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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Abstract

Exposure to DNA-damaging agents produces a range of stress-related responses. These change the expression of genes leading to mutations that cause cell cycle arrest, induction of apoptosis and cancer. We have examined the contribution of haploid and diploid DNA damage and genes involved in the regulation of the apoptotic process associated with exposure, The Comet assay was used to detect DNA damage and quantitative RT-PCR analysis (qPCR) to detect gene expression changes in lymphocytes and sperm in response to methyl methanesulfonate. In the Comet assay, cells were administered 0 -1.2 mM of MMS at 37 $^{\circ}$C for 30 min for lymphocytes and 32 $^{\circ}$C for 60 min for sperm to obtain optimal survival for both cell types. In the Comet assay a significant increase in Olive tail moment (OTM) and % tail DNA indicated DNA damage at increasing concentrations compared to the control group. In the qPCR study, cells were treated for 4 h, and RNA was isolated at the end of the treatment. qPCR analysis of genes associated with DNA stress responses showed that TP53 and CDKN1A are upregulated, while BCL2 is downregulated compared with the control. Thus, MMS caused DNA damage in lymphocytes at increasing concentrations, but appeared not to have the same effect in sperm at the low concentrations. These results indicate that exposure to MMS increased DNA damage and triggered the apoptotic response by activating TP53, CDKN1A and BCL2. These findings of the processing of DNA damage in human lymphocytes and sperm should be taken into account when genotoxic alterations in both cell types are produced when monitoring human exposure.

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. 2000).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, Kasten, and Giordano 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, Caron de Fromentel, and May 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, researcher revealed that the p53 protein is superfluous for normal progress but is essential in cellular response to DNA damage (Liu and Kulesz-Martin 2001; Liu, Chung, et al. 2010). 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^{CDKN1A} 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 by inhibition of DNA replication during relation with 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, Hou, et al. 2010). The members of the Bcl-2 family of proteins are included in the regulation of apoptosis pathways as inducer and inhibitor 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.

Materials and Methods

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 ± 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 $-80 \,^{\circ}C$ until analysis.

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 $-20 \,^{\circ}C$ overnight and then transferred to $-80 \,^{\circ}C$ for storage before use.

Cell treatment

Cell suspensions (1 ml, 10^6 cells/ml) were mixed with fresh Roswell Park Memorial Institute (RPMI) 1640 Medium (total volume 1000 µl). One hundred µl of cell suspension were then added to each treatment tube with, 890 µl RPMI medium, plus 10 µl 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).

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. 2000). For both lymphocytes and sperm, cell viability was measured by use of the Trypan blue exclusion test (10 µl of 0.05% Trypan blue added to 10 µl of cell suspension (Pool-Zobel et al. 1992). Viability was generally >90%, but always >75% (Henderson et al. 1998).

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 µl 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 µl of 0.5% LMP was added on top and slides were placed on ice for another 5 min. When using

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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_2EDTA, 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.

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 $OD_{260/280}$ 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.

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 = $2^{-\Delta CT}$ x100, where ΔC_T = C_T of target gene- C_T of an endogenous housekeeping gene. Evaluation of $2^{-\Delta CT}$ indicates the fold change in gene expression, normalized to the internal control (β-actin) which enables the comparison between differently treated cells.

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

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

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

total proteins, the β-actin was used. Figure 3 B and 4 B 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 cells 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 Figures 3 A and B and 4 A and B.

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). DSBs can also create various signal transduction pathways that can ultimately result in cell tumorigenesis, to programmed cell death (Suwaki, Klare, and Tarsounas 2011). These DNA strand breaks inducing programmed cell death is a crucial event for numerous regular chemotherapeutic agent applications (Waxman

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and Schwartz 2003). Programmed DNA lesions also form as intermediates through developmentally regulated genome rearrangements in germ cells and somatic cells (Tsai and Lieber 2010; Longhese et al. 2009).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 (Figure1). 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 (Russell et al. 1992; Ehling and Neuhauser-Klaus 1990; 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 double-strand breaks. 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 others 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; Pandir 2015; Migliore et al. 2006). 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, Eichhorn, and Kaina 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, Hayon, and Haupt 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 (Figures 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 BLC2, indicate that the accumulation of DSB contributes to the induction of mitochondria-dependent cell apoptosis under these experimental conditions.

