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**ORIGINAL ARTICLE**

# Coffee beverage reduces ROS production and does not affect the organism's response against *Candida albicans*

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## Abstract

Coffee is a mixture of substances with potential beneficial and adverse health effects. Several studies demonstrate the antioxidant effect of the phenolics compounds present in coffee. Neutrophils produce reactive oxygen species (ROS) by activating of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2), which plays a key role in organism defense against microbial pathogens. Diabetes mellitus patients are more susceptible to bacterial and fungal infections. The present study evaluated the influence of coffee beverage on NOX2 activity and ROS generation and the impact of this effect on phagocytosis and killing of *Candida albicans* by neutrophils from diabetic and non-diabetic animals. Diabetes mellitus was induced in male Wistar rats using 2% alloxan. Diabetic and non-diabetic animals were divided into groups treated and untreated with coffee drink (7.2 mL/kg/day) or apocyanine (16 mg/kg/day) for 50 days. After 50 days, the animals' glycemic profile was measured by blood glucose and glycated hemoglobin (HbA1c) tests. The generation of ROS in neutrophilic cells was measured by chemiluminescence and cytochrome C reduction assays. *C. albicans* phagocytosis and death were evaluated by optical microscopy using the May-Grunwald-Giemsa staining method. The coffee drink has not altered the glycemic profile and NOX2 activity of the animals. However, coffee reduced the ROS pool in non-diabetic and diabetic animals, but this activity did not harm the phagocytosis or killing of neutrophils. Treatment with apocyanin decreased ROS production and killing capacity of neutrophils from non-diabetic animals against *C. albicans*. We suggest that the coffee drink intake prevents oxidative damage and does not impair response of the organism against opportunistic microorganism.

**Keywords:** *Candida albicans*. *Coffea arabica* L. Diabetes mellitus. NOX2. Reactive oxygen species.

## How to cite

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## INTRODUTION

Coffee consumption is widespread in many countries and reached 162.12 million bags consumed in 2017/18 (Jeszka-Skowron et al., 2020). Its potential health effects have been widely studied, such as reduced risk of disease (type 2 diabetes mellitus, depression, cancer, hepatic injury, cardiovascular disorders, among others), regulation of blood glucose levels, beneficial action on the gastrointestinal tract and intestinal microbiota, increased mental alertness, high antioxidant activity (Costabile et al., 2018; Melo Pereira et al., 2020; Muñoz et al., 2020). The relationship between the consumption of coffee and human health and disease are complex as its composition, its preparation and the ingested dose may vary. Among the many bioactive substances that are present in the drink, two stand out: caffeine and chlorogenic acid, which are substances associated with reduced risk of developing several diseases such as arteriosclerosis, cancer and diabetes mellitus (Ludwig et al., 2014a; Muñoz et al., 2020; Reis, et al. 2019).

Diabetes mellitus is characterized by chronic hyperglycemia and metabolic changes in lipids, carbohydrates and proteins due to defects in secretion and insulin resistance or both or downgrade of insulin levels (Association, 2016; Mirza et al., 2019). A high glucose level is considered an important factor in the development of tissue damage. Some authors suggest that these damages are caused by hyperglycemia linked to increased flow through the polyol pathway, by activation of protein kinase C (PKC) isoforms and hexosamine biosynthetic pathway with increased formation of advanced glycation end products (AGEs) (Souza Ferreira et al., 2016; Ding, et al. 2019; Jud and Sourij, 2019; Shakeel, 2015). All of these mechanisms may result in an overproduction of reactive oxygen species (ROS) in mitochondria (Ding et al., 2019).

The ROS generation seems to be a common thread among these pathways, and a major source of ROS is the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 **(**NOX2) (de Souza Ferreira et al., 2016; Shah & Brownlee, 2016). The glucose level stimulates phagocyte-type NADPH oxidase activity that influences the functioning of pancreatic beta cells (Ding et al., 2019). The killing and microbicidal functions of neutrophils are directly related to the metabolic pathway that involves NOX2 and PKC and generates ROS, mainly superoxide radicals. Changes in ROS production by phagocytes have been identified as a major cause of immune system dysfunction in diabetics and of their increased susceptibility to bacterial and fungal infections (Souza Ferreira et al., 2016; Kempf et al., 2007).

