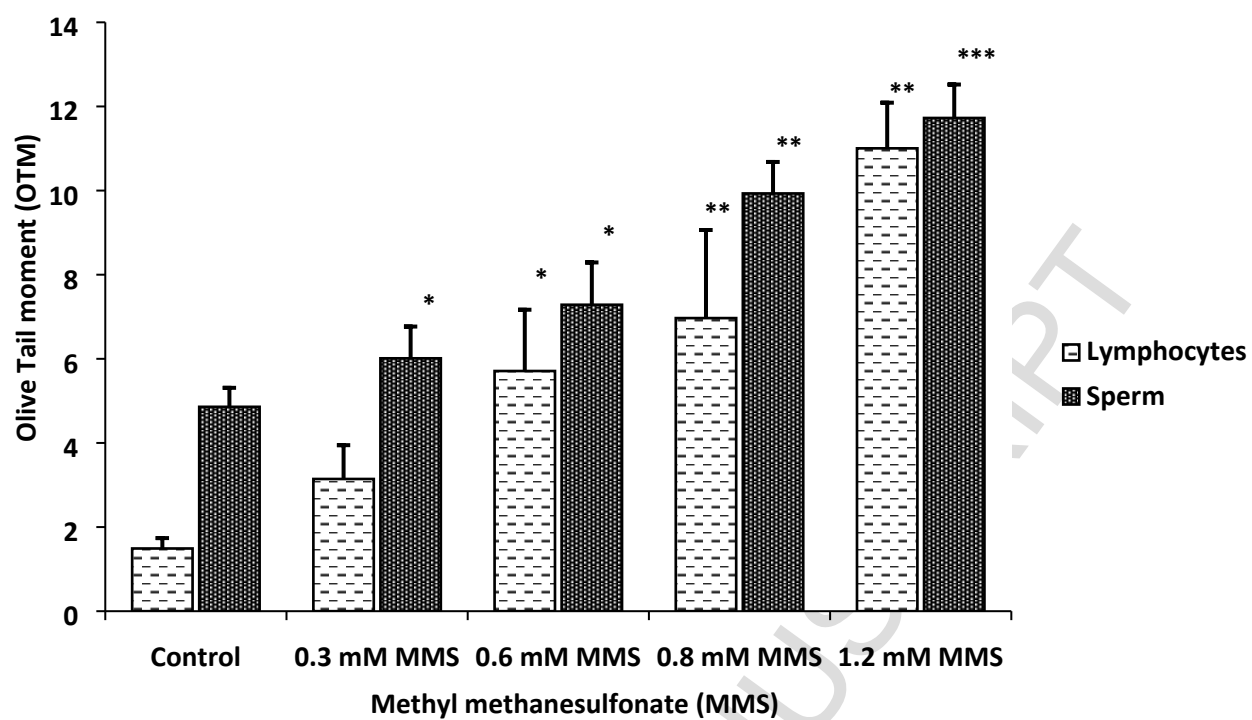


Fig 2

