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Short Note

Is the micronucleus test indicative of the chronic exposure to genotoxic agents in all mammals? Implications of the spleen in the results of this test

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Abstract

Environmental studies in several countries are including genotoxicity tests as tools to evaluate exposure to contaminants. Most of these tests are developed on sentinel species of mammals. The *Ctenomys* genus has been used as a sentinel for screening environmental contamination in several countries as Brazil, Chile, Argentina and Uruguay. One of the most used assays has been the *in vivo* micronucleus test which can be conducted easily on peripheral blood samples since the maturation of erythrocytes involves the loss of the major nucleus. The test potentially can detect genotoxic damage caused by chronic exposures. However, the results obtained may reflect chronic exposure depending on the spleen features that the species have. Until now, there are no studies to determine the role of the spleen in the *Ctenomys* genus. This note provides new data dealing with this subject and provides an anatomical interpretation of the results obtained. To determine chronic or recently exposure when a sentinel is used, is highly recommended to develop this kind of approaches.

During cell division (mitosis), the genetic material is replicated and divided equally giving rise to two identical daughter cells. However, if errors in replication or subsequent DNA cleavage (for being affected the mitotic spindle) or genotoxic agents generated breaks, chromosomal losses occur and the distribution of genetic material is not equitable. The areas of the genome that are lost cause one or more nuclei of smaller size than the primary nucleus called micronuclei (MN). Detached genetic material may be derived from whole chromosomes (loss caused by aneugenic processes), or more frequently from acentric chromosome fragments (loss caused by clastogenic events), being excluded from the primary nucleus of new mitotic cells during anaphase (Ribeiro et al., 2003; Zalacain et al., 2005; Murdy and Carballo, 2006).

Since mammalian erythrocytes have no nucleus, the MN can be easily detected and this is why the blood, in the case of mammals, it is the most widely tissue used for the MN test (Ribeiro et al., 2003; Murdy and Carballo, 2006).

Originally, MN test was developed to bone marrow cells of rodents by Schmid (1975). The use of bone marrow erythrocytes is used to detect recent genetic damage, while MN test in peripheral blood erythrocytes reveals chronic exposure to mutagens (Ribeiro et al., 2003; Murdy and Carballo, 2006; Udroiu, 2006a). However, most studies using MN as a molecular marker of genetic damage in mammals, do not analyze the role of the spleen in the removal of micronucleated erythrocytes (Udroiu, 2006a). A way to know, if we choose as sentinel, a species that has MN removal by the spleen, without performing splenectomy, is to compare the frequency of MN in erythrocytes from peripheral blood and the bone marrow. If the percentages of red blood cells containing MN in bone marrow significantly exceed the percentages in peripheral blood, this implies splenic removal of MN from the circulating cells (Udroiu, 2006b).

The vascular distribution is perhaps the greatest source of variation in interspecific splenic architecture. The variation in the structure and morphology of the venous sinuses forms the basis of the classification of the spleens into two groups: sinusal and non sinusal spleens. The former is found in rats and dogs and the latter in mice. Venous sinuses of sinusal spleens are larger, more numerous, have numerous anastomosis and a characteristic structure of the venous walls in relation to the non sinusal spleens. Venules pulp of the sinusal spleens are not small and have not anastomosis, they are characterized by an endothelium constituted by irregular flattened cells with fenestrations easily crossed through by erythrocytes (Cesta, 2006; Udroiu, 2006b).

Venous sinuses of the sinusal spleens are formed by rod-shaped endothelial cells with small gaps between them where erythrocytes accumulate, especially if a red blood cell has a rigid inclusion as a MN (Chen and Weiss, 1973; Chotivanich et al., 2002; Udroiu, 2006b). This type of spleen is only present in mammals, while in other taxa, erythrocytes have a nucleus, making impossible the cell circulation between these fine grooves. This is important, since in the event of finding differences between the percentages of MN from the bone marrow and peripheral blood, we could say that in the *Ctenomys* genus, MN test may be used as a marker of recent genetic damage.

The result of the MN test in animals as *Ctenomys*, which are fossorial rodents, is even more relevant than when it is used in mammals living on the earth's surface, given their special physiological characteristics.

The *Ctenomys* genus exhibit prevalence of hypoxia and hypercapnia, this fact, generated several physiological changes that are not found in other species, such as high concentrations of hemoglobin and hematocrit when compared with underground rodents (Stein et al., 2010).

Studies in *Ctenomys lami*, suggest that hemoglobin and hematocrit values are lower than those found for other subterranean rodents that feed on roots. It is important to note that, individuals of the genus *Ctenomys* feed mostly on the aerial parts of the plants that they eat, which implies a decrease in exposure to an atmosphere rich in carbon dioxide (CO_2) and low in oxygen (O_2) as they must leave their caves to feed (Stein et al., 2010).

