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Protective Effect of Propolis Extract on Pancreatic β Cell under Oxidative Stress *in vitro*

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Abstract **Introduction:** Oxidative stress is one of the most important mechanisms in the emergence of type 2 diabetes. It would therefore be important to increase the antioxidant potential to prevent the deleterious effects of oxidative stress. **Methods:** MTT assay was performed to assess cell viability in the murine β TC-6 beta cell line. TBARs (thiobarbituric acid reactive substances) and GSH (glutathione) were measured and apoptosis were assessed by flow cytometry. **Results:** Exposure to 150 µM of H_2O_2 and 100 µM of tert-butyl hydroperoxide (t-BOOH) significantly reduced cell viability. When cells were simultaneously incubated with propolis extract (PE) and oxidants, cell viability relative to control was maintained. Exposure of cells to oxidants increased TBARs levels and reduced GSH concentration, a condition that was reversed when incubated with PE. A significant increase in apoptotic cells was seen when exposed to oxidants, however simultaneous incubation with PE reduced the number of apoptotic cells. **Conclusion:** PE has a protective effect against oxidative stress.

Keywords: *Polyphenolic, Propolis, Antioxidants, Oxidative stress, pancreatic beta cell*

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1. Introduction

Diabetes is a chronic disease with worldwide increase in incidence and prevalence. In 2014 around 387 million cases were reported and it is predicted that this number will dramatically increase in the coming years resulting in a serious public health problem and an economic challenge for health providers [1,2].

Currently, it has been proposed through *in vitro* and clinical trials, that oxidative stress plays an important role in the pathogenesis of type 2 diabetes mellitus (DM2) [3,4]. This is defined as a loss of redox homeostasis in which oxidation exceeds antioxidant defense. Free radicals and other oxidants, disproportionately increase the state of hyperglycemia, exceeding the capacity that many antioxidant enzymes have to reduce them [5].

It has been reported that oxidative stress is one of the main causative agents of dysfunction of the pancreatic β cell, because it alters structures such as DNA, proteins and fatty acids in a process known as lipid peroxidation of biological membranes [6]. It has been shown that the final products of lipid peroxidation (measured as thiobarbituric acid reactive substances, TBARs) are elevated in diabetic patients, especially those with microvascular complications [7,8,9]. The increase of 8 -hydroxydeoxyguanosine (8OHdG), a marker of oxidative DNA damage and reduced glutathione (GSH), a tripeptide consisting of glutamine, cysteine and glycine that contains a thiol group as a reducing agent has also been reported [10,11,12].

Experimental studies have also reported that pancreatic β cell, under conditions of hyperglycemia and excess free fatty acids (FFAs) increase oxidative stress by stimulating the enzyme NADPH oxidase (NOX) [13] which catalyzes the transfer of electrons from NADPH to molecular oxygen to form superoxide anion. Moreover, it has been demonstrated the activation of inducible nitric oxide synthase [14], leading to uncontrolled increase nitric oxide levels in the cytoplasm, causing cellular dysfunction, impaired insulin secretion and apoptosis of β cell [15,16,17].

It is also important to emphasize that the pancreatic β cell is especially susceptible to the consequences of oxidative stress due to the low expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px) [18,19]. In this way it is possible that improved antioxidant defences of pancreatic β cells may reduce their vulnerability to oxidative stress.

The present study suggests that propolis may have a protective effect against oxidative stress in pancreatic β cell, due to chemical characteristics that account for the large number of bioactive components that it has. Propolis corresponds to a resinous product which is collected by bees (*Apis mellifera*) used to seal cracks in the hive, in order to maintain a stable temperature and humidity [20]. The bees collect it from buds, shoots and wounds in

deciduous trees, then it is mixed with wax and the mandibular gland secretions from the bees [21,22].

The composition of propolis depends on the geographic area [23], to date, about three hundred compounds have been identified including fatty acids, cinnamic acid, caffeic acid, phenolics, flavonoids (flavones, flavanones, flavonols include, dihydroflavonols, calcones), terpenes, β steroids, aromatic aldehydes, alcohols, vitamins, minerals and essential oils [24-30]. Because of this great chemical diversity, propolis has been attributed to a great pharmacological potential [31], in which we can highlight anti-inflammatory, anti-cancer and anti-microbial and anithypertensive effects including many more [27,32,33]. These properties might be explained, almost in part, because of the antioxidant capacity of its compounds. Some studies have found that propolis extracts have the great ability to neutralize free radicals [24,34] and this property is associated with the content of phenolic compounds [35]. However, there are few studies evaluating the protective potential of propolis on the β cell under oxidative stress [36].

