Caryocar brasiliense oil improves cardiac function by increasing Serca2a/PLB ratio despite no significant changes in cardiovascular risk factors in rats

Lidiane Guedes Oliveira¹, Lauane Gomes Moreno¹, Dirceu Sousa Melo¹, Liliane Vanessa Costa-Pereira¹, Mayara Medeiros de Freitas Carvalho², Paulo Henrique Evangelista Silva¹, Ana Maria Alves¹, Flávio de Castro Magalhães¹, Marco Fabrício Dias-Peixoto¹ and Elizabethe Adriana Esteves¹*

Abstract

Background: Caryocar brasiliense (pequi) oil is high in monounsaturated fat acids (MUFA), especially oleic, and in carotenoids, which have been associated with protection against cardiovascular disease. However, this food is poorly studied in this context, especially in the cardiac function. Therefore, we investigated the effects of a long-term intake of pequi oil in systemic cardiovascular risk factors and in the ex vivo cardiac function of rats.

Methods: Previously, we determined fatty acids and carotenoids in pequi oil. Next, male rats were divided in C – control group feed a standard diet, and PO – pequi oil group fed the same diet added pequi oil (+2.25 g.100 g⁻¹). After 15 weeks, plasma lipids, glucose, insulin, blood pressure, heart rate, hepatic lipids were accessed and visceral fat pads were harvested. Hearts were used for the ex vivo cardiac function, histologic assays, SERCA2a and phospholanban (PLB) determinations.

Results: In agreement with scientific data, pequi oil had expressive amounts MUFA, especially oleic acid, and carotenoids. Hepatic triglycerides (TG) were reduced by pequi oil intake (p < 0.05). All others cardiovascular risk factors were not changed. The intrinsic heart rate was lower in PO group (p < 0.05). SERCA2a content was higher in this group (p < 0.05), without affecting PLB. Also, SERCA2a/PLB ratio increased in PO group (p < 0.05).

Conclusion: Pequi oil intake improved cardiac function ex vivo, despite no significant changes in systemic cardiovascular risk factors. The higher lipid offer in pequi oil diet, its composition in oleic acid and carotenoids could be related to those effects.

Keywords: Caryocar brasiliense, Monounsaturated fatty acids, Oleic acid, Carotenoids, Cardiac function, Cardiovascular disease

Background

Cardiovascular diseases (CVD) are responsible for 30% of all deaths worldwide each year [1] being the high intake of lipids one of the major modifiable risk factor in the etiology of these diseases [2]. Therefore, it has been indicated the amount and quality of dietary lipid as a main guideline target for reducing mortality and morbidity by those diseases [2, 3]. In this context, scientific interest has been particularly directed to the main fatty acids found in foods: trans, saturated (SFA), polyunsaturated (PUFA) and specially of our interest, monounsaturated (MUFA) fatty acids [4].

Indeed, in foods, some vegetable oils have instigated interest because their content of MUFA, and, or, PUFA, which have been associated to health benefits [5]. In this perspective, pequi oil has a potential for reducing
cardiovascular risk since it is high in MUFA, beyond others bioactive compounds that also have been associated to cardiovascular protection.

This oil is extracted from the Caryocar brasiliense fruit (pequi) and its major fatty acid is oleic (54%), the main MUFA in the diet [6]. Scientific evidences suggest that MUFA are associated to coronary heart disease prevention [7], by favorably improving blood lipids [8], reducing blood pressure [9], and modulating insulin sensitivity and glycemic control [10]. Oleic acid is pointed out as the main responsible for those effects. In addition, MUFA has also been associated with improvements in cardiac function, since they are main components of cardiomyocyte phospholipid membranes, ameliorated endothelial function and reduced both apoptosis of vessel smooth cells and cardiomyocytes [11, 12].

This oil also has high content of palmitic acid (35%), a common SFA in animal foods [13]. SFA are more easily oxidized by cardiomyocytes and they were associated to improvements in systolic cardiac function [14]. Moreover, fatty acids in general, are cardiac important sources of energy, since almost 50-70% of ATP used by those cells, is derived from acetyl coenzyme A (Acetil CoA), the product of the fatty acids β-oxidation [15].

