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Evaluation of Genetic Damage and Antigenotoxic Effect of Ascorbic Acid in Erythrocytes of *Orochromis niloticus* and *Ambystoma mexicanum* Using Migration Groups as a Parameter

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Abstract: The comet assay system is an efficient method used to assess DNA damage and repair; however, it currently provides the average result and, unfortunately, the heterogeneity of DNA damage loses relevance. To take advantage of this heterogeneity, migration groups (MGs) of cell comets can be formed. In this study, genetic damage was quantified in erythrocytes of *Oreochromis niloticus* and *Ambystoma mexicanum* exposed to ethyl methanesulfonate (ethyl methanesulfonate (EMS) 2.5, 5, and 10 mM over two hours) and ultraviolet C radiation (UV-C) for 5, 10, and 15 min using the tail length, tail moment, and migration group parameters. Additionally, blood cells were exposed to UV-C radiation for 5 min and treated post-treatment at 5, 10, and 15 mM ascorbic acid (AA) for two hours. With the MG parameter, it was possible to observe variations in the magnitude of genetic damage. Our data indicate that MGs help to detect basal and induced genetic damage or damage reduction with approximately the same efficiency of the tail length and tail moment parameters. MGs can be a complementary parameter used to assess DNA integrity in species exposed to mutagens.

Keywords: comet assay; migration groups; bioassays; genetic damage; ascorbic acid

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1. Introduction

The comet assay system can evaluate DNA damage and repair [1–4] and, due to its great sensitivity, versatility, and rapidity [5,6], has become a valuable tool for understanding the mechanism of action of chemical, physical, or biological agents [7]. It can detect and quantify genetic damage in individual cells, allowing information to be obtained on the heterogeneity of DNA damage or repair within a subpopulation of cells [8–11]. The heterogeneity of genetic damage has been defined as the variance of DNA damage [12] and was first demonstrated by Ostling and Johanson [13] who observed that the variable cellular penetration of an anticancer substance caused the appearance of undamaged cells as well as cells with extensive DNA break. These studies indicated that the heterogeneity of genetic damage could reflect the variation of DNA lesions from one cell to another in the same population [14]. The heterogeneity in DNA migration allowed the observation of responses of small subpopulations to various treatments [12,15] as well as its relationship with cell size, differences in sensitivity to genotoxic agents, or differences in the type of manifested genetic damage [2,10,15–17].

Two classifications were reported in 1995 that used DNA damage heterogeneity to form cell groups with different degrees of damage by visual scoring [18,19]. Visual scoring implies the categorization of comets according to the intensity of the tail and among its advantages were its simplicity, speed, low cost, and the quantification of genetic damage without the use of sophisticated image analysis programs [20]. This proved to be comparable with computerized analysis programs [21]. However, despite these research advances,

it is currently considered that the full potential of the comet test to detect heterogeneity in response to genetic damage has not been exploited, since only the average result is used and heterogeneity loses relevance [10,22]. Reynoso-Silva et al. [22] proposed a new parameter to detect genetic damage, called MGs, which take advantage of the heterogeneity of genetic damage and do not use only the average migration of the tail. It is based on the formation of groups of comets with a similar tail length and allows the use of visual scoring analysis and computerized programs. It was also was observed that the number of MGs is directly proportional to the genetic damage as well as the concentration of the genotoxic agent. MGs have not been deeply studied and could be related to the different sensitivities of cells in response to DNA damage and/or repair due to a specific agent [22] so it is necessary to investigate the advantages that this new parameter can have. Here, the comet assay system was used to evaluate the genetic damage induced by EMS and UV-C, as well as the post-treatment protective effects of AA in UV-C-exposed blood cells of *Oreochromis niloticus* and *Ambystoma mexicanum* using the tail length, the tail moment, and the MG parameter.

