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International Journal of Plant Biology & Research

Research Article

Protective Effect of Anacardic Acid as Antioxidant in the Mucociliary Epithelium of Frog Palate

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Submitted: 19 June 2020

Accepted: 29 June 2020

Published: 30 June 2020

ISSN: 2333-6668

Keywords

- Anacardic acid
- Oxidative stress
- Mucociliary transport
- Ciliary beat frequency
- Histochemistry
- Nrf2

Abstract

Anacardic acid (AA), has been described as potential antioxidant and Nrf2 is knowing as transcription factor responsible for synthesis of antioxidants proteins. The objective this study was verify the power antioxidant of AA, utilized frog palate epithelium as model of mucociliary epithelium and H_2O_2 as potent oxidative specie:1) Control Group (CG) - Ringer's solution (RS)/90' (n=8); 2) H_2O_2 (100 mM)/90' (n=14; 3) AA (50mg/kg)/30' and RS/90' (n=4); 4) AA (50mg/kg)/30' and H_2O_2 (100 mM)/90' (HA) (n=4). It was studied the mucociliary transport (MT), ciliary beat frequency (CBF), mucus profile (MP) and expression of Nrf2 (nucleus and cytoplasm). MT and CBF were evaluated through Two-Way ANOVA from two factors: time and treatment. MP and Nrf2 localization were evaluated by One-Way ANOVA test or Kruskal Wallis ($p<0.05$). MT was significant to time and treatment ($p<0.001$), CBF only treatment ($p<0.001$). MP was significant between AH and H_2O_2 for acid mucus ($p = 0.008$) and vacuoles between H_2O_2 and CG ($p = 0.003$). Nrf2 was different with relation nuclei between H_2O_2 and CG ($p=0.048$) and H_2O_2 and AH related with cytoplasm ($p=0.003$). AA showed to have a protective effect against reactive oxygen species without requirements of transcription factor Nrf2 translocate to nucleus.

ABBREVIATIONS

AA: Anacardic Acid Group; AH: Study Group Exposed to Anacardic Acid before Hydrogen Peroxide Exposure; CBF: Ciliary Beat Frequency; CG: Control Group; H2O2: Hydrogen Peroxide Solution; MP: Mucus Profile; MT: Mucociliary Transport; Nrf2: Nuclear Factor-Erythroid 2-Related Factor 2; RS: Ringer Solution; 30': 30 Minutes; 90': 90 Minutes

INTRODUCTION

Anacardic acid, a substance found in cashews (species *Anacardium occidentale* native from northeastern Brazil), was described as a potential antioxidant. The phenolic alkyl found in cashew plant, as anacardic acids [1], may act in tissue protection against oxidative stress due to their ability to suppress pro-oxidative [2,3], to inhibit the generation of superoxide anion [1,4,5], and acting in the chelation of metal ions [5,6].

The airways are in direct contact with the environment and exposed to the action of harmful agents which can cause severe tissue damage, mostly because of oxidative stress caused by reactive oxygen species generation. Feldman et al. [7], demonstrated that oxidative stress plays a crucial role in mucociliary dysfunction in human respiratory epithelium, and can cause a reduction in mucociliary activity, airway inflammation and profound change in physiology and lung function. The mucociliary epithelium is the major constituent of the airway respiratory tract and its physiology is a determining factor for effectiveness of mucociliary transport and consequent protection of the respiratory system.

Natural protection of airways system against oxidative stress is performed by different mechanisms, which involve cells that contain antioxidants that chemically transform oxidants [8]. Besides, Nrf2 (nuclear factor erythroid 2-related factor) protein is a transcription factor that modulates mechanisms of antioxidant response against oxidative stress [9]. Cytoplasm Nrf2 binds to suppressor protein Keap1, but oxidative stress triggers the dissociation of Nrf2-Keap1 and Nrf2 translocation to the nucleus. In intranuclear medium, Nrf2 binds to promoter regions of genes encoding antioxidant and detoxifying proteins [10,11], such as heme oxygenase, NAD (P) H-quinone oxidoreductase, GST, superoxide dismutase and glucuronosilltransferase 3-1A6 [12]. In addition to antioxidants, mucus, fluid lining the airways respiratory epithelium and cellular barriers also have protective action against damage [13]. Although the respiratory epithelium has mechanisms to inhibit the effects due to oxidative stress, sometimes, there is an imbalance between the antioxidants defenses and the damage caused by oxidants require others substances to suppress this effect and to protect the system.

