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Article

Impact of thyroid hormones and grape juice on biochemical markers and metabolic control proteins in experimental pulmonary arterial hypertension

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Background- Pulmonary arterial hypertension (PAH) increases afterload in the right ventricle (RV), inducing adverse ventricular remodelling. Objective- This study explored the protective effects of thyroid hormones and grape juice on serum biomarkers and proteins related to intermediary metabolism in the RV in a model of PAH. Methods- PAH was induced in Wistar rats via the administration of monocrotaline (60 mg/kg i.p.) and they were subsequently treated with organic grape juice (GJ) and thyroid hormones (TH), administered separately or in combination. Results: The RV systolic diameter significantly increased (20%) in the PAH group compared to the control group ($P = 0.002$). Total CK, LDH, and ALT levels were reduced (~50%) ($P<0.001$) in the PAH+TH+GJ group as compared to PAH group. Glucose, albumin, triglyceride, and total cholesterol levels were reduced (~50%) ($P<0.001$) in the PAH group; however, these parameters returned to baseline in the PAH+GJ group. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) protein expression increased two-fold ($P<0.001$) and PDH protein levels were reduced (~25%) in the PAH+TH+GJ group compared to the PAH group ($P<0.001$). Conclusion: The data suggest that serum biomarkers can help in the evaluation of this disease, and that this therapeutic approach can attenuate maladaptive remodelling in the PAH model.

Keywords: PGC-1-alpha, Pyruvate dehydrogenase, Monocrotaline, Food intake.

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a cardiopulmonary disease related to increased vascular resistance due to adverse remodelling of the lung vasculature (Huber, Bye, Brock, 2015). This condition increases the afterload of the right ventricle (RV) and provokes ventricular hypertrophy and heart failure, a condition known as *Cor pulmonale* (Inampudi *et al.*, 2020).

In PAH, there is an important change in the metabolic pathways used in cardiac tissue called the Walburg effect. This features a shift from pyruvate oxidation in the mitochondria to glycolysis metabolism, including the modulation of pyruvate dehydrogenase (PDH) activity and reduction of mitochondrial biogenesis (Paulin, Michelakis, 2014). PDH is an enzyme complex responsible for converting pyruvate to acetyl CoA, promoting oxidative metabolism, and acting as a metabolic switch (Bertero, Maack, 2018). In this context, peroxisome proliferator-activated receptor gamma coactivator (PGC-1-alpha) plays a fundamental role because it stimulates mitochondrial biogenesis and adapts to the cellular environment for aerobic metabolism (Liang and Ward, 2006). Although

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there is a large number of therapies available to improve vascular function in PAH, pharmacotherapies that target right ventricular metabolism still require exploration.

The anti-inflammatory and antioxidant potential of grape and its derivatives has already been well established in *in vitro* and *in vivo* studies (Rasines-Perea and Teissedre, 2017). Bernal-Ramirez *et al.* (2021) demonstrated that the administration of resveratrol protected against the disruption of mitochondrial integrity in PAH-provoked RV remodelling. Another cardioprotective strategy is the administration of thyroid hormone (TH). There is a relationship between cardiac diseases and reduced TH levels, as well as diminished expression of thyroid hormone receptors in the myocardium. TH administration has a positive role for the cardiovascular system, modulating energy metabolism, increasing ventricular contractility, and reducing peripheral vascular resistance (Jankauskas *et al.*, 2021)

The detection of PAH-induced early negative signs would facilitate the adaptation of therapeutic interventions and achieve a better prognosis in the disease. The analysis of blood markers used in medical practice may be key in this context. Therefore, this study evaluates the impact of treatment with grape juice and TH on plasma markers of cardiac, hepatic, and renal function, lipid metabolism, as well as on the metabolic control proteins PGC1-α and PDH in monocrotaline-induced PAH.

MATERIAL AND METHODS

Animals

This study was conducted in accordance with the Ethical Principles in Animal Experimentation formulated by the Brazilian College of Animal Experimentation, as well as those contained in the International Guiding Principles for Biomedical Research Involving Animals from the Council for International Organizations of Medical Science (CIOMS). This study was approved by the Ethics Committee on the Use of Animals of the Federal University of Rio Grande do Sul (UFRGS), under protocol number 37372. Forty-six male Wistar rats (200 ± 20 g) from the Center for Reproduction and Experimentation of Laboratory Animals were kept under standard vivarium conditions of a controlled temperature (21 °C); 12-hour light-dark cycle; and 70% relative humidity. Water and commercial food were offered *ad libitum*. Overall mortality from the experimental protocol was approximately 23%. However, in the PAH group which received T3 and T4, this value was 30%.