*Pequi* oil also has a substantial amount of carotenoids (8,10 mg.100−1 g), especially violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, neoxanthin and β-carotene [16, 17]. These compounds also have been related to cardiovascular risk reduction. They are potent antioxidants in biological systems and protect against oxidative damage [18]. Also, Csepanyi et al. [19] demonstrated that, in lower doses, these bioactive compounds improved cardiac function in rat isolated hearts, by langendorff system.

Therefore, *pequi* oil is a potential cardioprotector food. It could favorably modulate cardiac function, and improve systemic cardiovascular risk factors. However, although there are many evidences from its chemical components related to cardiovascular health, this food has been poorly studied in this context. To our knowledge, there are some research showing healing [20], chemopreventive [21, 22], anti-mutagenic [23], antioxidant [24], anti-inflammatory, antihypertensive [25] and anti-cancer [26, 27] properties of *pequi* oil in humans or animal models. In addition, most of the information has not been obtained from its intake. In addition, so far, there is no research regarding the effects of this oil directly in cardiac function.

Therefore, the aim of this study was to evaluate the effects of a long term *pequi* oil intake in systemic cardiovascular risk factors and in the ex vivo cardiac function of rats. We also explored the involvement of key proteins that modulate cardiac contractility and relaxation.

**Methods**

**Preliminary analysis of pequi oil**

*Pequi* oil was purchased from the local market in Diamantina-MG, Brazil. Previously to the rat study, fatty acids were determined by gas chromatography (GC Agilent 6850 Series GC System) according to the AOCS Ce 1−62 method [28]. Total amount of carotenoids was determined according the AOAC Official Methods of Analysis [29], using a spectrophotometer (Spectrod 210, model Analytikjena), at 450 nm.

**Bioassay design**

Sixteen male *Wistar* rats, 25 days old, were housed in individual stainless steel cages and kept in a room at 22 ± 2 °C and at a 12 h light/dark cycle, with free access to food and water during all experimental period (15 weeks). In the first day, all animals were randomly assigned into two treatments: C-control, fed commercial chow (*n* = 8) (Rhoster-Lab®, energy density: 328.06 Kcal.100 g−1) or PO- *pequi* oil, fed commercial chow added *pequi* oil (*n* = 8). *Pequi* oil was added to increase by 50% the lipid chow content (+2.25 g.100 g−1), which also increased its energy density up to 348.31 Kcal.100 g−1.

During the experiment, body weight and food intake were monitored for Feed Efficiency (FER(g/g) = body gain/food intake) and Energy Efficiency (EER (g/Kcal) = body gain/energy intake) ratios [30]. In the last day, overnight fasted animals were anesthetized (quetamin + xilazin/50 mg/kg + 10 mg/kg), and their nose-anus length were measured for Lee Index (LI) calculation (LI = [3body weight (g) + nose = anus length(cm)] ×10) [30].

After that, all animals were euthanized by decapitation for blood, livers, hearts, and tissue harvesting. All retroperitoneal and epididimal fat pads were removed and weighted in an analytical scale (Shimadzu AX 200) for the Adiposity Index calculation (AdI% = (epididimal pad + retroperitoneal pad)/body weight – (Σepidimal pad + retroperitoneal pad) *100) [31]. Blood was centrifuged in heparinized tubes to obtain plasma, which were aliquoted in eppendorf tubes and kept at -80°C until analysis.

