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Genotoxicity Evaluation of Polystyrene Membrane with Collagen and Norbixin by Micronucleus Test and Comet Assay

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ABSTRACT: *The biocompatible membranes are widely applied in the medical field in order to stimulate tissue repair. The biological principle of this type of treatment is the repair and guided regeneration. In the literature, there are few reports of studies evaluating the effects and biological properties of norbixin in animal tissues. Thus, the present study was to evaluate the effect of polystyrene membrane with collagen and norbixin, through the micronucleus test and comet assay in rats, as part of the recommended test battery to evaluate the mutagenic potential. The research project was approved by CEP / FACID Protocol 069/2014. For this study, 15 rats were divided into 3 groups were used: A - the membrane was introduced into the peritoneum of the animals through a laparotomy; B - received cyclophosphamide at a dose of 50mg / kg intraperitoneally; C - were performed only one laparotomy. A peripheral blood sample was collected from the animals for conducting Comet assay and 72 hours after the start of the experiment were euthanized. It was collected bone marrow material of each rat to perform the micronucleus test. In conclusion, through the tests, the membrane is not genotoxic.*
Keywords: *Genotoxicity, Mutagenicity, Biomaterials, Norbixin.*

I. Introduction

Articular cartilage is a tissue with no vascularization and no nerve endings and low capacity for self-repair, a damaged time has no restorability, thus resulting in osteoarthritis [1]. Osteoarthritis (OA) is a chronic degenerative disease which involves the articular cartilage, synovial fluid, ligaments, bone, meniscus, tendon and periarticular muscle, however, the destruction of cartilage is a common feature of OA progression and results in inefficient operation the affected joint.

The normal joint cartilage is composed of large amounts of extracellular matrix (mainly type II collagen), produced and maintained by chondrocytes. Currently, there is no effective therapy in the treatment of OA, except those that relieve the symptoms of the disease until the time that the joints need replacement surgery [2].

The lack of repair mechanism is attributed to the lack of cell recruitment to the injured area [1]. To overcome this problem, a lot of research on cartilage lesions articular treatments are discussed, but until now no study reports the regeneration of hyaline cartilage altogether. Several alternatives are practiced, including, arthroscopic interventions, techniques, cell-based, autograft/allograft cartilage and gene therapy technique, however, compensation is always performed by fibrocartilage that does not have the same biomechanical characteristics that the hyaline cartilage, necessary for the normal functioning of joints [3, 4].

There is therefore a clear need for alternative strategies for the production of curative treatments. Tissue engineering is an interdisciplinary field of rapid development that involves both materials science and engineering to medical research to address clinical problems. Most tissue engineering strategies is based on the use of biocompatible materials to facilitate tissue regeneration [5].

In recent years, it has increased the search for substances that can be used in tissue engineering in order to develop biomaterials that can be applied to renovate or restore the function of diseased or traumatized tissues in the body. Generally, naturally occurring biomaterials are characterized with biocompatibility, ie, have a lower rate of rejection by the living tissue allowing the adhesion and migration of cells within their structures in relation to synthetic polymers and also a lower cost manufacturing [6].

Methods based on the principles of tissue engineering have emerged as the most promising approaches for the repair of joint damage. These methods utilize a combination of cell signaling molecules and scaffolds for increasing the regenerative capacity of the natural joint with the aim of restoring healthy structure and function

of the damaged tissue [4]. Tissue engineering has successfully produced a number of applications in soft tissue, but regeneration of the articular surface is a formidable challenge, in view of the articular cartilage has a limited capacity to regenerate and mechanical stimuli play a major role in structure and function of cartilage and subchondral bone [4, 6].

Thus, it was developed a polystyrene membrane with collagen and norbixin following the biological principle of such treatment, ie, tissue engineering, which is guided in repair and regeneration. Norbixin is a compound extracted from the surface of the annatto seeds, fruit Urucuzeiro, plant species *Bixa Orellana L*., native to Central and South America, which has great potential in obtaining biodegradable and biocompatible polymers. The norbixin (C24H2804) is a hydrophilic dicarboxylic carotenoid, antioxidant. In the literature, there are few studies of reports that evaluate the effects and biological properties of norbixin in animal tissues [8].

Little is known, however, about the biocompatibility and cytotoxicity in vivo of such biomaterial. Therefore, in this study, our aim was to evaluate the biocompatibility and cytotoxicity of this membrane using *invivo* methods such as the micronucleus rat bone marrow cells test and the comet assay using peripheral blood of animals [9 10].