Drug administration and experimental group

PAH was induced via a single intraperitoneal administration of monocrotaline (MCT) (60 mg/kg dose) (Singal *et al.*, 2000). TH treatment was administered by T3 (2 μg/100 g/day) and T4 (8 μg/100 g/day) gavage for 14 days (this treatment does not induce hyperthyroidism) (De Castro *et al.*, 2014). Red grape juice (GJ) was administered by gavage (7 μL/g of body weight) for 14 days (Dani *et al.*, 2008). Bordo organic whole red grape juice (2019 harvest) was provided by Uva'só Organic Products. Its phenolic composition includes anthocyanins (529 mg/L), flavonoids (3522.6 mg/L), and resveratrol (0.61 mg/L). Treatment began seven days after the administration of monocrotaline. The experimental groups consisted of control animals (n = 10), which received an injection of saline and water by gavage during the treatment period, and the PAH groups, which were subdivided into PAH (n = 10), which received water by gavage; PAH+GJ (n = 9), which received grape juice by gavage; PAH+TH (n = 7), which received T3 and T4 by gavage; and PAH+TH+ GJ (n = 10), which received T3 and T4 hormones and, one hour after this administration, grape juice, both by gavage. On the twenty-first experimental day, echocardiography was performed, followed by euthanasia. The RV and blood were collected for further analyses.

Echocardiographic and morphometric analysis

The animals were anesthetised (ketamine 90 mg/kg; xylazine 10 mg/kg, intraperitoneal), subjected to trichotomy of the thoracic region, and placed in the lateral decubitus position. Images were obtained using two-dimensional, M-mode, and pulsed Doppler (Philips HD7 Ultrasound System, Andover, MA, USA) using an S12-4 transducer (Philips, Andover, MA, USA). RV diastolic and systolic diameters (RVDd and RVDs, respectively)

were evaluated. Body weights and food intake were evaluated daily. RV hypertrophy was assessed using the ratio of RV weight to heat weight.

Evaluation of biochemical markers and of triiodothyronine (T3)

Serum triiodothyronine (T3) levels were measured using the electrochemiluminescence method in a clinical analysis laboratory (Diagnostics da América S. A.). Serum measurements were also made of creatinine, urea, uric acid, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglyceride, glucose, creatine kinase (CK-total), creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH) levels. All tests were performed using commercial kits (LABTEST, São Paulo, SP, USA) specific for each test.

Western blot analysis

Right ventricle samples were homogenized by Cell Lysis and Protein Extraction for Western Blotting, and centrifuged at 1000 × *g* at 4°C. Supernatants containing 100 mg of protein were subjected to electrophoresis on a polyacrylamide gel (8–14%). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P transfer membrane; Millipore) in a mini-trans-blot electrophoretic tank. Immunodetection was performed using the following antibodies: PGC1-α (H-300) sc-13067, Lot #E1413, Santa Cruz Biotechnology and Pyruvate dehydrogenase (C54G1), Lot 6, Cell Signaling Technology. Secondary antibodies (anti-mouse or anti-rabbit radish peroxidase conjugate) were used for chemiluminescence detection in the Image Quant LAS4000 system (GE Healthcare) and quantified with ImageJ software. The Ponceau method was used for normalisation (Klein, Kern, Sokol, 1995). Four animals were randomly chosen per treatment group.

Statistical analysis

The data distribution was determined using the Shapiro-Wilk test. For data with a normal distribution of homogeneous variance, one-way analysis of variance (ANOVA) was used, followed by Tukey's post-hoc test (F). For data with a normal distribution and non-homogeneous variance, Welch's analysis (W) of variance (ANOVA) was used, followed by the Games-Holmes post-test. For data that did not show a normal distribution, a Kruskal-Wallis (K) analysis with Dunn's post-test was used. The generalized estimating equations (GEE)test was used to analyse weight and food consumption. Statistical significance was set at $P < 0.05$. All analyses were performed using the SPSS Statistics version 18 software.

RESULTS

Evaluation of echocardiographic, morphometric, and food intake parameters

RV systolic diameter was significantly increased only in the PAH group compared to the control group [$W(4,13.84) = 7.306$, $P = 0.002$]. No significant difference was observed in the RV diastolic diameter [$K(4,38) = 9.054$, $P = 0.060$] (Table I). On the other hand, there were elevated RV hypertrophic indices (RV/total body weight) in all PAH groups compared to the control group. From the eighth experimental day, the control group gained more weight than the other groups, and obtained greater weights at the end of the experimental protocol ($P \leq 0.001$) (Figure 1A). Furthermore, the difference between the final and initial weights was lower in the PAH+TH+GJ group than in all other groups (Table I). The control group had higher food consumption than the other groups ($P \leq 0.001$). The lowest food intake was observed in the PAH+TH+GJ group ($P \leq 0.05$) as compared to PAH (Figure 1B).