**Cardiovascular risk factors assays**

Fasted plasma glucose levels (GLU) were measured by a commercial kit, according the procedures recommended by the manufacturer and using a semi-automatic biochemical analyzer (PIOWAY-3000). Fasted plasma insulin (INS) was determined using a commercially available Enzyme-Linked Immunosorbent Assay kit – ELISA (Linco Research Inc., St. Louis, MO, USA) and a microplate reader (Spectra MAX 190, Molecular Devices, USA). Insulin resistance was accessed by the homeostasis model assessment of insulin resistance (HOMA-IR index), from fasted glucose and insulin levels according to Matthews et al. [32].
Total plasma cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C), Low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) levels were determined using commercial kits according to the specifications of the manufacturer and a semi-automatic biochemical analyzer (PIOWAY-3000). Liver samples were oven-dried (60 °C ± 2 °C for 72 h), and their lipids were extracted according to Folch et al. [33]. CHOL and TG levels were determined using commercial kits, according to specifications of the manufacturer, and using a semi-automatic biochemical analyzer (PIOWAY-3000).

Systolic blood pressure (BP), as well as heart rate (HR), were measured at the last week prior to the end of the experiment by the tail-cuff plethysmography method (MLT1020PPG IR Plethysmograph, PowerLab). Additionally, the double product index was calculated using systolic blood pressure and heart rate values, as an indicator of cardiac work [34].

**Ex vivo analysis and Langendorff preparation**

In the last day of the experiment, animals were anesthetized (quetamin + xilazin/50 mg/kg + 10 mg/kg) and decapitated 10–15 min after a 400 IU intraperitoneal heparin injection. Hearts were perfused in a Langendorff apparatus (ML785B2, ADInstruments) and left ventricular pressure (± dP/dt) was continuously recorded according to the Langendorff technique [35], using the Labchart 8 software. Systolic tension, diastolic tension, coronary flow, heart rate, and ± dT/dt values were the average of the recorded 30 min. All the ± dP/dt measurements were normalized to heart weight.

**Heart/body weight ratio and histologic analysis**

At the end of the cardiac function analysis, wet heart weights were recorded, normalized for the body weight, and expressed as muscle mass index (mgg⁻¹), according to Almeida et al. [36].

For cardiomyocyte diameter, hearts were fixed in 4% Bouin fixative solution, embedded in paraffin, and sectioned to 4 µm thickness. To determine myocyte cross-sectional area, heart sections were stained with hematoxylin and eosin and examined at 40× magnification. Only myocytes longitudinally cut with the nucleus centrally located in the cell and with cellular limits visible were used. The cross-sectional diameter (um) of the myocytes was traced using ImageJ software (National Institutes of Health), and determined by averaging 50 to 100 individual cardiomyocytes within the ventricular free wall over 5 or 6 sections per animal. A single investigator blinded to the experimental groups performed the analysis.

**Western blotting**

Total protein content of left cardiac ventricles was quantified by means the Bradford protein assay [37]. Protein (80 µg) was loaded onto a 10% polyacrylamide gel for electrophoresis. After electrophoresis, proteins were transferred to a PVDF membrane, blocked with a phosphate-buffered saline, containing 0.1% Tween 20 and 5% bovine serum albumin. Membranes were incubated overnight at 4 °C with the following primary antibodies: monoclonal sarcoplasmic reticulum Ca²⁺-ATPase isoform 2 (SERCA2a) (1:1000 dilution; Cell Signaling); monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000 dilution; Cell Signaling); and phospholamban (PLB) (1:6000 dilution; Cell Signaling). Thereafter, a monoclonal anti-rabbit or anti-mouse secondary antibody conjugated with peroxidase (1:4000 dilution, Cell Signaling) were used. Immunodetection was carried out using enhanced chemiluminescence (Amersham Biosciences), and protein levels were expressed as a ratio of optical densities.

**Statistics**

The statistical analyses were carried out using the Statistics 10.0 software. The experiment was carried out in a completely randomized design. All data obtained from the experiment are expressed as mean ± standard error. Statistical differences were evaluated by using one-way ANOVA. P-values less than 0.05 were considered statistically significant.

**Results**

**Pequi** oil had expressive amounts unsaturated fatty acids, especially oleic acid, a monounsaturated fatty acid (MUFA), followed by the linoleic, a polyunsaturated fatty acid (PUFA). Among saturated fatty acids (SFA), palmitic acid was the highest (Table 1). Pequi oil also presented 32.18 ± 8 mg/g of total carotenoids.

