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Colloquium Vitae

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IN VITRO ASSESSMENT OF CYTOTOXICITY AND OXIDATIVE STRESS PARAMETERS OF NATURAL RUBBER LATEX MEMBRANES STABILIZED WITH DIFFERENT CONCENTRATIONS OF AMMONIA IN CHO-K1 CELLS

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ABSTRACT

The purpose of this study was to assess *in vitro* cytotoxicity of natural rubber (NR) latex membranes stabilized with different concentrations of ammonia, as well as evaluate possible changes in oxidative stress parameters. To this end, CHO-k1 cells were exposed to NR membranes for 24 h. Cytotoxicity was assessed by the MTT assay and the oxidative stress parameters were established by glutathione (GSH) and malondialdehyde (MDA) levels. The results show that the ammonia present in the NR latex membranes can deplete cell viability and enhance GSH consumption in the cells. NR latex membranes stabilized with 2.0% of ammonia were cytotoxic to the cell lineage used in this study. From these results, it is possible to infer that the use of ammonia to stabilize NR latex membranes with biomedical purposes has to be done carefully, to avoid possible harmful effects to human health.

Keywords: Natural rubber latex, membranes, ammonia, cytotoxicity, GSH.

AVALIAÇÃO *IN VITRO* DA CITOTOXICIDADE E PARÂMETROS DE ESTRESSE OXIDATIVO DE MEMBRANAS ESTABILIZADAS COM DIFERENTES CONCENTRAÇÕES DE AMÔNIA EM CÉLULAS CHO-K1

RESUMO

O objetivo deste estudo foi avaliar *in vitro* a citotoxidade de membranas de borracha natural (BN) estabilizada com diferentes concentrações de amônia, assim como avaliar as possíveis modificações de parâmetros de estresse oxidativo. Para este fim, células CHO-k1 foram expostas a membranas de BN por 24 h. A citotoxicidade foi avaliada pelo ensaio do MTT e os parâmetros de estresse oxidativo foram estabelecidos pelos níveis de glutationa (GSH) e malondialdeído (MDA). Os resultados mostraram que a amônia, presente nas membranas de BN podem diminuir a viabilidade celular e aumentar o consumo de GSH nas células. As membranas de BN estabilizadas com 2,0% de amônia foram citotóxicas para a linhagem celular utilizada neste estudo. A partir destes resultados, é possível inferir que o uso de amônia para estabilizar as membranas de BN com propósitos biomédicos devem ser feitas cuidadosamente, para evitar possíveis malefícios à saúde humana.

Palavras-chave: Látex de borracha natural, membranas, amônia, citotoxicidade, GSH.

INTRODUCTION

Natural rubber (NR) latex from *Hevea brasiliensis* is a colloidal anionic system formed by rubber particles stabilized in a shell of phospholipids and proteins[1,2]. Lately, NR membranes has been investigated as delivery system of proteins[3], drugs[4] and nanoparticles[5,6]. This material also is capable of enhancing soft tissue healing, being used in several medical applications like prosthetics and bone grafts[1,7,8].

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Since fresh latex can spontaneously coagulate and putrefy shortly after it leaves the tree, ammonia is used to preserve NR and so it can be used as membranes. Ammonia solution in NR can inhibit bacteria action mainly because it elevates the pH conditions, hydrolyzes fatty acids esters and stabilizes the NR system[9].

Although ammonia is used to preserve and stabilize NR latex, it is highly toxic since it can disturb the pH balance of the cytoplasm and body fluids[10], modify the secretion of cytokines and elevate the activity of lysosomal hydrolases[11]. Besides this effects, ammonium ions inhibit important enzymes involved in protein metabolism, which can lead to free radical generation, and reduction of the antioxidant enzymes[12].

Besides the NR latex membranes being implicated in several biomedical applications, only few studies in the literature evaluated ammonia associated with NR latex membranes toxicity[13,14]. Therefore, there is an urgent need to evaluate the use of ammonia as latex stabilizing agent for biomedical purposes: evaluation of possible harmful impacts that ammonia can cause to biological systems.

In vitro tests are important tools to assess biological responses to a certain material and to evaluate preliminary human health security[15]. These tests allow the analysis of potential cytotoxicity of a substance/material, and also allow the quantification of sublethal damages to biological systems, such as oxidative stress parameters[16].

Therefore, the aim of this study was to evaluate the cytotoxic potential of NR latex membranes stabilized with different concentrations of ammonia and to assess possible changes in oxidative parameters. These results are important to stablish safety incorporation of ammonia as stabilizing agent in NR latex membranes used for biomedical purposes.