II. Material And Methods

2.1 Polystyrene membrane and norbixin with collagen

The membrane components were dissolved in chloroform in a ratio of 0.20 grams of polystyrene for 0.05 g of norbixin and 0.05 g of collagen. After 2 hours of magnetic stirring the mixture was placed in a *petri* dish at room temperature to evaporate the solvent forming the membrane.

2.2 ForMicronucleus Test

The experimental procedure was carried out in Experimentation and Mutagenicity Laboratory (LABEMUT) and Laboratory of Molecular Biology and Biological Studies Injuries (LABMINBIO) of the State University of Piauí (UESPI) as approved (Protocol - 069/2014) in the Research Ethics Committee (CEP/FACID). The exposed group (E) consisting of 05 rats received the membrane introduced into the peritoneum through a laparotomy and were fully anesthetized. They were euthanized 72 hours after the beginning of the experiment. It was collected bone marrow material from each rat after euthanasia to perform the micronucleus test in polychromatic erythrocyte, to evaluate the degree of genotoxicity.

2.2.1 Negative Control (NC)

Consisting of 5 rats in which were performed only one laparotomy. The duration and application are processed in parallel with the comet assay.

2.2.2 Positive Control (PC)

Five rats received injections via the I. P. with a single dose of cyclophosphamide at a concentration of 50 mg/kg per animal. The duration and application are processed in parallel with the comet assay.

2.2.3 Processing, preparation of the slide and staining

Bone marrow cells were collected immediately after the sacrifice of animals. For this, we use a 01 ml syringe for collection. This syringe was filled with Fetal Bovine Serum (FBS). We introduce the needle into the opening of a femur and FBS injected through the channel, pushing the medullary component toward the other end positioned in a *Falcon* tube, previously marked with the animal code. Resuspend the FBS in bone marrow material, to achieve homogeneity. The suspension was centrifuged for 5 minutes at 1,000 rpm and the supernatant discarded at the end of the procedure with *Pasteur* pipette. Completed the sample with 0.5 ml of FBS and resuspend by homogenization. Dripping the prepared suspension 02 drops in smears dull edge of a slide (previously marked with the animal's code) and with the aid of other slide bent at a 45 degree angle, we smear. After making the smear, let air dry. Were prepared two slides per animal. Staining was performed 24 hours after preparation of the slides for 3 minutes in *Giemsa*.

2.2.4 Analysis of the slides

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 micronuclei in 2000 PCEs cells per animal. The entire protocol is based in several publications [11-15].

2.3 For Comet Assay

The experimental procedure was performed in the LABEMUT and LABMINBIO UESPI laboratories. The exposed group (E) consisted of 5 animals that received the membrane introduced into the peritoneum through a laparotomy.

2.3.1 Negative Control (NC)
Consisting of 5 rats in which were performed only one laparotomy. The duration and application are processed in parallel with the micronucleus test.

2.3.2 Positive Control (PC)
Five rats received injections via the I. P. with a single dose of cyclophosphamide at a concentration of 50 mg / kg per animal. The duration and application are processed in parallel with the micronucleus test. Samples were processed 4h and 24h after exposure.

2.3.3 Processing, preparation of the slide and staining
The comet assay was performed on peripheral blood taken from the tail of each animal after 4 hours of exposure (check the damage) and 24 hours of exposure (check the repair).At the end of each period it is collected40 uL, transferring them to microtubes containing120 uL of low melting-point agarose (1.5%) at 37ºC.The mixture was homogenized and transferred topre-covered with agarose slides,getting coverslips (to spread the content homogeneously) and stored at 4ºC for 30 minutes [16]. Then the coverslips were removed and immersed invertical glass cuvettes containing lysing solution [NaCl (2.5M); EDTA (100 mM) and 1.2 g of TRIS (10mM)].Upon use, was added 1% Triton X-100 and 10% of DMSO.The slides were placed in a vessel containing electrophoretic buffer pH> 13.0 (300 mM NaOH and 1 mM EDTA, prepared from a stock solution of 10N NaOH and 200 mM EDTA, pH 10.0), remaining at rest for 20 minutes.The "race" electrophoretic was performed with 25 V and 300 mA at a temperature of 4ºC lasting 15 minutes in the darkconditions.After electrophoresis, the slides were removed from the chamber and immersed in neutralizing solution (0.4 M Tris, pH 7.5, for 5 min).This process was repeated 3 times.Finally, they were rinsed with distilled water and air dried, flushing them with Gel Red (2.0 Dilution: 10,000 uL per 10 minutes).All slides were analyzed in immunofluorescence microscopy (increase of 40X) equipped with excitation filter (420-490 nm) and barrier filter (520 nm).The images were obtained by a Opton System (digital camera CCD 5.0 mega pixel for immunofluorescence).It evaluated the damage to DNA by DNA percentage of measurement in the tail (% DNA-measurement of the proportion of the total DNA present in the tail) and the time of the tail (TM- tail length times the percentage of DNA in the tail) [17].These parameters were calculated on 100 nucleoids/sample (two slides per individual). For this, we used the software OpenComet [18].