In the rat study, at first, pequi oil intake did not affect body weight and food intake, as well as FER and EER (Table 2).

We also found that hepatic triglycerides (TG) were reduced by pequi oil intake. All others cardiovascular risk factors were not changed (Table 3).

To address if the treatment with PO could directly alter cardiac function, we performed Langendorf analysis (Fig. 1). Rats showed increased basal cardiac function as evidenced by increased contractility (+dP/dt) [PO: 1640.7 ± 167.7 mmHg/s-1/g-1, C: 1366.9 ± 420.4 mmHg/s-1/g-1, Fig. 1a] and relaxation (−dP/dt) [PO: 878.5 ± 128.8 mmHg/s-1/g-1, C: 639.5 ± 271.3 mmHg/s-1/g-1, Fig. 1b] indexes. Furthermore, the intrinsic heart rate (PO: 198.3 ± 36.8 bpm, C: 244.3 ± 64.4 bpm) was lowered by pequi oil intake (Fig. 1c).
Table 1 Fatty acids profile of pequi oil (g.100 g\(^{-1}\))

| Fatty acid      | Carbon num. | Mean ± SD  |
|-----------------|-------------|------------|
| Lauric          | C12:0       | 0.04 ± 0.01|
| Myristic        | C14:0       | 0.10 ± 0.01|
| Palmitic        | C16:0       | 37.05 ± 0.04|
| Stearic         | C18:0       | 2.12 ± 0.01|
| Arachidonotic   | C20:0       | 0.20 ± 0.01|
| Behenic         | C22:0       | 0.07 ± 0.01|
| Lignoceric      | C24:0       | 0.09 ± 0.01|
| Palmitoleic     | C16:1       | 0.82 ± 0.01|
| Oleic           | C18:1       | 57.42 ± 0.03|
| Linoleic        | C18:2       | 1.38 ± 0.01|
| α-Linolenic     | C18:3       | 0.32 ± 0.01|
| Eicosenic       | C20:1       | 0.25 ± 0.01|
| Total saturated | —           | 39.73 ± 0.03|
| Total unsaturated| —          | 60.27 ± 0.03|

Since cardiac function was ameliorated by pequi oil, we investigated the involvement of key proteins with cardiac function modulation. We observed an increase in SERCA2a content in PO group (Fig. 2a). The same was not observed for PLB content (Fig. 2b). In addition, the SERCA2a/PLB ratio was also higher in PO group (Fig. 2c).

Discussion

Pequi oil, accordingly to data available elsewhere [6, 38, 39], had oleic acid as its major fatty acid, being higher than olive oil, the main dietary source of it [40]. Otherwise, the second major fatty acid was palmitic, an important dietary SFA [41]. High carotenoid content was also observed. According to Rodriguez-Amaya et al. [42], to be a carotenoid source, a food must have more than 20 μg/g, which is associated to health benefits. We found 10× more carotenoids in pequi oil, so it could be an excellent carotenoid food source.

Thus, for the rat study, we decided to add pequi oil in the chow turning its lipid content 50% higher, which added oleic acid by 1.29 g.100\(^{-1}\) and carotenoids by 7.2 μg.g\(^{-1}\). Also, it increased palmitic acid by 0.83 g.100\(^{-1}\).

- According to Hariri and Thibault [9], it is necessary to increase lipid content of an experimental diet up to 30% of its total energy to induce metabolic disturbance in rodents. Adding pequi oil to the chow provided 18.51% of lipid energy.

Overall, our data indicated that long term pequi oil intake was able improve the ex vivo cardiac function, by increasing cardiac relaxation and contractility. We also inferred that this effect occurred independently of changes in systemic cardiovascular risk factor, since just hepatic TG was changed by pequi oil.

