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On the Mechanism of Genotoxicity of Ethephon on Embryonic Fibroblast Cells

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Abstract

Ethephon is one of the most widely used plant growth regulator in agriculture that its application has been increased in recent years. Many reports have raised concern over the safety of this organophosphorus compound. The aim of the current study was to assess the potential genotoxic effect of ethephon on murine embryonic fibroblast (MEF)cell line, using two genotoxicity endpoints; γH2AX expression and comet assay. γH2AX served as an early and sensitive biomarker of genotoxic damage. Oxidative stress biomarkers, including reactive oxygen species (ROS), lipid peroxidation (LPO) and total antioxidant capacity were also examined. The results showed a significant increase in cell proliferation, 24h post-treatment with 10, 40,160µg/ml ethephon, while at the higher concentrations cytotoxic effect was observed. The γH2AX expression and γH2AX foci count per cell were significantly increased at non-cytotoxic concentrations of ethephon, accompanied with increased DNA damage as illustrated by comet assay. LPO and ROS levels were elevated only at 160 µg/ml and higher doses. The results interestingly showed that low non-cytotoxic doses of ethephon promoted DNA damage while induced cell proliferation; raising the possibility of ethephon mutagenicity. The genotoxic effect of ethephon at low doses might not relate to oxidative damage and that increased in the level of ROS and LPO generation at higher doses could account for the cytotoxic effect of ethephon. Taken together, our study provides strong in vitro evidence on potential genotoxicity of ethephon at low doses. More precise studies are needed to clarify the mutagenic effect of chronic exposure to ethephon.

Key words: Ethephon, DNA damage, γH2AX,Comet assay, Oxidative stress

Introduction

It is known for a long time that exposure to environmental genotoxicants is a major reason for the increased incidence of degenerative diseases in human (WHO, 2009). Genotoxic agents can cause different types of DNA damage in somatic and germ cells, resulting in induction of mutation and genomic instability that is the hallmark of cancer initiation (Friedberg et al., 2005).The induced mutations in germ cells, may pass on to the next generation; contribute to the birth of genetically defective offspring and negative consequence for human health. Therefore, evaluation of genotoxic potential of environmental chemicals is foremost for all toxicological studies.

Organophosphorus compounds are organic substances widely used for diverse applications in agriculture and industry. Considering their beneficial properties, it is noted that acute and chronic exposure to organophosphorus compounds could exert systemic neurological toxicity by inhibition of acetyl cholinesterase activity (AchE) and prevention of neural transmission (Abdollahi & Karami-Mohajeri, 2012; Balali-Mood & Abdollahi, 2014; Minton & Murray, 1988). Besides their inhibitory role on AChE, there are increasing *in vivo* and *in vitro* evidence, pointing to the genotoxicity and carcinogenicity effect of organophosphorus pesticides (OP) (Abbassy et al., 2005; Bolognesi & Morasso, 2000; Lifshitz et al., 1999; Mostafalou &Abdollahi, 2013). It was shown that acute exposure to pesticides can induce chromosomal aberration, gene mutation, and DNA damage (Bolognesi, 2003). Several OPs have electrophilic entity and are capable of interacting with nucleophilic residue of DNA by alkylating that serve as the mechanism of their action. There are also studies on OPs-induced oxidative stress and contribution to DNA damage (Soltaninejad & Abdollahi, 2009; Lu et al., 2012).

Ethephon, an organophosphorus compound, is one of the most commonly used plant growth regulator in the world. It serves a broad spectrum of application in ripening of vegetables, fruits and cereals, as well as induction of flowering and protecting against pest (Environmental Protection Agency, 1995; Palam et al., 2005).The global increase in the consumption of plant growth regulators in agriculture and industry is expected to increase the ethephon market in near future.

Similar to organophosphorus compounds, ethephon has anti-cholinesterase activity in mammals (Brock, 1991). The butyryl cholinesterase inhibition was shown to be the sensitive marker of ethephon exposure (Haux et al., 2002). Despite the fact that organophosphorus compounds could act as mutagenic and teratogenic agents, the related researches on genotoxic effects of ethephon is very limited and there is controversy between the results of studies. While ECHA reported no genotoxic activity for ethephon, there were relatively few studies showing that ethephon causes teratogenic effects in animal models and has potential mutagenicity for somatic and germ cells in mice (Bhadoria et al., 2015; El Raouf & Girgis, 2011; Yu et al., 2006).