2. Materials and Methods

2.1. Chemical and Physical Agents

EMS (CAS 66-27-3) y AA (CAS 50-81-7) were obtained from Sigma Chemical Co. (Guadalajara, Jalisco, México). Dimethyl sulfoxide (DMSO, CAS 67-68-5) and disodium salt EDTA (CAS 60-00-4) were obtained from J.T. Baker (Ciudad de México, México). UV-C radiation was generated from a 254 nm wavelength lamp with a force of 10 volts, which was placed at a distance of 70 cm from the slides containing the blood cells (BCs).

2.2. Obtaining and Preparing the Sample

2.2.1. *Ambystoma Mexicanum* Blood Cells

Eight specimens of *Ambystoma mexicanum* were acquired from an Environmental Management Unit and were kept in captivity following the recommendations of the Basic Manual for the captive care of the Xochimilco de González y Zamora axolotl [23]. The axolotls were acclimatized in 50 L fish tanks with tap water under dark conditions with the following physicochemical conditions: salinity—0, temperature—15 ± 2 °C, pH—7.3 ± 0.2, and dissolved oxygen—8.1 ± 0.5 mg/L. They fed red worm and brine shrimp on alternate days. From each specimen, 100 µL of the whole blood was collected through a cut of approximately 5 mm of the third right branchial arch [24] and placed in a test tube containing 4 mL of phosphate solution (PBS) (160 mM NaCl, 8 mM Na_2HPO_4, 4 mM NaH_2PO_4, and 50 mM EDTA, pH 7) and immediately centrifuged at 3000 rpm for 5 min. The supernatant was removed and the pellet was suspended in 1 mL of PBS and immediately at 4 °C until use. Positive and negative controls were used as recommended [25].

2.2.2. *Oreochromis niloticus* Blood Cells

The fish obtained from the National Genomic Bank were acclimatized in aquariums of 5000 L of tap water following the recirculation of air, under a natural photoperiod with the following physicochemical conditions: salinity—0, temperature—20 ± 1 °C, pH—7.3 ± 0.2, and dissolved oxygen—8.1 ± 0.5 mg/L. Fish were fed fish roe every other day. Blood was extracted by gill puncture from samples 15–23 cm in length. Then, 100 µL of blood sample was obtained from each individual and treated as mentioned in axolotls.

Use in animals was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996). The specimens were sacrificed following the recommendations of Close et al. [26] and NOM-062-ZOO-1999 [27]. All procedures in both species were approved by the Institutional Ethics Committee.

2.3. Blood Cell Treatment

2.3.1. EMS and UV-C-Induced Genetic Damage

Next, 50 µL of each cell suspension, previously obtained, was mixed in individual tubes *v/v* with EMS-PBS solution, taking care of the final concentrations of EMS 2.5, 5, and 10 mM and that of PBS, for 2 h at 4 °C. At the end of the time, two centrifugations were carried out at 3000 rpm for 10 min with PBS to completely eliminate the genotoxic residues. Finally, the precipitate was re-suspended in 0.5 mL of PBS and stored at 4 °C until it was used in the comet test.

To evaluate the genetic damage induced by UV-C, the slides were prepared with agar containing the BC placed at a distance of 70 cm from the UV-C lamp, exposing them separately at 1, 3, and 5 min. After the exhibition, the comet test was carried out.

2.3.2. Post-Treatment Antigenotoxic Effect of AA on Blood Cells Exposed to UV-C

To evaluate the post-treatment antigenotoxic effect of AA, the slides were prepared with agar containing the BC, as indicated above, and exposed to UV-C for 5 min, before being subsequently immersed in different concentrations of AA (5, 10 and 15 mM) for two hours. At the end of the time, three five-minute washes with distilled water were carried out to remove AA residues.