Indeed, the slightly higher lipid availability from pequi oil could have contributed to these results. To achieve a better cardiac function, it is necessary a correct oxygen supply and energy provision to meet the myocardium demands. Heart is known by its ability to produce energy from fatty acids because it is more capable to perform beta-oxidation, since it has high amounts and activity of enzymes related to. Heart ATP storage is limited and it can assure just a few seconds of beating. Because of that, cardiac muscle can adapt quickly to the energy demand and increases up to 100% its energy production from fatty acids, when there is higher availability of those nutrients [43].

Otherwise, the nature of the fatty acids in this oil may also have accounted to these changes. Palmitic acid, the second higher fatty acid in pequi oil, is oxidized rather than other fatty acids in heart [44]. Oleic acid, the major fatty acid in this oil, is able to up-regulate the

| Variables         | C        | PO       |
|-------------------|----------|----------|
| Body weight (g)   | 286.4 ± 55.9 | 277.9 ± 35.0 |
| Food intake (g)   | 2209.5 ± 237.0 | 2406.3 ± 230.7 |
| Energy intake (Kcal) | 7247.3 ± 895.5 | 8373.8 ± 802.9 |
| Feed efficiency ratio (g.g\(^{-1}\)) | 0.10 ± 0.02 | 0.09 ± 0.01 |
| Energy efficiency ratio (g. Kcal\(^{-1}\)) | 0.032 ± 0.006 | 0.027 ± 0.004 |

C chow, PO Chow added pequi oil; Values are expressed as mean ± standard error. * indicates statistical difference (p < 0.05) between means by One way-ANOVA
transcription of genes coding for proteins involved in cardiac fatty acid transport and metabolism. These changes correspond to a 60% increase in cardiomyocyte fatty acid oxidation capacity [45].

Also, *pequi* oil intake provided exogenous antioxidants, especially carotenoids, which also could have accounted for those effects. Csepanyi et al. [19] showed in a rat model that carotenoid intake improved heart function at lower reperfusion times. These authors assigned those effects to the antioxidant properties of these compounds.

Conversely, although hepatic TG reduction was a timely result, a previous study from our lab also showed a significant reduction of those parameters in rats fed *pequi* pulp providing a 50% increase of dietary oil [38]. In addition, despite this result could not be related directly to cardiac function, there is a great body of evidence connecting hepatic lipid accumulation to cardiovascular risk, independently of coexisting cardiometabolic risk factors [46].

MUFA may exert their beneficial effects on hepatic fat content through their influence on lipid metabolism in the liver or in the abdominal adipose tissue [47]. A high MUFA diet could avoid hepatic lipid accumulation by activating catabolic pathways. It may result in degradation of the insulin-induced gene-1 protein, and therefore, inactivation of the transcription factor sterol regulatory element binding protein which promotes, among some effects, fat oxidation [40]. Indeed, an increasing body of evidence indicates an increment in fat oxidation rate, specifically with higher dietary MUFA levels, in several tissues [48, 49]. More recently, Liu et al. [13] showed in mice that hepatic oleic acid, provided both by diet or endogenously, is pivotal to prevent or to solve hepatic stress and inflammation induced by lipogenic diets.

Based on the systemic findings, we could infer that the improved cardiac function in *pequi* oil group was a consequence of intrinsic cardiac adaptations. The reduced heart rate and the increased cardiac SERCA2/PLB ratio in *pequi* oil group were important changes that can explain that. It is well established that a decrease in heart rate increases the diastolic period, which favors improved contractility/relaxation efficiency. Nevertheless, we believe that the improved cardiac function in *pequi* oil group was a consequence of the increased SERCA2a/PLB ratio.

It is known that, in rats, 92% of Ca^{2+} fluxes during cardiac excitation-contraction is regulated by SERCA2a. It acts as a sarcoplasmic reticulum (SR) protein regulated by PLB, facilitating SR calcium storage [50]. During systole, the action potential induces calcium release from SR and, the higher calcium availability, the higher contractility. During diastole, SERCA2a increases calcium reuptake to SR increasing the efficiency of relaxation [51]. In STZ-induced diabetic rats, the increase of SERCa2a expression protected from diabetic cardiomyopathy [52].
PLB is an integral SR membrane protein, which regulates SERCA2a activity. An upregulation in SERCA2a-to-PLB ratio is an important indicative of increased SERCA2a activity [53]. Thus, the increased SERCA2a/PLB ratio in *pequi* oil animal hearts may be an important mechanism that explain the increased cardiac contractility/relaxation index.