Evidences suggest that high consumption of coffee may reduce the risk of diabetes mellitus by affecting glucose metabolism with respect to insulin tolerance and oxidative stress as a consequence of chronic hyperglycemia (Costabile et al., 2018; Mellbye et al., 2015). Despite numerous reports in the literature on the association between coffee consumption and diabetes mellitus, these data are still inconclusive (Melo Pereira et al., 2020; Kalschne et al., 2019; Lee et al., 2015; Stefanello et al., 2019). In this context, the present study aimed to verify the influence of coffee beverage on ROS generation by NOX2 and on the phagocytic function of neutrophils and microbicidal activity toward *C. albicans* in diabetic rats.

## MATERIAL AND METHODS

### The preparation, characterization and determining bioactive compounds of integral coffee beverage

Samples of Brazilian coffee (*Coffea arabica* L.) were provided from Fazenda Conquista (865 m altitude), belonging to Ipanema Agricola S.A., located in Alfenas, MG. Coffee beverage was prepared at a 10% (w/v) concentration and then was lyophilized to facilitate the characterization of the sample (Lima et al., 2010). The lyophilized powder was stored in a freezer (-20°C) and was only solubilized at the time of administration to the animals. This procedure ensured the reproducibility of the study and enabled the administration of equal amounts of soluble solids to the rats.

The characterization of the coffee drink was carried out by determining the total phenolic content and bioactive compounds. The levels of total phenolics present in the sample was measured using the Folin-Ciocalteu method, with galic acid as the reference standard. To determine the bioactive constituents such as caffeine, chlorogenic acid (CGA) and total phenol, hot water extraction procedures were used according to (Lima et al., 2010). The chromatographic separation of these compounds was performed using a High Performance Liquid Chromatography (HPLC) equipped with a data transmitter, with a high pressure pump (Code number PU-4086, Jasco LC- NetII/ADC, Japan), a refrigerated automatic injector and a photodiode detector (Code number 6832-J002B, Model MD-2015, Jasco AS-2057 Plus, Japan).

HPLC device used a Phenomenex C18 ODS (octadecysilyl) reverse phase column (250 x 4.60 mm; 2.5 µm), with a gradient elution process, whose flow rate was 1 mL/min for 30 minutes at temperature environment. The gradient technique employed (A) acetate buffer and (B) methanol (Code number 34860-1L-R, PA, Aldrich, Germany) (HPLC grade). The concentrations of 5-caffeoylquinic acid (5-CQA) (Code number SMB00131-1MG) and caffeine (Code number C1778-1VL) were calculated using calibration curves made with their respective standards (Sigma Chemical Co, St. Louis-USA).

## Induction of Diabetes mellitus

After 12 hours fasting, diabetes mellitus induction was performed in male Wistar rats by intraperitoneal administration of 2% alloxan (150 mg/kg) (Szkudelski, 2001). Alloxan was the compound of choice for cost reasons (cheaper than streptozotocin) and because it is the standardized protocol in the laboratory, in addition to being widely used in the induction of experimental diabetes mellitus (Anyanwu et al., 2019; Bacevic et al., 2020; Chunudom et al., 2020; Singh et al., 2020). In this study, animals with blood glucose levels ≥ 250 mg/dL were considered diabetic (7 days after induction) and included in the experiment. Blood glucose measurements were monitored weekly using the blood collection from the rats' tails to monitor the diabetic condition.

## Experimental delineation

All *in vivo* experiments were performed in accordance with the guidelines of the International Council for Laboratory Animal Science (ICLAS) and were approved by the Ethical Committee for Animal Experimentation of Federal University of Alfenal (Unifal-MG) (document nº. 420/2012) before the study was carried out. Twelve-week-old male Wistar rats (*Rattus norvegicus*) weighing 320 ± 20 g were obtained from the Unifal-MG *vivarium*. These animals were maintained in a temperature of 25°C under a 12 h:12 h artificial light/dark cycle and received commercial feed and water *ad libitum* during the entire experimental period.

After a week of acclimatization, the animals were divided into 6 groups (n = 10/group): non-diabetic animals treated with distilled water (non-diabetic), non-diabetic animals treated with apocynin (non-diabetic + APO), non-diabetic animals treated with arabica coffee drink (non-diabetic + coffee), diabetic animals treated with water (diabetic), diabetic rats treated with apocynin (diabetic + APO), and diabetic animals treated with arabica coffee drink (diabetic + coffee).

