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Original Research

Anti-inflammatory and antioxidant effects of using alpha-tocopherol in cell culture of the parotid gland under conditions similar to diabetes mellitus

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Abstract

Background and Aims: Hyperglycemia, one of the most common causes that leads to oxidative damage, is frequently observed in association with inflammatory infiltration mediated by mononuclear phagocytes and epithelial cells present in glandular tissues of diabetic patients. Actions that make it possible to reduce tissue damage caused by the oxidative process inherent in this metabolic disease can become an efficient alternative in helping to maintain the Quality of Life of millions of people. The present study aimed to analyze the effects of the use of alpha-tocopherol in cultures of primary cells of the parotid gland submitted to conditions similar to those that occur in diabetes mellitus (DM). **Methods:** For this, cells from the parotid glands of Balb/C/Unib mice were extracted using the Percoll® protocol. The cells were organized into five groups with different amounts of glucose diluted in the cell culture medium and with the presence or absence of lipopolysaccharide to induce the inflammatory process, as well as the presence or absence of alpha-tocopherol. **Results:** The observed results indicated an increase in cell viability with the use of alpha-tocopherol and reductions of this same variable when in the presence of oxidizing agents. The proteins involved with the mediation of antioxidant protection and inflammation, Nuclear factor erythroid 2-related factor 2 (NRF2), nuclear factor kappa B (NfkB), and glutathione peroxidase were increased in the groups submitted to a hyperglycemic and inflammatory condition, but the same behavior was not observed in the other groups of the experiment. **Conclusion:** It is concluded, therefore, that alpha-tocopherol showed anti-inflammatory and antioxidant effects in this type of cell culture, attenuating the harmful effects of the conditions in which the cells were subjected.

Keywords: Antioxidants, Diabetes Mellitus Inflammation, Oxidants, Primary Cell Culture, Vitamin E.

Background and Aims

Diabetes Mellitus (DM) is classified as a complex metabolic disease that affects the metabolism of carbohydrates, lipids, and proteins [1]. In this sense, there is an increase in accumulated lipoproteins and lipids in diabetic people, resulting in significant changes in cellular health [2]. In this way, many researchers seek to understand the factors that stimulate the development of diabetes. To do this, they are classified into different types, the most common of which currently are diabetes mellitus type 1 (DMTI) and diabetes mellitus type 2 (DM TII) (3].

Studies indicate that for the development of type 2 diabetes, lifestyle, diet, and physical inactivity are the main stimulating factors [4]. Type 1 diabetes, classified as an autoimmune disease, has no apparent cause for its development, however, genetic, epigenetic, and environmental aspects have strong scientific indications [5]. However, both conditions of diabetes present hyperglycemia as a predominant symptom [1-5]. This symptom stems from an abnormal condition of expression or recognition at the level of insulin receptors [6].

Insulin is an anabolic hormone with the ability to stimulate glucose uptake, mainly by muscle and liver cells. Also, insulin increases protein synthesis and signals the cell's pathways for survival. However, dysfunction in its secretion or its recognition results in a greater presence of glucose in the plasma or the extracellular environment [7, 8]. This presence of glucose, above normal values, leads to an increase in reactive oxygen species (ROS) and, therefore, oxidative stress and, later, inflammation [9, 10].

Thus, the inflammatory condition, established in diabetes, promotes several dysfunctions, such as cell death and parenchymal degeneration and increased gland stroma [11]. The pancreas is the organ frequently injured in this condition, however other glands also suffer from hyperglycemia and the inflammatory condition established by it [12-14]. The parotid gland, for example, produces less saliva, leading to a symptom known as xerostomia (presence of dry mouth), in addition to the tissue impairments already described [15]. In this sense, understanding the hyperglycemic and inflammatory effects on other glands can lead to a deeper understanding of these conditions and their tissue and cellular consequences.

On the other hand, several studies seek to develop efficient treatments in the process of interrupting or reducing the damage caused by oxidative stress or even by the inflammation that occurs through this mechanism induced by hyperglycemia [16-20]. Alpha-tocopherol, a molecule belonging to the family of fat-soluble vitamins (isoprenoids), appears as one of the main substances most studied in this perspective [21, 22]. Recognized as a potent antioxidant agent, it has been observed in other studies carried out in humans and animals [23-26].