Considering all the above information, we proposed to evaluate the effect of propolis extract on pancreatic β cells β TC-6 undergoing oxidative stress *in vitro*.

2. Materials and Methods

2.1. Extract Preparation

At the Clinical Biochemistry laboratory of the University of Talca, methanol/water and propolis samples were obtained from an apiary in the Maule region (seventh region, Chile) as well as the propolis extracts (PE) were prepared. The samples were macerated with methanol (90%) at room temperature and then they were filtered twice to remove impurities. Subsequently, the extract was dried using a rotary evaporator (Heidolph). Both dried extracts were resuspended in methanol (90%) to generate methanol extracts with an initial solution of 20 mg/ml of propolis. Then, this solution was diluted to 2 mg/ml (ten times) in distilled water and dimethyl sulfoxide (DMSO) 1% so that from this stock solution, the necessary volumes were taken to obtain the final concentrations used in the assays (200, 100, 40 and 20 µg/ml) taken into account that IC_{50} was 20 µg/ml, regarding previous studies in which inhibition assay of radical DPPH was measured (unpublished data).

2.2. Reactives

The oxidants hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (t-BOOH) and trichloroacetic acid (TCA) were purchased from Merck. The malondialdehyde (MDA), sodium chloride (NaCl), DNTB reagent, EDTA, metaphosphoric acid, DMSO, reduced GSH, propidium iodide (PI) and albumin were purchased from Sigma Aldrich. The MTT kit was purchased from Cayman Biochemicals Company. Thiobarbituric acid was purchased from MP Biomedicals Company. BCA Kit and fetal bovine serum (FBS), derived from Thermo Scientific. Annexin/V binding kit were purchased from BD Pharmingen Company. Dulbecco's Modified Eagle Medium high glucose (DMEM-H) and tripsna-EDTA were purchased from the Gibco Life Technologies and PBS was purchased from Calbiochem.

2.3. Cell Culture

Dr A. Quest from the Cellular Communications Laboratory of the Faculty of Medicine of the University of Chile, donated pancreatic β TC-6, which were cultured in DMEM-H, supplemented with 10 % FBS; streptomycin 100 ug / ml and penicillin 100 IU / ml. Cells were seeded in a 100 mm sterile culture plate containing a total volume of 7 ml of medium DMEM-H. The plate was incubated at 37°C with 5% CO_2 in culture oven (Hf 160 W).

A change of culture medium was performed every other day to maintain and expand the cell line and harvesting procedure (harvest) 1 ml tripsina-EDTA 0.25% was used to generate new cell passages.

2.4. Viability Assay

Viability assays were performed using the MTT formazan Kit, whereby 96 well sterile plates were used, at 25,000 cells/well were seeded in 100 µl were used in supplemented DMEM-H. The plate was incubated at 37°C and 5% CO_2 for 24 hours, then the medium or solution of interest was removed and 100 µl was added to fresh culture medium, followed by the addition of 10 µl of MTT. This reaction was incubated for 4 hours, after which 100 µl of solvent crystal solution was added to dissolve the blue crystals formed as specified in the kit. Once crystals were dissolved the plate was measured in a microplate reader at 570 nm (multiskan go microplate spectrophotometer, Thermo Scientific). To calculate the viability the following mathematical formula was applied:

$$\%\ \text{Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \text{x} 100.$$

2.5. Lipoperoxidation Assay

Lipoperoxidation products were determined by measuring the amount of thiobarbituric acid reactive substances (TBARs) produced in the culture medium [37]. 106 pancreatic β cells were seeded in a sterile 10 mm plate with 5 ml of supplemented DMEM-H. From each experimental condition 500 µl of culture medium was taken and added to 1000 µl of 0.67% thiobarbituric acid plus 150 µl of 50 % trichloroacetic acid. The samples were mixed using a vortex (VELP scientific, Lab Tec) and then incubated at 90°C for 30 minutes (Reciprocal Water Bath NB-304, N-Batek).

After incubation, this was mixed again in the vortex, the sample was centrifuged for 15 minutes at 500 g (Sigma) and the absorbance of the supernatant was measured at 530 nm (multiskan go microplate spectrophotometer, Thermo Scientific).