The animals were treated with a coffee drink or apocinin, a natural organic antioxidant with the ability to inhibit the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and increase ROS capture (Wang et al., 2019; Xin et al., 2018). The administration of apocynin (Sigma) (16 mg/kg/day) (Nam et al., 2009) or of filtered coffee drink (a dose of 7.2 mL/kg/day - equivalent to a daily human consumption of eight cups of 50 mL of coffee) started 7 days after induction and confirmation of the diabetic status and was performed by gavage for 50 days. The non-diabetic group received the same amount of water.

### Obtaining neutrophils

After 50 days of treatment, the neutrophil recruitment was performed by intraperitoneal administration of 2 mL of 12% (w/v) sodium caseinate (Sigma) diluted in sterile 0.9% saline. After 4 hours of the procedure, the rats were anesthetized by intraperitoneal injection of 40 mg/kg of sodium pentobarbital and the intraperitoneal cavity was washed with 15 mL of cold sterile phosphate-buffered saline (PBS). The cell suspension was collected by aspiration with syringe (40 x 12 mm needle) after massage of the abdomen. This suspension was centrifuged at 4°C (500 *g* for 10 min) and kept on ice until the testing (Souza Ferreira et al., 2016). The experiments were carried out in triplicates.

### Cell counting and viability assessment

The viable cells (> 98% neutrophils) were quantified using a Neubauer chamber under an optical microscope employing the Trypan blue exclusion test (Souza Ferreira et al., 2016).

### Glycemia and HbA1c levels

The blood sample was obtained by cardiac puncture. Blood glucose levels were determined by the enzymatic method using an automatic analyzer (HumaStar 80-In vitro Diagnostic Ltda) according to the manufacturers. Glycated hemoglobin (HbA1c) was assessed by HPLC using the D-10 Dual BIO-RAD ion exchange equipment (Souza Ferreira et al., 2016). Dosages were performed in triplicates.

### NOX2 activity

The NOX2 activity was determined by the quantification of its superoxide anions produced in neutrophilic cells. The test measured the reduction of cytochrome C. The assay used 2 x $10^6$ neutrophils/mL, 100 µmol/L cytochrome C and 20 mg/mL catalase in PBS containing 10 mmol/L glucose (pH 7.4) added to a plastic bucket. Superoxide production was initiated by the addition of PMA (12-myristate 13-acetate phorbol) (53 ng/mL) and the reaction was monitored by spectrophotometry for 3 min at a wavelength of 550 nm (Souza Ferreira et al., 2016; Jones & Hancock, 1994). The tests were performed in triplicates.

### Chemiluminescence amplified by Luminol

Neutrophils were activated by PMA (53 ng per test) and added to a reaction mixture containing 1 mmol/L of luminol and 1x$10^6$ neutrophils per milliliter. The chemiluminescence produced was quantified in a Geomax® TM 20/20 luminometer, and the results expressed as units of relative light per second (RLU/s). The reaction was performed in PBS (pH 7.4) and followed by 30 min at 37 °C (Souza Ferreira et al., 2016; Rodrigues et al., 2002). Assays were performed in triplicates.

### Yeast preparation

*C. albicans* yeast (American Type Culture Collection (ATCC) 5372) was opsonized by 10% (v/v) rat autologous serum in 0.01 mol/L of phosphate buffer (pH 7.2), containing 0.15 mol/L of sodium chloride (NaCl) in PBS for 30 min at 37°C with orbital shaking at 150 rpm. Viability was measured by the 0.05% methylene blue exclusion test (99.9%), and the number of *C. albicans* was quantified in the Neubauer chamber (Souza Ferreira et al., 2012; Souza Ferreira et al., 2016).

### Phagocytosis and candidacidal activity

A number of 1 x $10^6$ neutrophils were incubated at 37°C with opsonized *C. albicans* (1 x $10^7$) at a 1:10 ratio at 37°C in 1 mL of RPMI medium in a rotatory system (10 rpm). After 30, 60, 90 and 120 min of incubation, an aliquot of 50 µL of this suspension were adhered to cover slip

glass by cytocentrifugation (Sorocito FANEM). After centrifugation, cover slips were stained with Wright's and May–Giemsa stains. Neutrophils with one or more attached yeasts were scored as phagocytic cells. The percentage of phagocytic cells and the number of yeast cells attached per 100 randomly chosen neutrophils were counted (Souza Ferreira et al., 2012). For candidacidal activity determination, cell viability of attached cells was evaluated using the dye exclusion (Rosenfeld) test (Souza et al., 2001). The experiments were performed in triplicates.

