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ASSOCIATION BETWEEN METABOLIC SYNDROME AND THE DISTURBANCE OF THE HYPOTHALAMUS PITUITARY THYROID AXIS IN A MALE SPRAGUE-DAWLEY RAT MODEL

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Abstract: Metabolic syndrome (MetS) is a constellation of neuroendocrine entities that contribute to chronic pro-inflammatory states. Though the thyroid axis is responsible for the energetic maintenance, little is known about its role in MetS. This work evaluated the association between MetS and the disturbance of the hypothalamic pituitary thyroid (HPT) axis in male *Sprague-Dawley (SD)* rats. 32 5-week-old rats were divided into control and experimental groups. For 28 weeks, control group received a hypoglycemic diet and experimental group a high-carbohydrate and fat diet. Serial measurements of systolic blood pressure (SBP) and heart rate were taken simultaneously. Then, the latter was sub-classified as normotensive and hypertensive. Rats were sacrificed by heart puncture and blood analysis was done [glucose (GLc), triglycerides (TAG), cholesterol LDL (LDL), insulin, thyroid-stimulating hormone (TSH) and free tetra iodine thyroxine (Free T_4)]. Significant differences were found in weight between control and normotensive rats, and control and hypertensive rats ($p<0.05$). Final SBP measurement lower in control, and normotensive rats vs. hypertensive rats ($p<0.05$). GLc increased in normotensive, and hypertensive rats vs. control group ($p<0.05$); same in normotensive vs. hypertensive rats. Insulin higher among control group vs. normotensive, and hypertensive rats ($p<0.05$). LDL higher in normotensive, and hypertensive rats vs. control group; same in normotensive vs. hypertensive. TAG higher in normotensive, and hypertensive rats vs. control group; same in hypertensive vs. normotensive ($p<0.05$). TSH increased in normotensive, and hypertensive rats vs. control group ($p<0.05$). Difference also found in hypertensive vs. normotensive. HOMA-IR higher in control vs. hypertensive group ($p<0.05$). Free T_4 and TSH percentiles' critical values were calculated ($p<0.05$). In hypertensive rats, one developed hypothyroidism; four, primary hypothyroidism and three, subclinical hypothyroidism. There is no sufficient evidence to reject an association between hypothyroidism and MetS with hypertension in this male SD rat model.

Keywords: Metabolic Syndrome, thyroid hormone, Sprague-Dawley.

INTRODUCTION

The MetS consists of a wide range of pro-inflammatory, hypercoagulable and neuroendocrine entities that induce chronic endothelial damage that create a synergic condition, increasing morbidity and mortality (Pajuelo; Sánchez, 2007; Gonzales-Rivas, 2012). The risk factors together with the *modus vivendi* interact in order to model its installation and development (Damaso et al., 2011; Montenegro et al., 2005). Despite its preventive character, the worldwide incidence and prevalence have increased over the last years (Díaz-Cisneros et al., 2006; Scarsella; Despres, 2003; Pando-Álvarez, 2012; Pajuelo et al., 2012). Because of the intimate relation between the metabolism and thermogenesis, studying the latter allows for better understanding of the thyroid axis functioning (He et al., 2016; Hoerman et al., 2015). In hypometabolic states, just as with MetS, the reduction of energy usage lead to an increase of weight and LDL levels and a decrease of lipolysis and gluconeogenesis. These influence key metabolic pathways in different organs that control the energetic balance by regulating their input and output. This increase is also a risk factor for cardiovascular diseases and atherosclerosis (Hak et al., 2000).

There is no sufficient evidence to reject an association between hypothyroidism and an increase of the total LDL and lipoproteins of low density that improve the substitution of thyroxine. The alterations of HPT axis and its relationship with the physiopathology of

MetS are, so far, unclear.

In order to study the dynamics of the HPT axis, the thyroid stimulating hormone (TSH) is used together with the tetraiodothyronine (Free T_4). The alterations of TSH and Free T_4 in serum concentrations can generate hypothyroidism or hyperthyroidism. Despite contradicting results, no correlation can be rejected in humans according to the parameters in the blood of the MetS and the HPT axis (Gutch et al., 2017).