To calculate the TBARs, a calibration curve was prepared using a standard by Malondialdehyde whose linearity is 1 to 10 µM.

2.6. Reduced Glutathione Measurement

To determine the reduced GSH, 106 cells were seeded in a sterile 10 mm plate with 5 ml of supplemented DMEM-H. After the incubation period under experimental

conditions, the culture medium was completely removed and 1 ml of PBS was added.

The cells were removed using a scraper for cell culture (Thermo Scientific) and all content was transferred to an eppendorf tube. Subsequently, this was centrifuged at 50 g for 5 minutes and the supernatant removed. The pellet was resuspended in 1 ml lysis buffer (pH 7.4, 50 mM Tris, 5 mM EDTA, 0.1% SDS, 30 mM NaCl) and subjected to ultrasonic fragmentation (Ultrasonic mrc) of 40 V at 5 second intervals, depositing on ice between each one.

Samples were centrifuged at 1000 g for 10 minutes and the supernatant was used to measure proteins and reduce GSH.

For the measurement of reduced GSH [38], the samples were deproteinized using precipitating reagent (1.65 g metaphosphoric glacial acid, 0.2 g EDTA, 30 g of NaCl in 100 ml distilled water) and allowed to incubate for 5 minutes at room temperature. All samples were centrifuged at 1000 g for 10 minutes.

250 µl of the supernatant was collected and was added to 1 ml of phosphate buffer plus 125 µl of DNTB reagent. Samples were incubated for 5 minutes at room temperature and measured in a spectrophotometer at 412 nm (Multiskan go microplate spectrophotometer, Thermo Scientific).

A calibration curve was made using reduced glutathione using a linearity range of 1 to 30 mg / dl sample.

2.7. Protein Measurement

BCA Kit was used to measure protein concentration of the supernatant of the cell lysates. A calibration curve using a standard albumin 1 mg / ml within the range of 20 to 1000 g / ml was performed. A 25 µl sample and 200 µl of working reagent was loaded, the plate was agitated for 30 seconds (Heidolph Titramax 1000, Merck) and incubated at 37°C for 30 minutes. Once the procedure was completed, the absorbance at 562 nm (multiskan go microplate spectrophotometer, Thermo Scientific) was measured.

2.8. Apoptosis Assay

To determine apoptotic cell death 100.000 cells per well in 24-well sterile plates contained in a volume of 500 µl of supplemented medium DMEM-H were seeded. Cells were labeled with annexin V and propidium iodide (PI) and measured by flow cytometry (BD Accuri™ C6). Having incubated the cells under specific conditions they were transferred to an eppendorf tube in 50 mL of binding buffer (100 mM HEPES / NaOH, pH 7.5, 140 mM NaCl and 2.5 mM CaCl2) and 2.5 ul of annexin V (10 µg/ml) and 5 µl of IP (50 µg/mL) (Aldrich, USA) was added. For controls, only untreated and without staining, annexin V only, only with PI and treatment double staining cells were considered. Apoptotic cells were expressed as percentage of total cell number.

2.9. Statistical Analysis

Each cell assay was performed in triplicate on three independent assays. Variance Analysis of one way (Oneway ANOVA) was used for comparison between groups and post analysis post hoc Dunnett's was used to compare with the control group or post hoc Bonferroni comparisons between all groups. Differences in $p < 0.05$ were considered statistically significant. Data was analyzed and plotted using the computational statistical software GraphPad Prism 4.0.

3. Results

To determine the proper concentration of H_2O_2, t-BOOH, PE and to assess the final viability assay, an MTT assay with different concentrations of each oxidant for a period of 24 hours, was performed.

As H_2O_2 concentration increased, the cell viability was reduced (Figure 1A). At H_2O_2 concentrations over 50 µM significant statistical differences were seen (***$p < 0.001$). Reduced viability of more than 50 % over 150 µM H_2O_2 concentrations (***$p < 0.001$) was found, and these were considered to carry out the viability assay simultaneously exposing H_2O_2 and PE. Likewise, it was observed that as the concentration of t-BOOH increased, the cell viability reduced (Figure 1B). A reduction in viability over 50% at concentrations greater than 100 µM t-BOOH (*** $p < 0.001$), was considered for the viability assay, for exposition to t - BOOH and PE simultaneously.