### Statistical analysis

The results were expressed as the mean ± standard deviation (SD). The obtained data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test of multiple comparisons and the level of significance was set to $p < 0.05$.

## RESULTS AND DISCUSSION

Coffee has in its composition an infinity of chemical substances such as chlorogenic and caffeic acid, lactones, diterpenes, including cafestol and kahweol, niacin, trigonelline, caffeine, among others, all belonging to different classes and, therefore, with relevant pharmacological (Cano-Marquina et al., 2013; Romualdo et al., 2019). The profiles of the bioactive compounds evaluated in this work are shown in Table 1. The detected caffeine content was similar to the average levels found in beverages prepared from Brazilian Arabica beans using the filter method (Camargo et al., 1999; Jeszka-Skowron et al., 2020). Caffeine is thermally stable, unlike trigonelline and CGA, which are thermosensitive, so the processes of roasting, extraction and drying should have less influence on the final content of caffeine in the product (Kwak et al., 2017; Marcucci et al., 2013). However, roasting causes significant losses of trigonelline and, consequently, reduces its antioxidant potential (Kwak et al., 2017). The concentration of this compound in the beverages varies according to the type of coffee (Yoshinari et al., 2013).

**Table 1.** Determination of caffeine, total phenols and total chlorogenic acids levels present in the integral coffee drink. The results represent the mean ± standard deviation (n = 5).

| Bioactive compounds | Concentration |
|---|---|
| Caffeine (mg/dL) | 0.724±0.010 |
| Total phenol (mg gallic acid eq/mL) | 6.640±0.040 |
| Total CGA (mg/mL) | 1.335±0.017 |

CGA: chlorogenic acids.

Coffee is a major source of chlorogenic acid in the human diet; daily intake in coffee drinkers is 0.5–1.0 g; coffee abstainers will usually ingest < 100 mg for day. Several *in vitro* studies have suggested the potential beneficial effects of chlorogenic acid on human health, such as antioxidant, antidiabetic, anticarcinogenic, cardioprotective, antibacterial effects, etc (Ludwig et al., 2014b; López-Froilán et al., 2016; Tomac et al., 2020). The dose used in this study (7.2 mL/kg/day) is equivalent to a daily human consumption of 8 cups of 50 mL (400 mL) of boiled and filtered coffee. Thus, the coffee drink administered to the animals contains about 534 mg/day of CGA (Table 1).

To verify the installation of diabetes mellitus, the blood glucose level was determined seven days after the induction of diabetes mellitus and during the treatment of the animals, the glucose level was monitored weekly (values ≥ 250 mg/dL). The glucose levels were also quantified at the time of euthanasia, and a significant difference (an increase of 300%) was observed between the non-diabetic animals and diabetic groups (Figure 1A). Because blood glucose indicates only a momentary state, we evaluated the glycation of proteins by measuring HbA1c. After 50 days of treatment, the HbA1c levels were significantly higher (an increase of 150%) in diabetic than non-diabetic animals, and this effect was independent of treatment with APO or coffee (Figure 1B).