Experiments with animal models show that MetS can be recreated by different methods, being the hyper caloric diet the one that most resembles the lifestyle of humans, reproducing biological phenomena with high precision (Yunta, 2007).

Delving into the diagnostic criteria of the MetS, the insulin resistance (IR) is at the base of a pyramid. The model evaluation index of homeostasis for the resistance to insulin (HOMA-IR) is a mathematical formula used to evaluate its presence in humans. There is interest in evaluating the HOMA-IR as a practical and fast way of measuring the IR in the framework of a pre-clinical work. SD strain is sensitive to high-carbohydrate and fat diets; hence, MetS is induced easily in this strain (Ghibaudi et al., 2002). Nationwide, neither a model of MetS nor the disturbance of the HPT axis have been recreated successfully within the SD strain. Until now, this is one of the first animal models designed to recreate and comprehend MetS and HPT axis on a small scale in a Peruvian context. The aim of the present study, therefore, was to investigate the association between the MetS and the disturbance of the HPT axis in an experimental model with male SD rats.

MATERIALS AND METHODS

Design and place of study: Experimental study carried out in the bioterium belonging to the Neuroscience and Behavior Laboratory located in the Research and Development Laboratory building (LID) of Universidad Peruana Cayetano Heredia (UPCH).

Study population:

Universe: Male SD rats.

Inclusion Criteria:

- Five-week-old male rats.
- Rats with no clinic evidence of any physiopatological framework (Hubercht; Kirkwood, 2010).

Elimination Criteria: Rats that presented any indicator of disease or lack of well-being and any abnormal behavior not related to the experimental work, were separated from the study and euthanized.

Sample: The program Epiinfo 6.1 was used to calculate the sample size. Considering 5 rats for the control and 27 rats for the experimental group, a susceptibility frequency for the development of hypertension in the SD strain (in experimental conditions) of up to 80% and a 95% of reliability; a power of prediction of 95.21% was obtained for this model (Oron-Herman et al., 2008; Panchal et al., 2011).

Analysis Unit: 5-week-old male rats of the SD strain from the bioterium of the UPCH.

Operational definition of variables: Metabolic Syndrome. Simultaneous occurrence of at least three of the following medical conditions: arterial hypertension, hyperglycemia, obesity or dyslipidemia (Parikh; Mohan, 2012).

Insulin Resistance. Significant increase of insulin secretion of experimental group in relation to the control group. The HOMA-IR index was used as an indirect method of quantification.

$$\frac{\left[\text{GLc}\left(\frac{\text{mg}}{\text{dl}}\right) \text{x Insulin (Uuml)}\right]}{405}$$

Alteration of the hypothalamic thyroid pituitary axis. The TSH and Free T_4 were quantified post cardiac puncture and the

percentiles were calculated with the following criteria: low ($_{p<10}$), high ($_{p10-50}$) and very high ($_{p>90}$).

$$Pi = l + \frac{h}{f}\left(\frac{iN}{100} - \alpha\right).$$

h width of Percentile group

l lower boundary of Percentile group

f frequency of Percentile group

N sample size or total number of observations

α cumulative frequency preceding Percentile group

Procedures and techniques: The protocol was approved by the Institutional Committee of Ethics in Research for Animals of the UPCH.

Experimental Animals: Thirty-two 5-week-old male SD rats were divided into two groups; experimental group (n=27) and control group (n=5). The group of experimental rats was also divided into two subgroups according to their SBP results as normotensive (n=9) and hypertensive (n=18).

Maintenance: the animals were subjected to standard environmental conditions (12 hour inverted dark-light cycle, 20ºC ± 2 and humidity 50 ± 10%), with daily-made ad libitum food and water (16). Researches were prohibited to use perfumes and deodorant. Rats were housed five per cage and maintained on normal pellet diet for 2 weeks before dietary manipulation, for acclimatization.

Diet: A handmade diet, prepared by an expert, was based on the formula studied by Panchal, et al. and approved by the Ethics Committee of the UPCH (Panchal et al., 2011). The control rats received a low-carbohydrate and low-fat diet with cornstarch as its principal carbohydrate (carbohydrates 59%, fat 1%, protein 3%, water 25%, salt mixture 2% and fiber 10%; 1.6 kcal×g^{-1}) while the experimental group was fed with a high-carbohydrate and fat diet with fructose and GLc as main carbohydrates (carbohydrates 45%, fat 24%, protein 6%, water 5%, salt mixture 3% and fiber 17%; 4.5 kcal×g^{-1}). The proportion of food eaten was calculated by weighting the given food and subtracting the weight of the remaining food.