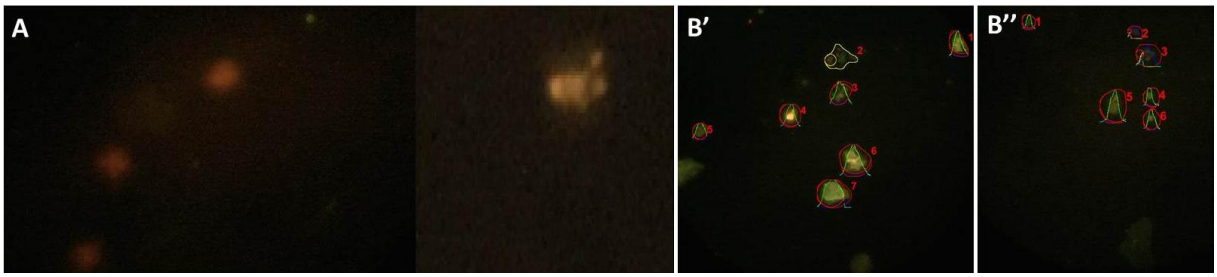

Figure 01. (A) Photomicrograph of comets. (B 'and B' ') processing the Open Comet software. Analysis of 100 comets per exposed individual.

2.4Statistical analysis
Data were analyzed using One-Way ANOVA and Tukey test. For all tests was considered a 5% significance level. The results were expressed as mean, or standard deviation of three independent experiments. The result was considered positive if there was a statistically significant increase ($p < 0.05$).

III. Results

3.1 For micronucleus test
In this paper we evaluate the genotoxic potential ofpolystyrene membrane with collagen and norbixina on bone marrow cells from *Rattus norvegicus*. Table 01 shows the mean micronuclei group E (Laparotomy + membrane) was 10.80 ± 7.56, PC group (cyclophosphamide) was 42.40 ± 11.93 NC group (laparotomy) was 3.6 ± 0.89. Process of exposure to the membrane did not increase significantly the mutagenicity when compared to the negative control ($p > 0.05$). When compared to the positive control, it detected a statistically significant difference ($p > 0.001$) (Fig. 02).

Table 01. Frequency MN in polychromatic erythrocytes in bone marrow *Rattus norvegicus* exposed to polystyrene membrane with collagen and norbixina

Group	Number of PCEs Analyzed	PCEMNs		Mean± standard deviation
		N^0	%	
CN	2.000	18	0,9	3,6 ± 0,89 [a]
CP	2.000	212	10,6	42,40 ± 11,93
E	2.000	178	8,9	10,80 ± 7,56 [a]

Legend: NC = negative control; PC = positive control (cyclophosphamide 50 mg/kg); E = Exposed; PCE = polychromatic erythrocytes and PCEMNs = Micronuclei in Erythrocytes polychromatic. "A" indicates a statistically significant difference (P <0.001) when compared to CP.