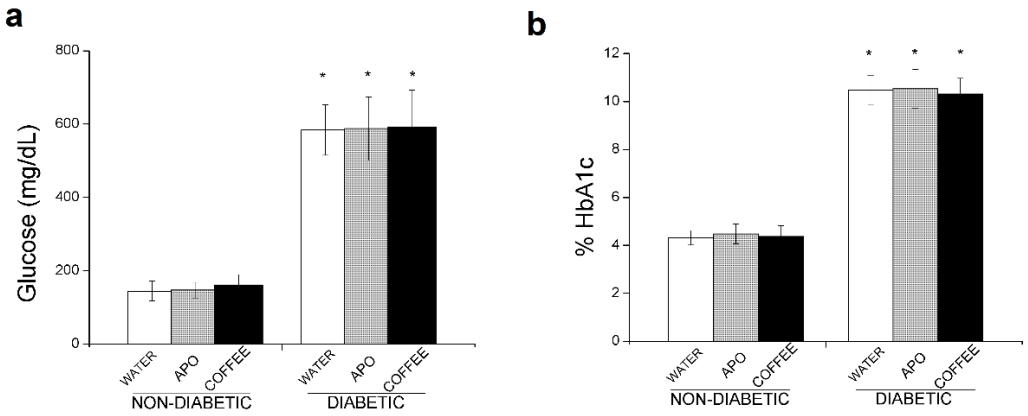


**Figure 1.** (**a)** Fasting blood glucose measurement (mg/dL) in the serum. (**b)** Blood HbA1c level measurement (%). Non-diabetics or diabetics corresponded to animals treated with: water, APO or coffee drink. The values are presented as means ± SD of the three experiments performed in triplicate. *$p < 0.001$ statistically significant difference compared diabetic with non-diabetic group (with the respective treatments). There was no statistical difference between treatments within each group, according to a one-way ANOVA followed by the Tukey test ($p < 0.05$). APO: apocynin.

The data regarding the influence of coffee on glycaemia in diabetic subjects are contradictory. Studies demonstrated a hyperglycemic acute effect, a decrease in insulin sensitivity and glucose tolerance, all related to caffeine action, but this effect can be modified when caffeine is consumed in a complex mixture, such as coffee. This is because other compounds of coffee can neutralize or antagonize the negative effect of caffeine on glucose metabolism, e.g., chlorogenic acid and trigonelline that have a hypoglycemic effect and responses to insulin (Di Girolamo et al., 2016; Jeszka-Skowron et al., 2020). Long-term caffeine intake promotes an increase in the secretion of the hormone adiponectin with insulin sensitizing properties. Coffee also stimulates the release of glucagon-like peptide 1 (GLP-1) and thus contributes to postprandial insulin secretion (Di Girolamo et al., 2016).

Components such as chlorogenic acid, melanoidins, quinides and N-methylpyridinium (PMN) can clearly affect glucose and insulin metabolism (Di Girolamo et al., 2016). An *in vitro* study demonstrated that cafestol stimulates insulin secretion and increases glucose uptake in muscle cells (Mellbye et al., 2015). *In vitro* tests also demonstrate that chlorogenic acid inhibits hepatic enzyme glucose-6-phosphatase which plays a critical role in glucose production in the liver (Van Dijk et al., 2009). A light roasting of the coffee beans provides a high concentration of CGA and trigonelline, which contribute to greater insulin sensitivity and secretion and better glucose uptake. While a more intense roasting process reduces the content of these components, changes their antioxidant activity and impacts on physiological effects (Di Girolamo et al., 2016).

Studies on the effect of apocynin and the NOX2 system on glycaemia are scarce and focus on the complications of hyperglycemia. Here, treatment of the animals with apocynin did not affect glucose and HBA1c levels (Figure 1), and this result is consistent with the descriptions of this effect in the literature (Thallas-Bonke et al., 2008). We evaluated the production of superoxide anion, which reflects the activity of NOX2 through the reduction of cytochrome C. Figure 2A shows no difference in the generation of superoxide anion among non-diabetic and diabetic animals. (Inoguchi et al., 1992) demonstrated that the production of ROS in cultured endothelial cells exposed to high glucose levels is similar to that of cells in response to stimulation by PMA, suggesting a greater translocation of PKC to the membrane and, thereby, providing an increased sensitivity to stimulation by PKC in neutrophils of diabetic patients. In this study, PMA was added to both the diabetic and non-diabetic groups.