Rats weight: Per week (wk.)

Systolic blood pressure measurement: A non-invasive blood pressure meter (LE 5002 Panlab, Harvard Apparatus, Barcelona, Spain) was used under sedation reducing the amount of stress as much as possible. It measured the pressure variation upon its tactile surface when placed on the rat's caudal artery. The SBP benchmark was 130 mmHg (Van Vliet; Montani, 2008).

Sacrifice by exsanguination via cardiac puncture: This technique was carried out 24 hours after the final SBP measurement (week 28). The food was removed from the cages 6 hours before the procedure, leaving only the water. The rats were sedated with a ketamine anesthesia solution (75g/kg) (ET-A-100®) and xylazine (4g/kg) (Dormi-Xil®). A layered cephalad-directed abdominal incision was made. The rib cage and the diaphragm were cut and two forceps were placed with intention to expose the heart. Immediately, a 10cc syringe with an embedded needle N°21 with 0.2 ml of 25 000 UI heparin (Wuhan Uni-Pharma, China®) crossed the left ventricle and blood was aspired. The volume of blood was poured slowly upon the walls of a tube with clot activator (Vacutainer®, Mexico). The tube was stirred and placed immediately in refrigeration for 20 minutes.

Sampling: The tubes were placed in a counterbalanced centrifuge (Eppendorf) at 4400 RPM and two rounds of 10-minute exposure were required to separate the serum from the cell bodies. Micropipettes were used to separate the serum and then placed in up

to three coded Eppendorf®. The samples were refrigerated at -20°C until further processing.

Processing of the blood samples: The analysis of the blood tests was performed in the Quality Control Laboratory, UPCH.

Euthanasia: The sick rats were euthanized by applying an intraperitoneal ketamine: xylazine solution, as indicated in AVMA Guidelines for Euthanasia of Animals (AVMA, 2007).

Analysis of Results: The Shapiro-Wilk test was used to evaluate the distribution of data with a 95% of reliability. If the result implied an adjustment to a normal distribution, parametric tests were used. If not, non-parametric tests were applied.

Also, the Difference in Means Test was chosen to assess the impact of the diet, SBP and the serum results between independent groups. The calculated statistic was compared to the acceptance or rejection regions with a reliability level of 95%. In addition, the Paired Samples t-Test was used to compare the means of the SBP, HR and weight of control, normotensive or hypertensive groups in different moments of the experiment. Finally, the serum tests analysis was done using correlations and a significant p-Value Test was applied.

Ethical aspects: No procedures were performed on the rats that inflicted pain on them. The work was approved by the Institutional Ethics Committee for Animals, UPCH.