The mechanisms of action of alpha-tocopherol are non-enzymatic, i.e.,, this vitamin works in a way to prevent reactive oxygen species from capturing electrons from other molecules, especially the phospholipids present in cell biomembranes [25].

However, few studies have observed the effects of alpha-tocopherol *in vitro* conditions [26], and even fewer studies have linked this vitamin to the harmful conditions observed in type 1 diabetes and other autoimmune diseases or that modify the metabolism glucose. The aim of this study was to observe the antioxidant, anti-inflammatory, and cellular cytotoxic effects of alpha-tocopherol in a culture of parotid gland cells submitted to a condition similar to DM.

Material and Method

Experimental design and cell isolation

This study was approved by the ethics committee for research in animal models of the Faculty of Medicine of Jundiaí (opinion No. 154/2016); after approval, 12 mice of the Balb/C/Unib strain were selected for this study. The animals were kept ad libitum until the fifth week of life, with a 12/12 light/dark cycle. Upon reaching the predicted week, they were euthanized using injectable ketamine (0.10 ml/body weight), intraperitoneally. The parotid glands of these animals were extracted and the process of cell isolation and cell culture of these glands was sequentially followed.

The cell isolation protocol was performed after the mechanical fragmentation of the tissue, a process performed by the researchers in a sterile environment and equipment with laminar flow appropriate for cell culture. Sequentially, the tissue was immersed in collagenase type 1 diluted in RPMI-1640 culture medium (1 mg/ml, Sigma Aldrich). During the tissue dilution time, the sample was stored in an incubator with a temperature of 37ºC and 5% Co_2.

After a period of three hours, the sample was removed from the incubator and subjected to the centrifugation procedure (Eppendorf

centrifuge - model 5804/R) for 10 minutes at a speed of 2000 revolutions per minute, at room temperature. After that, the supernatant was aspirated and the pellet was re-suspended in 5 ml of RPMI-1640 cell culture medium (Sigma Aldrich) plus 10% fetal bovine serum (Sigma Aldrich) and 1% antibiotics (penicillin and streptomycin) so that contamination of the material could be avoided. Once the re-suspension was completed, the material was added slowly to a falcon tube containing Percoll (Sigma Aldrich) in different densities to isolate the different types of cells present in the parotid gland. After the parenchymal cells were separated from the stromal cells, a process performed by the density of Percoll, the epithelial cells were added in cell culture plates to observe their development and proliferation.

Treatment of experimental groups

To observe the action of alpha-tocopherol on the cells of the parotid gland, it was divided into five groups, namely:

Group I: parotid gland cells without treatment, simulating normal conditions (with low glucose and without supplementary treatments in the culture medium);

Group II: cells of the parotid gland treated with lipopolysaccharides (100 ng/ml) to stimulate the inflammatory process and the formation of oxidizing agents, to observe the effects of the condition similar to diabetes;

Group III: cells of the parotid gland treated with lipopolysaccharides (100 ng/ml) and alpha-tocopherol (20 mMol) to observe the effects of the condition similar to diabetes and the action of the anti-inflammatory and antioxidant agent;

Group IV: cells of the parotid gland treated with anhydrous glucose (4.5 g/L) and lipopolysaccharides (100 ng/ml) to stimulate the hyperglycemic and pro-inflammatory condition, present in type 1 diabetes.

Group V: cells of the parotid gland treated with anhydrous glucose (4.5 g/L) and lipopolysaccharides (100 ng/ml) and alpha-tocopherol (20 mMol) to observe the effects of the condition similar to diabetes and the action of the anti-inflammatory and antioxidant agent.

Tests used to observe cellular effect

To observe the cellular effects caused by the use of the substances described in the above section "treatment of the experimental groups" of the present research, the immunoblotting tests for proteins involved in antioxidant and inflammatory processes were used, as well as the tests to verify the toxicity of alpha-tocopherol in cell culture, primaries of this type.