TABLE I - Thyroid hormone levels and morphometric and echocardiographic parameter data

Parameter	Control (n 10)	PAH (n 10)	PAH+GJ (n 9)	PAH+TH (n 7)	PAH+TH+GJ (n 10)	Value p
Hormone levels						
T3	42.85 [30.2-50.0]	30.50 [26.1-34.7]	42.20 [29.1-47.0]	84.90 [75.1-160.6]*§ł	92.95 [53.75-250.8]*§ł	0.003
Morphometric Data						
Final body weight (FBW) (g)	315 ± 36	259 ± 44*	257 ± 23*	241 ± 31*	224 ± 20*	≤0.001
Final weight – Initial weight (g)	91.50 ± 16.83	65.87 ± 9.20*	63.88 ± 13.43*	60.16 ± 16.08*	43.70 ± 17.25*§ł	≤0.001
Weight RV/Total Heart weight (g/g)	0.21 ± 0.01	0.29 ± 0.03*	0.28 ± 0.03*	0.32 ± 0.04*	0.29 ± 0.03*	≤0.001
Echocardiographic Data						
RVDd (cm)	0.35 [0.32-0.37]	0.44 [0.38-0.47]	0.39 [0.36-0.43]	0.45 [0.37-0.56]	0.46 [0.37-0.50]	0.060
RVDs (cm)	0.29 ± 0.02	0.40 ± 0.07*	0.38 ± 0.07	0.41 ± 0.11	0.41 ± 0.08	0.002**

PAH pulmonary arterial hypertension; GJ grape juice; TH thyroid hormones; FDW Final body weight; RV right ventricle; RVDd right ventricular diastolic diameter; RVDs right ventricular systolic diameter. One-way ANOVA (post Tukey test),

** One-way ANOVA Welch's (post Games Howell test), data presented as mean ± standard deviation. Kruskal-Wallis test (post Dunn's), data presented as median and 25th and 75th percentiles.