Considering the widespread use of ethephon and its potential hazard ,it is therefore essential to evaluate ethephon toxicity using different molecular biomarkers, particularly at DNA level. Phosphorylation of H2AX (γH2AX) is an early response to induction of DNA double strand breaks (DDB)(Celeste et al., 2003; Kuo & Yang, 2008). Following exposure to the genotoxic agents and formation of DNA damage, protein kinases recruited to the site of injury and trigger the activation of DNA damage signaling cascade. Accordingly, Histone H2AX, a member of core histone protein in chromatin structure, becomes highly phosphorylated by phosphatidyl inositol-4,5-bisphosphate 3-kinase activation that can be detected in the form of foci by immunocytochemistry; serve as a sensitive biomarker for DNA damage. The purpose of this

study was to investigate the genotoxic effects of ethephon on MEF cell line by evaluating the pH2AX expression and foci formation as well as direct measuring of DNA damage in individual cells using the comet assay. To the best of our knowledge, this is the first investigation in which ethephon genotoxicity is determined using γH2AX assay. Furthermore, this study examined cellular behavior after exposure to the non-cytotoxic concentrations of ethephon and the possible role of intracellular oxidative stressas a causative mediator of ethephon toxicity. The present study attempted to provide a basis for the in vitro toxicology data of ethephon.

Methods:

Chemicals:

Ethephon (≥96% purity; CAS no. 16672-87-0) was purchased from Sigma-Aldrich (UK). Ethylene diamine tetra acetic acid (EDTA), hydrochloric acid, acetic acid, sodium acetate, magnesium chloride, sodium sulfate, sulfuric acid, phosphoric acid, potassium dihydrogen phosphate, potassium hydrogen diphosphate, sodium carbonate were from the Merck Chemical Company (Germany). Unless otherwise stated, all other materials used in this study were obtained from Sigma-Aldrich (Germany).

Cell culture and treatment with ethephon

NIH-3T3 murine embryonic fibroblast (MEF) cell line was purchased from the Pasture institute of Iran, Tehran. Cells were cultivated at 37°C, under 5% CO2 condition and maintained in DMEM (high glucose, with sodium pyruvate, BioSera, France), supplemented with 10% FBS and 1% penicillin/streptomycin (BioSera, France). To examine the cytotoxic effect of ethephon, 3T3 cell line was treated for 24h with increasing concentrations of ethephon in medium containing no serum.

Cell proliferation

Cell proliferation was quantified by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium assay according to the method described previously (Gerlier & Thomasset, 1986). In brief, ethephon-treated cells in 96 well plate was exposed to MTT reagent (0.5 mg/ml in PBS) and incubated for 4h at 37°C, 5% CO2 in humid atmosphere. The living cells have the ability to reduce MTT to blue formazan crystals. Next, 100 µL of DMSO was added to each well and the plate was incubated at room temperature (RT) for 20 min with shaking. Finally, the absorbance intensity was measured at 570 nm and a reference wavelength of 630 nm, using multi-well plate reader (Biotech, USA).

Flow cytometry analysis of γ-H2AX

Ethephon-treated and control cells were examined for γ-H2AX nuclear expression. Approximately 500,000 cells were collected and fixed with cold 2% formaldehyde for 15min, followed by permeabilized with 0.1% Triton X-100 in PBS and incubated in blocking buffer containing 0.5% BSA for 30 min. Next, cells were stained with anti-pH2AX (Ser139) mAb (Cell signaling, USA) (1:500) for 2h at RT. After two washes with PBS, cells were incubated with anti-rabbit IgG Alexa Fluor 488 (Abcam) at 1:1000 dilution in blocking buffer, and shaken for 1h in the dark. 10,000 of the events were acquired for each sample using FACScan flow cytometer (Mindray, China). Data were analyzed by FlowJo software 7.6.2 version. Samples without primary antibody were used as the negative control.