All tests followed specific protocols based on the recommendations of the manufacturers of the products used in the present study.

Statistical treatment

The statistical treatment used in the present study was Anova one way, taking $p<0.05$ as statistically significant events. All analyses passed the Bonferroni verification test, assuming the same value for p.

Results

The results obtained in this study were classified in chronological order, presented as they were collected, analyzed and verified, always in triplicates. Initially, the evolution and development of cell culture will be presented, through the alpha-tocopherol cell toxicity test (IC-50). Subsequently, the results of the quantification of proteins involved in the processes of induction of inflammatory state, response and cellular antioxidant defense will be presented.

After obtaining the cells, extracted from the animals' parotid glands, it was possible to observe an adherence of the cells, in culture, from the zero hour until five days after the extraction.

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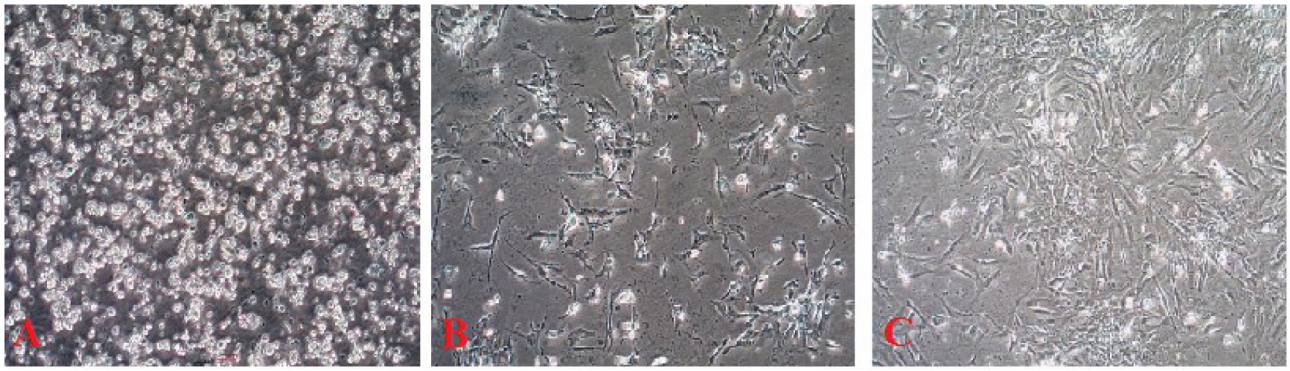
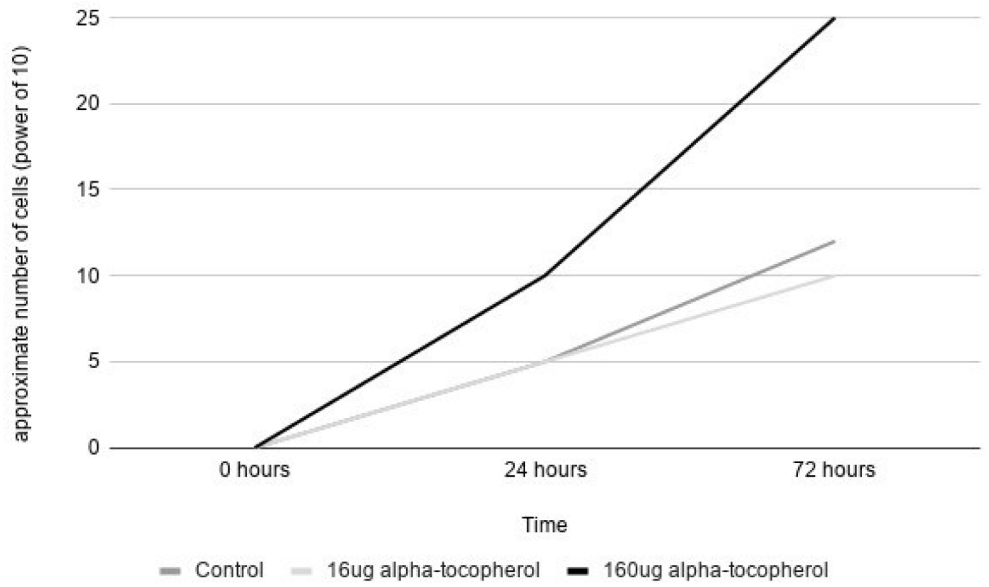