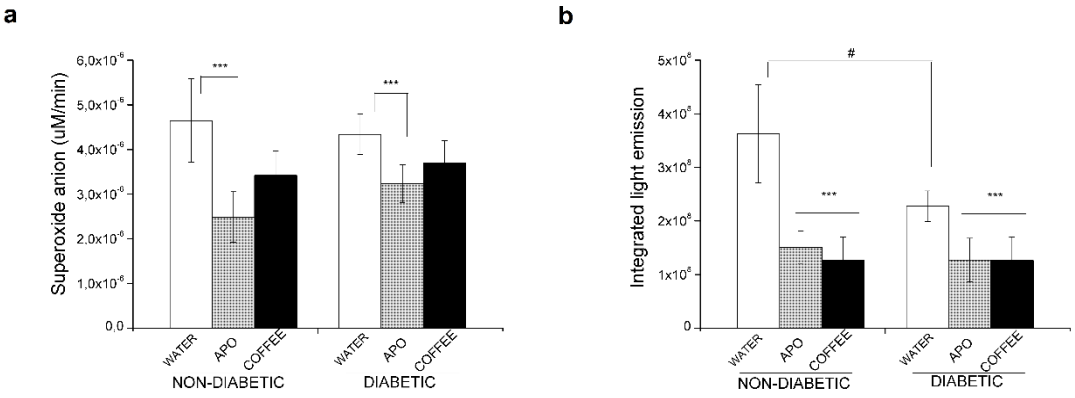


**Figure 2.** Measurement of reactive oxygen species (ROS). (**a)** Superoxide anions on the peritoneal neutrophils (1x10$^6$ cells) of rats stimulated with PMA (53 ng.mL$^{-1}$). ***$p$ < 0.001 means that there was a statistical difference. (**b)** Measurement of the pool of ROS obtained for the oxidation of luminol promoted by peritoneal neutrophils (1x10$^6$ cells). $^{\#}p$ < 0.001 statistically significant difference compared diabetic with non-diabetic group. ***$p$ < 0.001 means that there was no statistical difference. Non-diabetics or diabetics corresponded to animals treated with: water, APO or coffee drink. The values are presented as means ± SD of the three experiments performed in triplicate. PMA: 12-myristate 13-acetate phorbol, APO: apocynin.

Apocynin, as expected, significantly inhibited the production of superoxide anion in both groups and, consequently, reduced oxidative stress (Wang et al., 2019), but the inhibitory effect was more pronounced in the non-diabetic group. Apocinin is a potent NOX inhibitor that acts by inhibiting the translocation of p47phox to the cell membrane and prevents system assembly, and has been widely used as a non-toxic NOX2 inhibitor (Qiu et al., 2016). NOX2 inhibition can preserve pancreatic islets due to reduced ROS production and thereby regulate insulin secretion (Ding et al., 2019). The apocynin inhibition mechanism starts with its activation by hydrogen peroxide ($H_2O_2$) and myeloperoxidase (MPO) to form the diapocinin dimer, which oxidizes the NOX2 thiols and blocks the translocation of p47phox to the membranes and consequently the assembly of this enzyme (Pintard et al., 2020). Some studies have shown that the apocynin dimer is more efficient than apocynin itself (Almeida et al., 2012) and others suggest that only the dimer is able to block the activity of NOX2 (Van den Worm et al., 2001). Our group previously demonstrated that diabetic animals have lower MPO activity than non-diabetic animals (Souza Ferreira et al., 2012) which could explain the less pronounced effect of apocynin in the diabetic group.

The coffee did not affect the production of superoxide anion, independent of the diabetic state (Figure 2A). The superoxide anion originates from other species, e.g., $H_2O_2$, hydroxyl radical (OH$^{\bullet}$), hypochlorous acid (HOCl) and nitric oxide (NO) (Pintard et al., 2020). We also assessed the extracellular ROS production by chemiluminescence amplified by luminol. As observed in Figure 2B, the hyperglycemia decreased the pool of ROS in neutrophils. The myeloperoxidase catalyzes the reaction involving reactive species, such as hydrogen peroxide and chloride ion, generating HOCl. These species are involved in microbial killing (Galijasevic, 2019; Winterbourn and Kettle, 2013). When evaluating the ROS pool, our measurements include HOCl produced by MPO-dependent activity. We reported that neutrophil MPO activity is decreased in diabetic rats (Souza Ferreira et al., 2012). The reduced production of ROS observed in Figure 2 may reflect this decrease of HOCl, because of the low activity of MPO (Figure 2B).

Apocynin and coffee both inhibited neutrophil ROS production. The inhibitory effect of apocynin on ROS generation can be is well described. As for coffee, the studies are often in conflict. Numerous studies describe the antioxidant properties of coffee, while others show the opposite effect (AlAmri et al., 2020; Chu et al., 2011; Kwak et al., 2017; Tomac et al., 2020;

Wu et al., 2011). One of the most widely studied and proven properties of coffee is its antioxidant activity related to natural phenolic compounds such as chlorogenic acid, caffeine and melanoidins, which contributes to the improvement of the pathophysiological characteristics of type 2 diabetes mellitus (T2D) (Butt & Sultan, 2011; Chu et al., 2011). However, little is known regarding its anti-oxidative mechanism in the modulation of key cellular processes. (Chu et al., 2011) demonstrated that coffees (regular and decaffeinated) had high cellular antioxidant activity due to the availability of these compounds to the cells, which resulted in the protection of cellular components and consequently in the neutralization of cellular ROS. In present study, we observed that coffee reduced ROS production. Related to this point, the increase in expression and activity of the antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) in the livers of rats was recently described (Vicente et al., 2014). Neutrophils also express these enzymes, which could contribute to the reduction of ROS observed in the group treated with coffee. Furthermore, it has been described that caffeic acid reduced the activity of the GTPase protein Rac1 accompanied by a decrease in NOX2 regulation (Xu et al., 2005).