2.4. Alkaline Comet Test

The comet assay was carried out using the method of Speit and Hartmann [28]. Slides were covered with a 1% normal melting point (NMP) agarose, allowing the solidification and removal of a completely clean surface. Then, a 0.6% low melting point (LMP) agarose layer was placed on the slide. Once solidified, another layer of agarose was added (5 µL of the previously obtained cell suspension and 95 µL of the 0.5% LMP agarose). Finally, a third layer of 0.5% LMP agarose was added to cover the second layer [29]. Afterwards, the slides were immersed in lysis solution (2.5 mM NaCl, 10 mM Na_2 EDTA, 10 mM Tris-HCl, 1% lauryl sarcosinate, 1% Triton X-100, and 10% DMSO, pH 10) for 24 h at 4 °C. Subsequently, they were placed in a horizontal electrophoresis system with electrophoresis buffer (NaOH 300 mM and Na_2 EDTA 1 mM) for 45 min and electrophoresis was performed for 15 min (1.0 V/cm with an amperage of ~300 mA (Labconco, model 4333280). After electrophoresis, all slides were gently washed with distilled water to remove the alkaline solution, and all were immersed in neutralization buffer (0.4 M Tris nase, pH 7.5) for 5 min. The gels were stained with ethidium bromide (100 µL to 20 µg/mL) for 3 min and then washed three times with distilled water to remove possible bromide residues.

The slides were analyzed using a fluorescence microscope (Zeiss, model Axioskop 40) with an excitation filter of 515–560 nm. Comets were observed at 10X and their tail length was determined using the comet II test software (Zeeiz Sinoptic Mikro SA De CV México, 2012) that automatically indicates the start and end of the comet's length [30]. Approximately 100 comet cells were analyzed per sample (2 slides per experimental treatment).

To take advantage of the heterogeneity of genetic damage observed in the comet test, the grouping method proposed by Reynoso-Silva et al. was used [22]. Additionally, Microsoft Excel 2019 software was utilized to form MGs with comet cells.

2.5. Statistical Analysis

The Shapiro–Wilk and Levene tests were performed to analyze the normality of the data and its homoscedasticity. Analysis of variance based on Kruskal–Wallis ranks was performed. Dunn's posterior tests were then performed to identify which treatment made the difference. All statistical analyses were performed using the statistical software Sigma Plot 12.0 [31] and Stat-graphics of Nau [32]. $p \leq 0.05$ indicated significance.

3. Results

Basal and induced genetic damage by EMS and UV-C in erythrocytes of *Oreochromis niloticus* and *Ambystoma mexicanum* are presented in Figure 1. In *Oreochromis niloticus*, the tail moment (Figure 1A) and the tail length (Figure 1C) showed significant genetic damage ($p < 0.05$) induced by EMS and UV-C compared to the negative control. EMS showed dose-proportional genetic damage in both parameters, although the 10 mM concentration did not increase as expected, which could be attributed to excessive DNA destruction. In the UV-C case, the tail length was visually proportional to the exposure time, although the difference between UV-3 and UV-5 was not significant. Both tail moment and migration groups were also proportional to UV-1 and UV-3; however, UV-5 did not show significant differences with UV-3. The behavior can be considered uniform in the three parameters since these are not 100% compatible and lead to slight statistical variations. Similar to what was observed with tail moment and tail length, the MG (Figure 1E) shows significant genetic damage ($p < 0.05$) induced by EMS and UV-C with respect to the negative control. It was observed that the concentration of EMS 10 mM decreases the number of MGs; however, the difference is not significant with respect to EMS 2.5 and 5 mM. In the case of UV-C, differences were observed in the number of MGs with an increasing dose.