METHODOLOGY

Membrane preparation

Natural rubber (NR) latex membranes were prepared using latex collected from rubber trees Hevea brasiliensis (RRIM 600 clone) in an experimental farm in Indiana City, in the region of Presidente Prudente, São Paulo State, Brazil. The latex was obtained by the tapping method and stored at a low temperature. Samples of latex were stabilized with 0.25%, 0.5%, 1.0%, and 2.0% of ammonium hydroxide (v/v). NR membranes were prepared by casting at temperature of 65°C and they were annealed for 10 h. The NR membranes used in the cell toxicity tests were sterilized with ethylene oxide.

Qualitative chemical characterization of NR membranes using FTIR spectroscopy

For the chemical characterization of natural rubber membranes stabilized with different concentrations of ammonia, Fourier Transform Infrared (FTIR) technique was used. These spectra were compared with the spectrum observed for the natural rubber membrane without ammonia.

Cell Line

For the experiments, CHO-k1 (Chinese hamster ovary) cell line was cultured in 10 mL of DMEM-F10 (1:1) medium supplemented with 10% fetal bovine serum in 25 cm^2 flasks and kept in an incubator with 5% CO_2 atmosphere at 37^oC.

Cell Exposure Protocol

In vitro evaluation of cytotoxic effects of the NR latex membranes stabilized with different concentrations of ammonia were carried out using extract dilution and direct contact method.

For extract dilution method, liquid extracts of the membranes NR (0% ammonia), 0.25%, 0.5%, 1.0% and 2.0% were obtained following ASTM F619 standards[17]. Briefly, the NR membranes samples were crushed into pieces, mixed with PBS (phosphate buffer saline pH 7.4) extraction solution at a ratio of 60 cm^2/20 mL, and maintained under these conditions for 24 h without agitation. Thereafter, the solid pieces of composite material were removed and the liquid extracts were filtered, pH adjusted to 7.4 with NaOH, and used to perform biological assays. After the preparation of the extracts, the cells were cultured in 24-well plate with 200 µL of culture medium and exposed to 200 µL of the different extracts, using PBS as negative control (NC), for 24 h.

For the direct contact method, ethylene oxide sterilized 100 mm^2 square pieces of COF and NR latex membranes were used. The cell suspension was distributed in a 12-well plate at 37ºC for the formation of the cell monolayer. Then fragments of the membranes or nontoxic filter fragments (NC) were placed on the center of the well plate in contact with the cells for a period of 24 h.

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After the exposure period, the cytotoxic potential and biochemical parameters of oxidative stress were evaluated.

Evaluation of in vitro cytotoxicity

For cytotoxic potential, MTT reduction method was performed according to Mosmann[17]. Briefly, 0.3 mg/mL of MTT solution was added and cells were incubated at 37°C for an additional 4 h. The absorbance of each sample was determined using a microplate reader at 492 nm. The absorbance of the NC was considered to represent 100% of cell viability (CV). The CV of the other samples was determined using the following formula: CVE = [(AE – AB) / (ACTR – AB)] × 100, where CVE is the viability of cells exposed to the extract, AE is the absorbance of the cells exposed to the different extracts, ACTR is the absorbance of cells exposed to PBS and AB is the absorbance of the blank (well containing a culture medium only).

Measurement of glutathione (GSH) levels

GSH levels were determined by addition of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), as described by Ellman[18]. DTNB, a symmetric aryl disulfide, reacts with free thiols to form disulfide and 2-nitro-5-thiobenzoic acid; the latter product can be quantified by its absorbance at 412 nm. The measurements were conducted with a spectrophotomer (Quimis, São Paulo, Brazil) and concentration of total proteins was measured as described by Bradford[19]. Results are expressed as nmol/g of protein.

Measurement of malondialdehyde (MDA) levels

MDA levels were determined by the addition of thiobarbituric acid (TBA), as described by Uchiyama and Mihara[20], with modifications. Briefly, 200 µL of cell homogenates were mixed with 400 µL of the TBA solution (1% TBA, 50 mM NaOH and 0.1 mM BHT) and 200 µL of 7% phosphoric acid. The mixtures were incubated in a boiling bath for 15 min. After cooling the tubes on ice, 1.5 mL of n-butanol was added and the reaction mixture was centrifuged at 5000 × g for 10 min. The absorbance of the supernatant was read at 532 nm, and the concentrations of MDA were calculated using tetraethoxy propane as a reference standard.

