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EFFECTS OF PRENATAL EXPOSITION TO TESTOSTERONE ON STRUCTURAL PARAMETERS OF ADIPOSE TISSUE IN SHEEP HEMBS

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Abstract: Prenatal exposure to excess testosterone (EPT) has shown a reprogramming effect on the metabolic function of the offspring, although there is no clarity on the direct impact on the adipose tissue, an essential organ in the regulation of energy in response to the insulin. The aim of this study was to determine whether EPT has an impact on the morphology and gene expression of subcutaneous (GSC) and visceral (GV) adipose tissue. Samples of adipose tissue from female sheep EPT were evaluated in fetuses of 120 days of gestation (120 dg) and in pubertal individuals (38 weeks of age). Histological sections of samples were evaluated for a morphometric analysis considering density, area, perimeter and larger diameter. After eso, the gene expression of the elements of the insulin signaling pathway was analyzed through qPCR assays. The EPT females did not show changes during the fetal stage, however, during the pubertal stage there was evidence of hypertrophy of adipose tissue cells in GV ($P<0.05$), and in GSC as well. While the insulin pathway expression profile, EPT in the fetal stage showed a suppressor effect of the pathway factors in GSC and GV, while at 38 weeks a suppressor effect of the GLUT4 expression in GSC ($P<0.05$) and promoter in GV ($P<0.05$), with a marked increase in the expression of Akt. These results suggest that EPT has repercussions on the metabolism of adipose tissue in females, with GV being more sensitive to the effects of testosterone.
Keywords: Fetal reprogramming, testosterone, adipose tissue, insulin, sheep.

INTRODUCTION

Adipose tissue represents between 3 and 70% of body weight in adult humans [10]. One of its main characteristics is the sensitivity that it presents in the face of changes in the balance of energy and nutrient intake, because changes in these factors from an early age could have repercussions on its development and functioning process.

Adipose tissue forms part of connective tissue and is composed of adipocytes, cells that store lipids in their cytoplasm. They are surrounded by highly vascularized and innervated connective tissue and at the same time contain macrophages, fibroblasts and precursor cells. Within its functions, adipocytes play a critical role in energy homeostasis, in the soil storing triglycerides, which also controls thermogenesis under neuroendocrine signals, food intake, immunity and neuroendocrine function [1]. The white adipose tissue is composed histologically by adipocytes with a single vacuole (unilocular), scarce cytoplasm and eccentric nucleus, its main function is to be a reserve of energy. It is the predominant tissue in adult human beings, in mammals this tissue is located at the general subcutaneous level, but specifically in the hind limbs and abdominal area [10, 8,27]; and surrounding viscera, mainly omentum, mesentery, kidneys and pericardium[1,23]

The process of adipogenesis begins on the 30th day of gestation in sheep, after the formation of the pancreas, and its increase is pronounced at the end of the gestation [26]

The adipose tissue actively participates in energy homeostasis. On the one hand, it constantly responds to changes in the balance of energy and nutrient intake, which alters certain parameters such as proliferation of preadipocytes and their differentiation into mature adipocytes, hypertrophy of these and finally apoptosis [10]. On the other hand, it fulfills an endocrine function in secreting leptin, adiponectin and resistin, among a series of other factors involved in metabolism, for example, the biological action of insulin on adipose tissue depends primarily on the interaction with its membrane receptor (IR), which once combined with the ligand, the

protein manifests a conformational change that leads to the activation of its substrates: Insulin Receptor Substrate IRS-1, IRS-2, IRS-3, IRS-4, IRS-5 and IRS -6. These molecules produce the recruitment of the enzyme phosphatidylinositol-3-kinase (PI3K) which promotes the second messenger of the membrane protein to activate a downward signaling through the protein kinase B (PKB/Akt). Finally, protein kinase C (PKC) is activated, which results in a migration of intracellular vesicles that contain the glucose transporter GLUT4, which is synthesized and its fusion with the plasma membrane for glucose uptake and internalization[4].

Insulin Resistance is defined as the deficient uptake of glucose by different tissues and the inability to maintain euglycemia, with the suppression of glucose synthesis at the hepatic level and lipolysis, resulting in an increase in insulin requirements [12,3,18]. An activity outside the signaling route could lead to disturbances in metabolism and homeostasis [20]. At the same time, this route is sensitive to external signals and depends on different extracellular stimuli for its correct functioning [4]. It has been determined that prenatal exposure to androgens acts as a fetal reprogramming factor that has triggered adverse effects on reproductive and metabolic function [21, 15, 11, 14]. In relation to the aforementioned background, it is considered prudent that testosterone could have a reprogramming effect on fatty tissue, altering glucose uptake both in the fetal and postnatal stages, being similar to the pathological situation of polycystic ovary syndrome (PCOS) in pregnant women, a condition characterized by a dysfunction of ovarian steroidogenesis resulting in hyperandrogenism [24].