With the aim of evaluating the appropriate incubation time for performing the cell viability assay exposed to oxidants (H_2O_2 and t-BOOH) and PE, a MTT assay at different incubation times was determined, considering the concentrations at which a reduction in viability over 50 % was obtained compared to control cells. For this, both cell lines were exposed to oxidant assays and viability was evaluated at 0, 4, 8, 16 and 24 of hours of incubation.

When viability of β TC-6 cells exposed to 150 µM of H_2O_2 was evaluated. It was observed that with increased exposure time, cell viability was reduced (Figure 1C). After 8 hours of incubation there was a statistically significant reduction (*** $p < 0.001$ Figure 1C). Reductions over 50 % of viability were observed at 16 and 24 hours of incubation. No statistical significant differences between the 16 and 24 hours of incubation were observed. Similar results were obtained using t-BOOH as an oxidant, the only significant differences were observed after 8 hours of incubation (*** $p < 0.05$ Figure 1D).

To determine the optimum propolis extract dosage (PE) to be used without causing toxicity in pancreatic β cells, a cell viability assay at increasing PE concentrations was done. For this, the cells were exposed to an extract containing vehicle (DMSO and methanol) or PE at a concentration equal to the IC_{50} (20 µg/ml), or at a concentration twice IC_{50} (40 µg /ml), or at a concentration showed five (100 µg/ml) and 10 times (200 µg/ml) IC_{50} for 24 hours.

No statistical significant differences were seen when cells were incubated with vehicle at IC_{50} concentration and twice IC_{50} with respect to the control (Figure 1E). However, when the extract concentration ranged between five to ten times the IC_{50} concentration, significant decreases in cell viability were observed (***$p<0.001$; Figure 1E) however, in order to evaluate antioxidant potential *in vitro*, these concentrations were discarded.

To perform the final viability, assay 150 µM of H_2O_2 and 100 µM of t-BOOH oxidants were used for 24 hours

and the antioxidant potential of PE at concentrations one and two times the IC_{50} was evaluated.

