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Evaluation of the toxic potential of ethyl methanesulphonate (EMS) on *Hydra vulgaris*

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A R T I C L E I N F O

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A B S T R A C T

The effect of EMS at final concentration of 0.09, 0.18, 0.27 and 0.37 mM was studied on *Hydra vulgaris* using morphological, regeneration, oxidative stress markers and DNA damage as parameters. The morphological scores showed a significant dose dependent difference in the *Hydra* exposed to 0.18, 0.27, and 0.37 mM of EMS for 24, 48, 72 and 96 h. The regeneration scores also showed a significant difference in the gastric region of *Hydra* exposed to 0.37 mM of EMS for 48 h. A significant difference in the scores of regeneration was observed for the mid body portion exposed to 0.18, 0.27 and 0.37 mM of EMS for 72 and 96 h of duration compared to control. A dose-dependent significant increase in the activities of glutathione-S-transferase (GST), catalase (CAT), and superoxide dismutase (SOD) was observed compared to control. The thiobarbituric acid reactive species (TBARS) levels were also significantly increased compared to control. The genotoxic damage was assessed in the cells of gastric region of the *Hydra* exposed to 0.09, 0.18, 0.27 and 0.37 mM of EMS for 48 h by performing comet assay. A significant dose-dependent increase in the DNA damage was observed compared to control.

1. Introduction

Hydra is a fresh water polyp, having a regenerative capability which makes it an ideal model for not only studying developmental aspects, but also the impact of various environmental toxicants [9]. It is used as a as a bio-indicator for fresh water ecosystems [27]. The simple organization of cells in two epithelial layers, the ectoderm and endoderm with mesoglea in between [6] is useful to evaluate the potential of environmental pollutants, nanomaterials, industrial and municipal effluents and other toxicants [32]. Sewage effluents and land-fill leachates are responsible for polluting natural water body and therefore, can impose a potential risk to the aquatic species [31]. Ethyl methanesulphonate (EMS) is a monofunctional ethylating agent and has been reported to exhibit mutagenic effects in viruses to mammals [20]. The *Hydra* can be easily cultured in lab and due to the property of regeneration we can get genetically similar colonies. The compounds for testing can be dissolved in water at desired concentrations and the effect can be easily studied. Although the toxicity as well mutagenic effect of alkylating agents have been studied on various experimental models (*in vitro* and *in vivo*), studies on aquatic animals are warranted [2,11,16,34,37,36,35]. In the present study the effect of EMS at various concentrations was studied on the *Hydra vulgaris.*

2. Materials and methods

2.1. Culture of Hydra

The Hydra medium having 1 mM calcium chloride, 0.1 mM magnesium sulphate, 0.1 mM potassium chloride, 1 mM sodium chloride and 1 mM Tris Base (pH 7.4) was used to culture *Hydra* [42]. The *Hydra* were allowed to grow in culture bowls. The temperature was kept 18 °C, with the conditions of 12 h light and 12 h dark. The freshly hatched *Artemia salina* nauplii were fed to *Hydra* for maintaining the culture. Polyps starved for 24 h were picked from the stock culture for all assays.

2.2. Toxicity testing

The final concentrations of 0.09, 0.18, 0.27 and 0.37 mM of EMS

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were established in the Hydra medium. Non-budding healthy adult polyps 5 per treatment (5 replicates per treatment) were exposed to EMS and the changes in morphology (Fig. S1), if any, was observed under a stereo-zoom microscope after 24, 48, 72 and 96 h, with renewal of medium at every 24 h interval. Morphological changes were recorded and scored according to Wilby (1983) [24] given below. Score 10 means healthy animal; scores 9–6 represent grades of morphological changes that are not lethal; and scores 5 and down represent lethality [43].