Immunofluorescence staining for γ-H2AX foci formation

Cells grown on cover slips, were fixed with 2% formaldehyde and permeabilized in 0.1% Triton X-100 for 3 min followed by overnight incubation with 3% BSA/PBS at 4°C. Pre-treated cells

were labeled with anti-pH2AX (Ser139) mAb (Cell signaling, USA) (1:200) for 2h at RT. After two washes with PBS, cells were incubated with 1:500 diluted anti-rabbit IgG Alexa Fluor 488 (Abcam) for 1h in the dark. DAPI (BioStatus) was applied to nuclear staining. Cells were then mounted in glycerol/PBS (1:1) and analyzed by fluorescence microscope (Zeiss, Germany). The number of foci signals per cell nucleus was counted using particle analysis tool of ImageJ software. Samples without primary antibody were used as the negative control.**Alkaline comet assay**

The alkaline single cell comet assay was performed as previously described (Narayanaswamy et al., 2014). Briefly, 24h after exposure to ethephon, cells were trypsinised and counted. For each sample, 10000 cells were mixed with 0.7% low melting agarose in PBS at 37°C and allowed to solidify on 1% normal agar pre-coated slides for 20min at 4°C. Next, cells were lysed in buffer containing 1% Triton X-100 in 10 mM Tris, 100 mM EDTA for 1 h. The slides were placed in a horizontal gel electrophoresis chamber (Bio Rad Sub Cell GT, Germany), covered with alkaline running buffer (10 N NaOH, 200 mM EDTA, pH.13). After 20 min exposure time, DNA was electrophoresis at 300 mA, 25 V for 20min. Following 5 min neutralization in 0.4 M Tris pH-7,5 and 100% ethanol washing, the slides were allowed to dry overnight at 4°C. SYBR green at 1:10000 concentration were applied to visualize the comet tail formation under fluorescence microscope. Around 50–100 cells were analyzed for different parameters of the comet, including comet tail length and comet olive tail moment [(tail mean-head mean) x % of DNA in the tail] as calculated by CASP software (CaspLab, Poland). Hydrogen peroxide (100μM, 20 min) was used as a positive control**.**

Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS was measured using dichlorofluorescin diacetate (DCFH-DA) as described previously (Mostafalou et al., 2015). In brief, ethephon-treated and control cells (8000 cells/well in 96-well plate) were lysed in 75 µl extraction buffer (0.25 mM sucrose, 20 mM KCl, 1 mM EDTA, 5 mM HEPES and 0.5mM DTT solution) for 5min, followed by centrifugation at 5000g for 10 min, 4ºC. Next, 50µl of supernatants were collected and added to a new plate containing 80 µL of assay buffer per well (130 mM KCl, 20 mM NaH2PO4, 5 mM MgCl2, 20 mM, Tris-HCl, 0.1 mM FeCl3, 1.7 mM ADP, and 0.1mM NADPH). After adding 5 µL DCFH-DA (5 µM), the plates were incubated for 15 min at RT. Oxidation of DCFH-DA to DCF was measured continuously every 5 min for 1 h using fluorometer (Biotech, Tecan US, Inc); set at excitation of 485 nm and emission of 528 nm. Final mean data were normalized to total protein level.

Measurement of lipid peroxidation (LPO)

To measure LPO, the amount of thiobarbituric acid (TCA)-reaction substances (TBARS) were assessed by TBA reaction in ethephon-treated and control cells (Fakhri-Bafghi et al., 2016). For this aim, cell suspension in PBS were homogenized and mixed with 28% (w/V) TCA (1:2), followed by centrifugation at 3000g for 30 min. To 600 µl aliquots of the supernatant, 150 µL TBA 1% (w/v) was added and the resultant mixture was heated in boiling water for 15min. After adding 4 µL n-butanol, the contents were recentrifuged for 15min and the absorption was measured at 532 nm with a double beam spectrophotometer. The extinction coefficient of 155 mM^{-1} cm^{-1} were used to calculate the concentration of TBARS.

Measurement of ferric reducing antioxidant power (FRAP)

In this experiment, the ability of antioxidant to reduce $Fe^{3+}$to Fe^{2+} was measured (19). 100µl of homogenized cell suspension in PBS obtained from the previous LPO test, were added to a FRAP

reagent containing 2,4,6-tripyridyl-s-triazine (TPTZ), sodium acetate buffer (300 mM, pH 3.6) and FeCl3.6H2O (20 mM) at 10:1:1 ratio, followed by incubation for 10 min at 37 °C. Finally, the absorbance was measured at 593 nm. The values were calculated as µmol/mg protein. Hydrogen peroxide was used as a positive control**.**

Statistical analysis

All experiments were carried out at least three times to ensure reproducibility and the data are presented as mean ± SEM (standard error of mean). Statistical analyses were performed using IBM Statistics 20. For comet assay and H2Ax Foci analysis, statistical significance (*P* value<0.05) was determined by one-way analysis of variance followed by Dunn's multiple comparison tests. The results of MTT assay and oxidative analysis were statistically analyzed using student's t Test.