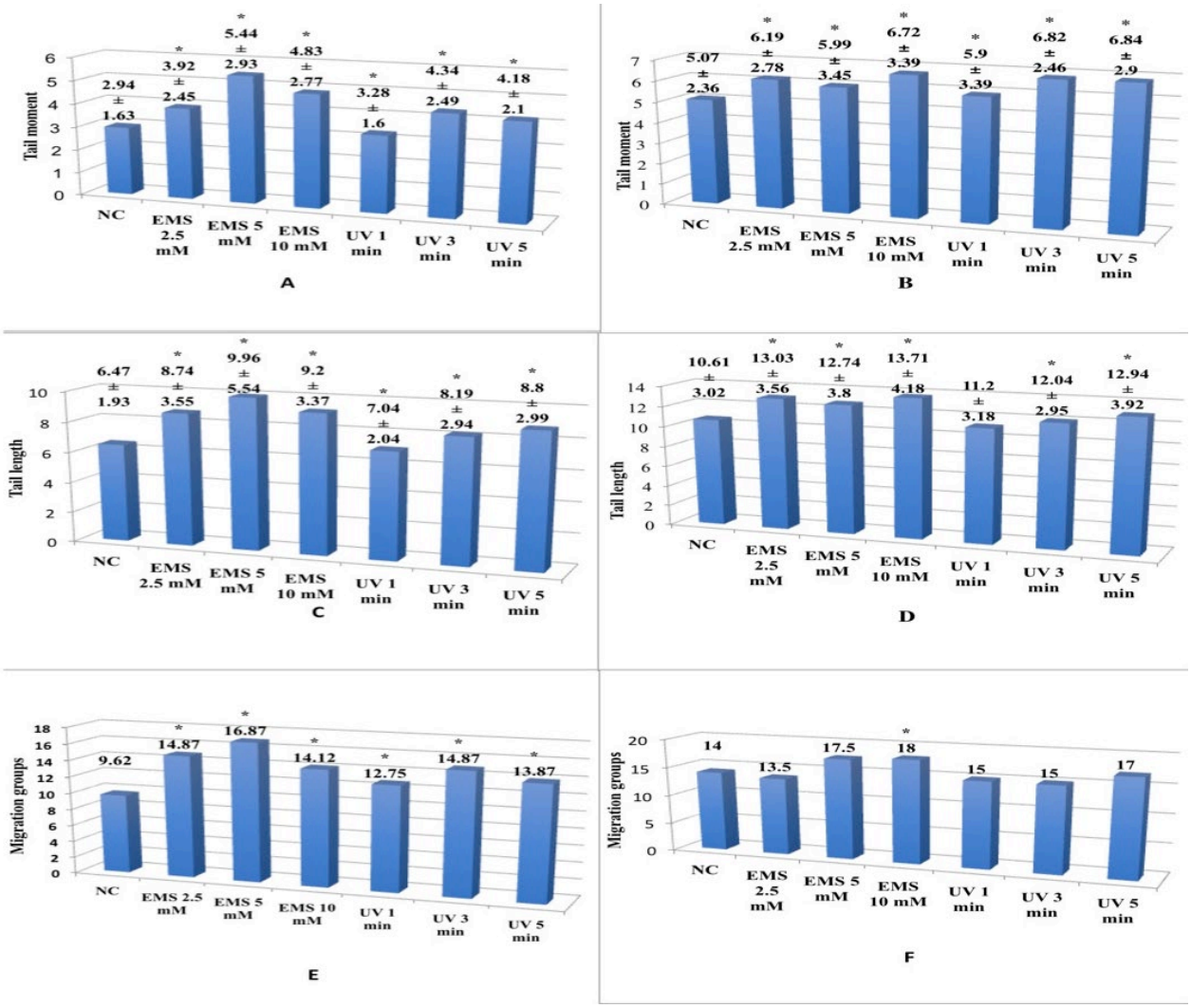

Figure 1. Induced genotoxic effect by different times or concentrations of UV-C and EMS evaluated with the parameters tail moment, tail length, and MGs in comet cells of *Oreochromis niloticus* (**A**,**C**,**E**) and *Ambystoma mexicanum* (**B**,**D**,**F**). * Significant differences ($p < 0.05$) regarding the negative control (NC).