Statistical Analysis

The results are shown as mean ± SD. All testes were statistically significant at 95% of confidence level in Graph-Pad Prism® 6.01. For cytotoxicity, comparisons between multiple groups were performed using One-way ANOVA with Dunnett. For biochemical parameters, comparisons between multiple groups were performed using One-Way ANOVA with Tukey.

RESULTS

Qualitative chemical characterization of NR membranes using FTIR spectroscopy

FTIR spectra showed a small band at 1562 cm^{-1} in all membranes stabilized with ammonia, indicating the formation of amide groups R-$CONH_2$. The results are shown in Figure 1.

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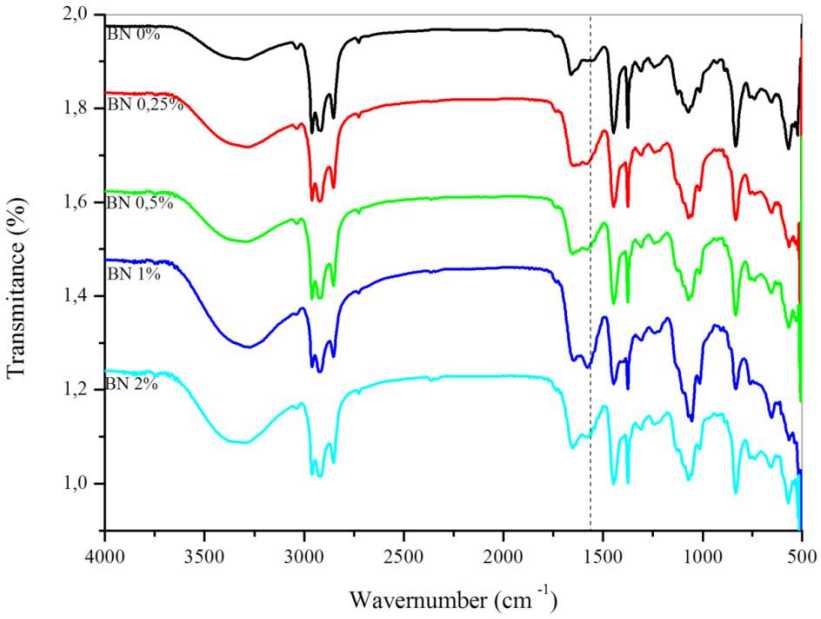

Figure 1. Absorbance spectra of NR membranes stabilized with different concentrations of ammonia.

Evaluation of in vitro cytotoxicity

Cytotoxicity evaluation was assessed by the MTT assay and the results are shown in Figure 2. In Figure 2A is shown that that the extracts obtained from the membranes of NR alone and the membranes of NR stabilized with 0.25%, 0.5%, 1.0% and 2.0% of ammonia did not alter cell viability of CHO-k1 cells after 24 h of exposure. In Figure 2B, the assessment of the cell viability from direct contact showed that membranes stabilized with 0.5% and 2.0% of ammonia differed statistically from the negative control (NC). The cells exposed to NR membranes with 0.5% of ammonia presented an increase in the cell viability while NR membranes with 2.0% of ammonia depleted cell viability. It is possible to infer from these results show that the presence of ammonia in the NR membranes can influence the metabolic activity of CHO-k1 cells.

Measurement of glutathione (GSH) levels

It can be seen in Figure 3 that the different concentrations of ammonia caused significant reduction of the cellular levels of GSH in a dose dependent manner ($p \leq 0.05$).

Measurement of malondialdehyde (MDA) levels

In Figure 4 it is possible to verify that the extracts of NR latex membranes with different concentrations of ammonia didn't raise significantly the levels of MDA in CHO-k1 cells.

