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Estimation of the Role of Different Staining Protocols on Micronucleus Test Accuracy in Gamma-Irradiated Rats

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Abstract

The micronucleus (MN) assay is widely used to assess the genotoxicity and cytotoxicity of various agents in different species. One of the key factors that can affect the accuracy of micronuclei (MNi) scoring is the staining protocol. Thus, this study aimed to investigate the effect of different staining protocols on the scoring accuracy of micronuclei induced by gamma irradiation in male rats. Male rats were exposed to a single dose of 6Gy were γ-irradiation and euthanized 24 hours later. Bone marrow cells collected and an MN assay was prepared using different staining protocols, including Giemsa (G), Feulgen (F), Hematoxylin & Eosin (HE), and a combination of Feulgen and Hematoxylin & Eosin (F-HE). The results revealed significant differences between the scored MNi frequencies using different stains as compared with each other. In conclusion, there is an interaction between staining techniques and the scoring accuracy of MNi and aberrant cell frequencies. Therefore, standardization and choice of staining protocols are critical for reliable and consistent results in micronucleus assays.

Keywords: Micronucleus, Gamma irradiation, Genotoxicity, Giemsa, Feulgen, Hematoxylin & Eosin

Introduction

MN assay was proposed in the early seventeenth. It is a simple technique for assessing genotoxicity in animal bone marrow erythrocytes. Afterward, it was demonstrated that peripheral blood lymphocytes could be applied for the MN test and advocated for its use as a biomarker (Adhikari, 2019). MN could develop during the mitotic metaphase/anaphase transition (Guo *et al*., 2020). It might be caused by a chromosomal fragment that didn't integrate into the daughter nuclei upon breaking (Fenech, 2020). The MN assay may be considered the most sensitive technique for detecting DNA damage (Mousavikia *et al*., 2023). Therefore, the MN assay has emerged as one of the most widely used assays for determining the genotoxicity of various chemical and physical variables, including ionizing radiation-induced DNA damage (Sommer *et al*., 2020).

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Ionizing radiation is used for thereby of various types of cancer (Yi *et al*., 2021). Ionizing radiation damages DNA, causing single and double-strand breaks as well as DNA protein crosslinks, and may result in cell death (Bushmanov *et al*., 2022). Considering the close relationship between DNA damage and carcinogenesis (Rezatabar *et al.*, 2019), early recognition is critical for successful therapy and a favorable prognosis in many cancer cases (Ginsburg *et al.*, 2020). Consequently, MN can be utilized as a screening technique for early detection of cancer. Particularly the MN test can detect both numerical chromosomal changes and chromosome breaks (Setayesh *et al.*, 2020).

In the MN test, several stains are utilized. Some stains, such as Feulgen, Acridine Orange, 4',6-Diamidino-2 Phenylindole (DAPI), and Propidium Iodide, are DNA-specific. On the other hand, non-specific stains like Giemsa, May-Grünwald-Giemsa, Papanicolaou, Orcein, and Hematoxylin and Eosin (Singam *et al.*, 2019). However, the impact of various staining protocols on the outcomes of MN tests has received very little consideration (Yarmohammadi & Naderi, 2023).

The purpose of this study was to see how different staining techniques affected the scoring accuracy of MNi and aberrant cell frequencies in γ-irradiated rats. MN frequencies were examined using four distinct staining protocols: Giemsa (G), Feulgen (F), Hematoxylin and Eosin (HE), and Feulgen followed by Hematoxylin and Eosin (F-HE).

Materials and Methods

Animals Grouping

Acquired from the National Center for Radiation Research and Technology, Cairo, Egypt, sixteen mature male rats weighing between 110 and 120 g. Prior to the experiment, the animals were placed in metal cages in a room with good ventilation and given a week's acclimation period. The animals were given water and a typical commercial pellet meal. The animals were split up into two groups, each with eight animals. Control group: Not irradiated rats and Irradiated group: Rats were exposed to (6Gy) γ-rays. The samples from each animal were sub-divided into 4 Groups (group for each stain). All the study's protocols, animal precautions, and treatment were in agreement with the ethical guidelines allocated by the Research Ethics Committee (REC-NCRRT) with approval No. (24A/20).

γ-Irradiation

The rats were exposed to (6Gy) whole-body gamma-irradiation at the National Centre for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority, Cairo, Egypt, using a (Cesium-137) Gamma Cell-40 biological irradiator at a dose rate of 0.42Gy/min.