We hypothesized that anacardic acid can be used as an adjuvant in antioxidant protection in the respiratory system [14]. Thereby, we analyzed the anacardic acid as an antioxidant agent using hydrogen peroxide as a powerful oxidizing agent in frog palate epithelium, a model of mucociliary epithelium [15]. We studied the mucociliary transport, ciliary beat frequency, mucus profile and cellular expression of Nrf2 protein in epithelial cells. We aim to test the protective effect of anacardic acid in the frog palate epithelium to understand better its effects in the mucociliary epithelium as antioxidant protection.

MATERIAL AND METHODS

This study was approved by Ethical Committee of Research of the São Paulo University Medical School (#374/11).

Frog palate preparation

Adult *Lithobates catesbeianus* frogs were used as biological model, which according to (Carvalho-Oliveira et al., 2005), are suitable study models by having epithelium similar to that found in mammals. Using hypothermia as anesthesia, the frogs were quickly decapitated and palates removed disrupting their jaws from the junction of the pharynx and the esophagus to the skin of the back. The palates were stored in a glass container with gauze impregnate with frog Ringer solution and placed in a refrigerator at 4°C for 24 hours. All experiments were performed 24 hours after the dislocation of the frog palate and a maximum of 5 days, according to the procedures described by Macchione et al. [16,17]. Under these conditions, the ciliary activity is preserved [18].

Study Groups

Control Group: frog palate samples after 90 min immersion in Ringer's solution (n=8). Experimental Group H_2O_2: frog palate samples after immersion 90 min in hydrogen peroxide at concentration of 100 mM. (n=14). Experimental Group AA: Samples of frog palate after 30 min immersion in anacardic acid at a concentration of 500 µL (50mg/kg) (n=4). Experimental Group HA: Samples of frog palate after 30 min immersion in solution containing anacardic acid at concentration of 500 µL (50mg/kg) and after immersion at concentration of 100 mM hydrogen peroxide for 90 minutes (n=4).

Mucociliary transportability

In order to not dehydrate the mucus samples of each frog palate, these were collected and stored in Eppendorf tubes immersed in ice and containing Vaseline oil. Thereafter, for removing vaseline oil they are immersed in petroleum ether before mucociliary transport to be measured. This procedure, according to Rubin et al. [19], does not alter the rheological and mucus transport properties. Next, the frog palates are kept in acrylic chamber with 100% humidity provided by ultrasonic nebulizer, with solution ½ Ringer 73.8 mEq / L Na +, 2 mEq / L K + 2.3 mEq / l Ca2 + and 78 mmol / L Cl. Under these experimental conditions the frog palate preparation is influencing only by the physical properties of mucus [16]. The mucociliary transport is then determined by measuring the rate of displacement of autologous mucus samples placed on the surface of the frog palate epithelium using a stereoscopic equipped with a magnifying objective reticulated. The time offset (d) between two points of the sample (d = 6 mm) is measured in seconds, and measurements are made 5 times and for each dose of the study.

Ciliary beat frequency

The frequency of ciliary beating was evaluated with the aid of a light microscope (Olympus BX50, Tokyo, Japan) at 40X magnification, connected to a video camera (Sony Trinitron 3CCD, Tokyo, Japan) and a monitor video. We used for such a technique adapted from the technique described by Braga et al [20].

We focused groups of ciliated cells of interest in the epithelium of frog palate. Then a source of strobe light (Machinne Vision Strobe, Cedarhurst, NY), was placed in front of the mucociliary epithelium, emitting light flashes at a rate ranging from 0 - 33 Hz. The ciliary beat frequency is measured by reference to the frequency the strobe light that shines in the epithelium of the frog palate: this is manual decreased gradually, until we have the impression that the displayed eyelashes group had stopped its movement. At this point, we can conclude that the ciliary frequency is the same as the frequency of light incident on the epithelium.

Optical microscopy and morphometry

Samples of the epithelium of all palates were collected for histological study. Samples were removed near the displacement region of the palate, with 15 by 15 mm and fixed in buffered 4% formaldehyde solution and processed according to routine histologic procedures for paraffin embedding and cutting [21].