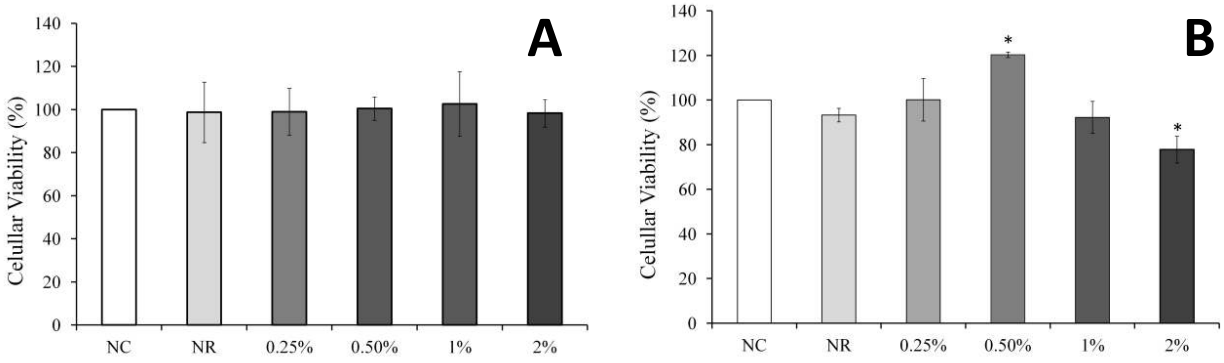

Figure 2. Impact of treatment of Natural Rubber membranes stabilized with 0.25%, 0.5%, 1.0% and 2.0% of ammonia on cell viability of CHO-k1 cells. (A) Cell viability of the extracts obtained from the membranes. (B) Cell viability assessment from direct contact to the membranes. Cell cultures were exposed for 24 h. Bars represent means ± SD of results. * Indicate statistical difference between control group and treated cell groups. ANOVA and Dunnett's test ($p \leq 0.05$).

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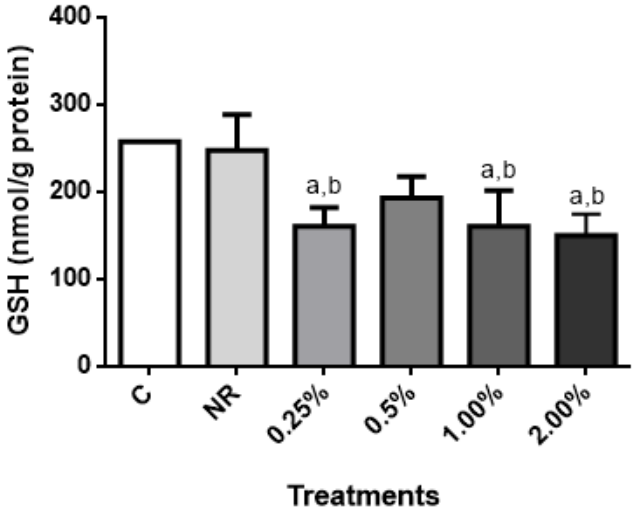

Figure 3 – Quantification of GSH in CHO-k1 cells after exposure to Natural Rubber membranes stabilized with 0.25%, 0.5%, 1.0% and 2.0% of ammonia. Cell cultures were exposed for 24 h. Bars represent means ± SD of results. [a] Statistically different from control group (C) and [b] statistically different from NR control group. ANOVA and Dunnett's test ($p \leq 0.05$).

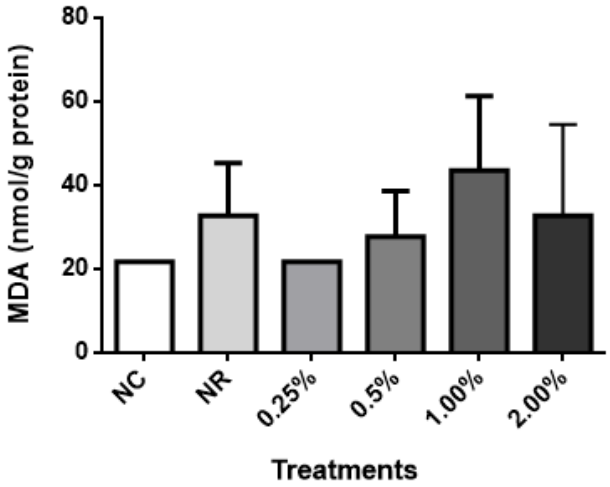

Figure 4 – Quantification of MDA in CHO-k1 cells after exposure to Natural Rubber membranes stabilized with 0.25%, 0.5%, 1.0% and 2.0% of ammonia. Cell cultures were exposed for 24 h. Bars represent means ± SD of results. ANOVA and Dunnett's test ($p \leq 0.05$).

DISCUSSION

Natural rubber (NR) latex from *Hevea brasiliensis* is a raw material that after polymerization acquires important distinct characteristics at low costs such as elasticity, flexibility, and strength[21-23]. NR has been proven to be biocompatible, naturally stimulating angiogenesis as well as cell adhesion and extracellular matrix formation[21,24,25].