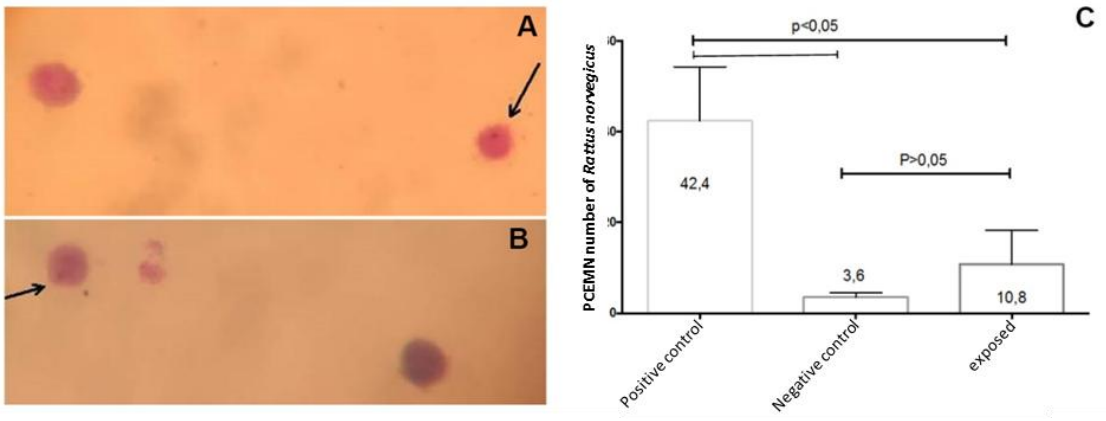

Figure 02.In (A) and (B) polychromatic erythrocyte with micronucleus in bone marrow of *Rattus norvegicus*(arrow). (C) Results found after the MN test: meanpolychromatic erythrocyte with a micronucleus.

3.2 For Comet Assay

The results presented in Table 02 show that the membrane did not induce DNA damage and genotoxicity of animal cells. The toxic potential of the membrane was investigated and reviewed comet indicated an increase of damage to the 1st 4h exposure evaluated by the% DNA in the tail. However, after 24 h, there was a statistically significant decrease compared to the first few hours, indicating a repair of the initial genotoxic effect. This finding is consistent with other parameters analyzed, as the% of DNA in the head, TailMoment and the length of the tail.

Table 02. DNA damage found per group after exposure to polystyrene membrane with collagen and norbixina.

Parameters	Positive control	Negative Control	Membrane 4h	Membrane 24h
Head DNA %	3,2 ± 0,4	12,3 ± 0,89 [a]	5,0 ± 0,42 [a]	13,2 ± 1,2 [a]
Tail DNA %	4,5 ± 0,8	0,8 ± 0,02 [a]	7,3 ± 1,2 [a]	0,3 ± 0,07 [a]
Tail length (μM)	16,0 ± 1,7	14,0 ± 2,0 [a]	18,0 ± 2,0 [a]	3,0 ± 1,3 [a]
TailMoment	1.4 ± 0,56	0,8 ± 0,63 [a]	1,7 ± 0,21 [a]	0,93 ± 0,25 [a]

Results expressed as mean and standard deviation. "A" indicates a statistically significant difference (P <0.001) when compared to CP.

IV. Discussion

A variety of biomaterials formed by natural polymers, synthetic polymers and derivatives of acellular matrix is huge [19], however, they are only classified as biomaterials are free of causing local or systemic adverse biological response may not be toxic, carcinogenic, antigenic and mutagen [20]. Under the biotechnological perspective obtaining water-soluble derivatives of carotenoids is interesting in commercial perspective. The use of synthetic materials for replacement or repair of biological tissues, has always been a major concern in the medical field, which justifies the investigation of new materials in tissue engineering.

The selection of the biomaterial to be used should take into account the physical, chemical and mechanical properties of the material [21, 22]. The membranes are designed to provide a favorable microenvironment of repair of articular cartilage, formed to the diffusion of nutrients, growth factors and other biologically active agents in the field of tissue regeneration [23].

In this study, toxicity was assessed inpolystyrene membrane with collagen and norbixin through in vivo experiments in rats. To determine the genotoxic potential of the membrane, two experiments were conducted,

micronucleus test and comet assay. The micronucleus test showed that extracts from the membrane does not alter the incidence of polychromatic erythrocyte suggesting that the membrane has no genetic toxicity in rats.The Comet Assay showed an increase of damage in the first 4 hours, and for 24 hours of exposure time, showed a statistically significant decrease from the early hours suggesting repair genotoxic effect. Taken together, our results suggest that the membrane does not have biological toxicity. It is known that DNA repair is essential to providing the maintenance of homeostasis and, therefore, cell and tissue survival. In most cases, this is correctly handles, restoring the original molecule [24].

In summary, the findings indicated no damage after exposure generated thepolystyrene membrane with collagen and norbixin,suggesting that there was adequate or sufficient time to repair the same, showing that the membrane is not cytotoxic, and therefore, is safe for in vivo applications. Thus, future studies will be conducted to investigate the effects of the membrane in the repair of articular cartilage.

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