Histochemical Stain

The slides were stained with Shiff's periodic acid (PAS) and Alcian Blue (AB) at a pH 2.5. The use of these stain was chosen because neutral and acidic glycoproteins are stained in red and blue, respectively [21]. For the morphometric analysis were captured ten digital photos for every palate, using microscope Leica DMR, an increase of 400x, and camera Zeiss Axio Cam MRC5. After the acquisition of the images, the analyses of the photos were made through the program Image Pro Plus, with manual dialing the following variables concerning the PAS/AB:

1) number of matching points in acid mucus;

2) number of matching points in neutral mucus;

3) number of matching points in mixed mucus;

4) number of matching points in vacuoles;

5) number of matching points in "other" (portions of the epithelium corresponding to the extracellular matrix, cell nuclei and cilia);

6) number of total points (sum of matching points in mucus acid, neutral, mixed, vacuoles and "other").

Quantification of Nrf2

Five-µm thick sections were subjected to immunohistochemistry to identify cells expressing Nrf2. Briefly, sections were deparaffinized, and 0.5% peroxidase solution in methanol was applied for 5 minutes to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 20 minutes. Sections were incubated overnight with

the anti-Nrf2 (1:2000) (ab31163; Abcam plc., Cambridge, UK) antibody, and 3,3 diaminobenzidine (Sigma Chemical Co., St Louis, MO, USA) was used as a chromogen. The sections were counterstained with Harris hematoxylin (Merck, Darmstadt, Germany). All primary and secondary antibodies were applied to negative and positive controls and the specificity of primary antibody was tested in human tracheal epithelium slide. The slides were coded, and the researcher who performed the morphometrical analyses was blinded to the study groups. The expression of Nrf2 was determined by counting cell with positive cytoplasm and nucleus. The number of Nrf2 expression in nucleus of epithelium was utilized to quantity of the activation of this protein. To this, it was identified the number the positive cytoplasm and nucleus divided by total number of cytoplasm and nucleus in the epithelium [22]. The results were expressed by percentage of stained nuclei (+)/total nuclei; stained cytoplasm (+)/ total cytoplasm.

Statistical Analysis

Analysis of variance was performed for repeated measures analysis considering the exposure group (Ringer-Frog, H_2O_2, anacardic acid, anacardic acid + H_2O_2). For the evaluation of normality was used the Kolmogorov-Smirnov test. To Mucociliary Transport and Ciliary Beat Frequency were evaluated with two-way ANOVA to two factors: time and treatment with anacardic acid. To histochemistry and Nrf2 localization normal distribution

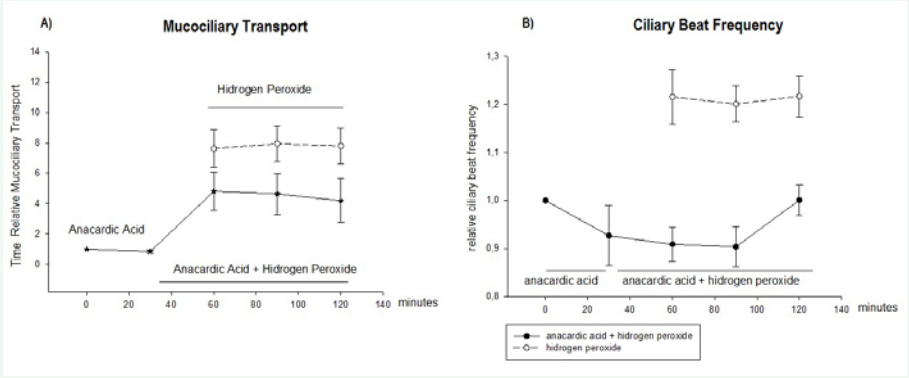

Figure 1 Graphical representation of relative changes on the mucociliary transport rate showed significant difference to time ($p<0.001$) and treatment ($p<0.001$) and ciliary beat frequency showed significant difference to treatment ($p<0.001$) in a frog palate exposed to a concentration of 100 mM hydrogen peroxide in time intervals of 30 minutes. Comparing the group exposed only to hydrogen peroxide and previously exposed group anacardic acid at a concentration of 500 µL (50mg/kg).