In *Ambystoma mexicanum*, the tail moment (Figure 1B) and tail length also indicated genetic damage. Both EMS and UV-C induce significant genetic damage with respect to the negative control ($p < 0.05$). EMS presented a behavior similar to that reported in *Oreochromis niloticus* only in the tail moment parameter. No dose-response relationship is observed in the tail length. Regarding UV-C, the tail moment and the tail length were proportional to the exposure time. MGs did not reveal EMS or UV-C-induced genetic damage (Figure 1F). Only the 10 mM EMS treatment presented significant differences compared to the negative control ($p < 0.05$).

The genetic damage induced by EMS and UV-C and the protective effect of ascorbic acid after the exposure of *Oreochromis niloticus* erythrocytes to these agents is presented in Table 1. The genotoxic treatment with UV-C in *Oreochromis niloticus* erythrocytes increases significantly ($p < 0.05$) and in direct proportion to the exposure time, the average length of the tail, and the number of MGs with respect to the negative control. No significant differences were observed with respect to the negative control (NC) in the migration group containing the highest number of comet cells. The EMS showed the same behavior except for the 10 mM EMS concentration where the number of MGs (6) and the average tail length (9.2 µm) were obtained. A significant decrease ($p < 0.05$) compared to NC was also shown in the number of cells in the migration group that contained the highest number of comet cells. The antigenotoxic treatment with different concentrations of AA after exposing the cells to 5 min of UV-C showed significant differences in the number of MGs only in UV-C 5 min + 5 mM and in the case of tail length in UV-C 5 min + 5 mM and 15 mM AA. The tail length detected an increase in genetic damage compared to positive control (PC), but not a decrease, while MGs detected a significant decrease at least for UV-C 5 min + AA 5 mM. The number of MGs containing the highest number of comet cells increases significantly ($p < 0.05$) compared to the PC.

Table 1. Genotoxic and antigenotoxic treatments in *Oreochromis niloticus* erythrocytes. The genetic damage was observed using the number of MGs, migration groups with the highest comet cell number, and the mean tail length observed in each treatment.

	Genotoxic Treatment			**Ascorbic Acid Antigenotoxic Post-Treatment**		
	Number of Migration Groups	**Migration Group Containing the Highest Comet Cells Number**	**Mean Tail Length in µm**	**Number of Migration Groups**	**Migration Group Containing the Highest Comet Cells Number**	**Mean Tail Length in µm**
NC	9.6	155	6.47	9.62 *	155	6.47
EMS 2.5 mM	14.87	108 *	8.7 *			
EMS 5 mM	16.87	118 *	9.96 *			
EMS 10 mM	14.12	106	9.2 *			
UV-C 1 min	12.75	182	7.04 *			
UV-C 3 min	14.87	130	8.19 *			
UV-C 5 min	13.87	153	8.8 *			
PC (UV-C 5 min)	13.87			10.87	153	7.04
UV-C, 5 min + AA 5 mM				9.6 *	178 *	7.43 *
UV-C, 5 min + AA 10 mM				10.25	251 *	7.33
UV-C, 5 min + AA 15 mM				13.87	260 *	8.28 *

* Significant differences ($p < 0.05$) regarding the negative control.

The antigenotoxic effects of AA on blood cells *Oreochromis niloticus* and *Ambystoma mexicanum* exposed to UV-C are shown in Figure 2. In *Oreochromis niloticus*, both the tail moment (Figure 2A) and tail length (Figure 2C) show that the AA treatments at 5, 10, and

15 mM are significantly different from the negative control ($p < 0.05$), but a reduction in genetic damage with respect to the PC is observed in the parameter tail length. In the MG (3E), UV-C treatment 5 min + AA 10 mM caused a statistically significant reduction in genetic damage compared to the positive control ($p < 0.05$), even at the basal level, since no statistical difference was observed with respect to the negative control ($p < 0.05$). In *Ambystoma mexicanum*, the tail moment and tail length (Figure 2B,D) showed significant differences in the different treatments with respect to the negative control ($p < 0.05$), but only the post-treatment AA 15 mM reduced the genetic damage compared to the positive control ($p < 0.05$), although it did not reach the level of basal genetic damage. In the MG, no significant differences were observed between the treatments.