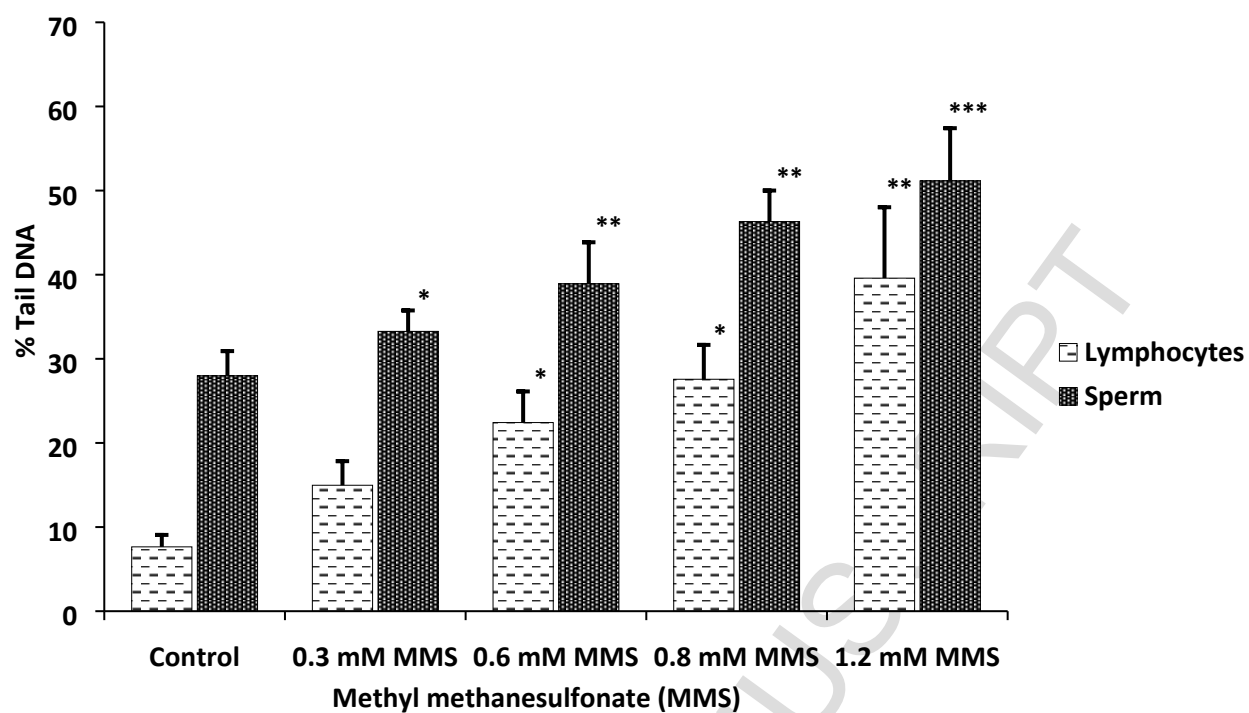
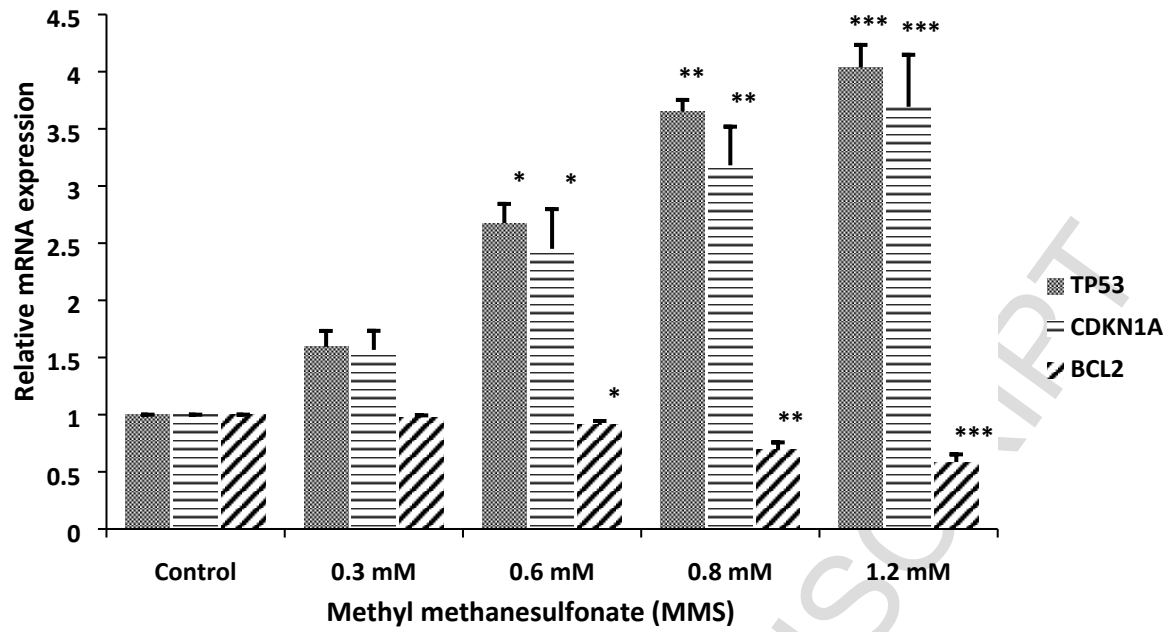