* significant difference compared to the control group

§ significant difference compared to the PAH group

ł significant difference compared to the PAH+GJ group

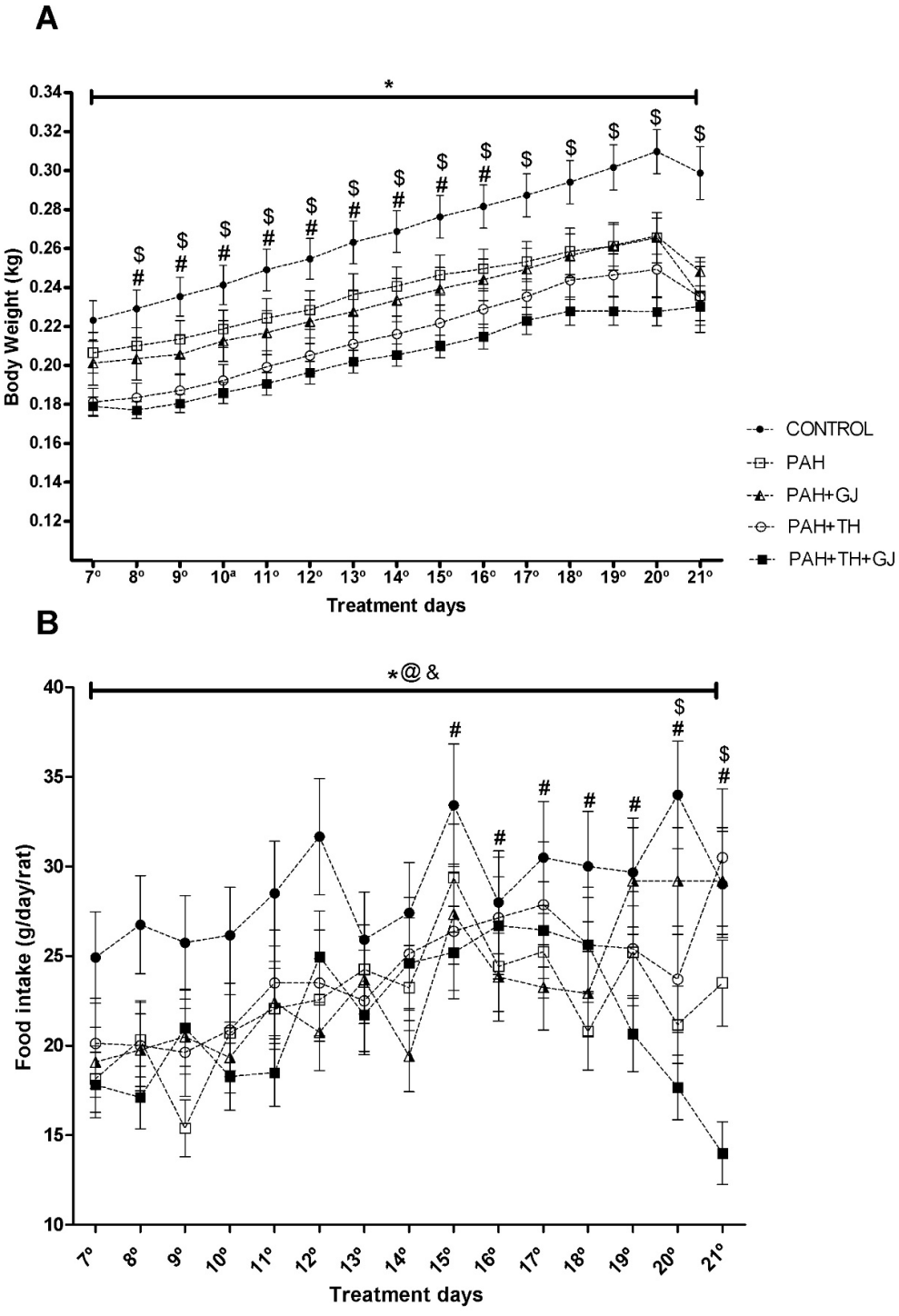

FIGURE 1 – A – Body weight assessment. * difference between the PAH+TH or PAH+TH+GJ group and the control group. # difference between moments in relation to the initial day. $ difference in the interaction treatment and moment between the control group and the PAH+TH+GJ group.

B – Control of food consumption. * Difference between the control and PAH groups; PAH+GJ; PAH+TH; PAH+TH+GJ. @ difference between the PAH+JUCE and PAH+TH+GJ groups. & difference between the PAH+TH and PAH+TH+JUCE groups. # difference between moments in relation to the initial day. $ difference in the treatment and moment interaction between the control group and PAH+TH+GJ. Statistical analysis by GEE, $P \leq 0.05$.

Measurements of serum parameters: TH levels and cardiac, hepatic and renal markers

T3 levels increased in both groups that received hormone treatment [K(4.39) = 15.940, P = 0.003] (Table 1). The PAH+TH+GJ group showed reduced CK-NAC levels compared to the control and PAH+GJ groups [W(4,13.46) = 11.03, P = 0.0003]. A similar profile was observed in CK-MB [W(4,13.94) = 7.063, P = 0.0025], LDH [K(4,34) = 14.22, P = 0.0066], and ALT levels (Figure 2A–D). The PAH, PAH+TH, and PAH+TH+GJ groups showed decreased AST levels compared to the control group [K(5,32) = 22.44, P = 0.0002] (Figure 2E). No significant changes were observed in the ALT/AST ratio (Figure 2F). Glucose levels decreased in the PAH and PAH+TH+GJ groups compared to the control group [K(5,31) = 23.99, P = 0.0001] (Figure 3A). Albumin levels decreased in the PAH, PAH+TH, and PAH+TH+GJ groups compared to the control and PAH+GJ groups [W(4,12.72) = 35.74, P = 0.0001] (Figure 3B). Triglyceride levels were significantly reduced in the PAH, PAH+TH, and PAH+TH+GJ groups; this reduction was more pronounced in the PAH+TH+GJ group [K(4,34) = 14.22, P = 0.0066] (Figure 3C). The same profile was observed for total cholesterol [W(4,12.11) = 30.90, P = 0.0001] (Figure 3D). There was no significant difference in creatinine levels [K(5,27) = 1.196, P = 0.8788] (Figure 4A). Alternatively, decreased urea levels were observed in the PAH, PAH+TH, and PAH+TH+GJ groups compared to the control and PAH+GJ groups [F(4,28) = 27.57, P = 0.0001] (Figure 4B). Similarly, a decline in uric acid levels was observed in the PAH and PAH+TH+GJ groups compared to the control and PAH+GJ groups [W(4,12.03) = 11.86, P = 0.0004] (Figure 4C).

