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Assessment of Genotoxicity PHB/Norbixin/Ethyleneglycol Membrane by Micronucleus Test and Comet Assay

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Abstract:*Biomaterials, synthetic or natural origin substances that replace and / or stimulate biological systems that no longer have their functions, require the biocompatibility characteristics, biodegradability and biofunctionality for the restoration of tissue function, for example. Given the antioxidant potential norbixin (Bixaorellana L.) and biodegradability and biocompatibility of polyhydroxybutyrate (PHB) (natural polymer), aimed to prepare a membrane from these two constituents with the addition of ethylene glycol reagent (PHB/norbixin/ethyleneglycol) to evaluate their genotoxic effects through the micronucleus test and comet assay. For this study, 15 animals Rattusnorvegicus were divided into 3 groups: A - the membrane was introduced into the peritoneum of the animals through a laparotomy; B - just a laparotomy with distilled water; C - single dose of cyclophosphamide injection of 50 mg/kg intraperitoneally.They were collected from each rat bone marrow and peripheral blood to perform, respectively, the micronucleus test and comet assay. In conclusion, both tests suggested that the membrane is not genotoxic.*

Keywords:*Biomaterials, PHB/norbixin/ethyleneglycol, Micronucleus test, Comet assay.*

I. Introduction

Biomaterials are substances of natural or synthetic origin which replace and/or stimulate biological systems that have lost functions such as tissue repair, that is, loss of continuity of the skin in wounds. These substances must present physical, chemical and biological properties necessary for treatments that lead a better repair and regeneration guided satisfactorily [1,2].

The good performance of these materials in living organisms requiresimportant characteristicssuch as biocompatibility, biofunctionality, biodegradability and the ability to dissolve in organisms [3] in order to obtain good tissue interaction consistent with the healing process.

The polyhydroxybutyrate (PHB), a natural polymer with unique feature of biodegradable thermoplastic,produced by bacteria from renewable carbon sources such as sugarcane, is being used in membranes for biological purposes [4]. PHB is linear saturated polyester, partially crystalline, with properties similar to plastics derived from petroleum, such as polyethylene and polypropylene. The consistency as a biomaterial has polyhydroxybutyrate and bioresorbable and biocompatible action polymeric plasticizercan be used in models of biological membranes [5-8].

The PHB into membranes may be associated with several biomaterials with important properties in tissue healing and bone repair. The polyhydroxybutyrate can be associated with hydroxylapatite, collagen, and other natural materials. The norbixin, an extracted apocarotenoide of annatto (*Bixaorellana L.*), a native plant of Tropical America, has great potential for use in biodegradable and biocompatible polymeric membranes due to antioxidant, antimicrobial and antitumor properties it presents. compounds extracted from*Bixaorellana L.*are widely used in the food and textile industry as natural colorants and dyeing fabric; they have the ability to counteract free radicals and even several studies aimed to evaluate its properties in animals through interactions with biological environment, they have reported the absence of genotoxic effects, teratogenic or mutagenic by these materials [9-13].

This research aimed to develop polyhydroxybutyrate membranes and norbixin (*Bixaorellana L.*) with the addition of ethyleneglycol to evaluate genotoxic effect through the micronucleus test and comet assay, as part of the recommended test battery for assessment of his mutagenic potential for further biological application with healing purposes.

II. Materials and Methods

The study was approved by the Ethics Committee on Animal Use - CEUA / FACIME, protocol number 09241/2016 on 08/08/2016. The whole procedure and handling of the animals were conducted strictly in accordance with the Guiding Principles for the Care and Use of Laboratory Animals and theAroucaFederal LawNo. 11.974 2008 - Animal Experimentation.

2.1 Synthesis Membrane

In the membrane preparation initially polyhydroxybutyrate (0.5 g) was dropped into chloroform. Then, the PHB in chloroform was heated under stirring and the norbixin pigment (0.250 g) andethyleneglycol (0.45 ml)was addedto the PHB and after the solution to reach the ambient temperature, the mixture was then poured into petri dish and allowed to stand for 24 hours. Ethylene glycol was added to dose for better conformation of the biomaterial.