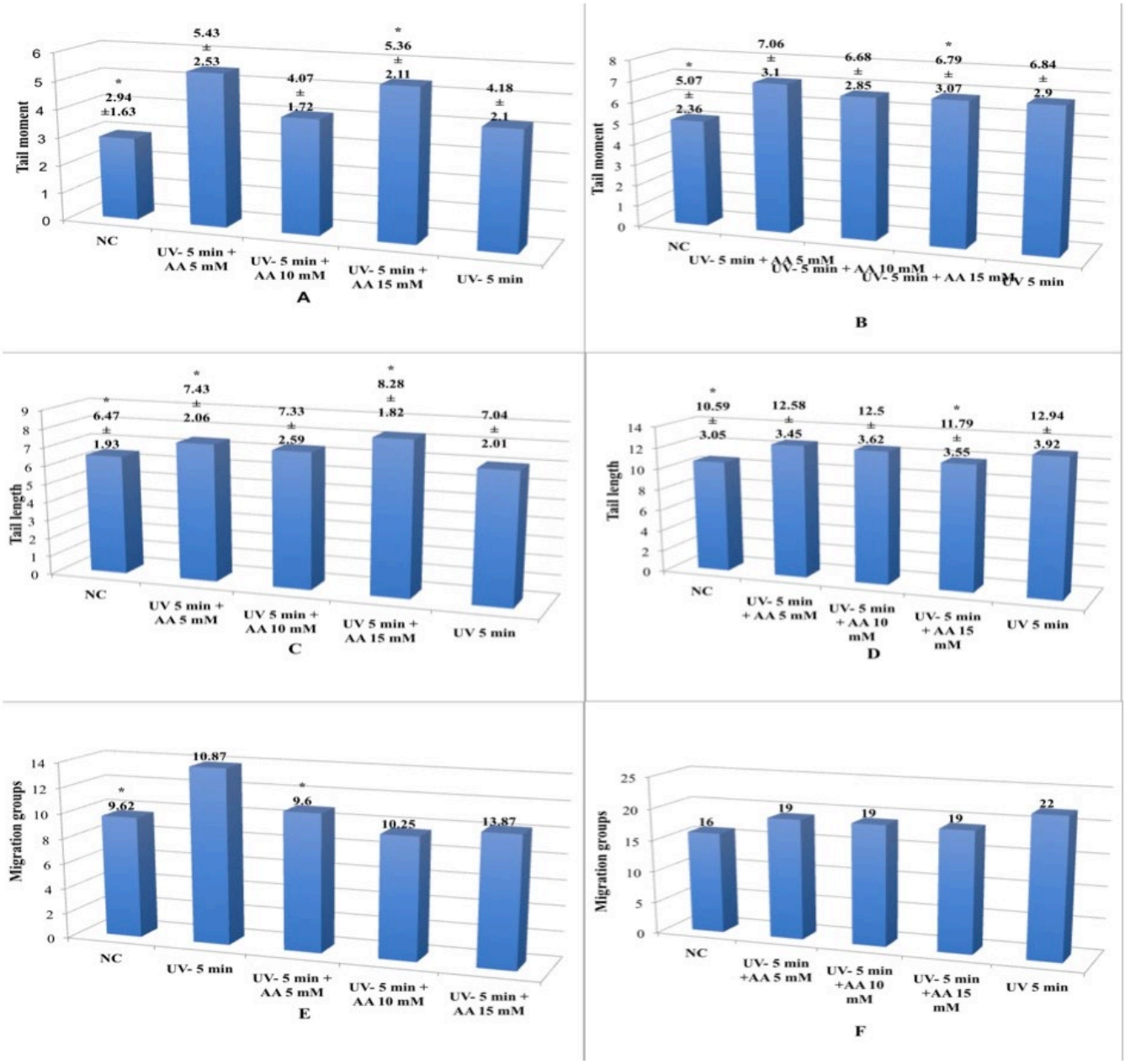

Figure 2. Genetic damage observed using the parameters: tail moment, tail length, and MGs in comet cells of *Oreochromis niloticus* (**A**,**C**,**E**) and Ambystoma mexicanum (**B**,**D**,**F**) exposed post-treatment to UV-C 5 min (PC) and UV-C + AA. * Significant differences ($p < 0.05$) regarding the negative control.

The genetic damage induced by EMS and UV-C and the protective effect of ascorbic acid after exposure of *Ambystoma mexicanum* erythrocytes to these agents is presented in Table 2. UV-C genotoxic treatment in *Ambystoma mexicanum* erythrocytes significantly increased ($p < 0.05$) the average length of the tail, but not the MG with respect to the NC. No significant differences with respect to NC were observed in the MG containing the highest number of comet cells. The EMS showed practically the same behavior, but in the 10 mM EMS, a significant decrease was observed ($p < 0.05$) with respect to the NC in the number of cells of the migration group that contained the highest number of comets.

Table 2. Genotoxic and antigenotoxic treatments in *Ambystoma mexicanumn* erythrocytes. The genetic damage was observed using the number of migration groups, the migration groups with the highest comet cell number, and mean tail length observed in each treatment.

	Genotoxic Treatment			Ascorbic Acid Antigenotoxic Post-Treatment		
	Number of Migration Groups	Migration Group Containing the Highest Comet Cells Number	Mean Tail Length in μm	Number of Migration Groups	Migration Group Containing the Highest Comet Cells Number	Mean Tail Length in μm
NC	13	123	10.61	16	123	10.59 *
EMS 2.5 mM	14	90	13.03 *			
EMS 5 mM	11	105	12.74 *			
EMS 10 mM	14	88 *	13.71 *			
UV-C 1 min	11	132	11.2			
UV-C 3 min	11	127	12.04 *			
UV-C 5 min	11	95	12.94 *			
PC (UV-C 5 min)				22	95	12.94