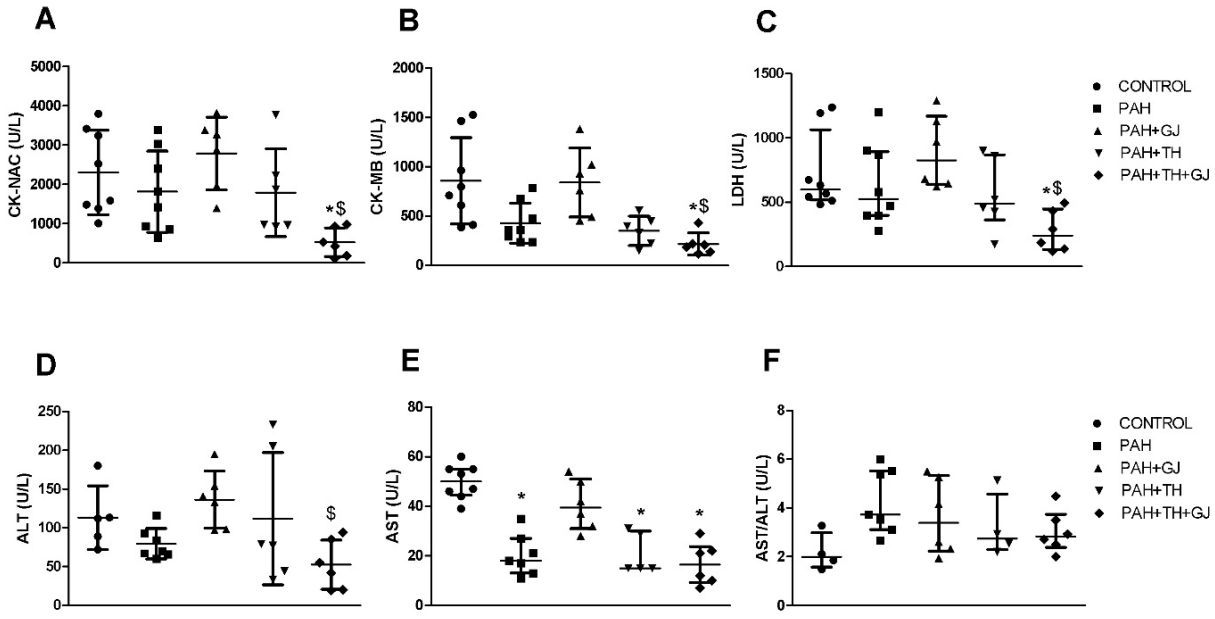

FIGURE 2 - Analysis of cardiac markers and liver enzymes (A) CK-total, (B) CK-MB, (C) LDH, (D) AST, (E) ALT, and (F) AST/ALT. Data are expressed as mean ± standard deviation. * significant difference compared to the control group. $ significant difference compared to the PAH+GJ group. Statistical analysis: Welch's analysis of variance (ANOVA), followed by the Games-Holmes post-test (CK-total, CK-MB); Kruskal-Wallis analysis with Dunn's post-test (LDH, AST, ALT, and AST/ALT), $P \leq 0.05$.

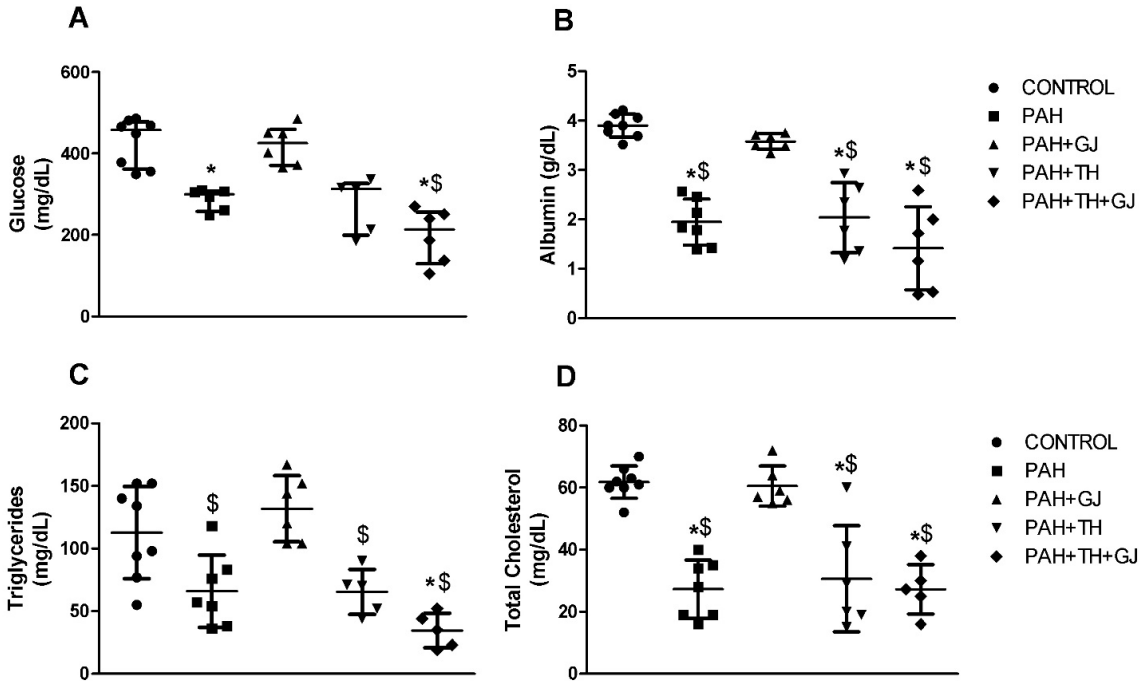

FIGURE 3 – (A) Glucose, (B) Albumin, (C) Triglycerides, and (D) Total Cholesterol. Data are expressed as mean ± standard deviation. * significant difference compared to the control group. $ significant difference compared to the PAH+GJ group. Statistical analysis: Welch's analysis of variance (ANOVA), followed by the Games-Holmes post-test (Albumin, Total Cholesterol); Kruskal-Wallis analysis with Dunn's post-test (Glucose, Triglycerides), $P \leq 0.05$.