Scores according to Wilby [24]

Score	Morphology of polyp
10	Extended tentacles; body reactive
9	Partially contracted; slow reactions
8	Clubbed tentacles; body slightly contracted
7	Shortened tentacles; body slightly contracted
6	Tentacles and body shortened
5	Totally contracted; tentacles visible
4	Totally contracted; no visible tentacles
3	Expanded; tentacles visible
2	Expanded; no visible tentacles
1	Dead but intact
0	Disintegrated

2.3. Regeneration assay

The potential of affecting the regeneration of *Hydra* by EMS was studied by exposing the gastric region to 0.09, 0.18, 0.27 and 0.37 mM of EMS. Using a fine needle, the polyps were cut below the hypostome and above the budding region to separate the gastric regions (1.5 mm) placed individually in the *Hydra* medium having the selected final concentrations containing the desired concentration of EMS. The separated gastric regions were (five/treatment group) (Fig. S2). The medium was renewed every 24 h until 96 h. The extent of regeneration was examined microscopically as per the method described by Wilby [24].

Scores according to Wilby (1983)

Score	Scoring of inhibition of regeneration
10	Mouth, 4–6 tentacles and peduncle
9	Mouth, 4–6 tentacles
8	Mouth, 4 tentacles and basal disc
7	Mouth and 4 tentacles
6	Tentacle buds and basal disc
5	Tentacle buds only

2.4. Oxidative stress markers

2.4.1. Preparation of homogenate for biochemical assays

For the preparation of homogenate (10 %) 100 Hydra per treatment (5 replicates/treatment) were homogenized in 0.1 M of phosphate buffer.

2.4.2. Determination of glutathione (GSH) content

The determination of GSH content was performed as per the colorimetric method of Jollow et al. [17]. The optical density (OD) was read at 412 nm and the results were expressed in µmoles of GSH/gram tissue.

2.4.3. Determination of glutathione-S-transferase (GST) activity

The activity of GST was estimated according to the method of Habig et al. [13]. The OD was read at 340 nm and the activity was expressed as µmoles of CDNB conjugates/min/mg protein.

2.4.4. Determination of thiobarbituric acid reactive species (TBARS)

TBARS were determined according to the method suggested by Ohkawa et al. [26]. The results were expressed as µmoles of thiobarbituric acid reactive species (TBARS) formed/hour/gram tissue.

2.4.5. Superoxide dismutase (SOD) activity

The activity of SOD was determined as per the method of Marklund and Marklund [19]. The OD was read at 420 nm. An increase in the OD was read for three minutes at the interval of 30 s. The activity was expressed in units per milligram of protein.

2.4.6. Catalase (CAT) activity

The activity of CAT was determined by the kinetic approach of Beers and Sizer [5]. The activity of catalase was expressed as µmoles of H_2O_2 consumed per minute per milligram of protein.

2.5. Analysis of DNA damage by comet assay

The method of Mukhopadhyay et al. [21] was used to perform comet assay. The gastric region from 100 hydra per treatment (3 replicates/group) was removed in Poel's Salt Solution (NaCl - 0.086 %; KCl - 0.313 %; $CaCl_2H_2O$ - 0.116 %; NaH_2PO_42 H_2O - 0.088 %; $KHCO_3$ - 0.018 %; $MgSO_47$ H_2O - 0.513 %). The gastric region were placed in 300 µL of collagenase (0.5 mg/mL in PBS, pH 7.4) kept at 25°C for 15 minutes. The prepared slides were placed in chilled electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH > 13). After completion of the electrophoresis the slides were stained for 10 minutes in the dark with ethidium bromide (20 µg/mL; 75 µL/slides) and randomly 25 cells per slide (3 replicates/group) were selected to score the tail length (Comet ScoreTM v1.5 Software, TriTek Corporation, Sumerduck).

2.6. Statistical analysis

The data was analyzed by using one-way analysis of variance (ANOVA) with post hocTukey test using GraphPad Prism software [version 5.0]. The level of significance was kept at $p < 0.05$. The values were expressed as mean ± SEM.