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 BCL2 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.

Acknowledgement

Conflict of Interest Statement

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Figure and table legends

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

Figure 1. 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 ± SE. * = comparison with negative control. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 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 ± SE. * = comparison with negative control. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 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. β-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. β-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.

Figure 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.

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β-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. β-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 *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 1

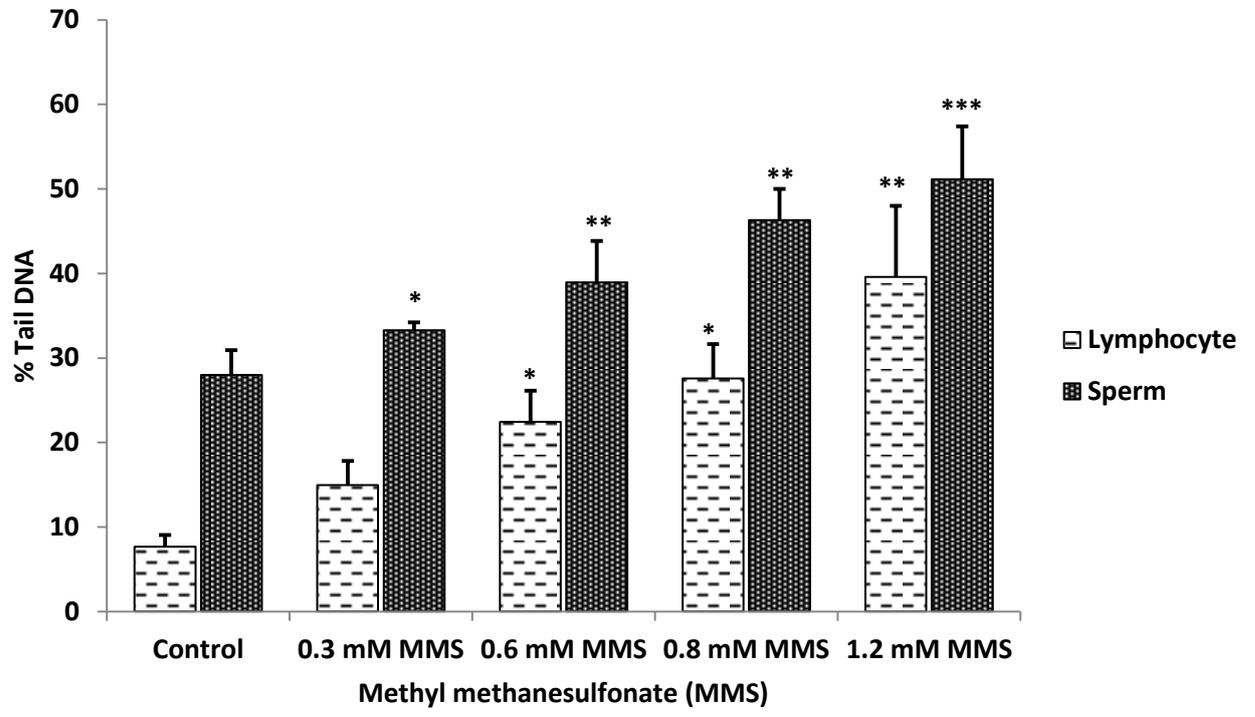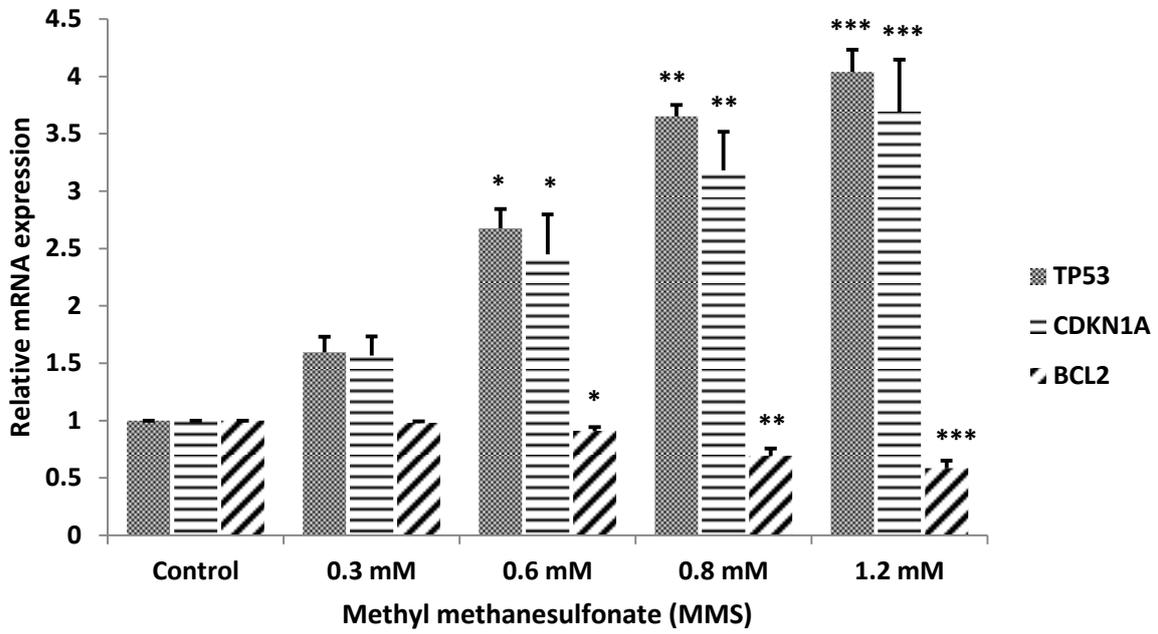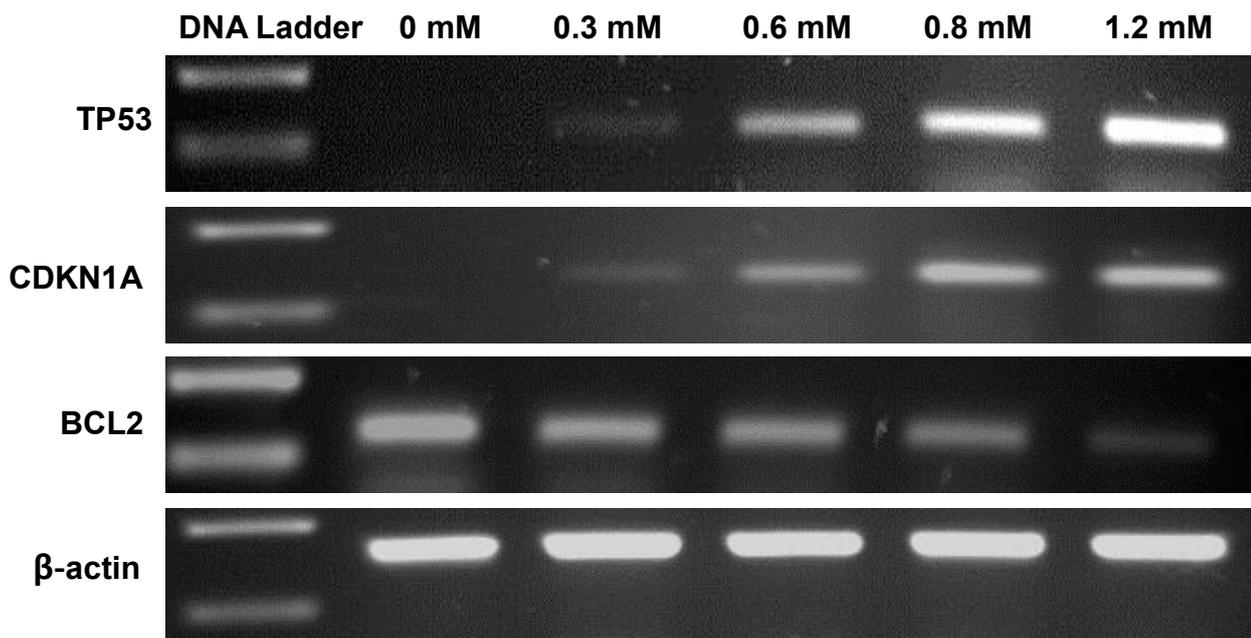| Different exposure concentrations of lymphocytes	Olive Tail moment Mean ±SE	%Tail DNA Mean ± SE
Control	1.49 ± 0.14	7.65 ± 0.82
0.3 mM	3.14 ± 0.46	14.97 ± 1.65
0.6 mM	5.71 ± 0.84 *	22.42 ± 2.14 *
0.8 mM	6.97 ± 1.21 **	27.57 ± 2.36 *
1.2 mM	11.00 ± 1.34 **	36.71 ± 3.73 **
Different exposure concentrations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