Fig 3

A



B

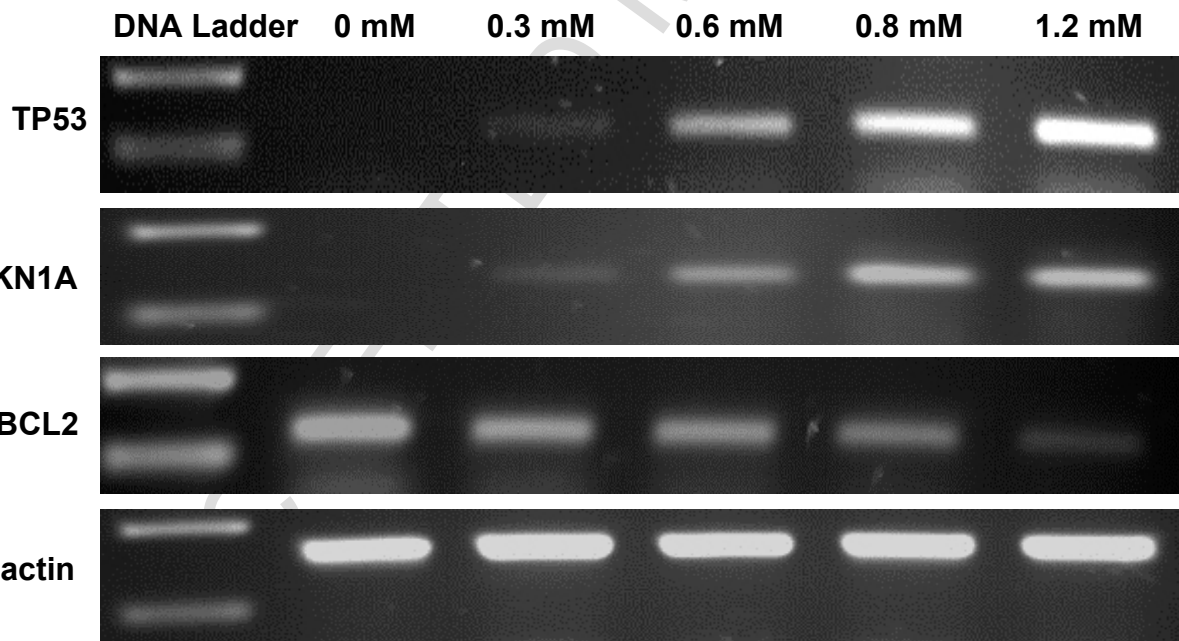
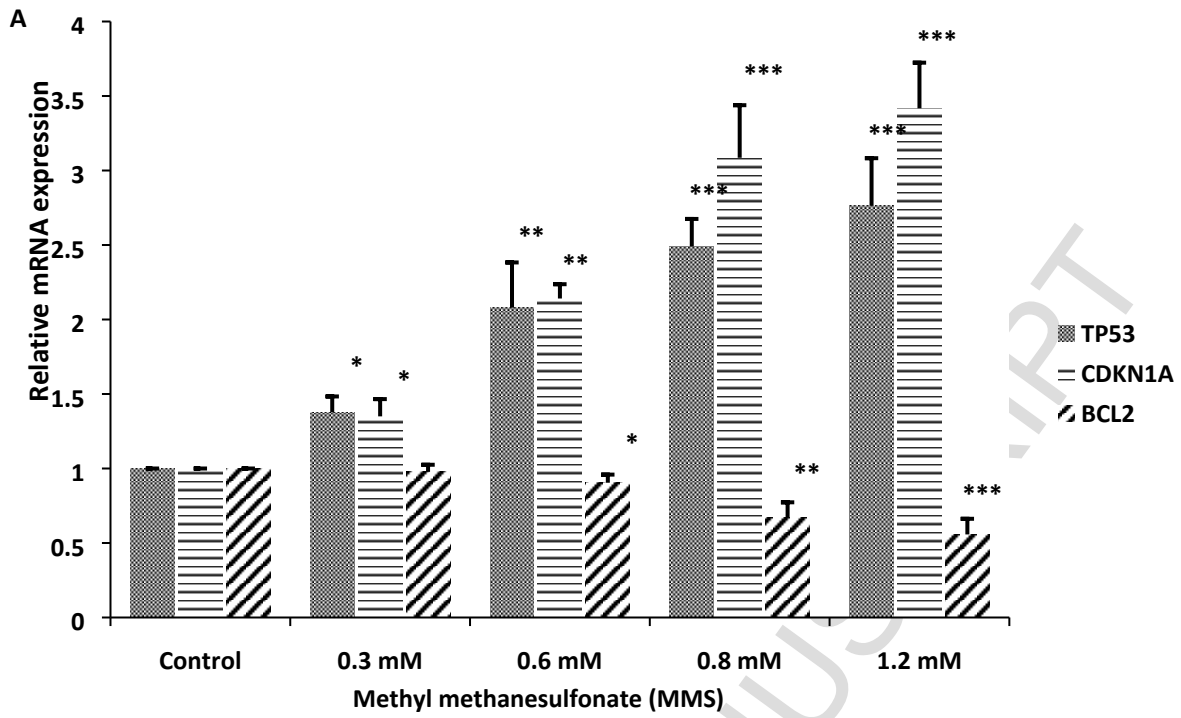