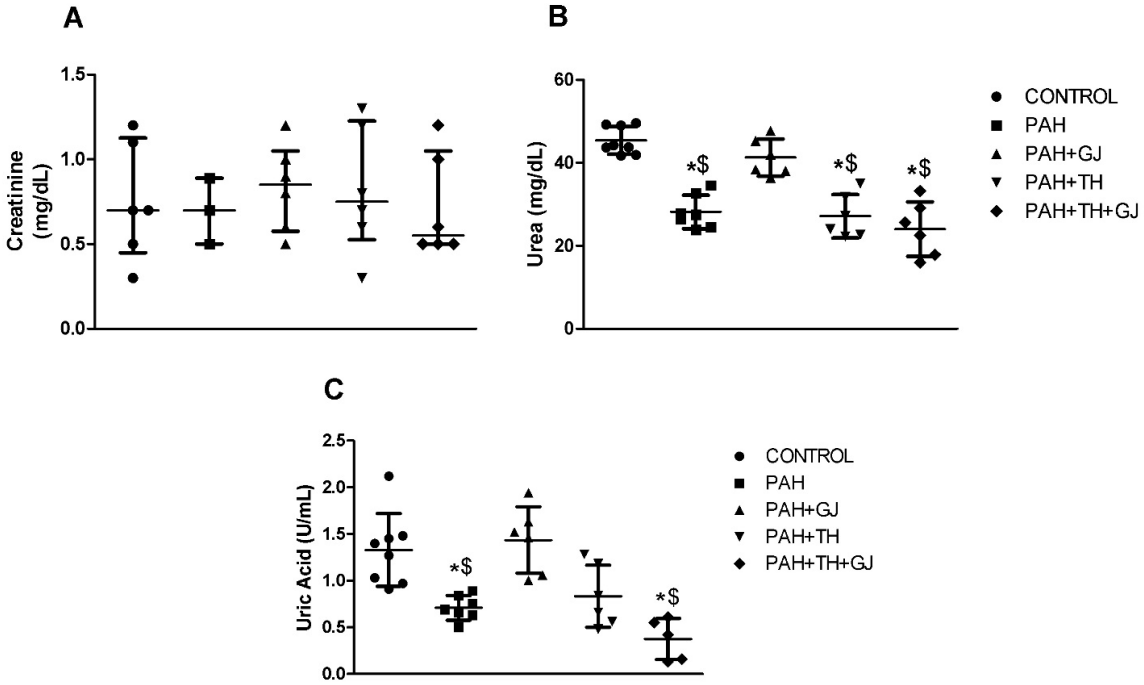

FIGURE 4 – Analysis of renal function markers. (A) Creatinine, (B) Urea, (C) Uric acid. Data are expressed as mean ± standard deviation. * significant difference compared to the control group. $ significant difference in relation to the PAH+GJ group. Statistical analysis: one-way analysis of variance (ANOVA), followed by Tukey's post-hoc (Urea); Welch's analysis of variance (ANOVA), followed by the Games-Holmes post-test (Uric acid); Kruskal-Wallis analysis with Dunn's post-test (Creatinine), $P \leq 0.05$.

PGC1-α and PDH protein expression

PGC1-α protein expression was higher in the co-treated group than in the control and PAH+GJ groups [F(4.15) = 5.454, P = 0.006] (Figure 5A). However, the PDH immunocontent decreased in the co-treated group compared to the PAH group [F(4,15) = 4.632, P = 0.012) (Figure 5B).