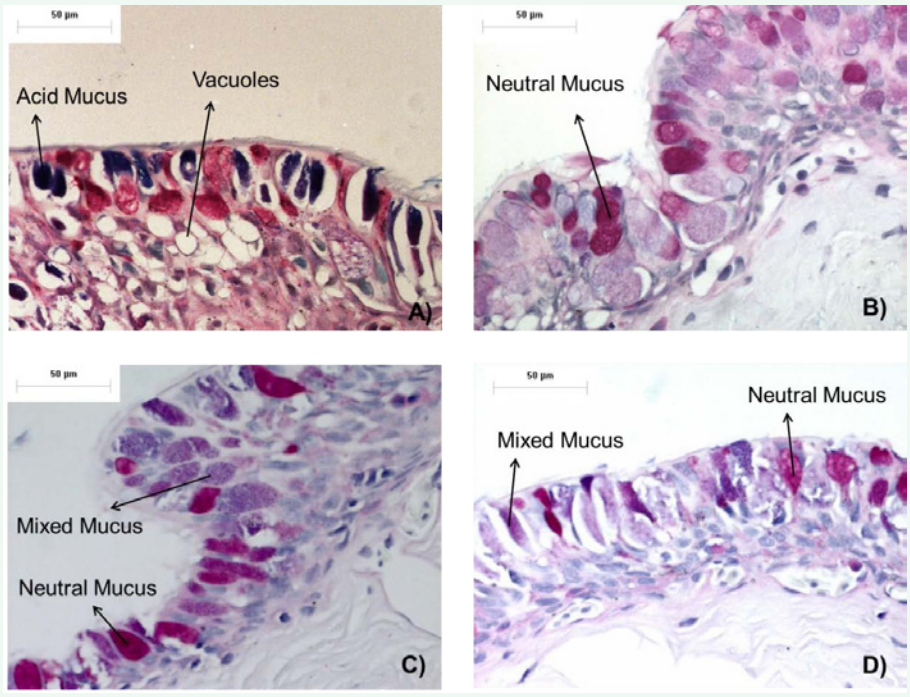

Figure 2 Photomicrographs (400X) of the mucociliary epithelium from frog palates (PAS/AB) of the Hydrogen Peroxide (A), Anacardic Acid (B), Anacardic Acid pre-treatment (C) and Control (D) groups after 90 minutes of exposure.

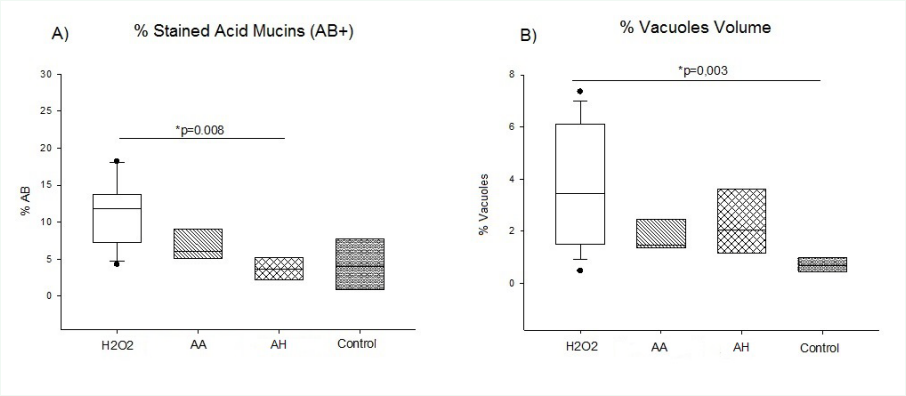

Figure 3 Graphical representation of acid mucus percentage (A) and vacuoles percentage (B) obtained in the H2O2 (immersion in hydrogen peroxide, 100 mM for 90 minutes), AA (immersion in anacardic acid for 30 minutes at a concentration of 500 µL - 50mg/Kg), AH (immersion in anacardic acid at a concentration of 500 µL - 50mg/kg, followed by immersion in 100 mM hydrogen peroxide for 90 minutes) and Control.