Fig 4



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645 **B**

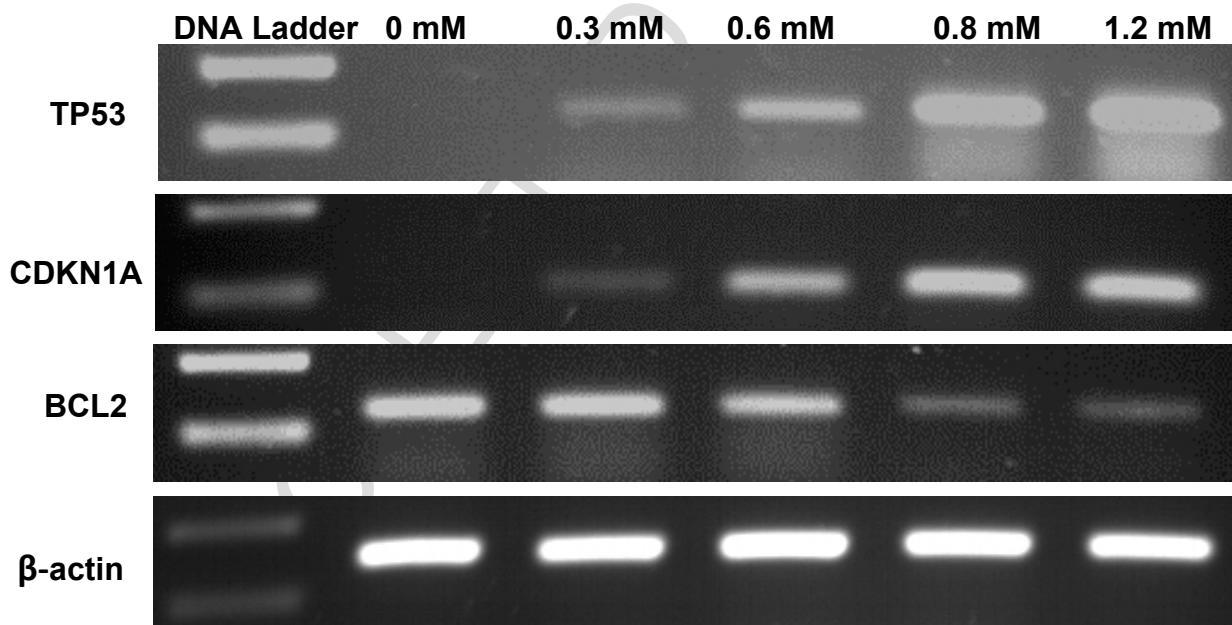


Table 1. Concentration-responses of MMS in human lymphocytes and sperm were measured using the alkaline Comet assay with Olive tail moment (OTM) and % tail DNA. Data shown represents group values (mean  $\pm$  SE) of three experiments (100 cells per experiment). Ns not significant, \* $P < 0.05$ , \*\*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