3. Results

The *Hydra* exposed to 0.09, 0.18, 0.27 and 0.37 mM of EMS for 24 h showed a reduction of 4 %, 12 %, 16 % and 20 %, respectively, in the size of tentacles compared to control (Fig. 1; $p < 0.05$). The *Hydra* exposed to selected concentrations of EMS for 48 h showed a decrease to 10 %, 20 %, 36 % and 40 %, respectively, in the size of tentacles compared to control (Fig. 1; $p < 0.05$). The *Hydra* exposed to selected concentrations of EMS for 72 h showed a reduction of 12.24 %, 24.48 %, 44.89 % and 59.18 %, respectively, compared to control (Fig. 1; $p < 0.05$). The *Hydra* exposed to selected concentrations of EMS for 96 h showed a decrease of 18 %, 34.69 %, 55.10 % and 67.34 %, respectively, compared to control (Fig. 1; $p < 0.05$). The results obtained for regeneration assay are shown in Fig. 2. The dissected gastric region of *Hydra* exposed to 0.09, selected concentrations of EMS for 24 h showed no significant difference compared to growth in control (Fig. 2; $p < 0.05$). After 48 h of exposure to 0.27 mM of EMS showed a significant delay of 19 % in the regeneration compared to control (Fig. 2; $p < 0.005$). No significant difference was observed in the regeneration of gastric region exposed to 0.09, 0.18 and 0.27 mM of EMS for 48 h compared to control (Fig. 2; $p < 0.05$). A significant delay of 15.62 %, 31.25 % and 37.5 % was observed in the gastric region exposed to 0.18, 0.27 and 0.37 mM of EMS, respectively, for 72 h compared to control (Fig. 2; $p < 0.005$). The gastric region exposed to 0.18, 0.27 and 0.37 mM of EMS for 96 h showed a significant delay of 23.80 %, 40.47 %, and 47.61 %, respectively compared to control (Fig. 2; $p < 0.05$).

Hydra exposed to selected concentrations of EMS for 48 hrs showed a significant increase of 49 %, 121.95 %, 193 % and 258 %, respectively, in the TBARS compared to control (Fig. 3c; $p < 0.05$). *Hydra* exposed to selected doses of EMS for 48 hr showed a significant decrease of 22 %, 36 %, 46 % and 61 %, respectively, in the GSH content compared to control (Fig. 3a; $p < 0.05$). *Hydra* exposed to selected concentrations of

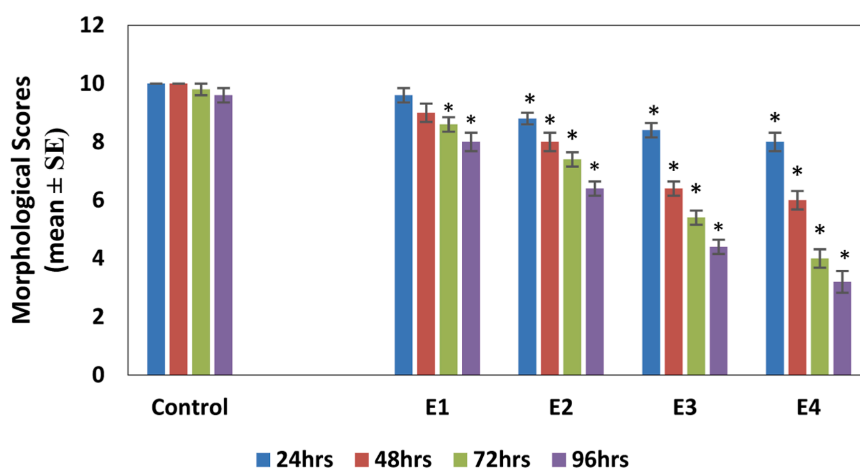

Fig. 1. Morphological scores measured in *Hydra vulgaris* exposed to various doses of EMS for 24, 48, 72, and 96 h of duration. *significant at $p < 0.05$ compared to control. E1 = 0.09 mM; E2 = 0.18 mM; E3 = 0.27 mM; E4 = 0.37 mM; EMS: Ethyl methanesulphonate.