UV-C, 5 min + AA 5 mM				19 *	115	12.58
UV-C, 5 min + AA 10 mM				19 *	113	12.5
UV-C, 5 min + AA 15 mM				19 *	119 *	11.79 *

* Significant differences ($p < 0.05$) regarding the negative control.

The antigenotoxic treatment with different concentrations of AA after exposing the cells to 5 min of UV-C showed a significant decrease ($p < 0.05$) with respect to PC in the number of MGs in all concentrations. The tail length decreased genetic damage compared to PC only for 5 min UV-C + 15 mM AA. The number of MGs containing the highest number of comet cells significantly increased ($p < 0.05$) compared to PC.

4. Discussion

The comet test is a popular tool used to detect genetic damage [1] and there are several parameters used in this test such as tail length and tail moment [33]. Although the comet test is a well-established test in genetic research [3], its full potential to quantify heterogeneity in response to genotoxic agents has not been exploited [10]; for this reason, Reynoso-Silva et al. [22] proposed a new parameter to detect genetic damage, called the MG. In an effort to assess the ability of the MG as a parameter to detect genetic damage, *Oreochromis niloticus* and *Ambystoma mexicanum* erythrocytes were exposed in vitro to the genotoxic agents EMS and UV-C; additionally, the antigenotoxic effect of AA was assessed. The use of EMS and UV-C radiation as genotoxic models was previously reported in different test systems [34–39] and our data with tail moment and tail length confirm these reports.

The evaluation of MGs, using the tail length, as a product of the heterogeneity in the length of the comets, allows characteristic genetic damage to be evaluated in response to

specific genotoxic agents [22]. For example, UV-C generates MGs with comet cells between 130 and 182, while EMS does it between 106 and 118.