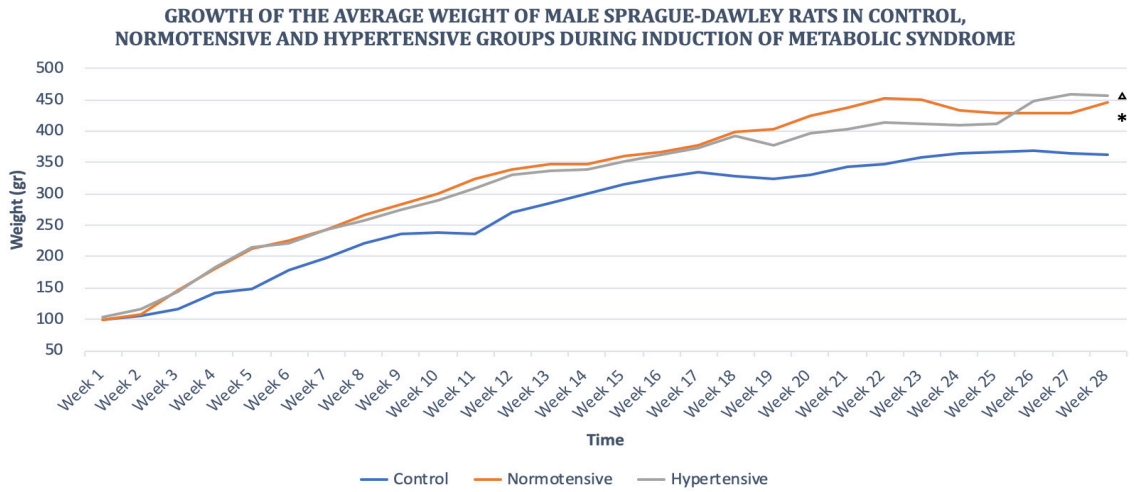
RESULTS

EVALUATION OF METS VARIABLES

Weight: The variation of the weight measured weekly was also significantly different among the normotensive and hypertensive rats vs. control but not when comparing the normotensive and hypertensive rats (Graph 1). By week twenty-eight, 55% of the rats fed with high-carbohydrate and fat diet had survived, whereas in the control group the survival rate was 100%.

Systolic blood pressure: The blood pressure measurement was evaluated synchronically with the HR, as well as with weight measurements (Table 1).

Serum Blood Samples: The results obtained by evaluating the serum blood samples using the mean difference test to compare

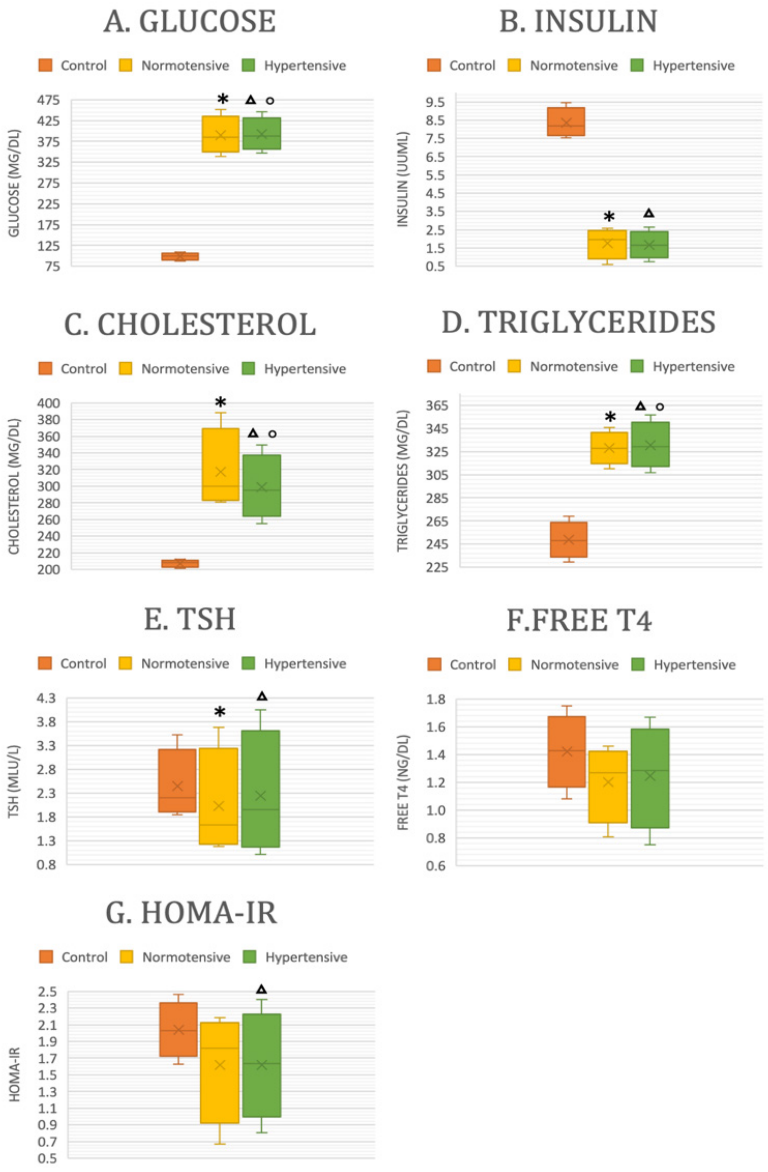


Graph 1: Growth of average weight of male SD rats in control, normotensive and hypertensive groups during MetS induction. Data shown are means ± SEM, n=4-13 rats per group. *p < 0.05 (control and normotensive); ▵ p < 0.05 (control and hypertensive); *p < 0.05 (control and normotensive) significantly different as indicated.