Results:

Exposure to non-cytotoxic concentrations of ethephon induces cell proliferation

Incubation of cell culture with different concentrations of ethephon after 24h, showed significant increase in cell proliferation at 10, 40,160 µg/ml (P< 0.05) compared to the control group as demonstrated by increased rate of MTT reduction using tetrazolium reduction assays (Fig1). The results were confirmed by trypan blue staining and cell counting.

However, higher concentrations of ethephon (640 and 2560 µg/ml), significantly reduced cell proliferation and showed cytotoxic effect.

Ethephon induces a genotoxic effect

To investigate ethephon-induced DNA damage responses, we first analyzed the expression of γH2AX as a specific indicator of DNA damage. 24h after exposure to different concentrations of ethephon, the expression of γH2AX increased significantly in a concentration dependent manner as was analyzed by flow cytometry. The histograms related to 10, 40 and 160µg/ml shifted to the right, reflecting the higher γH2AX intensity compared to non-treated control group (Fig2A). Accordingly, the fluorescence mean intensity of γH2AX was increased after ethephon treatment (1.84, 1.97 and 2.59 as of the response to 10, 40 and 160 µg/ml, respectively), which indicated the induction of DNA damage response (DDR).

The quantitative immunofluorescence analysis of γH2AX foci showed that ethephon induced accumulation of γ-H2AX in nuclei of 3T3 MEF (Fig 2B) and the mean numbers of foci per cell were increased even at low non-cytotoxic concentrations (Fig 2C). There were no significant differences regarding γ-H2AX foci size between ethephon-treated and non-treated cells (Fig2C)

To gain insights into the consequence of ethephon-induced DDR, the comet assay was performed to monitor the emergence of single and double strand DNA breaks. Cells containing damaged DNA, acquired the appearance of the comet with a head and tail. Whereas, undamaged DNA appears as a round intact DNA with no tail (Fig 3, A-D). Significant increase in comet olive tail moments was observed between non-treated control group and 40, 160 µg/ml ethephon (up to 1.5, 1.98 fold, respectively) as shown in Fig3-E. However, there was no significant difference between 10µg/ml ethephon and control. Similar results were obtained when comparing comet tail length values ($P<0.0001$) (Table 1).The present data further confirmed the induction of DNA strand breaks after ethephon treatment in cultured cells.

Ethephon induced oxidative stress at high concentration

The potential of ethephon to induce oxidative stress and impair antioxidant capacity as an underlying mechanism of ethephon-induced DNA damage was assessed by measuring the oxidant and antioxidant parameters in 3T3 embryonic fibroblast cells. The results showed that ethephon significantly induced ROS production at doses of 160 and 640 µg/ml after 24h, whereas there were no significant changes in ROS content between control and other ethephon treatments (Fig 4). Similar results were obtained for LPO, as demonstrated by a dramatic increase in concentration of 160 µg/ml; from 12.55 ± 0.8 to 18.80 ± 0.8 µM.

FRAP assay is a potent method to assess the antioxidant power. The data showed no significant differences in antioxidant power at all tested concentrations ($P>0.05$) (Data were not shown).

Discussion

Although ethephon has been classified as a non-harmful chemical, many environmental and regulatory agencies are still uncertain about its toxicity and adverse effects on human health. Facing the uncertainty about the safety of ethephon and its residual issues, US-EPA established the maximum residue levels and tolerance limits for this chemical in different raw agricultures. Indeed, studies on cytotoxicity and particularly genotoxicity of ethephon are very limited and further experiments are needed to evaluate the in vitro and in vivo side effects of ethephon. Accordingly, the present study attempted to investigate the impact of exposure to ethephon on genotoxicity and cytotoxicity of embryonic fibroblast cells using sensitive biomarkers of DDR. Embryonic fibroblast cell line have been implicated in many classical toxicology tests and were the cells of choice for the assessment of developmental toxicity with the characteristics similar to bone marrow-derived mesenchymal stem cells (Dastagir et al., 2014). To assess DNA

damage, we measured phosphorylation of H2AX, as a well-known sensitive and early indicator of histone modifications in response to genotoxic agents. The obtained data showed that ethephon within 24h exposure time, significantly induced the expression of γH2AX. This finding indicated that cell exposure to ethephon, even at low doses (10µg/ml) stimulated DDR molecules through phosphorylation of H2AX.