Treatment with both genotoxic agents in *Oreochromis niloticus* and *Ambystoma mexicanum* erythrocytes showed that MG is as efficient parameter as tail length can be used to assess genetic damage [22]. The decrease in tail length observed in *Oreochromis niloticus* erythrocytes with 10 mM EMS treatment can be attributed to excess DNA damage, leading to the underestimation of tail length [40] (Alvarez et al., 2001) and a decrease in the number of MGs. This excess damage and the significant decrease in the number of cells in the MG containing the highest number of comet cells in *Ambystoma mexicanum* suggest that although the average genetic damage increases, the number of cells with high damage decreases. This behavior has not been previously reported. The formation of MGs makes it possible to identify cells with different levels of genetic damage and compare them in the same sample, so that they do not lose relevance as a consequence of the average effect [22].

The large amount of MGs observed in *Oreochromis niloticus* erythrocytes, after applying different treatments, is due to the fact that not all cell nuclei suffer the same damage [41]. The MG helps to identify groups with the highest number of comets and the number of cells per group [22], which could be the subpopulations most affected by each treatment and could be related to the specific damage that provokes each agent. In the erythrocytes of *Ambystoma mexicanum*, we did not observe this behavior with MGs; despite the genetic damage observed, the erythrocytes did not show great heterogeneity of genetic damage, which could be due to the fact that this species has a large number of mechanisms to preserve genome stability [42]; however, we currently have little information about it. This is of great importance [10] because a genotoxic agent might damage only a small fraction of the cells in a tissue, but it is those cells that are most likely to cause a tumor.

AA has been reported as an antigenotoxic agent [43,44]; therefore, it was used in this study and the MG was able to detect a decrease in genetic damage. In *Oreochromis niloticus*, a reduction in genetic damage was observed through the tail length and MG parameters, as has been reported for other bioassays or comet test parameters. Cases where no reduction is observed could be related to poor DNA repair in fish compared to mammals [45–48]. In *Ambystoma mexicanum* erythrocytes, it was not possible to evaluate differences between treatments and MGs also showed the ability to detect UV-C-induced genetic damage and post-treatment using AA in erythrocytes from both organisms. Furthermore, tail length could not detect a decrease in tail magnitude with respect to PC. These results agree with what was reported by Reynoso-Silva et al. [22]. The MG containing the highest number of comet cells significantly increased ($p \leq 0.05$) the number of cells compared to the PC. This may be due to the specific damage that is manifested by the action of high-concentration AA [49]. AA antigenotoxicity is notable at concentrations as low as 5 mM, as observed using MGs, but AA concentrations equal to or greater than 10 mM do not show an antigenotoxic effect.

Despite the average effect of the comet test, the number of MGs observed through the tail length parameter is directly proportional to the genetic damage, which coincides with that reported by Reynoso-Silva et al. [22], indicating that the frequency of comets per group is related to the damage and depends specifically on the genotoxic agent and not only on the concentration.

A limitation of the comet assay is that the cellular response to a genotoxic treatment not only depends on the average amount of DNA damage in the cells; it also depends on the response of a small population of cells [10]. This work shows that MGs allow the subpopulations most affected by agents that damage the genetic material to be observed, which increases the sensitivity of the comet test and complements the existing parameters.

5. Conclusions

Although there are established methods to detect DNA damage, it is necessary to make modifications to the procedures in order to increase their sensitivity and versatility. To use the MG parameter, it was possible to observe induction and a reduction in genetic damage in *Oreochromis niloticus* erythrocytes, but the same effectiveness was not observed in *Ambystoma mexicanum*, which suggests that differences in the sensitivity to genotoxic agents in the organisms studied or the presence of subpopulation cellular highly affected each treatment. MGs can be used as a complementary parameter to assess DNA integrity in species exposed to mutagens.

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