Group	Control (n=5)	Normotensive (n=4)	Hypertensive (n=13)
Food intake (g/rat/day)	20.00 ± 3.01	34.86 ± 4.52*	40.71 ± 7.14 ▵
Wk. 1 weight (g)	115.40 ± 42.95	100.50 ± 11.47	104.38 ± 11.38
Wk. 14 weight (g)	269.60 ± 28.53	314.25 ± 34.91*	337.15 ± 55.73▵◦
Wk. 28 weight (g)	361.80 ± 26.10	445.78 ± 65.88*	457.83 ± 49.92▵◦
Wk. 1 HR (s^{-1})	342.00 ± 11.02	332.25 ± 16.46	335.54 ± 22.55
Wk. 14 HR (s^{-1})	341.40 ± 18.08	342.00 ± 8.83	345.54 ± 13.15
Wk. 28 HR (s^{-1})	317.40 ± 32.72	371.00 ± 57.81	355.54 ± 48.99
Wk. 1 SBP (mmHg)	107.20 ± 6.46	107.75 ± 2.22	106.77 ± 18.74
Wk. 14 SBP (mmHg)	109.80 ± 8.07	122.50 ± 7.05*	139.46 ± 6.33▵◦
Wk. 28 SBP (mmHg)	109.00 ± 4.85	115.60 ± 2.08*	145.38 ± 8.06▵◦
GLc (mg/dl)	99.31 ± 7.78	388.62 ± 46.49*	390.93 ± 32.32▵◦
Insulin (Uuml)	8.36 ± 0.93	1.83 ± 0.87*	1.67 ± 7.78▵
Cholesterol (mg/dl)	207.58 ± 4.15	311.42 ± 51.37*	298.99 ± 28.50▵
TAG (mg/dl)	248.12 ± 14.40	327.86 ± 16.10*	329.96 ± 14.97▵◦
TSH (mlU/L)	2.51 ± 0.69	1.85 ± 1.19*	2.24 ± 1.18▵
Free T_4 (ng/dl)	1.43 ± 0.24	1.22 ± 0.29	1.24 ± 0.39
HOMA-IR	2.062 ± 0.36	1.69 ± 0.70	1.58 ± 0.46▵

Data shown are means ± SEM. 0.05 *p < 0.05 (control and normotensive); ▵p < 0.05 (control and hypertensive); ◦p < 0.05 (normotensive and hypertensive) significantly different as indicated.

Table 1. Clinical and laboratory principal characteristics of SD rats



Graph 2: Plasma levels of blood samples after 28 weeks. Data shown are means ± SEM, n=4-13 rats per group. ▵p < 0.05 (control and hypertensive); ◦p < 0.05 (normotensive and hypertensive) and *p < 0.05 (control and normotensive) significantly different as indicated.

Group	Statistic test	GLc vs. Insulin	GLc vs. LDL	GLc vs. TAG	GLc vs. TSH	GLc vs. Free T_4	Insulin vs. LDL	Insulin vs. TAG	Insulin vs. TSH	Insulin vs. Free T_4	GLc vs. GLc
Control	Covariance	- 29.56	1,654.05	488.05	38.19	- 8.88	- 32.19	- 7.74	- 0.73	0.18	1,621.34
	Correlation	- 0.04	0.01	0.00	0.05	- 0.02	- 0.06	- 0.01	- 0.21	0.08	0.01
Normotensive	Covariance	- 13.23	629.26	17.62	28.58	- 9.69	- 11.28	- 0.03	- 0.52	0.17	964.44
	Correlation	- 0.70	0.68	0.04	0.75	- 0.77	- 0.68	0.00	- 0.76	0.73	0.92
Hypertensive	Covariance	4.44	15.28	- 11.58	3.25	1.39	1.26	- 2.82	0.32	0.09	48.44
	Correlation	0.63*	0.47*	- 0.10	0.60*	0.74*	0.33	- 0.22	0.51	0.44	0.80*

*$p < 0.05$ significantly different as indicated.