During an infectious process, neutrophils induce the destruction of the infectious agent by activating NADPH oxidase NOX2 which results in the generation of ROS (Pintard et al., 2020). Thus, the phagocytic and killing activities of peritoneal neutrophils toward *C. albicans* were evaluated in this study.

Phagocytosis and *C. albicans* death by peritoneal neutrophils from diabetic animals were significantly compared to those of neutrophils from non-diabetic animals (Figure 3 and Figure 4), as described in our previous work (Souza Ferreira et al., 2016). Chronic hyperglycemia can decrease chemotactic migration of neutrophils, diapedesis, phagocytosis and bactericidal action of neutrophils, can also reduce the mobilization and phagocytic activity of polymorphonuclear leukocytes, in addition to preventing vasodilation resulting in less blood flow at the site of infection. These data demonstrate that the hyperglycemic state adversely affects the phagocytic and killing activities of peritoneal neutrophils exposed to *C. albicans*. It is known that the addition of ROS and proteins in the phagosome, such as lysozyme, neutral and acid proteases, phospholipases and MPO, also play an important role in the killing of pathogens. It has been shown that animals that are deficient in cathepsin G and elastase but produce superoxide anion normally are less resistant to infection by *Staphylococcus aureus* and *C. albicans* (Thévenod, 2008).

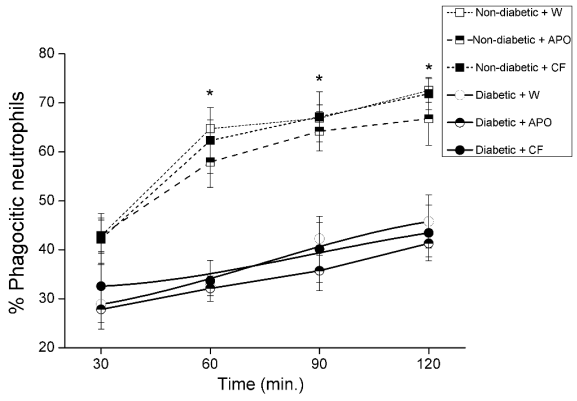


**Figure 3.** Evaluation of the effect of the coffee beverage and APO on the ability of neutrophils to phagocytize *Candida albicans* (% phagocytic neutrophils). Neutrophils were incubated with opsonized yeast *C. albicans* for 30, 60, 90 and 120 min. Non-diabetics or diabetics corresponded to animals treated with: water, APO or coffee drink. The values are presented as means ± SD of the three experiments performed in triplicate. **p* < 0.001 statistically significant difference compared diabetic with non-diabetic group (with the respective treatments). There was no statistical difference between treatments within each group, according to a one-way ANOVA followed by the Tukey test (*p* < 0.05). W: water; APO: apocynin; CF: coffee drink.

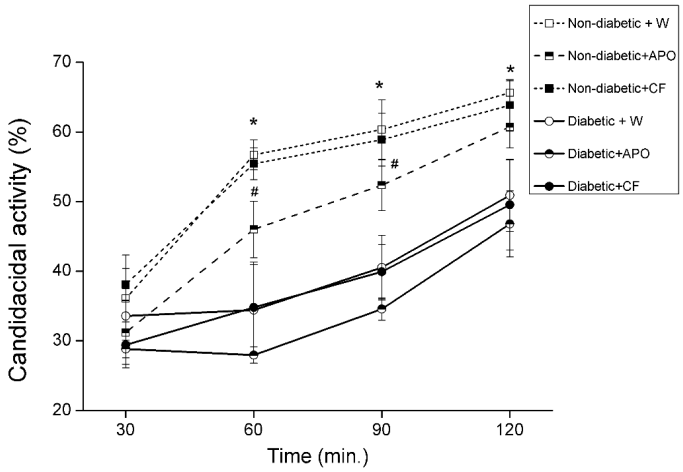


**Figure 4.** Evaluation of the effect of coffee beverage and of the APO on the killing of *Candida albicans* (candidacidal activity). Neutrophils were incubated with opsonized yeast *C. albicans* for 30, 60, 90 and 120 min. Non-diabetic or diabetic treated with: water, APO or coffee drink. Non-diabetics or diabetics corresponded to animals treated with: water, APO or coffee drink. *$p < 0.001$ statistically significant difference compared diabetic with non-diabetic group (with the respective treatments). #$p < 0.05$ statistically significant difference compared non-diabetic + APO with non-diabetic + CF and diabetic + W. W: water, APO: apocynin, CF: coffee drink.