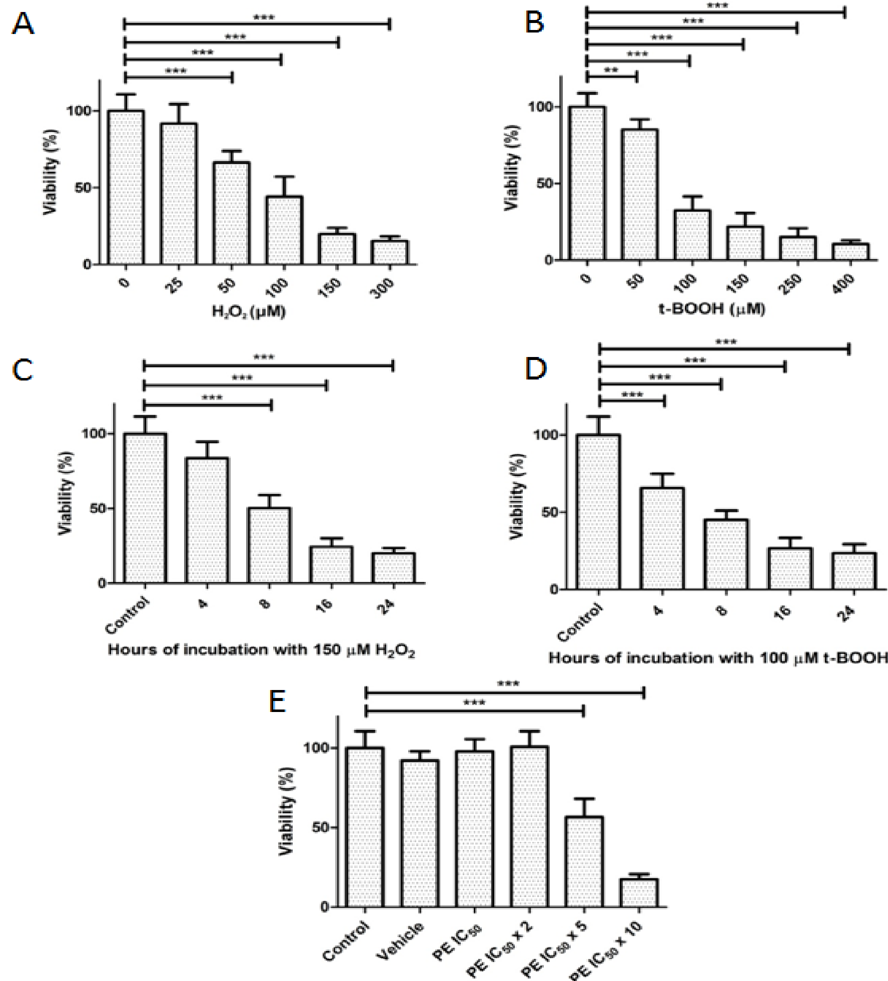

Figure 1. Viability assay of β TC-6 cells exposed to oxidant and PE. A and B Fig. show percentages of viable cells exposed to a different concentration of H_2O_2 y t-BOOH. C show percentages of viability cells incubate for 0 (control), 4, 8, 16 and 24 hours with H_2O_2 150 µM and t-BOOH 100 µM (D). E represents a cytotoxicity assay of β TC-6 cells exposed to a control Dulbecco's Modified Eagle Medium high glucose (DMEM-H medium), vehicle (methanol/water) and different concentration of PE. Result are presented as means ± SD (n=3). One way ANOVA, $p < 0,001$, Dunnett's post hoc test ***$p < 0.001$.

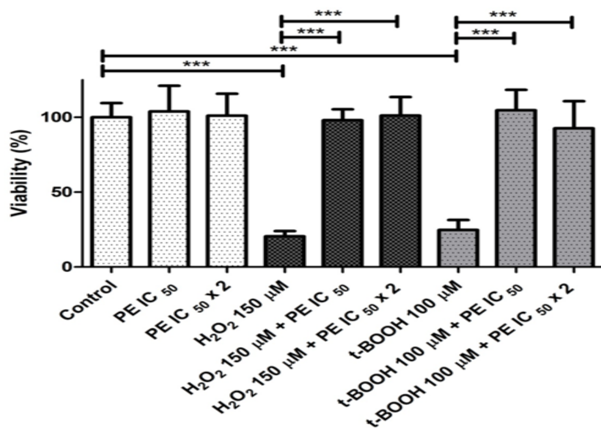

Figure 2. Viability assay of β TC-6 cells exposed to oxidants in presence and absence of EP. Figure show percentage of β TC-6 cells incubated under control and PE (White bars), H_2O_2 and H_2O_2 plus PE (black bars) and t-BOOH and t-BOOH plus PE (gray bars). Result are presented as means ± SD (n=3). One way ANOVA, $p < 0,001$, Dunnett's post hoc test ***$p < 0.001$

When cells were incubated in the presence of 150 µM H_2O_2 and 100 µM of t-BOOH, a significant reduction of viability (*** $p <0.001$, Figure 2) was observed. However, when cells were simultaneously incubated in the presence of the oxidant and PE, a viability percentage similar to the control was observed with no statistically significant differences (Figure 2).

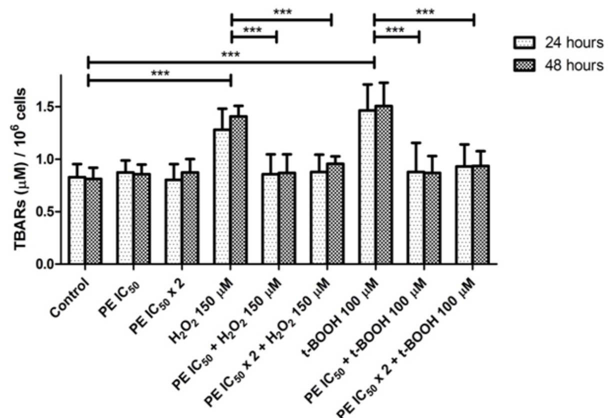

Figure 3. TBARs concentration from β TC-6 cells exposed to oxidant condition in presence and absence of EP. Figure show concentration of TBARs from β TC-6 cells generated to the medium at 24 and 48 hours of incubation under control condition, in presence of PE, oxidants and oxidants plus PE. Result are presented as means ± SD (n=3). One way ANOVA, $p < 0,001$, Dunnett's post hoc test ***$p < 0.001$

To determine the lipoperoxidation as an indicator of oxidative damage occurring on cell membranes, a TBARs assay in culture medium were measured. Regarding the results, no significant statistical differences between 24 and 48 hours of incubation under all evaluated conditions were observed. Increased TBARS was observed when β TC-6 cells were exposed to H_2O_2 and t-BOOH when compared to controls (*** $p < 0.001$ Figure 3) however, when cells were simultaneously incubated in the presence of PE, similar results were observed respect to the control condition (Figure 3).