In animals who require efficient oxygen transport, the presence of MN in their erythrocytes may affect the transport capacity of this molecule, the erythrocyte concentration in the blood and hence their physiology.

Another aspect to be considered when the MN test is performed is the effect that different staining procedures have on this assay. The most

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commonly used dye is Giemsa; nonspecific dye which according to Casartelli et al. (1997) generates false positives. Nersesyan et al. (2006) recommend the use of nucleic acid specific stains such as propidium iodide (PI) and 4',6-diamidino–2–phenylindole (DAPI).

For all relevant questions above mentioned, two aspects of the MN test in *Ctenomys* are studied here: 1. Differences generated by specific (PI) and nonspecific (Giemsa) staining and, 2. Comparison of the percentages of erythrocytes with MN and total MN between smears from bone marrow and peripheral blood to determine what type of spleen have the species used in this work.

The percentages of erythrocytes that present MN (one or more) and total MN (one erythrocyte can have more than one MN) are compared among smear made from bone marrow and from peripheral blood. Through these comparisons are expected to determine what type of spleen has the *Ctenomys* genus, to determine if the MN test can detect long-term chronic exposure or it must be limited to recent exposure to mutagens. Moreover, it is attempted to compare the percentage of erythrocytes presenting MN and total MN using specific and nonspecific staining for nucleic acids to determine whether the type of dye causes differences in the results obtained.

Samples from twenty animals of *Ctenomys pearsoni* from the south of Uruguay were used. Smears were made following the procedure described by Fenech (2007). To calculate the percentages of erythrocytes with MN and the total micronuclei (MN), Infinity Analyze™ software was used. The total erythrocyte counted was 2000 by smear (which were performed in duplicate and the final values were averaged for each individual). Percentages were compared by parametric statistics (Student's t test) by Statistica 6.1 software, since the data were normally distributed. The observations were made in epifluorescence microscope coupled to a camera Olympus. Two types of staining were used: nonspecific (Giemsa) and nucleic acids specific (PI) (2 μg/ml). Procedure followed Nersesyan et al. (2006).

The number of erythrocytes containing MN (cells containing one or more MN) and the total MN (more than one MN can be present in each cell) were registered on the basis of 2000 individual cells and the percentage was calculated for each variable. Regardless the staining used (PI and Giemsa), the differences among smears of peripheral blood and bone marrow in the percentages of erythrocytes with MN (2.87‰ peripheral blood -4.7‰ bone marrow for PI and 3.25‰-5.1‰ for Giemsa) and total MN (3.7‰-5.83‰ peripheral blood- bone marrow for PI and 4.55 peripheral blood -6.74 bone marrow for Giemsa) were statistically significant ($p<0.05$). The percentages of erythrocytes with MN and total MN when different dyes (PI or Giemsa) were compared did not show statistically significant differences. The percentages of erythrocytes with MN in peripheral blood were 2.87‰ for PI and 3.7‰ for Giemsa whereas that the percentages of total MN were 3.25‰ for PI and 4.55 for Giemsa. For bone marrow, the values obtained for erythrocytes with MN were 4.7‰ for PI and 5.83 for Giemsa whereas that for total MNs the values were 5.1‰ for PI and 6.74 for Giemsa. Although, an increasing of percentages were observed, differences were not significant ($p>0.05$).

These results confirm that the spleen of *Ctenomys pearsoni* populations used in this study is sinusal, i.e. possess venous sinuses that retaining MN as described for other mammals (Cesta, 2006). This implies that individuals undergoing to either aneugenic or clastogenic agents, could exhibit a decreasing of the mean corpuscular volume (MCV) due to the removal of MN in the spleen. This coincides with the findings of da Silva et al. (2000) for *Ctenomys torquatus*, exposed to pollution from coal mining in southern Brazil, where the frequencies of erythrocytes with MN in bone marrow exceeded those from peripheral blood.

This indicates that for the MN test, these sentinels as well as *Canis lupus*, *Rattus norvegicus*, *Cavia porcellus*, evaluate recently exposure, since micronuclei of red blood cells are removed from the circulation by the spleen (Cesta, 2006; Udroiu, 2006a,b). This does not occur in *Mus musculus*, felines and deer. In these groups, there are non sinusal spleen, therefore, the MN of erythrocytes are not retained in the spleen and remain for long periods in the bloodstream. This does not invalidate the use of the test in species with sinusal spleens as *Ctenomys* (Udroiu, 2006a), except for the fact that only young erythrocytes have to be considered (as well called polychromatic erythrocytes, PCE).

Regarding staining techniques, it is noteworthy that no differences were found between specific staining for DNA-RNA and non-specific staining. If Giemsa solution is properly prepared, it does not precipitate and if the processes of fixing and washing of the preparations are done correctly, there should be no differences for these tissues. It is important to note, that the greatest differences between staining procedures in the literature reviewed were detected on oral mucosal cells (Rajeswari et al., 2000; Nersesyan et al., 2006) rather than blood cells.

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