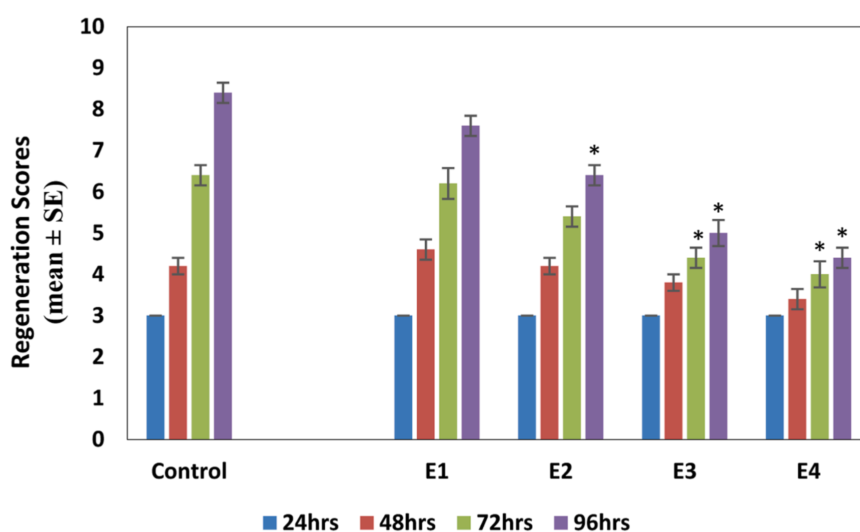

Fig. 2. Regeneration capacity of gastric region measured in *Hydra vulgaris* exposed to various doses of EMS for 24, 48, 72, and 96 h of duration. *significant at $p < 0.05$ compared to control. E1 = 0.09 mM; E2 = 0.18 mM; E3 = 0.27 mM; E4 = 0.37 mM; EMS: Ethyl methanesulphonate.

EMS for 48 h showed a significant increase of 50 %, 100 %, 138 % and 228 %, respectively, in the GST activity compared to control (Fig. 3b; $p < 0.05$). *Hydra* exposed to selected concentrations of EMS for 48 h showed a significant increase of 25 %, 81 %, 108 % and 136 %, respectively, in the activity of SOD compared to control (Fig. 3d; $p < 0.05$). *Hydra* exposed to selected concentrations of EMS for 48 h showed a significant increase of 22 %, 48 %, 66.08 % and 86 %, respectively, in the catalase activity compared to control (Fig. 3e; $p < 0.05$). The results of the comet assay performed on the cells of mid-body region of the hydra exposed to 0.09, 0.18, 0.27 and 0.37 mM of EMS are shown in Fig. 4(a–e). *Hydra* exposed to 0.09, 0.18, 0.27 and 0.37 mM of EMS for 48 h showed a significant increase of 342 %, 1028 %, 2342 % and 3685 %, respectively, in the DNA damage in a dose dependent manner compared to control (Fig. 4f; $p < 0.05$).

4. Discussion

EMS is an anti-cancer drug but is capable of alkylating biomolecules especially DNA which leads to mutation, chromosomal damage (genotoxicity), cancer and foetal abnormalities [22]. In the present world scenario it could damage the aquatic fauna and flora [38]. *Hydra* has been proved to be an excellent model for assessing not only the impact of anthropogenic pollutants, but also any harmful agents present in the aquatic system [32]. The presence of alkylating agents in the environment cannot be ignored [8]. In the present study we have reported the toxic effects of EMS on *Hydra*. The results suggest that the toxic impact of EMS depends on the duration of exposure and the concentration of the EMS. EMS also delayed the duration of regeneration in *Hydra*. Several compounds such as polychlorinated biphenyls, triclosan, bisphenol A, bisphenol diglycidyl ether, noniphenol and 17 α-Ethinylestradiol have been evaluated for their toxic potential on *Hydra*. All compounds produced toxic effects at certain doses and resulted in the inhibition of regeneration [1,28,29,30]. *Hydra* is well known for its regenerative ability and sensitivity towards aquatic chemicals/pollutants [10,28]. More detailed assessment (dose-as well as time-dependent) can be obtained by taking morphological changes as parameter (Karntanut and Pascoe, 2000). A significant dose-dependent increase in the micronucleus frequency was observed in the erythrocytes of *Clarias lazera* upon exposure to EMS [25]. Similarly the DNA damage was observed in the freshwater mussel (*Unio pictorum*) due to the exposure to EMS [38]. Alkylating agents are well-established anti-neoplastic agents that can enter the aquatic environment through human excretion and wastewater [41]. Our present study on *Hydra* clearly demonstrates that the effect of EMS is duration- and dose-dependent. Even in developed