Micronucleus Test

According to Jain & Pandey (Jain & Pandey, 2019), rat femur bone marrow samples were collected at the time of sacrifice. The femurs were washed with 2 ml of fetal calf serum (Sigma) in centrifuge tubes. The bone marrow samples were homogenized and the cell suspensions were centrifuged at 1000 rpm for 10 min. The supernatant was then partially discarded to leave a few drops of fetal calf serum to re-suspend the cell pellet in it. Then the samples were smeared on clean, dry slides, which were then fixed with absolute methanol for 10 min. Eight sample slides were made for each animal to be used in the micronucleus assay. From each animal 1000 cells were scored (for each 2 observers) to count the number of micronucleated polychromatic erythrocytes (MNPCEs) and micronucleated normochromatic erythrocytes (MNNCEs) **(Figure 1)**.

Cytological Staining and Experimental Design

All the stains were purchased from the Biodiagnostic Company (Egypt). The cytosmears were divided into 4 groups (two slides each) and individually stained with G, F, HE, and F-HE as follows:

Giemsa Staining (G-group)

Slides were stained with G at a concentration of 5% (v/v) and diluted in phosphate buffer (Na_2HPO_4 0.06 M and KH_2PO_4 0.06 M, pH 6.8) (Johnson *et al.,* 2010).

Feulgen Staining (F-group)

Slides were immersed in 5mol/L HCl for 10 minutes, double washed with distilled water, stained with Schiff's reagent for 10 minutes, the strain was drained without washing and fixed with a fixative solution for 2 minutes, and then slides were washed in running tap water for 5 minutes, and finally counterstained with 1% light green for 2 minutes (Zhang *et al.,* 2015).

Hematoxylin & Eosin Staining (HE-Group)

Slides were stained in Hematoxylin for 20 minutes, then washed thoroughly in running tap water, differentiated in acid-alcohol, again washed in running tap water for 10 minutes, and then counterstained in Eosin for 2 minutes. Finally, the slides were washed in running water until the excess eosin was removed (Bancroft & Layton, 2012).

Combination Staining between Feulgen and Hematoxylin & Eosin (F-HE-Group)

The slides are stained first with F then immersed in Hematoxylin for 3-5 minutes, and Eosin for a few seconds in the last step after staining the slides with light green (JalayerNaderi, 2018).

Criteria for Scoring

Screening of each slide was made in a Zigzag manner from one end to the other end of the slide. For each slide, 1,000 erythrocytes with integral cell borders were counted according to Holland *et al.* (2008) criteria for defining an additional nuclear body as an MN. A LeitzWetzlar-Orthomat binocular optical microscope was used for scoring cells. All of the slides were examined at low magnification x125 for screening and high magnification x1250 for MNi counting. MNi was scored blindly twice by two distinct observers at different intervals.

Statistical Analysis

As the total number of MNi (MNPCEs and MNNCEs) scored in 1000 cells, the MNi frequencies were calculated. The data that were acquired were presented as mean ± standard error, or M±SE. Version 20 of the Statistical Package for Social Science (SPSS) program for Windows was used to conduct the statistical analysis. One-way analysis of variance (one-way ANOVA) was utilized to assess significant differences between groups, and for multi-group comparisons, the least significant difference (LSD) was employed. Additionally, to assess the significant impact of stain type on the obtained data, use a two-way ANOVA and then Tukey's multiple comparison test (Festing & Altman, 2002). Cohen's kappa value was used to evaluate the inter-observer agreement (IOA), and the approximate 95% confidence interval (CI) was then computed. P-values of less than 0.05 were regarded as noteworthy (Viera & Garrett, 2005).

Results and Discussion

When compared to the matching control group, the percentages of total MNi in the irradiated group stained with the four distinct techniques and scored by two observers show a significant increase by ≈9-fold. While these results demonstrated significant variation between investigated stains among the same group (whether control or irradiation group) **(Figure 2)**.

In the same manner, for the 4 different stains, the MNPCEs and MNNCEs frequencies in the irradiated group, as scored by the 2 observers, were significantly higher than in the corresponding control group. However, there is a significant variation in the observed results when comparing different stains. G had the highest scored MN frequency followed by F-HE, whereas F had the lowest value **(Table 1)**. In addition, the results revealed that all of the staining procedures had a good level of agreement. F-HE had the highest inter-observer agreement (kappa = 0.9) followed by HE (kappa = 0.87), which represents outstanding agreement. G exhibited substantial agreement with (kappa = 0.77). F had the lowest inter-observer agreement (kappa = 0.70) **(Table 2)**.

Both of the major effects (irradiation and staining method) were statistically significant, and the staining methods and groups had a significant interaction impact. **Figure 3** depicts the interaction impact between the four distinct staining techniques and the acquired findings in the different groups using two-way ANOVA profile plots of the estimated marginal means.