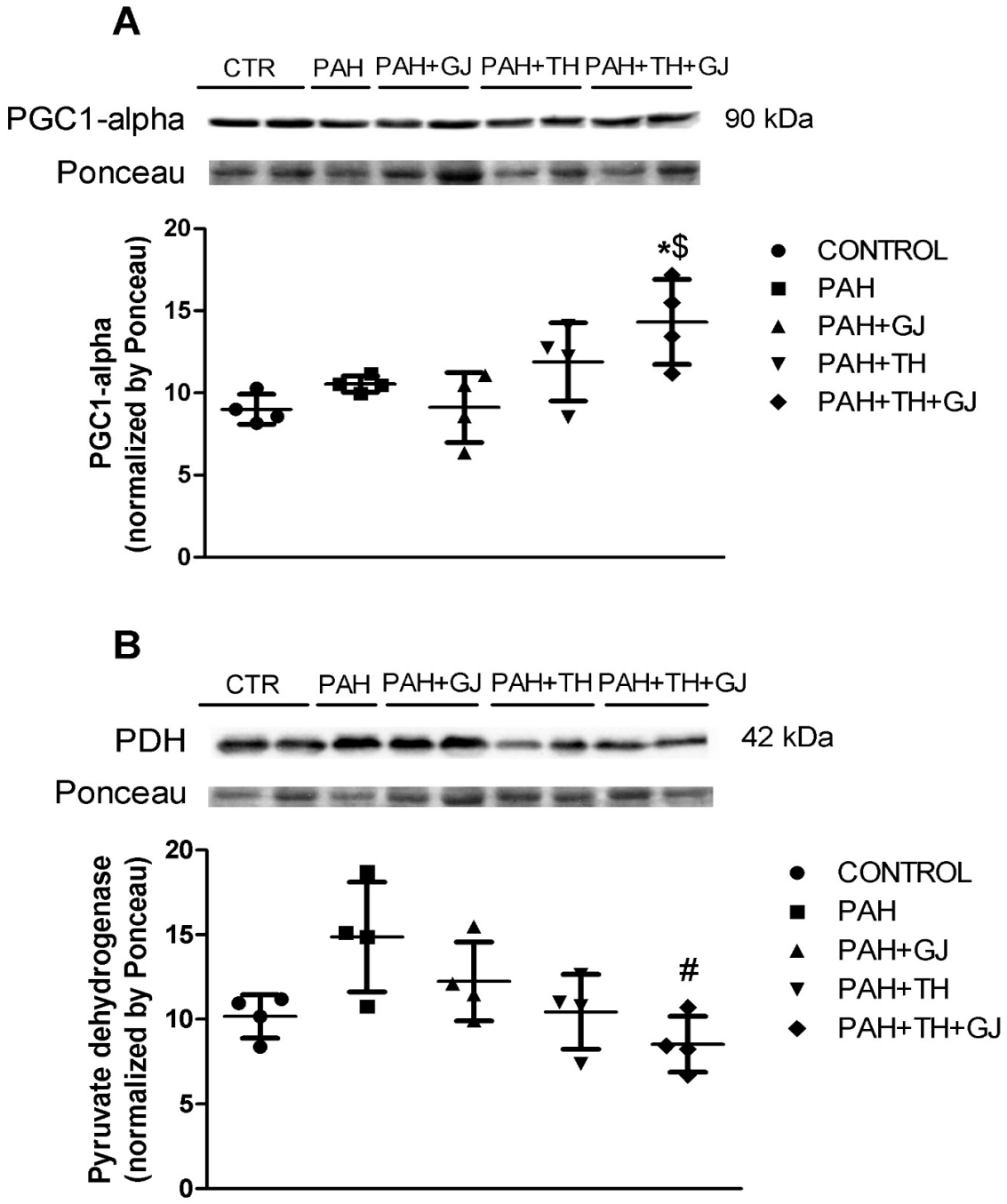

FIGURE 5 – Protein expression by Western blot. (A) PGC 1-alpha, (B) Pyruvate dehydrogenase (PDH). * significant difference compared to the control group. $ significant difference in relation to the PAH+GJ group. Statistical analysis by one-way ANOVA with Tukey's post-test, $P \leq 0.05$.

DISCUSSION

To the best of our knowledge, this study is a pioneering attempt to explore the effects of the administration of organic grape juice and thyroid hormone on metabolic and cardiac morphometric parameters in an experimental PAH model. PAH decreased albumin, triglyceride, and total cholesterol levels, associated with a decrease in body weight. This condition is according to the process of PAH-induced cachexia, a key marker of poor prognosis. Additionally, these biochemical alterations had repercussions for the RV, as observed by the PAH-induced elevation in the systolic diameter. Nevertheless, grape juice and thyroid hormones alone, as well as their co-administration, are promising treatments for mitigating damage to the RV caused by PAH. This protective action was verified by evaluating total CK, CK MB, and the protein expressions of PGC -1 alpha and PDH.

Malnutrition and cachexia are often observed in PAH patients. However, few studies have investigated the nutritional status of these patients (Vinke *et al*., 2018). Our study observed both weight loss and reduced food consumption, which worsened as the disease progressed. Zimmer *et al.* (2021) reported similar data indicating the impact of PAH on energy metabolism and nutritional status. Serum biochemical analyses corroborated the state of weight loss. There were decreased albumin, total cholesterol, and triglyceride levels in the PAH group, which was likely associated with the reduced feeding behaviour of these animals. Hypoalbuminemia is frequent in patients with PAH and results from malnutrition, inflammation, and cachexia (Arques, Ambrosi, 2011). Several mechanisms have been proposed for the development of cachexia in PAH. However, growth and differentiation factor 15 (GDF 15), a protein that plays a regulatory role in energy metabolism, has been highlighted as a cytokine associated with weight loss in PAH (Albuquerque *et al*., 2022). Although the treatments did not reverse the weight loss and anorexic behaviour caused by PAH, grape juice administration was promising for the recovery of albumin, triglycerides, total cholesterol, and glucose levels in the PAH+GJ group. Penedo-Vázquez *et al.* (2021) demonstrated that the administration of curcumin and resveratrol ameliorated body and muscle weights and reduced muscle damage and proteolysis in cachectic mice, and was associated with increased sirtuin-1 levels. PAH-induced abnormal weight loss can critically impact cardiac morphometry and function.