Group	Statistic test	LDL vs. LDL	TAG vs. TAG	TSH vs. TSH	Free T_4 vs. Free T_4	Insulin vs. Insulin	LDL vs. TAG	LDL vs. TSH	LDL vs. Free T_4	TAG vs. TSH	TAG vs. Free T_4	TSH vs. Free T_4
Control	Covariance	13.81	166.04	0.38	0.05	0.65	- 6.44	0.44	0.44	- 5.02	- 0.61	0.11
	Correlation	0.80	0.80	0.80	0.80	0.80	- 0.11	0.15	0.44	- 0.50	- 0.17	0.64
Normotensive	Covariance	1,979.21	194.61	1.07	0.06	0.58	484.83	45.88	- 10.71	11.86	- 2.11	- 0.24
	Correlation	0.02	0.00	0.31	0.04	0.75	0.00	0.08	- 0.03	0.02	0.00	- 0.11
Hypertensive	Covariance	753.45	206.83	1.29	0.14	0.31	15.82	26.05	- 8.16	- 2.38	- 1.02	- 0.35
	Correlation	0.92*	0.92*	0.92*	0.92*	0.92*	0.04	0.77*	- 0.73*	- 0.13	- 0.175	- 0.76*

*$p < 0.05$ significantly different as indicated.

Table 2. Covariance and correlation of blood samples

the control, normotensive and hypertensive groups are shown in Graph 2.

Also, correlations and covariance were calculated (Table 2).

EVALUATION OF THE VARIABLES ASSOCIATED TO THE HPT AXIS WITHIN THE CONTROL AND EXPERIMENTAL GROUPS

To classify the disturbance of HPT axis, the 10th, 50th and 90th percentiles for TSH and T_4 hormones were calculated. A significant difference ($p<0.05$) was found between the calculated critical values (Table 4). The results in the hypertensive rats were dissimilar. 23% of hypertensive rats reached normal values ($TSH_{p10\text{-}50}$ and Free $T_{4\ p10\text{-}50}$). One hypertensive rat developed hypothyroidism ($TSH_{p>90}$ and Free $T_{4\ p<10}$). Four out of thirteen rats developed primary hypothyroidism ($TSH_{p>90}$ and Free $T_{4\ p<10}$) and three out of thirteen hypertensive rats, subclinical hypothyroidism ($TSH_{p>90}$ and Free $T_{4\ p10\text{-}50}$).

A statistical difference in the control vs. normotensive rats was found, as well as in normotensive vs. hypertensive rats (Table 3).

DISCUSSION

A handmade diet based on the results found by Panchal, et al. was used for 7 months (Panchal et al., 2011). The stablished time to induce MetS (8 to 40 weeks) varies according to the diet and rat strain (Wong et al., 2016). In this study, it was 2.25 times longer than the chronic-exposure time stated by Lanzoni, et al. (Lanzoni et al., 2016). which is necessary to induce chronic toxicity in experimental conditions. Hence, the metabolic effect in the rats and their survival rate would be a result of the composition and continuity of the diet used.

The rats fed with the high-carbohydrate and fat diet had a survival rate of 55% corresponding to 104-week-old rats (Keenan et al., 1994; Esquivel-Solís; Gómez-Salas, 2007). Statistical difference was found between the amount of food consumed by the control vs. hypertensive rats fed with the high-carbohydrate and fat diet; the latter, composed of GLc and fructose. The metabolic effect in the rats fed with the high-carbohydrate and fat diet, as well as their survival rate, would be a result of the composition and continuity of the diet. In energy-rich diets, a decreased

Group	Control	Normotensive	Hypertensive
10th percentile TSH	2.13	1.47	1.04
50th percentile TSH	2.20*▵	3.00*▵	1.33*▵
90th percentile TSH	6.37◦	4.15◦	4.24◦
Group	**Control**	**Normotensive**	**Hypertensive**
10th percentile Free T_4	1.41	0.89	1.28
50th percentile Free T_4	1.82*▵	1.76*▵	1.73*▵
90th percentile Free T_4	2.91◦	1.52◦	2.13◦

Data shown are means ± SEM. 0.05 *p < 0.05 (p10 and p50); ▵p < 0.05 (p50 and p90); ◦p < 0.05 (p10 and p90) significantly different as indicated.