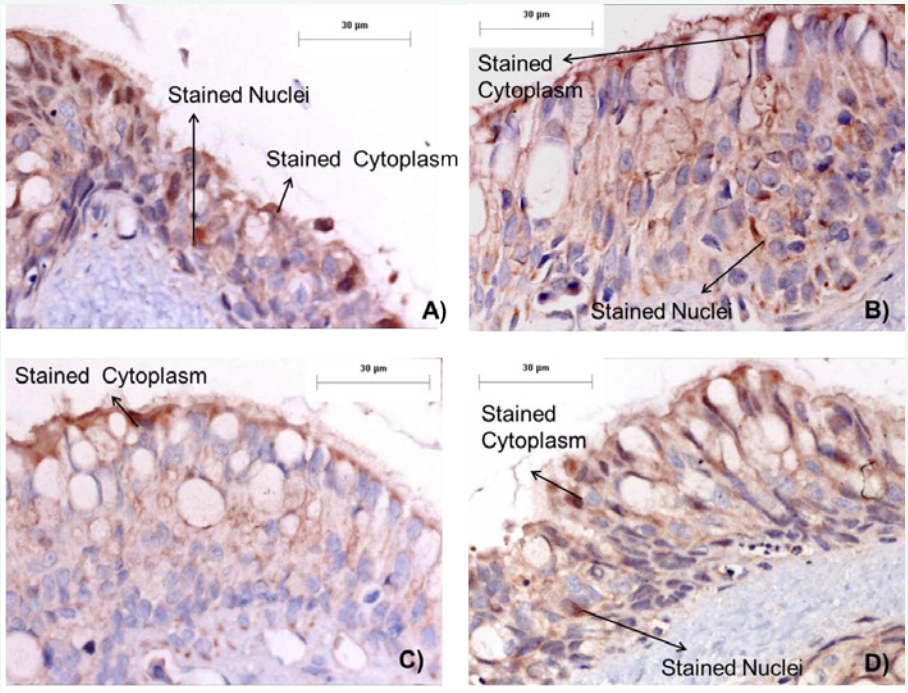

Figure 4 Photomicrographs (400X) of the frog palates mucociliary epithelium showing Nrf2 localization in the cytoplasm and nucleus of Hydrogen Peroxide (A), Anacardic Acid (B), Anacardic Acid pre-treatment (C) and Control (D) groups.

were evaluated by one-way ANOVA test and the data with non-normal distribution, we used the Kruskal Wallis. The significance level was set at 5%.

RESULTS AND DISCUSSION

Mucociliary transportability and Ciliary beat frequency

The Figure 1A shows mucociliary transport, in which there was a statistically significant difference on two factors in which was seen statistically significant difference in two factors: time and treatment ($p<0.001$). Figure 1B shows ciliary beat frequency, in which only treatment was statistically significant difference ($p<0.001$).

Histochemistry Study

The Figure 2 shows the result of staining with PAS/AB, while the Figure 3 shows the graphic results of histochemistry, in which Figure 3A presents statistically significant difference between AH and H_2O_2 for acid mucus ($p = 0.008$). In addition, in Figure 3B was observed an increase statistically significant difference in vacuoles between H_2O_2 and control groups ($p = 0.003$).

Nrf2 cell localization

The Figure 4 shows the stained results of Nrf2 localization, while Figure 5 shows the graphic results of slides analysis, showing significant difference between H_2O_2 and control group, related with positive nuclei ($p=0.048$), but not with others groups,