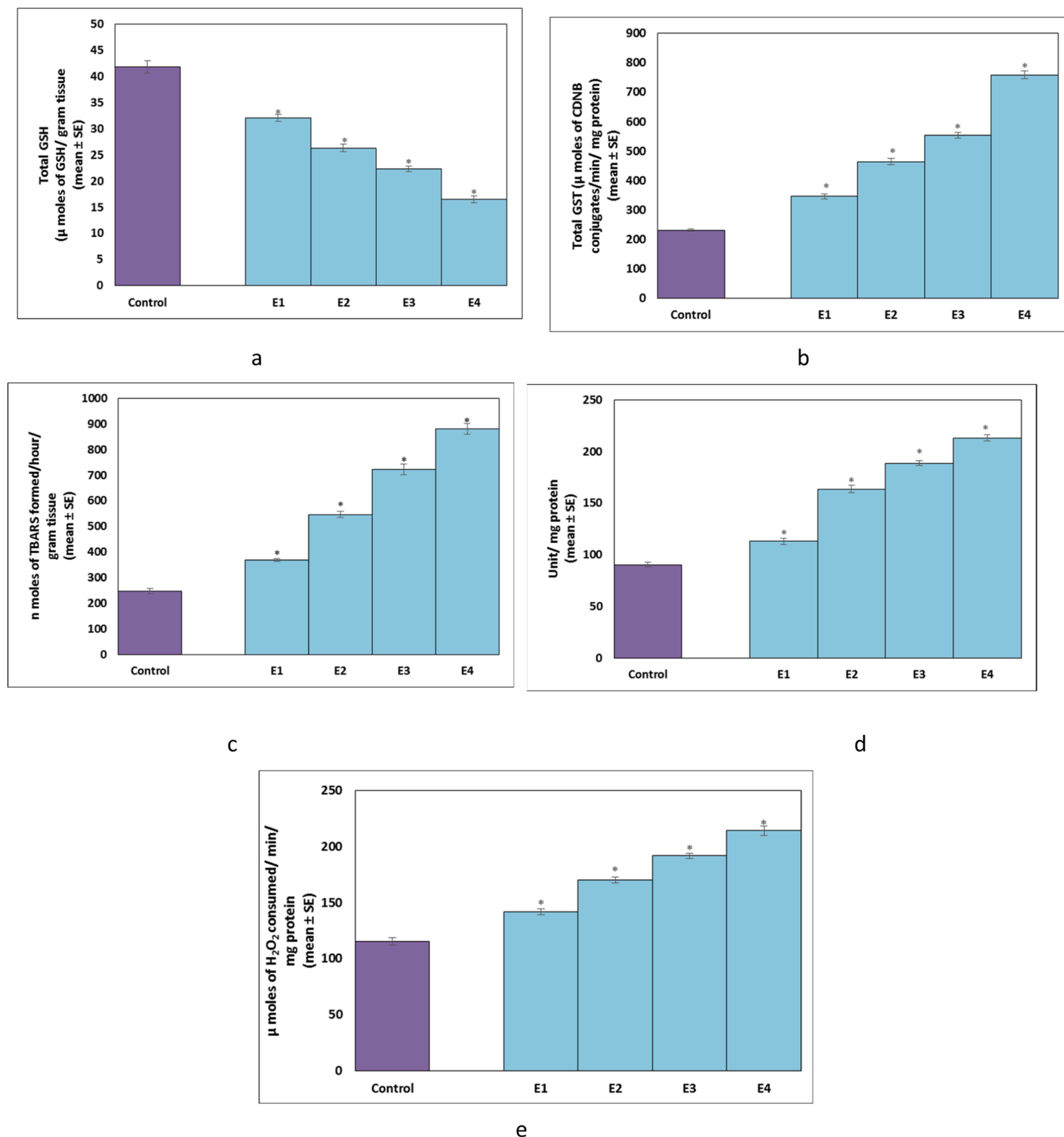

Fig. 3. Glutathione content (GSH) (a), glutathione-S-transferase (GST) activity (b), TBARS levels (c), superoxide dismutase (SOD) activity (d), and catalase (CAT) activity (e) measured in the *Hydra vulgaris* exposed to various doses of EMS for 48 h. *significant at $p < 0.05$ compared to control; E1 = 0.09 mM; E2 = 0.18 mM; E3 = 0.27 mM; E4 = 0.37 mM; EMS: Ethyl methanesulphonate.

countries about 30 % of municipal and industrial waste water remain untreated [41]. Being diploblastic, both ectoderm and endoderm are directly exposed to the toxicants present in the medium [42]. DNA damage has been reported among various animal models exposed to EMS [3,12,38]. EMS acts as a mutagenic agent by alkylating guanine residue and to form base lesion O^6-ethylguanine [4]. GSTs are the main enzymes for the detoxification and perform functions in conjugation with glutathione [39]. The tripeptide glutathione (GSH) plays an important role in taking care of reactive oxygen species (ROS) [18]. An increase in the TBARS due to the exposure of EMS in *Hydra* supports the production of ROS [4]. *Hydra* reduced the generated oxidative stress with the help of SOD, CAT and GSTs [7]. In our study the exposure of EMS resulted in the increase of LPO, SOD, CAT activities and decrease the levels of GSH content which was also observed when the *Hydra* was exposed to other toxicants such as Bisphenol A, copper sulphate, cobalt and various elements [14,23,29,42,43]. The delay in the head regeneration in *Hydra* could be due to the disruption of cell differentiation and inhibition of proliferation by the exposure of EMS. EMS is able to break chromosomes, although the mechanisms involved are not well understood. The plausible hypothesis is that DNA bases are ethylated by EMS (mostly the N-7 position of guanine) that are gradually hydrolyzed from the deoxyribose on the DNA backbone leaving behind an apurinic (or possibly an apyrimidinic) site which is unstable causing single-strand breakage of the DNA [33]. It has been reported that different alkyl methanesulfonates, including EMS, lead to the formation of 7MeG in addition to their own specific alkylation DNA adducts. The ester group of the sulfonate determines the specific types of DNA adducts produced, and the sulfonate might undergo transesterification with the methyl donors that commonly exist in eukaryotic organisms such as SAM, resulting in the formation of MMS, which induce the generation of methyl DNA adducts after EMS exposure [40]. It has been proposed that *Hydra* regeneration is greatly affected by the extent of DNA damage. The damaged DNA will ultimately affect the gene expression of the essential genes [14,15]. Our results of comet assay performed on gastric region