2.2 Test Micronucleus

The experimental procedure was performed in the Experimental Surgery Laboratory (LACE) of FACIME / CCS.The sample consisted of 15 animals *Rattusnorvegicus*, divided into three groups, such as: A - negative control, B - exposure to the membrane and C - positive control.Each experimental group consisted of 05 animals anesthetized with ketamine (1.0ml/kg) and Xylazine (1,1ml/kg) intramuscularly. The rats were sacrificed 72 hours after the start of the experiment. Micronuclei were measured in 2,000 polychromatic erythrocytes (PCEs) / animal in bone marrow of adult *Rattusnorvegicus*, totaling 10,000 PCE's/group. In group A, there was no treatment was made just a peritoneal laparotomy for surgical simulation process. In group B, with exposure to biomaterial, proceeded with laparotomy for implantation of the membranein the peritoneal region (Fig 1). In group C, it was administered in single dosean injection i.p. cyclophosphamide(known to be genotoxic and inducing DNA damage of bone marrow cells) at a concentration of 50 mg/kg per animal.

Figure 1: Membrane implantation in the peritoneum of Rattusnorvegicus.

2.2.1 Processing, making the blade and end staining

Bone marrow cells were collected immediately after the sacrifice of animals. For this, we used one 01ml syringe for collection. This syringe was filled with fetal bovine serum. The needle was introduced in the opening of one end of the femur and the fetal bovine serum was injected through the channel, pushing the medullary component toward the other end, in a Falcon tube previously marked with the animal code. Bone marrow material was resuspended fetal bovine serum until homogeneous.

The suspension was centrifuged for 5 minutes at 1,000 rpm, discarding the supernatant the end of the procedure with Pasteur pipette. The sample was added with 0.5 ml of fetal bovine serum and suspended by homogenization.

Smears were prepared dripping off 02 drops suspension on the tip of a slide (previously labeled with the animal's code) and with the aid of another slide bent at a 45 degree angle to make the smear. After preparation of the smear slides were air dried. They were made two slides per animal.Staining was performed 24 hours after preparation of the slides for 3 minutes in Giemsa. The analysis was performed in blind field in an increase of 100x (immersion objective) in a short time by the same observer. We determined the frequency of micronucleiin 2000 PCEs cellsper animal.

The entire protocol was based on publications [14-18].

2.3 Comet Assay

Each experimental group consisted of 5 animals, according to the division and procedures performed in the aforementioned groups, lasting and parallel application to the micronucleus test. In each group, the analysis were processed 4h after exposure (acute) (n=five animals) and 24h after treatment (chronic study) (n=five animals).

2.3.1 Processing, preparation and slide staining

At the end of each exposure (4h or 24h) blood samples were collected from the tail of the animal and 40µl of this sample were transferred to microcentrifuge tubes containing 120mL of low melting point agarose (1.5%) at 37ºC. This mixture was homogenized and transferred to slides pre-coated with 5% agarose. Then the slides were covered with cover slips and placed at 4ºC for 30 minutes. Coverslips were removed and slides were immersed in lysis solution [NaCl (2.5M), EDTA (100mM) and 1.2 g of TRIS (10mM), 1% Triton X-100 and 10% DMSO].

For DNA denaturation, the slides were placed in an electrophoresis tank containing a buffer solution pH>13(300mM NaOHand 1mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) for 20min. Electrophoresis was performed at 25V and 300mA at a temperature of 4°C for 15 minutes. The slides were then immersed in a neutralizing solution (0.4M Tris, pH 7.5, 3 cycles of 5 minutes each).

The analysis was performed in immunofluorescence microscopy inincrease40xequipped with filter 420- 490nm and 520nm barrier filter. The images were obtained with a digital camera CCD 5.0 megapixel for immunofluorescence. It evaluated DNA damage by measuring the percentage of DNA in the tail and the height/length of the tail. These parameters were calculated at 100 nucleoids/sample (two slides per individual). For this, we used the software OpenComet. The entire protocol was based on publications [19-21].