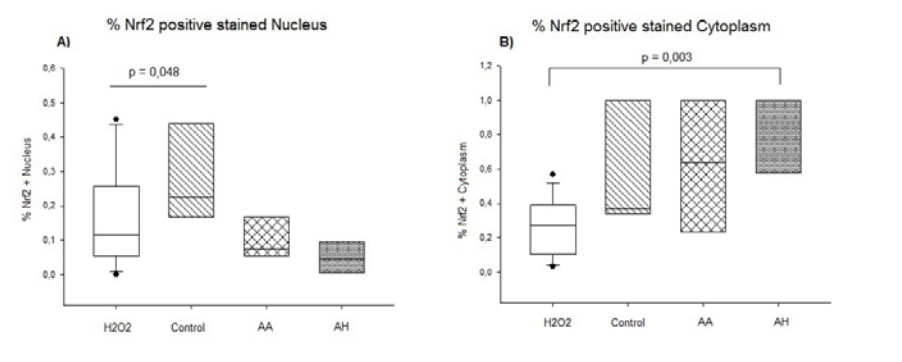

Figure 5 Graphical representation of positive nuclei percentage (A) and positive cytoplasm percentage (B) for Nrf2 satin obtained in the H_2O_2 (immersion in hydrogen peroxide, 100 mM for 90 minutes), AA (immersion in anacardic acid for 30 minutes at a concentration of 500 µL - 50mg/kg), AH (immersion in anacardic acid at a concentration of 500 µL - 50mg/kg, followed by immersion in 100 mM hydrogen peroxide for 90 minutes) and Control.

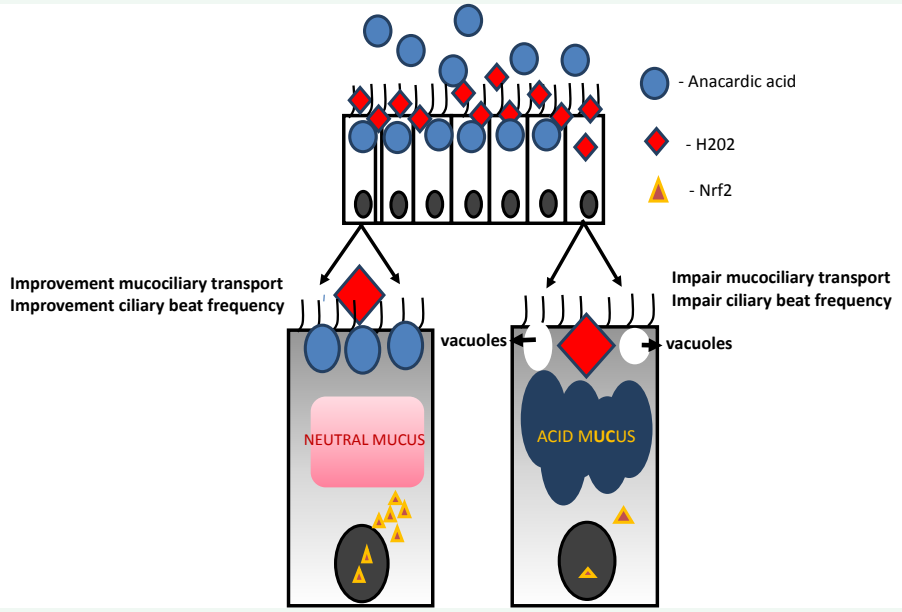

Figure 6 Schematic drawing showing the effect of Anacardic Acid on the mucociliary epithelium preventing the action of the oxidant inside the cell avoiding the consumption of the Nrf2 protein in the cytoplasm and nucleus.

while H_2O_2 and AH showed a significant difference related with positive cytoplasm (p=0.003).

This study aimed to verify the protective effect of anacardic acid in the epithelium of frog palate exposed to oxidative stress as a model of mucociliary apparatus. The results showed that changes in mucociliary transport rate and ciliary beat frequency was statistically significant to hydrogen peroxide (100 mM; 90 minutes) versus AH group [anacardic acid (500 µL - 50mg/kg) before hydrogen peroxide (100 mM; 90 min)]. The exposed group prior to anacardic acid showed less change in mucus transport and ciliary beat when compared to the group exposed only to hydrogen peroxide, suggesting a possible benefit in pretreating the palate with the anacardic acid to prevent, in part, the damage caused by oxidative stress. Furthermore, immunohistochemical analysis of the mucus showed a higher concentration of acid mucins (AB+) on the samples exposed only to hydrogen peroxide, compared to epithelium previously treated with anacardic acid. These results seem to be compatible with Majima et al. [23], studies, showing that AB+ concentration would be one of major factors determining the viscosity and elasticity of the mucus, i.e., mucus acidification changes mucins physicochemical properties, taking to cell edema and increasing mucus viscosity, which can disrupt mucociliary system integrity and consequently elicit changes in the defense mechanisms [24-27]. However, when the frog palate epithelium was pre-treated with anacardic acid before H_2O_2, the mucociliary system maintained the same behavior as when not subjected to H_2O_2.