Moreover, intracellular GSH concentration in β TC-6 was evaluated. Based on these results, there were no significant statistical differences when the cells were exposed to PE, but a statistically significant decrease in reduced GSH concentration was observed when cells were exposed to H_2O_2 and t - BOOH relative to PE and the control condition (*** $p < 0.001$; Figure 4). However, when incubated in the presence of the oxidant and PE, a concentration of GSH similar to the control condition was obtained (Figure 4).

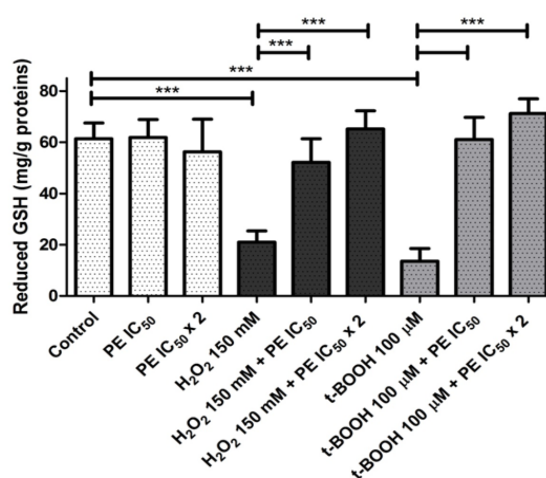

Figure 4. Reduced GSH concentration of β TC-6 cells exposed to oxidant condition in presence and absence of EP. Figure shows GSH concentration of β TC-6 cells, incubation under control condition, in presence of PE, oxidants and oxidants plus PE. Result are presented as means ± SD (n=3). One way ANOVA, $p < 0.001$, Dunnett's post hoc test ***$p < 0.001$

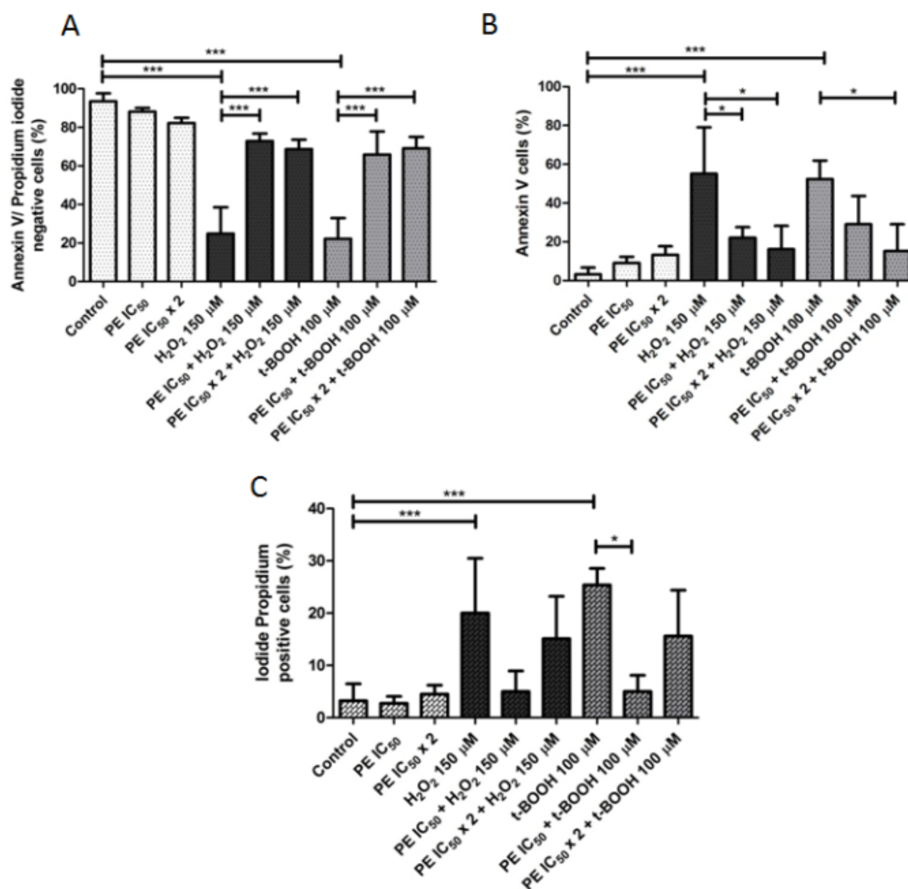

Figure 5. Percentage of viable, apoptotic and necrotic β TC-6 cells exposed to oxidant condition in presence and absence of EP A show viable β TC-6 cells (anexin V and PI negative), B represent percentage of apoptotic cells (anexin V-positive) and C show percentage of necrotic cells (PI positive). Result are presented as means ± SD (n=3). One way ANOVA, $p < 0,001$, Dunnett's post hoc test * $p < 0.05$; ***$p < 0.001$