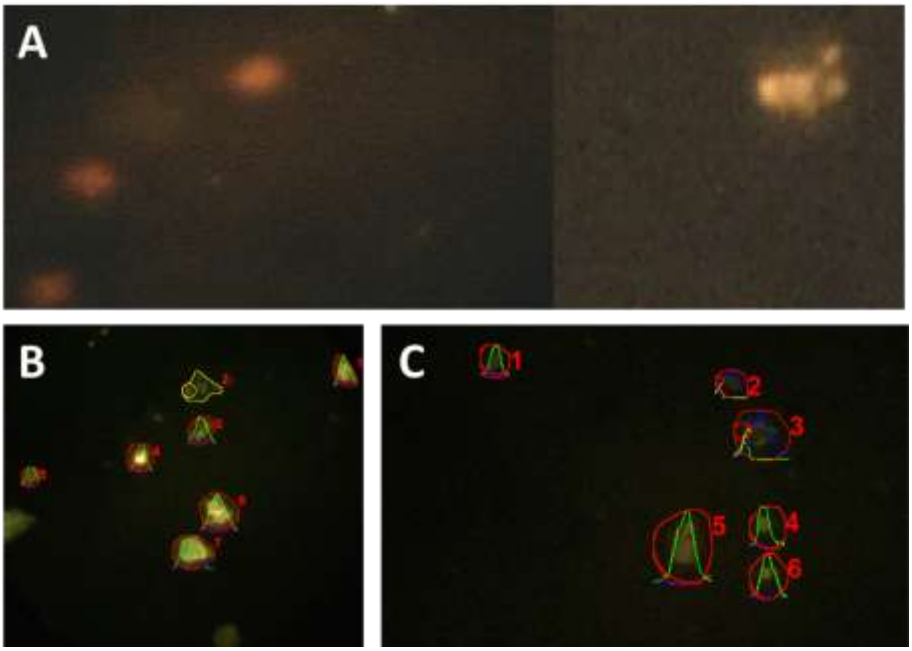

Figure 2: A) Example of analyzed comets. B) and C) processing by software OpenComet - analysis of 100 comets by exposed individual.

2.4 Statistical analysis

Data were analyzed using one-way ANOVA program and test T Student. A significance level of 5% ($p<0.05$) was considered. The results were calculated and expressed as mean and standard deviation.

III. Results

Fig 3 shows the presence of polychromatic erythrocytes with micronucleus in bone marrow *Rattusnorvegicus*.

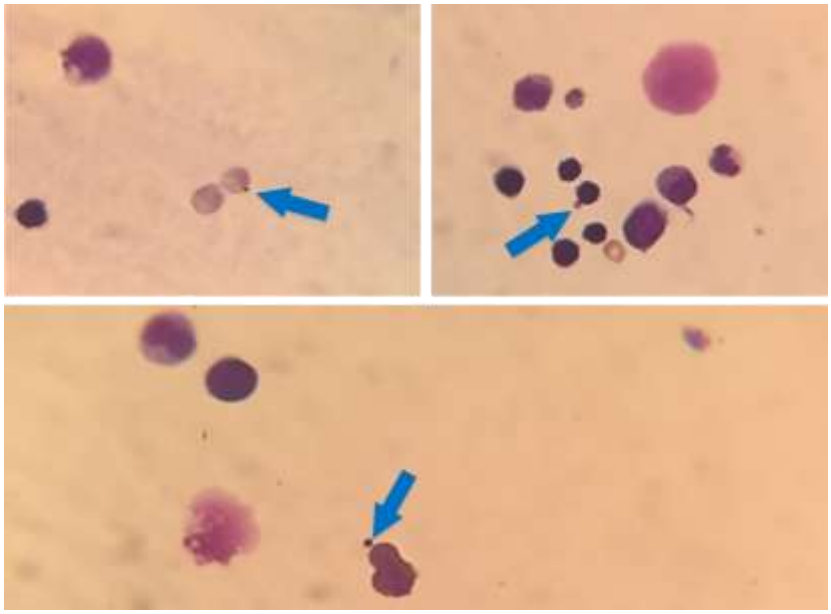

Figure 3: Image obtained by optical microscopy at 100x objective of showing the presence of micronuclei on the arrows.

Table 1 and Fig 4 shows the absolute frequency and PCEMNsmean of micronuclei with your representative percentage in the analyzed groups, as well as the mean and standard deviation of the same.

In the process of exposure to the membrane after 72 h of the experiment, there was no significant increase in genotoxicity by the micronucleus test when compared to the negative control because there was no statistical difference in the incidence of polychromatic erythrocytes in rats ($p>0.05$) . When compared to the positive control represented by cyclophosphamide, known to be genotoxic, there was a significant difference ($p<0.001$).

Table 1: Frequency of micronucleated polychromatic erythrocytes (PCEMNs) in bone marrow *Rattusnorvegicus* exposed to membrane PHB/Norbixin/Ethyleneglycol (chronic exposure - after 72 h).