The respiratory mucus possesses rheological properties of viscosity and elasticity and is important determinant of the efficiency of mucus by mucociliary transport device. Its viscoelasticity depends largely on the concentration of proteins, especially the glycoproteins, ionic composition and

pH [25]. Currently it is known that acid mucus, present in situations of aggression to the epithelium, such as acute and chronic respiratory diseases and also in continued exposure to environmental pollution [28], tend to cause an increase in mucus viscoelasticity, associated with increased airway resistance, in order to hinder the attack of ciliated epithelium itself. These changes, however, have as a consequence a greater difficulty in mucus to be transported by the ciliary beat, as observed in this study.

The histology analysis of frog palates regarding the nuclear and cytoplasmic concentration of Nrf2 was studied to allow verify the availability of this protein in the intercellular space. Nrf2 response to oxidative stress through of up-regulation the antioxidant inside the nucleus. Therefore, Nrf2 expression might be essential in decrease oxidative stress damage and maintaining the homeostasis in the human respiratory epithelium. The results obtained showed that Nrf2 in the treated anacardic acid, was not necessary, since there was a higher Nrf2 nuclear concentration of the group exposed only to hydrogen peroxide and lower Nrf2 nuclear concentration in group previously treated anacardic acid suggesting that the protective mechanism of anacardic acid may be sufficient to protect the epithelium and inhibiting the generation of ROS [14], no existing requirement to Nrf2 migrate to the nucleus where this would connection with the promoter of genes of antioxidant enzymes. Anarcardic Acid inhibit Histone Acetyl Transferase (HAT) through inhibition of nuclear NF-kB activation and the ability to form metal chelation in order to reduce the concentration of free transition metal content [28]. Others studies are necessary to verify this hypothesis.

CONCLUSION

The results of this study are consistent with the literature showed the potential effect protective of anacardic acid against oxidative stress and suggesting that is sufficient to avoid reactive oxygen species without the necessity of Nrf2 translocate to the nucleus for transcription of antioxidant enzymes (Figure 6).

ACKNOWLEDGEMENTS

This work was supported by *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP).