But after extraction, NR can clot which makes the use of ammonia solutions common before its transformation into different kinds of products. Depending on the storage time, concentrations ranging from 0.2% to 2% are used for the preservation of latex. However, ammonia is considered an irritant substance and the use of NR latex membranes as biomaterial presents the concern of hypersensitivity reactions in the host.

Analyzing the FTIR spectra of natural rubber membranes it was possible to observe the appearance of a small band at 1562 cm^{-1} in all rubber membranes stabilized with different concentrations of ammonia, but this did not occur in the membrane without ammonia. This result corroborates to the results found by Floriano et al.[23] that also viewed the appearance of the same band in rubber membranes stabilized with 2.0% ammonia. According to these authors,

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this band is linked to the reaction of ammonia with the membrane, leading to the formation of amide groups R-$CONH_2$.

The test used to assess cell viability in CHO-k1 cells was the MTT reduction assay that can evaluate indirectly the level of metabolic activity in eukaryotic cells[26,27]. Therefore, it is possible to verify that the ammonia presented at 0.5% in the NR membranes somehow interfered in the metabolism of the exposed cells, causing a higher reduction of the tetrazolium into formazan. This raise in the metabolism could be an adaptative cell response to a stressful change in the environment provoked by the presence of ammonia. Martinelle and Häggström[28] showed that the presence of ammonia causes an enhance in the cell demand for energy, since the presence of ammonia can alter ions gradients in the cell membranes.

However, to the cells that were exposed to a higher amount of ammonia (2.0%) it is possible to infer that the adaptive capacity of dealing with a stressful environment was saturated, causing the death of the cells[29]. Floriano et al.[23] also found that NR membranes that were stabilized with 2.0% of ammonia at harvest are more cytotoxic that the NR membranes prepared without the ammonia. Corroborating with this data, our work also found that NR membranes stabilized with 2.0% of ammonia presented cytotoxic potential to the cell lineage used in this study.

In addition to the cytotoxic potential of the membranes, measurements of two biochemical parameters to monitor the redox status of the cells were performed. Lately, GSH has become an important factor of research activity due to its multiple roles in cell function and cell survival. Bobermin et al.[30] demonstrated that ammonia treatment can decrease GSH content in rat neurons, indicating enhanced GSH consumption. GSH has also been indicated as supply counteract in ammonia-induced cell death[31]. Our work, using CHO-k1 cells shows that different concentrations of ammonia associated with NR latex increased the consumption of GSH in these cells, especially the concentration of 2.0%. One possible explanation for the depletion of intracellular GSH found in this study could be assigned to the use of the glutathione for obtainment of glutamate, the latter used in the cells to detoxify from ammonia[32,33].

Another analyzed biochemical parameter was the formation of malondialdehyde (MDA), an indicative of lipoperoxidation. Although it can be seen an increase in the MDA levels of the cells treated with the extract of NR latex membrane and in the cells treated with the extract of NR latex stabilized with 1.0% of ammonia, it was not statistically significant. A slightly increase in MDA concentrations in the cells was also found by Davuluri et al.[34], that studied the effects of hyperammonemia in skeletal muscle. They verify that an elevation in the concentrations of ammonia in the cells increased mitochondrial matrix enzyme manganese dependent superoxide dismutase (MnSOD) expression, which provoked an increase in ROS and also in lipoperoxidation. This increase in the MnSOD could also be another explanation for the raise in the GSH consumption in the cells, since the metabolism of MnSOD generates hydrogen peroxide, one of the substrates of glutathione peroxide, an enzyme that uses glutathione for its reduction. But probably in our study, the contact of CHO-k1 cells with the extracts of NR latex membranes stabilized with ammonia didn't raise the levels of ROS in the cells enough to provoke lipid damage.

Therefore, it is possible to conclude based on the results found in this work that the ammonia present in the natural rubber (NR) latex membranes can cause stress in CHO-k1 cells, influencing the cell metabolic activity. In lower concentrations of ammonia (0.25% and 0.5%) the cells were able to adapt to the stressful environmental, but in higher concentrations (2.0%) this adaptive capacity was suppressed, leading the cells to death and being cytotoxic to the cell lineage used. It was also verified that the ammonia presented in the NR latex membranes enhanced the consumption of GSH in the cells exposed to the extracts of these membranes. From these results, it is possible to assure that the use of ammonia for *Hevea brasiliensis* latex stabilization, which could be used to produce membranes with biomedical application, has to be done with caution avoiding possible harmful effects to human health.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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