Figure 1
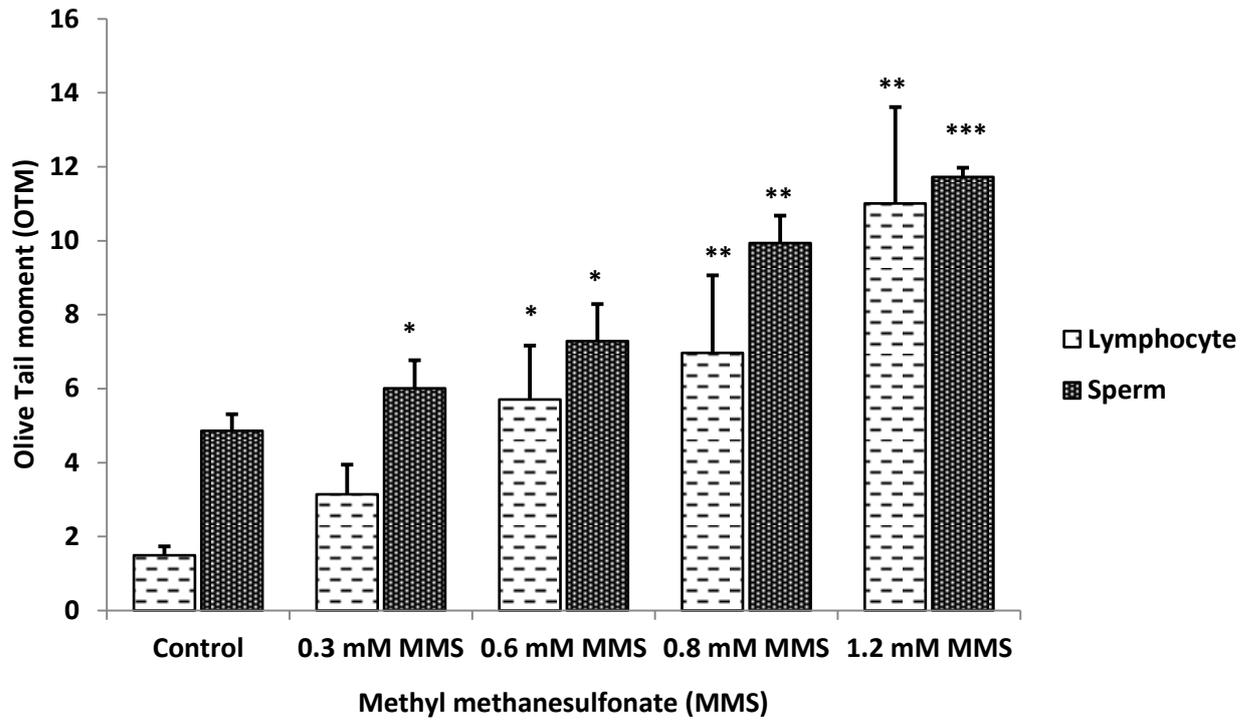
Figure 2

Figure 3

A

B

Figure 4