We further investigated the γH2AX foci as it was reported previously could reflect the number of DNA double strand breaks (Firsanov et al., 2011; Kinner et al., 2008). The results showed increased in the average number of foci per nucleus in ethephon-treated cells compared to the control group, supporting the notion that they introduce true DNA breaks. We also detected DNA damage in cells of control group, which was reasonable as DNA damage occurs naturally under physiological conditions during cell developmental process and dynamically removed by DNA repair machinery. Although our results revealed no significant difference in mean foci size between treated and non-treated cells, there was a clear difference in foci patterns of two groups in which γH2AX foci distributed homogeneously in ethephon samples compared to the interspersed pattern of the foci in control cells.

The findings from alkaline comet assay further confirmed ethephon-induced DNA damage, as demonstrated by increased in olive tail moment values and tail length. This finding strongly supported the in vitro genotoxic capability of ethephon in cell culture.

The genotoxic concentrations of ethephon were observed at doses as low as 10µg/ml in γH2AX assay and 40µg/ml in comet assay. Interestingly, the observed genotoxic doses were in lower range of those evaluated in in vitro mutagenicity assays by European Chemicals Agency (ECHA)(European Chemicals Agency, 2012); raising more attention towards the potential chronic toxicity of ethephon.

Indeed the mutagenic activity of ethephon has been previously reported in bacteria and yeast using human liver S9 fraction in the Ames test (European Chemicals Agency, 2012). It was shown that ethephon at defined concentrations, induced point mutations in S. typhimurium, TA1535 strain (with and without S9 fractions). However, no mutations were reported in mammalian CHO cells after exposure to ethephon.

Furthermore, Yu et al. (2006) showed increased micronucleus rate of bone marrow PCE cells in mice exposed to sub-chronic doses of ethephon (Yu et al., 2006). Our results were also in line with studies providing evidence on teratogenicity and mutagenicity of ethephon in animal models. Accordingly, decreased in RNA and DNA content as well as induced structural chromosomal aberrations were demonstrated in mice treated with selected doses of ethephon (Al-Twaty, 2006; El Raouf & Girgis, 2011; Nada & Alakilli, 2008). Indeed, the number of studies on chronic and sub-chronic toxicity of prolonged exposure to ethephon are limited, even less is known about the chronic genotoxic effect of this widely used chemical. Our results emphasized on the necessity of further researches in this area.

We also quantified the effect of ethephon on cell viability and proliferation rate by MTT assay and cell counting. The results indicated the increased cell proliferation at low doses of ethephon during 24h exposure time. Modulation of cell proliferation by ethephon was previously studied by Perovicet al., (2001) showed that various mammalian cell lines react to ethephon by up regulation of cell cycle-associated protein and induction of cell division (Perovic et al., 2001).

Besides, we found out that in proliferating cells the level of DNA strand breaks increased in response to ethephon. This indicated that DNA repair pathways are inefficient to remove the DNA damage while support the continuous growth of cells with defect DNA. DDR as a key signaling network has an important role in protecting the cells from genomic instability and DNA

damage accumulation, a precursor for cancer. In response to DNA damage, different signaling proteins including those involved in cell cycle checkpoints and DNA repair machinery become activated to elicit cell cycle arrest and repair the site of injury. Only those cells capable of repairing the DNA are allowed to enter cell cycle. In case of irreparable damage, apoptotic responses become activated to remove cells harboring genomic instability; act as a barrier towards cancer development (Bartkova et al., 2005). Based on the above notions, our observations on ethephon-induced DNA damage accompanied with increased cell proliferation may raise the possibility of mutagenic and carcinogenic effect of ethephon.

To find out the underlying mechanism of ethephon induced genotoxicity, we examined OS status parameters including ROS level, antioxidant capacity and lipid peroxidation. Measurements of intracellular ROS and LPO showed dramatic increased only at high doses of ethephon, while there were no significant changes in low concentrations, indicating the involvement of other mechanisms for genotoxicity of ethephon. The increased OS at high doses might at least partly related to the low pH of ethephon solution leading to acidification of cell culture medium at high doses.