To evaluate the effect of propolis on apoptosis induced by the oxidants in pancreatic β cells, the number of annexin V positive cells, indicative of apoptosis and iodide from propidium-positive, indicative of necrotic cells, was analyzed. Based on these result, exposure to 150 μM H_2O_2 or 100 μM t-BOOH, reduced the percentage of viable cells and significantly increased the percentage of annexin V positive and iodide propidium - positive cells, as we expected (*** $p < 0.001$; Figure 5). Notably, simultaneous incubation with the PE and oxidants, attenuated both apoptotic (* $p < 0.05$) and necrotic cell death (* $p < 0.05$) of the β TC -6 cells, both as IC_{50}, IC_{50} x 2 (Figure 5).

4. Discussion and conclusion

In the present study, PE was used to evaluate the effect on culture of β pancreatic β TC -6 cells subjected to

chemically induced oxidative stress using H2O2 and t-BOOH. It has been demonstrated that H_2O_2 induces an increase of superoxide over basal levels via stimulation of enzymes iNOS [39] and NOX, generating damage in the proteins, DNA and cell membranes [40]. Moreover, t-BOOH induces oxidative stress through the decrease of intracellular GSH and NADPH, glutathione peroxidase substrates and glutathione reductase, favoring an increase of endogenous H_2O_2 [41].

Based on the obtained results, a reduction of cell viability of over 50 % at concentrations of 100 µM of t-BOOH and of 150 µM of H_2O_2 was observed. It is has been reported that MIN6N pancreatic β cells exposed to 70 µM of H_2O_2 for a period of 4 hours reduced viability to approximately 60% [42], moreover a viability reduction of 40% when cells were incubated with 150 µM of H_2O_2 for a period of 45 minutes [43]. Gonzalez C et al. (2014) reported a reduction in cell viability of 60% by using MIN-6 cells exposed to 150 µM of H_2O_2 for the same period of incubation [44], lower than found in this study. Moreover, Martin M. Á. et al (2013) showed a reduction in viability of 40% of β Ins-1 cells incubated with 50 µM of t-BOOH over a 2 hour period [45], on the other hand, a reduction of approximately 70% were obtained when cells were incubated with 100 µM over an incubation period of 24 hours. This suggests that the variability of the results depends on the protocols used; cell line and the incubation time, even when the same oxidants are used, and all of these are key factors in the standardization of viability assay.

It has been widely reported that oxidative stress damages cell membranes through the lipid peroxidation of fatty acids, generating oxidative stress biomarkers such as TBARs. This measurement is useful to assess the overall impact of oxidative damage induced by free radicals. Arne Linden et al (2008) revealed an increase in TBARs in culture of C6 glioma cells exposed to t-BOOH, however no significant changes in the generation of such markers occurred when cells were treated with H_2O_2 [46], unlike what we report in this study (Figure 3).

Moreover, it has been shown that exposure to equimolar concentrations of oxidants such as t-BOOH, H_2O_2 and menadione, HepG2 cells generated greater TBARs than Caco-2 (intestinal) [47]. Similar results have been obtained in oligodendroglia cells when exposed to 100 µM of H_2O_2, showing an increase in TBARs concentration produced in the culture medium [47]. Therefore, although concentrations may vary by cell type, TBARs generation is a common characteristic to many cells exposed to oxidants, as observed in this work. This concentration significantly increased when the cells were exposed to oxidants, regardless of type, which validates the effect of oxidative stress in pancreatic β cells and correlates with clinical trials which demonstrate the increase of these lipid peroxidation markers in patients with diabetic pathology [8,48].

Another phenomenon associated with an oxidative stress biomarker studied in this work corresponds to the decrease of reduced GSH. Glutathione in a reduced state is the main intracellular antioxidant, conferring a protective effect against oxidative stress exposure. It is a substrate of the enzyme glutathione peroxidase, which catalyzes the detoxification of organic peroxides [49].