Still, our study shows some limitations. As there is no information about human intake of *pequi* oil as well as a few ones about doses used previously in animal models (as a whole food), it is possible that the amount of *pequi* oil added has not been sufficient to promote clear effects, especially in systemic cardiometabolic risk factors. However, we chose to increase dietary lipids by 50% using *pequi* oil because we tried to associate some basic results gotten from pre-tests and in this way, the diet did not turned into a high fat [9]. Besides, our research group and others showed, previously, improvements in systemic cardiovascular risk factors in rats and humans fed *pequi* pulp or *pequi* oil pills providing 600 mg/d of *pequi* oil [23–25, 38, 39]. In this way, we would be offering at least, 600 mg/d of *pequi* oil. Also, we were unable to address the observed effects to MUFA, or carotenoids or both. However, at this point of investigation, we were more interested, in knowing if the whole food intake exerted any effect. In addition, a complete characterization of the mechanism and the reasons by which the long term intake of *pequi* oil led to lowering hepatic triglyceride deposition, bradycardia and increased SERCA2a/PLB ratio is beyond the scope of the present study and requires future investigations. Moreover, it is important to point out that this is the first paper showing cardiovascular effects of long-term *pequi* oil intake, especially on cardiac function.

Moreover, future research will include more profound molecular analysis not only on calcium transient but also on the cardiac redox state [54] in hearts of animals fed *pequi* oil. It also would be necessary to evaluate if cardioprotective effects of *pequi* oil happens to be in other situations, such as, in different doses of *pequi* oil that mimic human servings and, or, in pathological conditions (non-alcoholic fatty liver disease, obesity, insulin resistance), or at minimal cardiometabolic disturbances, since food bioactive compounds may not show clear effects in healthy conditions.

Conclusions

Taken together, our data indicates that *pequi* oil was able to improve rat ex vivo cardiac function, by increasing cardiac relaxation and contractility, despite no significant changes in systemic cardiovascular risk factors. The higher availability of lipids associated to the higher content of oleic and palmitic acids and carotenoids provided by *pequi* oil diet could be related, at least, in part to those findings.

Abbreviations

C: Control diet; CHOL: Total cholesterol; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GLU: Glucose; HDL-C: High-density lipoprotein; INS: Insulin; LDL-C: Low-density lipoprotein; MUFA: Monounsaturated fatty acids; PO: *Pequi* oil diet; SERCA2a: Sarcoplasmic reticulum Ca2+ ATPase; SFA: Saturated fatty acids; TG: Triglyceride

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Availability of data and materials

All data generated or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

Conceived and designed the experiments: EAE, MFDP, FCM. Performed the experiments: LGO, LGM, DSM, LVCP, MMFC, PHE, AMA. Analyzed the data: LGO, LGM, EAE, MFDP. Wrote the paper: LGO, LGM, EAE, MFDP. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

The study protocol was approved by the Ethic Committee on Animal Use/Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, MG, Brazil. Animal welfare and experimental procedures were performed strictly in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the US National Institutes of Health.

Author details

1 Programa Multicêntrico de Pós-Graduação em Ciências Fisiológicas, Sociedade Brasileira de Fisiologia (SBFis) – Universidade Federal dos Vales do Jequitinhonha e Mucuri – UFVJM, Rodovia MG 367 – Km 583, n° 5000, Alto da Jacuba, Diamantina, MG, Brazil. 2CEP: 39100-000. Programa de Pós-Graduação em Ciências Biológicas, Universidade Federal de Ouro Preto – Campus Universitário, Morro do Cruzeiro, Ouro Preto, MG, Brazil. CEP: 35400-000.

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