In summary, our results showed that embryonic fibroblast respond to ethephon by inducing DNA damage. The findings of this study are important, as they contradict to the EPA's report imply that ethephon could induce genetic damage at very low concentrations. The present study, further implicate the potential genetic hazards of ethephon particularly in chronic exposure. Such data of in vitro genotoxicity in mammalian cells provide the basis of researches for understanding the toxicity of ethephon at the molecular level and needs to be supplemented by further studies. In general, more attention should be paid towards the chronic risk posed by ethephon to human and animal health.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Table

Table1. The average comet tail length, 24h after exposure to concentrations of ethephon	
Ethephon µg/ml	Tail length
0	12.02±4.9
10	17.21±12.7^{ns}
40	23.99± 14.66****
160	32.84±22.8****
*ns: no significant,****P< 0.0001,**P < 0.001*	

Table1. The average comet tail length, 24h after exposure to concentrations of ethephon. Results are the mean ± SEM of three independent studies

Figures:

Fig1:

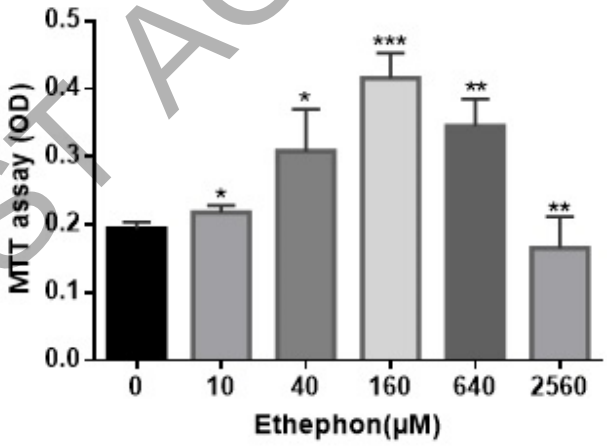


Fig2:

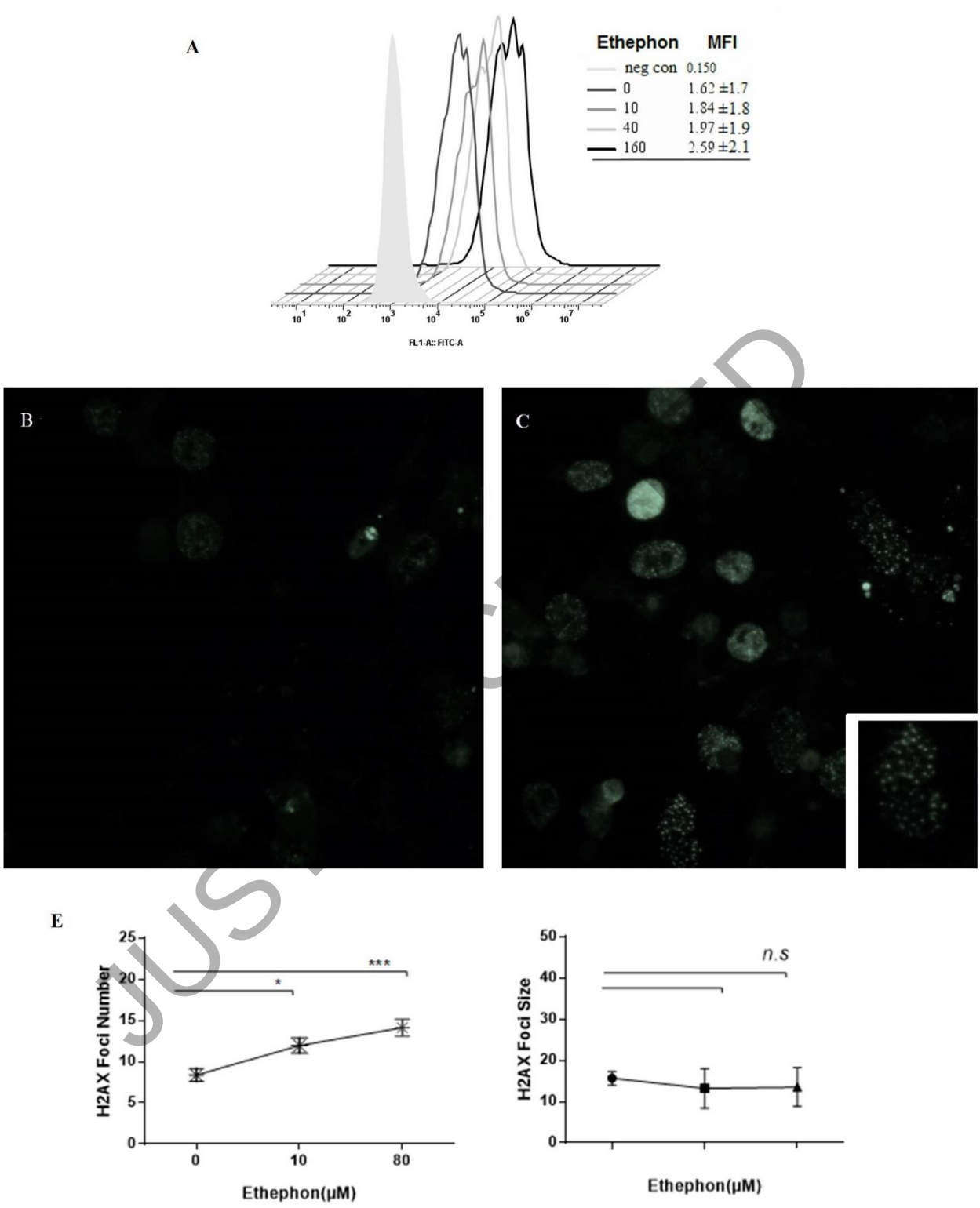
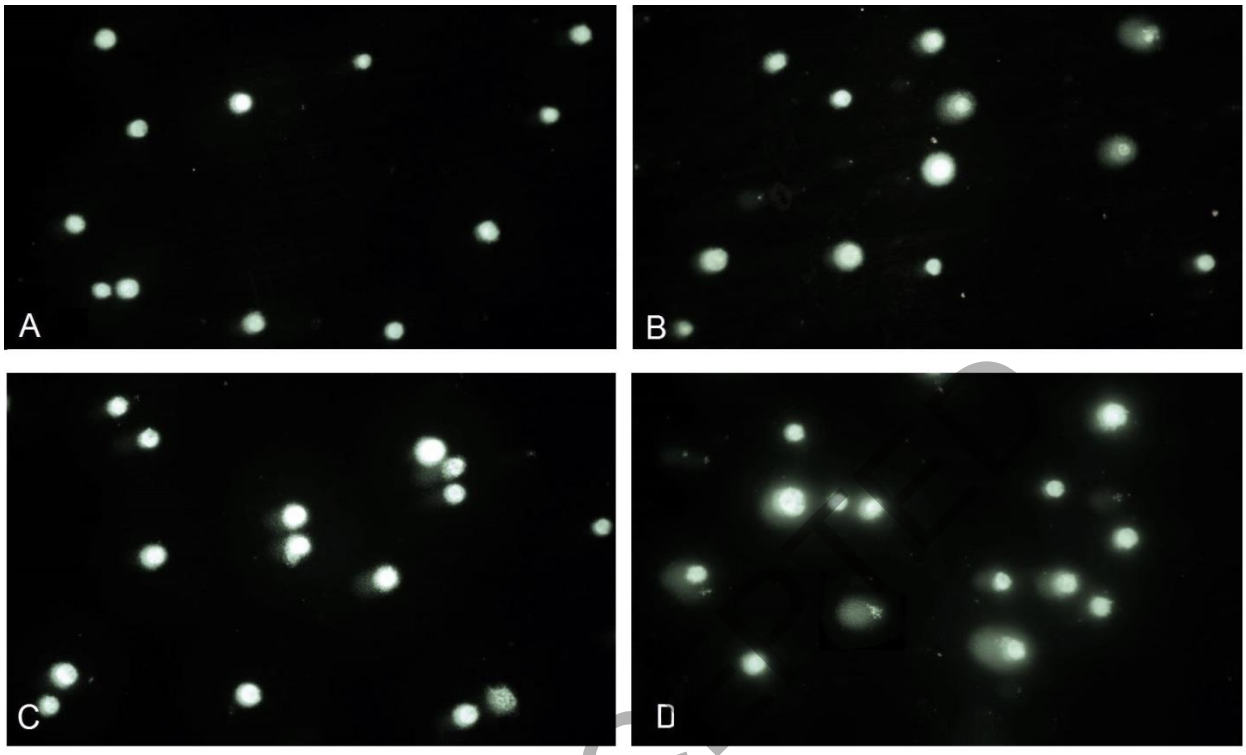

A
Ethephon MFI
neg con 0.150
0 1.62 ±1.7
10 1.84 ±1.8
40 1.97 ±1.9
160 2.59 ±2.1
FL1-A:: FITC-A
B
C
E
H2AX Foci Number
***
*
0
10
80
Ethephon(μM)
H2AX Foci Size
n.s
Ethephon(μM)

Fig3:



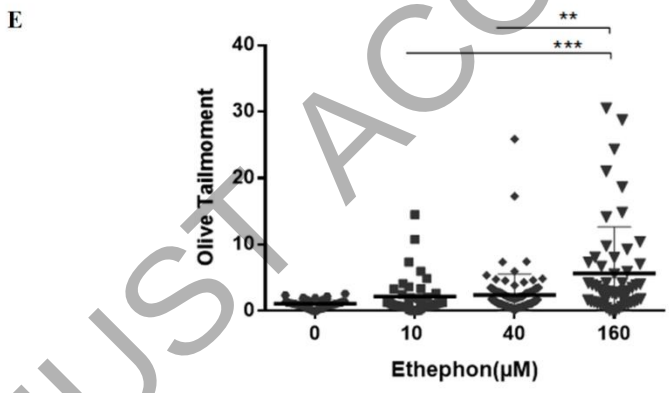


Fig4:

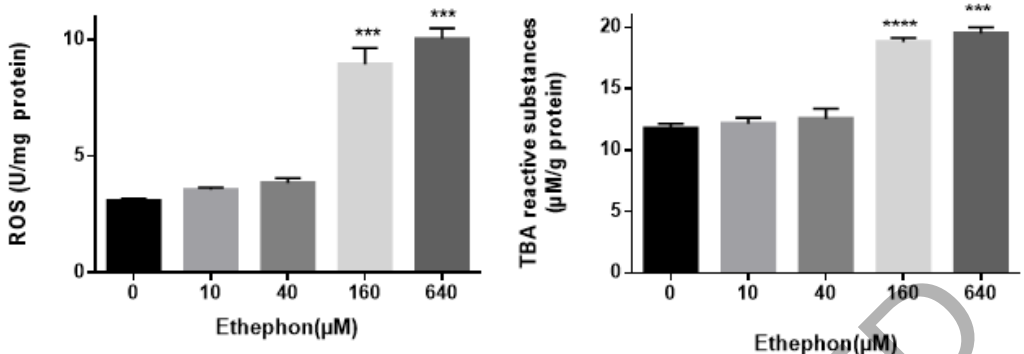


Fig 1. Cell proliferation was evaluated by MTT assay after exposure to the increasing concentrations of ethephon. Ethephon induced cell proliferation at 10, 40 and 160µg/ml after 24h exposure time (*P < 0.05, **P < 0.01, ***P < 0.001). Results are the mean ± SEM (error bars) of three independent studies each performed in triplicate.

Fig 2. Flow cytometry analysis of γH2AX expression and immunofluorescence analysis of γH2AX foci after exposure to increasing concentration of ethephon. (A) FACS histograms shifted to the right showed how increasing concentrations of ethephon induced γH2AX expression after 24h exposure time; shaded histogram represent negative control, Mean fluorescence intensity (MFI) increased in response to ethephon (B) Image of immunofluorescence staining of γH2AX in embryonic 3T3 fibroblast cells (left: non-treated control group and right: 24h after ethephon treatment) (C) Immunofluorescence analysis showed increase in γH2AX foci number per cell in ethephon treated cells. Foci number per cell were counted using ImageJ (NIH) software. The data represent the average of three independent experiments (*P < 0.05, ***P < 0.001). There were no significant differences in foci size between the groups (ns: no significant, P>0.05).

Fig 3. Evaluation of ethephon-induced DNA damage by comet assay. A typical image of 3T3 MEF comet; cells were exposed to the increasing concentrations of ethephon: (A) non-treated control cells, (B) 10 µg/ml, (C) 40 µg/ml and (D) 160 µg/ml ethephon. Round intact nucleus represents intact DNA while damaged DNA had a tail and fade head (E) Graph depict the Olive tail moment after exposure to ethephon treatment. Comet parameters were quantified by CASP software. Ethephon induced olive tail moment in cells treated with 40 and 60 µg/ml concentrations (**P < 0.01, ***P < 0.001).Values shown (horizontal lines) are the mean from 50-100 of randomly selected images of each sample.

Fig 4. The level of reactive oxygen species (ROS) and lipid peroxidation in ethephon-treated cells. Intracellular ROS were quantified by dichlorofluorescein diacetate (DCFDA) staining and calculated as a unit per total protein (Left). ROS increased dramatically at 160 and 640µg/ml. Similar results were obtained for lipid peroxidation measured by TBARS formation (Right) (***P < 0.001). Results are the mean ± SEM (error bars) of three independent studies each performed in triplicate.