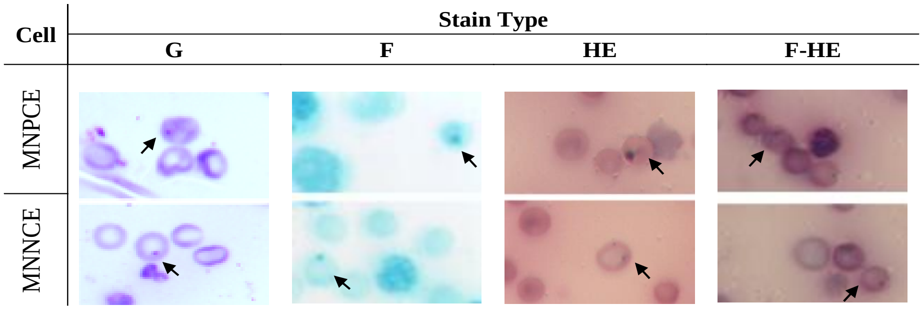


Figure 1. Photomicrographs of MNPCEs and MNNCEs using four distinct staining methods (G, F, HE, and F-HE)

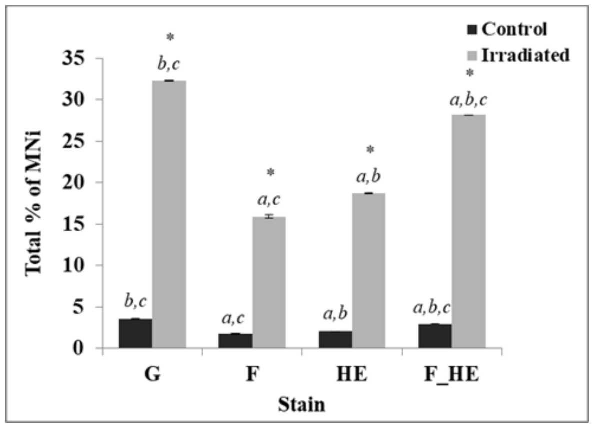


Figure 2. Histogram of total micronucleated cells in the bone marrow of control and 6Gy γ-irradiated rats using four different stains: Where, a: significant as compared with G, b: significant as compared with F, and c: significant as compared with HE, and *: significant as compared with corresponding control group. P-values ≤ 0.05 were considered significant.

Table 1. Comparison of MNPCEs and MNNCEs frequencies in the bone marrow of control and 6Gy γ-irradiated rats scored by two different observers using (G, F, HE, and F-HE)

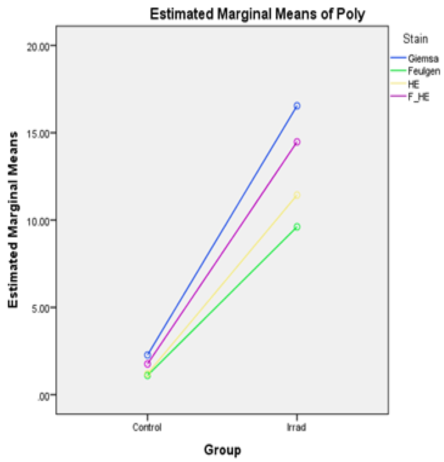
Observer	Group	Stain Type							
		G		F		HE		F-HE	
		MNPCEs	MNNCEs	MNPCEs	MNNCEs	MNPCEs	MNNCEs	MNPCEs	MNNCEs
A	Control	1.95± 0.11	1.56± 0.11	0.91± 0.09*	0.85± 0.09*	1.10± 0.09*#	0.95± 0.06*#	1.63± 0.06*#$	1.33± 0.04*#$
	6Gy	16.59± 0.41 a	15.73± 0.44a	9.84± 0.23a*	6.29± 0.12a*	11.41± 0.17 a*#	7.23± 0.20 a*#	14.48± 0.99 a*#$	13.64± 1.11 a*#$
B	Control	1.96± 0.12 b	1.63± 0.14b	0.93± 0.09b*	0.88± 0.08b*	1.11± .10 b*#	0.96± 0.05 b*#	1.65± 0.07 b*#$	1.34± 0.04 b*#$
	6Gy	16.50± 0.43 ac	15.68± 0.47 ac	9.39± 0.42ac*	6.33± 0.13 ac*	11.45± 0.16 ac*#	7.28± 0.20 ac*#	14.48± 0.99 ac*#$	13.69± 1.12 ac*#$

Note. Where, a: significant as compared with A-Control, b: significant as compared with A-6Gy, and c: significant as compared with B-Control. And *: significant as compared with the corresponding group and cell type stained with G, #: significant as compared with the corresponding group and cell type stained with F, and $: significant as compared with the corresponding group and cell type stained with HE. P-values ≤ 0.05 were considered significant.