Figure 1: Progression of adherence and cellular development of the cells of the parotid gland. Image A presents immediate conditions after extraction and the Percoll protocol procedure. Image B indicates cell condition after 72 hours in an RPMI-1640 culture medium. Image C shows the condition of the cells after five days in RPMI-1640 medium. 10X magnification.



Graph 1: Proliferation of cells of the parotid gland treated with minimal dosages and above the recommendations of the literature. The number of cells approximate times the power of 10^5.

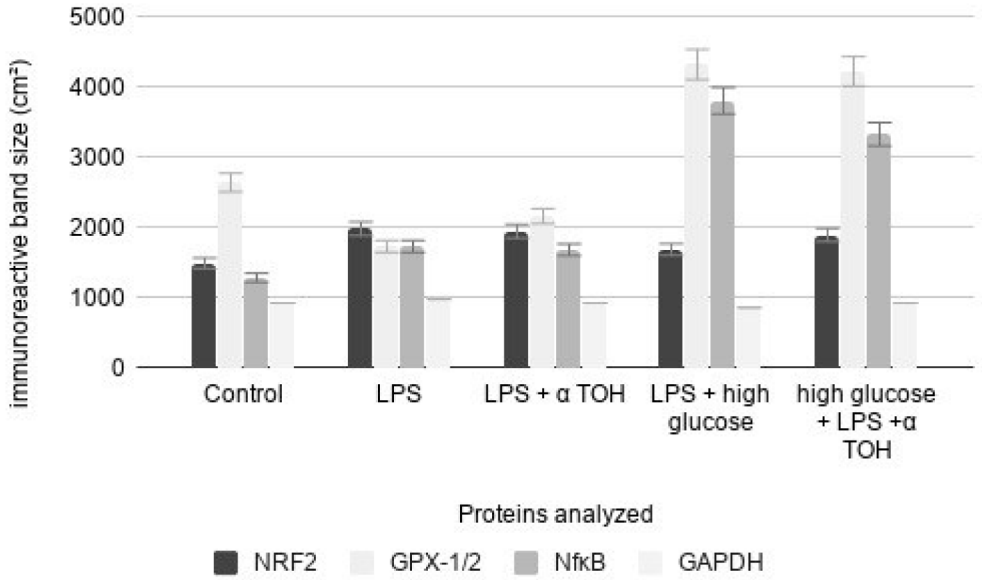
The results presented so far reflect only the effects of cell isolation and alpha-tocopherol as a non-harmful antioxidant to the cell culture studied in this research. The results below, in turn, express the data collected to observe the treatments used in the study.

Discussion

DM is a chronic metabolic disease that affects the gland tissues involved in insulin secretion and glucose metabolism. One of the main symptoms related to diabetes is the presence of hyperglycemia, which is an important cause of a chronic inflammatory state and stimulates reactive oxygen species, among other radicals, which are highly harmful to cellular structures [27,28].

Thus, the preservation of organelles and biomembranes of the cells affected by the harmful conditions generated by hyperglycemia, in addition to blocking the inflammatory condition, consists of an attempt to delay the harmful progress of this condition [29]. In this sense, alpha-tocopherol is an efficient complementary treatment in several experimental contexts [21-23, 30].

Wallert and collaborators [31] observed that alpha-tocopherol reduced the infiltration of monocytes and neutrophils in the cardiac tissue of C57BL/6 mice that suffered an acute



Graph 2: Immunoblotting of proteins involved in cellular metabolism as an antioxidant and inflammation. NRF2 is short for type 2 nuclear erythroid factor protein. GPX-1/2 is short for type 1 and 2 glutathione peroxidase protein. NF-kB is short for nuclear factor-kappa B. GAPDH is the control protein for this type of test.

myocardial infarction. Besides, reductions in the levels of reactive oxygen species and peroxidated lipids were also observed. In the same perspective of improvement, Özqül and collaborators [32]] found that the administration of 30 mg/kg intraperitoneally, via alpha-tocopherol diluted in saline to Sprague-Dawley animals with acute pancreatitis, attenuated the harmful effects of the disease and showed improvements in the levels of lipase and amylase expressed in blood plasma.