Treatment	**Number of pces analyzed**	**PCEMNs**		**Mean ± Standard deviation**
		Nº	%	
Distilled water	2.000	61	3,05	12,2 ± 1,92
CPA (50 mg / kg)	2.000	226	11,3	21,0 ± 4,74
Membrane	2.000	105	5,25	45,2 ± 8,25

In Fig 4, the mean values were arranged MM found by treatment group after 72h of the experimentwith the data in Table 1.

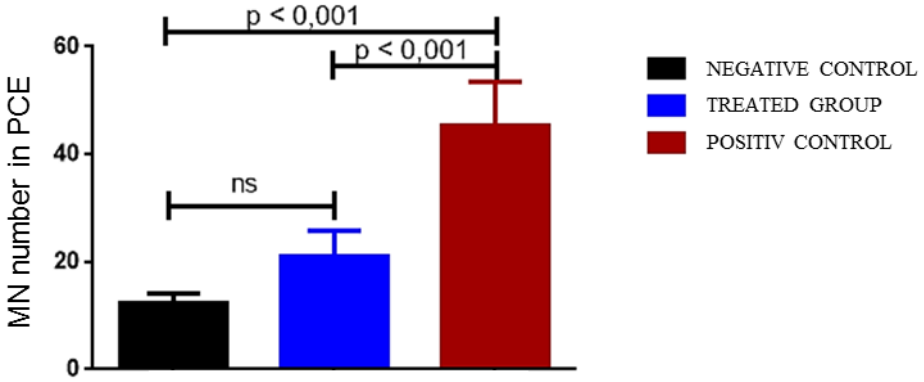

Figure 4: Mean of MM found in 2000 polychromatic erythrocytes for exposure 72h.

Regarding the assessment by the comet assay, it showed an increase of damage to the exposure of the 1st 4h evaluated by the% of DNA in the tail. This result significantly decreased after 24h of exposure. The genotoxic effect was apparently repaired. This finding is consistent with other parameters analyzed, as TailMoment and the length of the tail.

Table 02: DNA damage found per group after exposure to membrane PHB/Norbixin/Ethyleneglycol.

Parameters	Positive Control	Negative Control	MEM 4h	MEM 24h
% DNA cauda	6,8 ± 1,1	1,1 ± 0,21	5,8 ± 1,2	1,2 ± 0,23
Comprimento da cauda(μM)	17,0± 2,0	7,6± 2,1	14,3 ± 0,4	7,0 ±1,0
TailMoment	1.9 ± 0,1	0,8± 0,6	1,0± 0,32	0,54± 0,2

IV. Discussion

In this study we tested the genotoxicity of the membrane PHB/norbixin/ethyleneglycol in an experiment in vivo with *Rattusnorvegicus* animals through the micronucleus test and comet assay. The micronucleus test suggested that the biomaterial has no biological toxicity in order to not change the incidence of polychromatic erythrocytes. But the comet assay, although it revealed an increase in the DNA damage in the first 4h, suggested a repair of the same in 24 hours.

Similar results were obtained in the study of Monte et al., in which the membrane polystyrene-collagen-norbixin showed no mutagenic effect by means of micronucleus test and comet assay, bone marrow *Rattusnorvegicus* after 72h under similar conditions for this exposure. Despite the promising results, there are few studies that evaluated the effects and biological properties of norbixin in animal tissues [22].

Researchers like Esposito et al conducted in vitro assay with PHB. Blends of poly (p-dioxane) / poly (hydroxybutyrate) (PPD / PHB) have been developed and tested using fibrochondrocyte for application to cartilage tissue, and showed no cytotoxicity. It has been found by MTT analysis that blends allowed adhesion and fibrochondrocyte proliferation in several variations of its composition, and also maintainability Synthesis extracellular matrix collagen.They concluded that such blends may be recommended for cell culture [6].

The in vivo assays used in this study are important because they bring biological and physiological information detailedand they are officially approved and considered by the Organization for Economic Cooperation and Development (OECD) as part of the security protocol analysis [18,14]. Potential to carcinogenic studies of promising products and materials for biomedical use utilizing genotoxicity tests are on the rise, as a given DNA may be associated with any carcinogenic potential. Therefore, it is necessary to evaluate them [23,24].

V. Conclusion

In summary, the micronucleus test and comet assay,known genotoxic tests,showed results that corroborate the adequacy of the membranePHB/Norbixin/Ethyleneglycol for biological purposes and these will contribute to future studies on the effects of the membrane on the healing of skin wounds.

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