Table 3. TSH and Free T_4 Percentiles

absorption of GLc suppresses the hepatic gluconeogenesis stimulated by insulin, which develops hyperinsulinemia, IR and hyperglycemia and disrupts the HPT axis (Dergal, 2013). Moreover, energy-rich food is more desirable than low glycemic index food and generates a lower satiety due to the effect of the fat, contributing to hyperphagia and increases of weight, as found in this study (Ciranna, 2006; van der Klaauw; Farooqi, 2015; Avena et al., 2008). However, in the hypertensive group, a notorious weight loss is registered since week 18 up to week 26.

Multiple factors including hypermetabolism, excessive free radicals, and a chronic inflammation, have been implicated in tubulointerstitial lesions in diabetic nephropathy (Zheng; Zheng, 2016). The work load in tubular cells in diabetes is greatly elevated, as a result of an accumulated high serum glucose and related metabolites that increase osmolarity gradient and promotes hyperfiltration. Accordingly, energy generation and consumption are increased to meet the functional need, leading to a state of hypermetabolism that provokes cell stress and free radical overgeneration. An increased production of pro-inflammatory interleukins secretion conducts to cachexia in diabetes (Rehman; Akash, 2016). These mechanisms partially explain the weight loss registered in some of the hypertensive rats during this period, in contrast to the weight gained by others.

β cell failure is key to the onset and progression of any type of diabetes as well as an interplay of genetic and acquired factors that contribute to abnormal mechanisms, impaired function and reduced mass leading to inappropriately low blood insulin levels (Marchetti et al., 2020). These concepts are aligned with the HOMA-IR index results obtained in our study, which were significantly higher in the control group than in hypertensive group ($p<0.05$).

The hypertensive rats fed with the high-carbohydrate and fat diet met the MetS definition and presented a significant difference in the glycemic serum samples. Since insulin secretion is a very dynamic process, this result could be a consequence of a sympathetic activation without a counter-regulation during the rats' sacrifice which favored the increase of glycemia during the cardiac puncture. Nevertheless, new information is being published and it is now known that, when IR is well stablished, destruction of pancreatic B-cell population occurs (Lasheen et al., 2015). Furthermore, an early reduction of 30-50% of the islet volume could occur because of an increased death and reduced regeneration (Marchetti et al., 2020). Control rats showed normal values and the high-carbohydrate and fat diet fed rats showed a lower HOMA-IR index. Once again, oxidative stress could be another important mechanism through which an overload of glucose levels and/or free fatty acids lead to β cell impairment (Marchetti et al., 2020).

In the present study, a high and direct correlation between TSH and LDL cannot be rejected after 28 weeks of MetS induction; congruent with Yang et al. findings (Yang et al., 2016). Nevertheless, an inverse and high correlation between cholesterol and Free T_4 cannot be rejected. A high-cholesterol diet can induce hypercholesterolemia and LDL accumulation in pituitary tissue, leading to a pituitary disfunction. Hypertriglyceridemia associated with higher concentrations of TSH, as seen in our study, is known to contribute to chronic cellular dysfunction and to the disturbance of the mitochondrial membrane as a result of the free-radical action (Tallapragada; Karpe; Tikoo, 2015). The sensitizing action of thyroid hormones over catecholamines may explain the reason why only the above-mentioned findings developed

in hypertensive rats.

Thermogenesis implies the capacity to maintain an *ad hoc* range of body temperature in homoeothermic beings to perform optimal cellular processes. The thyroid hormones have a regulating role in this process increasing energy expenditure and thermogenesis. Metabolic status with energy excess, like in MetS, may inadequately stimulate the HPT axis due to a resistance to thyroid hormones; failing to maintain sufficient uncoupled thermogenic oxidative processes, and with it, increasing oxidative stress (Silva, 2006; Lacaustra et al., 2018). The changes obtained in thyroid hormones secretion could be a dynamic response that SD rats have developed as a consequence of a chronic exposure to high-energy diets.

In conclusion, there is not sufficient evidence to reject a positive association between hypothyroidism and MetS with hypertension in this male SD rat model.

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