In the present study, the observed results corroborate those available in the literature [21-26, 30-32]. It was found that cell cultures that were in contact with the prescribed dosages of alpha-tocopherol 100 ng/ml) suffered less cell death than the groups that were exposed to harmful agents, LPS and hyperglycemia.

Another result of great importance was the increase in the protein glutathione peroxidase (GPX-1/2), available in intracellular medium and involved in antioxidant processes of conversion of hydrogen peroxide into water and oxygen, a process mediated in conjunction with the enzyme intracytoplasmic catalase, in groups submitted to stressors [33]. In this sense, the LPS group and hyperglycemia, group four, had the highest levels of this protein.

Corroborating this, the same group showed high levels of nuclear factor kappa B (NfkB) expressed by the immunoblotting assay. The NfkB is an important protein involved in the inflammatory process cycle that induces the transcription of the genes responsible for encoding the cytokines interleukin 1, interleukin 6, and the tumor necrosis factor in its most diverse isoforms [34]. This is because, once NfkB is expressed, a process that is mediated by the presence of stress, free radicals, ultraviolet radiation, and harmful microscopic agents, the ReLA p50 protein is stimulated, which can penetrate the cell nucleus and trigger the action of the RNA polymerase protein on the transcription of genes related to pro-inflammatory proteins [35]. The cellular effects observed after the installation of this complex system are associated with greater cell death and tissue necrosis, as well as decreased parenchyma and increased tissue stroma [36].

From the observed results, it appears that the cells of the parotid gland responded to the presence of inflammatory agents by increasing the intracellular antioxidant defense and by signaling local inflammation, a fact that in an organic perspective would trigger the advance of mononuclear phagocytes to the signaled places to fight the sources of inflammation [37].

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Once the alpha-tocopherol was administered, according to the experiment carried out with group five, the expression of NfkB decreased, without affecting the reduction of GPX-1/2. It assumes that this behavior results from a joint action of antioxidant molecules, with alpha-tocopherol acting in an extracytoplasmic environment, preventing the stress of biomembranes, mainly cytoplasmic, and glutathione peroxidase acting inside the cell.

The importance of alpha-tocopherol is recognized as an antioxidant that acts in the cellular environment in an extracellular way [30]. Its main action as an antioxidant is to donate electrons to unpaired radicals and thereby stabilize them before they oxidize cell membranes [24]. Subsequently, once the alpha-tocopherol is oxidized, it is possible to reduce it again through the union with other tocopherols present in the extracellular medium or through the relationship with ascorbic acid [38].

In a study published by Ausili and collaborators [39], it was identified that alpha-tocopherol is closely related to the lipid portion of biomembranes so that variations in the electronic density profile of these cell structures are not identified. Still, it was observed by the authors that the alpha-tocopherol is located very close to the lipid-water interface, a fact that corroborates the observations made by other authors, such as Quin, Yu, and Yu, who observed that the alpha-tocopherol is located in this region of the biomembrane. but it oscillates horizontally along with the structure so that it is not restricted to a single area [40].

Thus, it appears that alpha-tocopherol showed antioxidant effects comparable to those already documented in the literature, however, the effects of this molecule were not known in detail under the conditions experienced in this study and the cells used.

Conclusion

Alpha-tocopherol showed antioxidant effects and attenuated the inflammatory progression by decreasing the expression of the nuclear factor kappa B (NfkB) protein in cells of the parotid gland of mice submitted to high doses of glucose and lipopolysaccharides. Therefore, further studies are needed to understand the effects of this vitamin on animal and human conditions.

Conflict of Interest

The authors declare no conflict of interest.

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