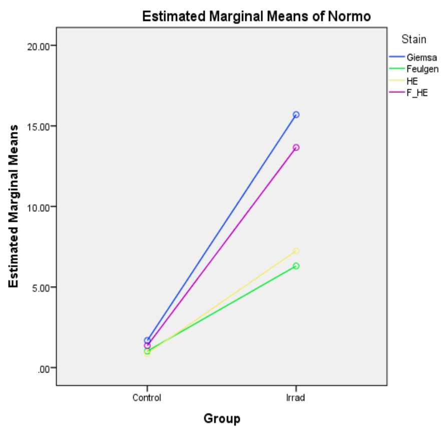
Table 2. The symmetric measures to assess the inter-observer agreement (IOA) using Cohen's kappa value and the approximate 95% confidence interval (CI)

Stain Type	Value of kappa	SE	95% CI
G	0.77*	0.075	(0.919, 0.625)
F	0.70*	0.082	(0.866, 0.544)
HE	0.87*	0.061	(0.988, 0.748)
F-HE	0.90*	0.05	(0.999, 0.803)

Where,
* indicate significant agreement between observers (P-value <0.05),
A value of kappa >0.7 indicates a good level of agreement;
0.4≤kappa≤0.59→ moderate agreement,
0.6≤kappa≤0.79→ substantial agreement,
0.8≤kappa≤0.99→ outstanding agreement.



a)



b)

Figure 3. Two-way ANOVA profile plots of the estimated marginal means of MNPCEs a) and MNNCEs b) frequencies

The most vulnerable organ to the cytotoxic effects of ionizing radiation is bone marrow. Ionizing radiation causes many forms of DNA damage in bone marrow cells, some of which may go unrepaired. Unrepaired DNA damage, in this case, may result in cell death or chromosomal instability (Bagheri *et al.*, 2018). Micronuclei (MNi) are tiny, extra nuclei generated by the exclusion of lagging chromosomal segments or whole chromosomes during mitosis. MNi frequency, thus indicates chromosomal breakage or mitotic damage indirectly. MNi quantification is commonly employed in cytogenetic damage analysis (Bochtler *et al.*, 2019).

The F stain's strong DNA specificity and clear translucent appearance of the cytoplasm, which allows easy detection of MNi, might explain the lowest count (Dave *et al.*, 2019). Apart from being a very sensitive technique, one limitation of this staining procedure is that it is somewhat protracted, and might result in the underlining of MNi (Bertolino *et al.*, 2023). The higher MNi incidence reported with nonspecific DNA stains (G and HE in the present study) might be attributed to nuclear abnormalities such as karyorrhexis, karyolysis, and condensed chromatin are misinterpreted as MNi (Setayesh *et al.*, 2021). Keratin granules (spherical cytoplasmic aggregates), that formed in degenerated cells with nuclear abnormalities as a result of cell damage and lack DNA, may be identified as MNi using nonspecific stains (Kohli *et al.*, 2017). An additional communal source of confusion is the presence of tiny dye granules that might sometimes mimic MNi (Sabharwal *et al.*, 2015). F-HE improves nucleus visualization by increasing ground contrast. The form and contour of the nucleus are quite distinct and exact when using this procedure. This combination might be a modified F staining procedure for enhanced nucleus visualization (JalayerNaderi, 2018).

These parameters were carefully considered in the current study to reduce the likelihood of counting these mimickers. The reason for the substantially larger count found with G and F-HE compared to F stain deserves more investigation. Because the scientific literature lacks adequate appropriate data on the use of G and F-HE stains for MNi count, more extensive studies are needed to confirm the cogency of these stains. Using each of the four staining procedures, whether by a DNA-specific stain or a DNA-nonspecific stain, the mean total MNi frequency in the irradiated group was considerably greater than that in the controls **(Figure 2)**. These findings are consistent with those obtained in several researches, such as those conducted by (Shao *et al.*, 2018). This study anticipates laying the groundwork for more detailed comparative studies to confirm the validation of DNA nonspecific stains in MN assays and the role of stain type in the accuracy of the results. Additionally, we must keep in mind that the type of stain is a significant factor when comparing the results from different studies.

Conclusion

In conclusion, the staining protocol used can have a significant impact on the scoring accuracy of MNi induced by gamma irradiation in male rats. In this model, our findings revealed an interaction effect between staining techniques and the scoring accuracy of micronucleated cells. Staining protocol standardization is critical for reliable and consistent results in MN assays. Furthermore, and based on our examination, which included more than one type of stain. We recommend that the assay not be limited to monitoring micronuclei in erythrocytes alone, as there are numerous other measurements with unique alerts and connotations for any experimental study. Examples include screening myelocytes in addition to erythrocytes and recording their MNi or the presence of morphological disorders, as well as screening for some cytological processes that indicate the expression of apoptotic and necrotic cells.

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Conflict of interest: None

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Ethics statement: The authors declared that all study protocols, animal precautions, and treatment were in accordance with the ethical guidelines assigned by the Research Ethics Committee (REC-NCRRT) with approval No. (24A/20).

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