A significant reduction of GSH in β Ins-1 cells exposed to oxidative stress-induced conditions with t–BOOH has been shown [45]. Similar results were observed in this study, as the same effect on β TC- 6 cell line when exposed to H_2O_2 and t-BOOH was obtained. This reduction is probably due to the cell need to counteract levels of free radicals generated during the induction of oxidative stress.

There are a number of extracts that have antioxidant capacity and therefore have potential therapeutic value against diseases in which oxidative stress is involved [50,51,52]. Therefore, a PE was resorted to evaluate the antioxidant effects already described, however, it was first necessary to assess the cytotoxic effects that could induce cell damage [53]. In the present study, it was found that concentrations of between 20 and 40 µg/ ml of phenolic compounds present in PE did not alter the viability of β TC -6 cells (Figure 1E). However, concentrations over 100 µg/ml and 200 µg/ml significantly reduced cell viability.

After selecting adequate PE concentrations, viability and biomarkers of oxidative stress in the presence of propolis extract were evaluated. When cell lines were exposed to oxidants in the presence PE of viability conditions similar to the controls were observed (medium culture only) so the compounds present in the extract protected the β cells in culture from the cytotoxicity of the oxidants (Figure 2). Moreover, a concentration of TBARs and seeded GSH to the control condition in presence of the extract and oxidants was obtained (Figures 3 and 4). These results are comparable to those obtained in numerous studies evaluating the protective effect of extracts with antioxidant capacity in cell lines under oxidant conditions [45,54].

The ability to display PE to reduce oxidative damage in pancreatic β –cells may be due to an indirect or direct effect. The first one is based on the ability of compounds such as acid phenethyl ester (cape) to activate nrf2 transcription factor [55]. The nrf2 transcription factor is a protein that positively regulates the expression of a number of antioxidant enzymes such as heme oxygenase -1 and enzymes involved in GSH metabolism [56], so propolis extract through its phenolic compounds can activate nrf2 and increase the ability to neutralize excess oxidants. The direct effect, on the other hand, is due to the ability of propolis extracts to directly neutralize free radicals or other oxidants in the culture medium [57]. This is due to the presence of numerous flavonoid and phenolic compounds capable of neutralizing radical by yielding electrons.

It has been suggested that oxidative stress is involved in the initiation of apoptosis. Within this context it is known that ROS alters the mitochondrial membrane releasing cytochrome c which is a central event in aggregation of the molecular adapter Apaf-1 and consequently caspase activation [58]. Moreover, Bcl-2, a protein involved in the regulation of apoptotic cell death through the control of pore formation in the mitochondria that allows the exit of cytochrome c and pro-apoptotic proteins [59], can protect of Bcl-2 cells from apoptosis induced by ROS when it is overexpressed, but the molecular mechanism is unclear. Bcl-2 alone does not exert any antioxidant effects, but it has been suggested that it acts indirectly by increasing endogenous antioxidant levels [60].

In this research it was shown that propolis extract protects β TC-6 cells from induced apoptosis by H_2O_2 and

t- BOOH, reducing the number of positive annexin-V cells when exposed to these oxidants (Figure 5). The same occurred when evaluating IP permeability, (levels of necrotic cells), which are permeable to this compound.

While there is much evidence that PE activate the apoptotic pathways of tumor cells [61,62,63], other studies show that in normal cells subjected to oxidants, propolis have the ability to neutralize oxidative stress through its phenolic compounds, thus preventing activation of the apoptotic pathways [64]. However, the molecular mechanisms by which PE protects the β cells from apoptosis induced by oxidative stress, has not been clearly described. However, probably both, direct and indirect mechanisms are involved, as discussed above. Finally, there is a good agreement between cell viability assay and the evaluation of apoptosis under the conditions studied in this research.

Taken together, it was observed that oxidants reduced cell viability and increased apoptosis, increasing the levels of the oxidative stress marker TBARs, and reducing antioxidant defenses such as GSH. Most important, the current results suggest that PE exerts a significant protective effect on β cells subjected to oxidative stress. So, it would be of great interest to study the molecular mechanisms, by which this effect is produced, and then by carrying out bioavailability assays on an animal diabetes model *in vivo* and finally evaluating the antioxidant potential in clinical trials with patients.

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