REFERENCES

1. Trevisan MTS, Spiegelhalder B, Bartsch H, Owen R, Wurtele G, Pfundstein B, et al. Characterization of alkyl phenols in Cashew (Anacardium occidentale) products and assay of their antioxidant capacity. Food Chem. Toxicol. 2006; 44: 188-197.
2. Ha TJ, Kubo I. Lipoxygenase inhibitory activity of anacardic acids. J Agric Food Chem. 2005; 53: 4350-4354.
3. Sun Y, Jiang X, Chen S, Price BD. Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation. FEBS Lett. 2006; 580: 4353-4356.
4. Masuoka N, Kubo I. Characterization of xanthine oxidase inhibition by anacardic acids. Biochim. Biophys. Acta. 2004; 1688: 245-249.
5. Kubo I, Masuoka N, Ha TJ, Tsujimoto K. Antioxidant activity of anacardic acids. Food Chem. 2006; 99: 555-562.
6. Tsujimoto K, Hayashi A, Ha TJ, Kubo I. Anacardic acids and ferric ion chelation. Z. Naturforsch C. J Biosci. 2007; 62: 710-716.
7. Feldman C, Anderson R, Kanthakumar K, Vargas A, Cole PJ, Wilson R. Oxidant-mediated ciliary dysfunction in human respiratory epithelium. Free Radic. Biol. Med. 1994; 17: 1-10.
8. Kirkham P, Rahman I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. Pharmacol. Ther. 2006; 111: 476-494.
9. Cullinan SB, Diehl JA. Coordination of ER and oxidative stress signaling: the PERK/Nrf2 signaling pathway. Int J Biochem Cell Biol. 2006; 38: 317-332.
10. Shih AY, Imbeault S, Barakauskas V, Erb H, Jiang L, Li P, Murphy TH. Induction of Nrf2 driven antioxidant response confers neuroprotection during mitochondrial stress in vivo. J Biol Chem. 2005; 280: 22925-22936.
11. Cho HY, Jedlicka AE, Reddy SP, Kensler TW, Yamamoto M, Zhang LY, et al. Role of NRF2 in protection against hyperoxic lung injury in mice. Am. J. Respir. Cell Mol. Biol. 2002; 26: 175-182.
12. Cross CE, Halliwell B, Allen A. Antioxidant protection: A function of tracheobronchial and gastrointestinal mucus. Lancet 1984; 1: 1328-1330.
13. Carvalho ALN, Annoni R, Torres LHL, Durão ACCS, Shimada ALB., Almeida FM, et al. Anacardic Acids from Cashew Nuts Ameliorate Lung Damage Induced by Exposure to Diesel Exhaust Particles in Mice. Evid. Based Complement Alternat. Med. 2013: 549879.
14. Carvalho-Oliveira R, Saiki M, Pires-Neto RC, Lorenzi-Filho G, Macchione M, Saldiva PHN. Anti-oxidants reduce the acute adverse effects of residual oil fly ash on the frog palate mucociliary epithelium. Environ Res. 2005; 98: 349-354.
15. Macchione M, Guimarães ET, Saldiva PHN, Lorenzi Filho G. Methods for studying respiratory mucus and mucus clearance. Braz. J. Med Biol. Res. 1995; 28: 1347-1355 (a).
16. Macchione M, Oliveira AP, Gallafrio CT, Muchão FP, Obara MT, Guimarães ET, et al. Acute effects of inhalable particles on the frog palate mucociliary epithelium. Environ. Health Perspect. 1999; 107: 829-833(b).
17. Rubin BK, Ramirez O, King M. Mucus-depleted frog palate as a model for study of mucociliary clearance. J. Appl. Physiol. 1990; 69: 424-429 (a).
18. Rubin BK, Ramirez O, Finegan B, King M. Analysis of airway mucus collected from healthy subjects at the time of surgery. Am. Rev. Respir. Dis. 1990; 141: 1040-1043(b).
19. Braga, PC. In vivo observation and counting methods forciliary motion. In: Braga, PC, Allegra, L. (Eds.), Methods in Bronchial Mucology. Raven Press, New York, 1988; 269-276.
20. Macchione M, Lorenzi-Filho G, Guimarães ET, Junqueira VBC, Saldiva PHN. The use of the frog palate preparation to assess the effects of oxidants on ciliated epithelium. Free Radic Biol Med. 1998; 24: 714-721(c).
21. Yoshizaki K, Brito JM, Moriya HT, Toledo AC, Ferzilan S, Ligeiro de Oliveira AP, et al. Chronic exposure of diesel exhaust particles induces alveolar enlargement in mice. Respir. Res. 2015; 16: 18.
22. Majima Y, Harada T, Shimizu T, Takeuchi K, Sakakura Y, Yasuoka S, et al. Effect of biochemical components on rheologic properties of nasal mucus in chronic sinusitis. Am J Respir Crit Care Med. 1999; 160: 421-426.
23. Giddens WE Jr, Fairchild GA. Effects of sulfur dioxide on the nasal mucosa of mice. Arch. Environ. Health 1972; 25: 166-173.
24. Holma B. Effects of Inhaled Acids on Airway Mucus and Its Consequences for Health. Environ. Health Perspect 1989; 79: 109-113.

25. Lemos M, Lichtenfels AJ, Amaro Júnior E, Macchione M, Martins MA, King M, et al. Quantitative pathology of nasal passages in rats exposed to urban levels of air pollution. Environ. Res. 1994; 66: 87-95.

26. Pires-Neto RC, Lichtenfels AJ, Soares SR, Macchione M, Saldiva PHN, Dolhnikoff M. Effects of São Paulo air pollution on the upper airways of mice. Environ. Res. 2006; 101: 356-361.

27. Brant TC, Yoshida CT, Carvalho T de S, Nicola ML, Martins JA, Braga LM, et al. Mucociliary clearance, airway inflammation and nasal symptoms in urban motorcyclists. Clinics 2014; 69: 867-870.

28. Hamad FB, Mubofu EB. Potential biological applications of bio-based anacardic acids and their derivatives. Int. J. Mol. Sci. 2015; 16: 8569-8590.

Cite this article

Frias DP, Nakayama JT, Yoshizaki K, Carvalho-Oliveira R, Trevisan TS, et al. (2020) Protective Effect of Anacardic Acid as Antioxidant in the Mucociliary Epithelium of Frog Palate. Int J Plant Biol Res 8(1): 1119.