Despite the reduced generation of ROS by diabetic and non-diabetic groups, the coffee drink did not affect the neutrophils capacity of phagocytosis or death in our model (Figure 3 and Figure 4). Information related to the effects of coffee on the body's defense mechanism against pathogens is scarce. Experimental evidence indicates that phenolic compounds can regulate cellular processes that lead to the inflammatory response and can increase defense or not cause any type of effect (AlAmri et al., 2020; Chu et al., 2011). This controversy indicates that the response to the activity of these defenses depends on the etiology of oxidative stress, the experimental protocol, the antioxidant defense in question and the type and source of dietary antioxidant. Many of the physiological benefits of coffee have been attributed to multiple and important functions related to the ability of phenolic compounds to bind to cell receptors and membrane transporters and influence gene expression. The signaling and cell adhesion mechanisms triggered by these compounds may exert a direct action (eliminating ROS) or indirect (increasing the activity or expression of antioxidant enzymes with activation of immune defense mechanisms) (Costabile et al., 2018; Vicente et al., 2014).

In this study, apocynin was used as a standard to evaluate its action on the microbicidal ability of neutrophils and its effect was compared with the possible antioxidant activity of coffee. As observed in Figure 2B, apocynin was effective in inhibiting the production of superoxide anion and, consequently, the ROS pool. As seen in Figure 3, apocynin did not interfere with the phagocytic ability of these cells toward *C. albicans*. However, a small decrease in neutrophil microbicidal capacity of animals treated with apocynin occurred, at 60 and 90 minutes, most likely due to the decreased ROS produced by these cells (Figure 4). (Stolk et al., 1994) reported that apocynin did not interfere with the polymorphonuclear defense because it did not affect phagocytosis or intracellular killing of *Staphylococcus aureus*.

In this study, we observed that apocynin inhibited the microbicidal ability of neutrophils only in the non-diabetic group (Figure 4). As stated earlier, diabetic animals had lower MPO activity, which compromises apocynin dimer formation and its activity (Souza Ferreira et al., 2012). These data support other studies which have demonstrated the importance of MPO to the activity of apocynin (Bedard and Krause, 2007; Almeida et al., 2012). It is worth noting that coffee contains many bioactive components, including chlorogenic acid, caffeine, lignin, isoflavonoids and caffeic acid which are cited as the main compounds responsible for its antioxidative action (Akash et al., 2014). The levels of these components may be high or low, depending on the species of coffee and the type of treatment (roasting process, procedure

for extraction and other variables), which modifies the physicochemical properties of the coffee and, consequently, the effects that were described in other studies.

## CONCLUSIONS

Our data showed that intake of coffee beverage did not affect the glycemic profile of non-diabetic or diabetic animals. However, coffee reduced the generation of ROS in non-diabetic and diabetic animals and did not affect neutrophil phagocytosis or killing against *C. albicans*. Treatment with apocynin decreased the production of superoxide anion by NOX2 in neutrophils from non-diabetic and diabetic rats. In addition, the apocynin decreased the killing capacity of neutrophils from non-diabetic animals against *C. albicans*, emphasizing the importance of NOX2 system for this neutrophil activity. We suggest that coffee drinking prevents oxidative damage regardless of the pathway NOX2. Finally, it is important to note that this study showed that coffee beverage consumption does not affect the response of the organism against opportunistic pathogens such as *Candida albicans*.

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## Authors' contributions

ASDS, CSF and TFOL performed the experiments, collected and analyzed the data; RGFAP, SMSD and MRR designed the study and performed the data evaluation; EBF statistical analysis; ARL assisted in the analysis of coffee; FBAP and THA performed the data evaluation; ASDS and MRR wrote the paper; SAF analysis and evaluation of data and wrote the paper.

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