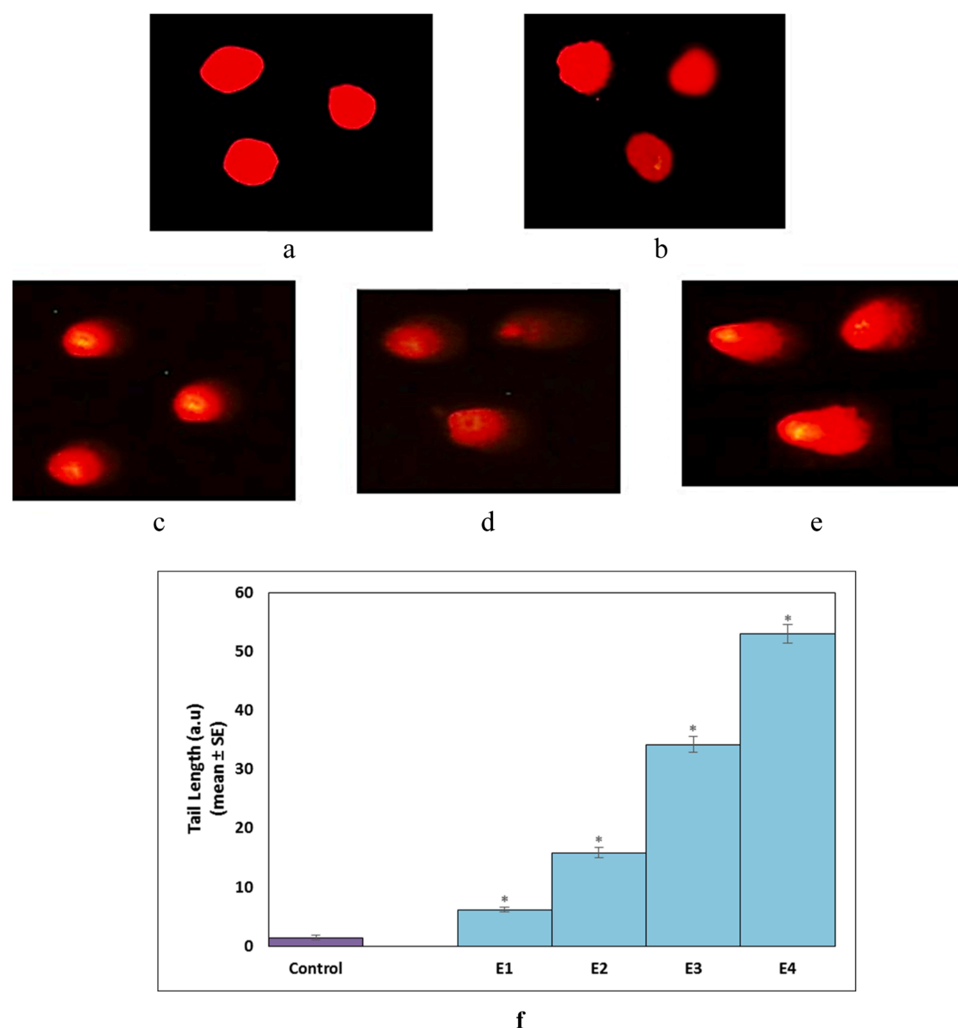

Fig. 4. Comet assay performed on the gastric region cells of *Hydra* (a–e) and tail length (f) measured in the gastric cells of *Hydra vulgaris* exposed to various doses of EMS for 48 hrs. *significant at $p < 0.05$ compared to control. a- Control; b- *Hydra* exposed to 0.09 mM of EMS; c- *Hydra* exposed to 0.18 mM of EMS; d- *Hydra* exposed to 0.27 mM of EMS; e- *Hydra* exposed to 0.37 mM of EMS; EMS: Ethyl methanesulphonate.

cells strongly support that on exposure to EMS the DNA is damaged. EMS has been reported to produce ROS and increased lipid peroxidation [44]. The generated lipid hydroperoxides which can cause damage not only to protein but also to DNA and may affect the expression/functioning of essential genes responsible for regeneration in *Hydra*.

5. Conclusion

The results suggest that *Hydra* is not able to cope up with the stress caused by EMS. EMS is commonly used in various laboratories and is also a well-known anti-cancer agent. It can reach water bodies and can be detrimental to the animals living in water. *Hydra* is useful in assessing the water quality of the aquatic bodies. Our present study measures the impact of various concentrations of EMS on aquatic animal model and suggests the use of *Hydra* as a powerful model for the studying the effect of aquatic contamination.

CRediT authorship contribution statement

yasir siddique: Writing – original draft, Methodology, Data curation, Conceptualization. **Himanshi Varshney:** Investigation. **Iqra Subhan:** Investigation. **Kajal Gaur:** Investigation. **Javeria Fatima:** Investigation. **Smita Jyoti:** Software.

Ethical Statement

The work on *Hydra* does not require ethical clearance.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data Availability Statement

All data generated or analysed during this study are included in this published article [and its Supplementary information files].

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101839.

Data availability

Data will be made available on request.

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