The morphometric and echocardiographic parameters analysed identified PAH-provoked RV hypertrophy and ventricular dilation during systole. This indicates structural remodelling of the RV due to the increase in the afterload characteristic of PAH. Türck *et al.* (2022) observed an association between decreased RV output and pulmonary artery outflow acceleration/ejection time ratio, with increased RV diameters and RV hypertrophy. Moreover, Dos Santos Lacerda *et al.* (2017) showed that a reduced tricuspid annular plane systolic excursion (TAPSE), a RV contractility index, was associated with an increased RV hypertrophy index. Zimmer *et al. (*2020*)* highlighted that the increased RV diastolic and systolic diameters in PAH were correlated with the inflammatory process, as a significant inflammatory infiltrate was observed in the RV of PAH animals. The known anti-inflammatory action of the phenolic compounds present in grape juice could explain the partial mitigation of the ventricular dilation process observed in our results (Maleki, Crespo, and Cabanillas, 2019). Although the total CK and LDH levels were not altered in the PAH group, the treatment positively influenced the profile of cardiac damage markers, as the combined treatment of grape juice and TH was more effective in reducing the total CK, CKMB, and LDH levels. As these biomarkers indicate potential cellular damage, their reduction suggests that this treatment approach may prevent cellular injury (Komolafe *et al*., 2017; Parsanathan and Jain, 2020).

A reduction in deiodinase activity is associated with impairment of TH metabolism in cardiac tissue, as demonstrated by Olivares *et al.* (2007). These authors showed a decrease in hepatic type 1 deiodinase activity and an increase in cardiac type 3 deiodinase in infarcted rats. These conditions increase the removal of TH, leading to myocardial ischaemia-induced hypothyroidism (Low T3 Syndrome) (Olivares *et al*., 2007). Cardiac damage is a consequence of augmented TH catabolism, justifying cardiac disease treatment with these hormones. Corssac

et al. (20216) evaluated the effects of TH on the RV in an acute myocardial infarction (AMI) model, in which the p-Akt/total Akt ratio, stimulated by TH treatment, increased. Activation of the AKT pathway results in cardioprotection against cellular death, because this protein is related to cell growth and survival (Corssac *et al.*, 2016). However, to the best of our knowledge, there have been no studies that have used TH to treat Cor Pulmonale. Data from the literature show that both hypothyroidism and hyperthyroidism are associated with PAH. Low TH levels, in hypothyroidism, can diminish ventricular inotropism, decrease cardiac output, and increase vascular resistance, both of which are conditions that would be associated with the development of PAH. Nevertheless, Al Husseini *et al*. (2013) demonstrated that, in a sugen-chronic hypoxia (SuHx) model, thyroidectomy and propylthiouracil treatment reduced PAH development in rats, and replacement TH worsened the disease. Conversely, hyperthyroidism, especially Graves' disease, has been identified as the main trigger for PAH. In this context, the autoimmune mechanism that leads to hyperthyroidism could also induce endothelial damage and an increase in pulmonary vascular resistance (Vrigkou *et al*, 2022). This therefore shows that TH treatment needs be administrated with caution in a PAH model.

In parallel, PAH-induced changes in energy metabolism also occur (Bertero and Maack, 2018). In a healthy heart, oxidative metabolism prevails, and ATP production relies on approximately 70% fatty acids and 30% glucose as sources for the oxidation process (Spyropoulos *et al.*, 2021). Nevertheless, heart failure provokes pivotal changes in metabolic programming, inducing cardiomyocytes to switch fatty acids to glucose as their main energetic substrate (Nakai *et al.*, 2019). PGC-1-alpha plays a key role in metabolism control because it promotes mitochondrial biogenesis and leads to the oxidative pathway instead of glycolic metabolism (Liang, Ward, 2006). We observed that co-treatment (grape juice plus TH) increased PGC-1-alpha in PAH rats, suggesting maintenance of the oxidative pathways to the detriment of the glycolytic, providing a cardioprotective effect. The change from oxidative to glycolytic metabolism appears to be detrimental to the cardiac tissue, even though this shift in the pathway is a way to compensate for the energy deficit due to adverse ventricular remodelling. Nakai *et al.* (2019) demonstrated that monocrotaline-induced RV damage is associated with decreased Krebs cycle intermediates and increased glycolysis metabolites. One enzyme that links the glycolytic pathway and the Krebs cycle is PDH. The combined treatment (grape juice plus TH) promoted a reduction in PDH protein expression, which could indicate preservation of the healthy metabolic environment in the RV. However, further studies are required to confirm this hypothesis.

STUDY LIMITATIONS

Although the objective of the study was to evaluate cardiac, hepatic, lipid, and renal plasma parameters and associate them with metabolic control proteins in monocrotaline-induced PAH, and evaluate the impact of TH and GJ treatments on these parameters, assessing functional cardiac and TSH levels is important to better understand the effects of this therapeutic approach in this experimental model. The lack of these evaluations is therefore a limitation of this study.

CONCLUSION

PAH causes a series of alterations in the RV structure, function, and metabolism. Our study demonstrated the cardioprotective effect of the association between thyroid hormones and grape juice for the first time. This effect is mainly demonstrated by the increase in the expression of PGC-1a, a key protein in the metabolic regulation of the heart. In addition, isolated administration of grape juice demonstrated beneficial effects on biochemical parameters in the hearts of animals with PAH, preventing metabolic changes linked to the cachexia process. Despite these findings, further studies are necessary to understand the mechanisms involved in this therapeutic approach for RV failure.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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