MATERIALS AND METHODS

The sheep model has been a tool validated for these studies, and during the last few years this species has been used to demonstrate the effects of prenatal exposure to testosterone considering changes in endocrine-reproductive aspects with the woman and the easy handling of the species. The sheep model has concluded reproductive and metabolic characteristics in agreement with the PCOS [22].

This study considers the unrestricted use of research animals in which the procedures indicated will be carried out following the guidelines established by the Bioethics Committee of the Faculty of Veterinary Sciences of the Universidad de Concepción.

ANIMALS

40 adult eggs of Suffolk Down breed will be used. They will be distributed into groups of 20 treated (T-female) and 20 control (C-female) animals. After the selection, a synchronization of estrus and crosses with males of proven fertility will be carried out. Estrus synchronization will be performed with an intravaginal progesterone implant for 7 days (Eazy Breed, Zoetis) followed by a single intramuscular injection of 75 µg of prostaglandin PGF2 alpha (Ovolute, Dragpharma). Finally, diagnosis and control of pregnancy will be carried out through ultrasound control after the 28th day of the mountain.

An androgenization protocol validated by Sir-Petermann et al., 2002 will be carried out; Recabarren et al., 2008.

The samples of omentum (visceral grease, GV) and femoral region (subcutaneous grease, GSC) will be extracted.

For the morphological analysis of the adipose tissue, 3 representative microphotographs (40x) will be taken and selected randomly from the sections of each animal using a Leica DM2000 microscope and DF295 camera (Leica). The images obtained will be analyzed with the ImageJ software (NIH, USA) to

determine the number, diameter, perimeter and area of each adipocyte. The results will be expressed as average ± standard error of the average (average ± SEM).

MOLECULAR ASSAYS

Between 25 and 50 mg of subcutaneous (GSC) and visceral (GV) grease will be homogenized with a BioLogics 300 UT ultrasound sonicator (Biologics Inc. USA) in 5 pulses at 70% power in hielo. The RNA extraction will be performed in 1 mL of TRIzol (Invitrogen) following the manufacturer's instructions. The expression assays of the elements of the insulin signaling route (IR, IRS-1, IRS-2, PI3K, Akt, PKC and GLUT4).

RESULTS

Both at the fetal stage and at 38 weeks of age, a similar behavior is observed between control and treated individuals in terms of density, area, perimeter and diameter of adipocytes.

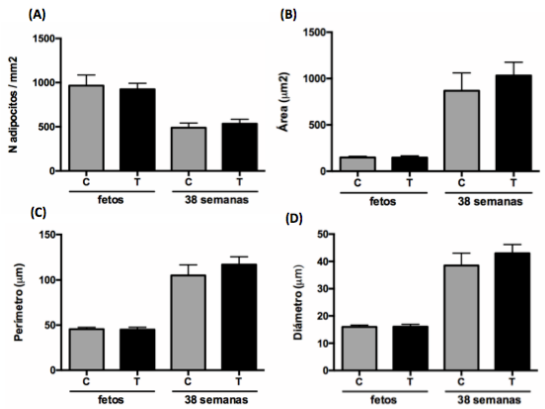

Figure 7. Comparison of morphological parameters in subcutaneous fat. A, Number of adipocytes (mm2). B, Area of adipocytes (µm2). C, Perimeter adipocytes (µm). D, Larger diameter of adipocytes (µm). C, control; T, EPT.

With respect to visceral adipose tissue, during the fetal stage a similar trend is observed in morphological parameters. At 38 weeks of age there is an increase in these parameters with statistically significant differences between density, area, perimeter and diameter ($p = 0.0083$, $p < 0.0001$ $p = 0.0149$ and $p = 0.0206$, respectively)

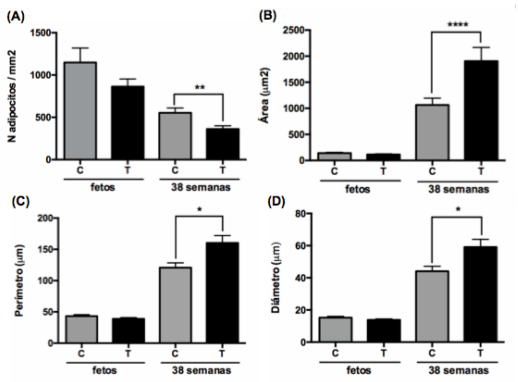

Figure 9. Comparison of morphological parameters in visceral fat. A. Number of adipocytes (mm2). B. Area of adipocytes (µm2). C. Perimeter adipocytes (µm). D. Larger diameter of adipocytes (µm). C, control; T, EPT* Statistically significant differences.

At the fetal level, the EPT group shows an increase in the expression of IR, IRS-1, IRS-2, PKC, PI3K and Akt genes; being significant only the expression of Akt (p=); mean that a significant decrease in the final expression of GLUT4 (p=). At 38 weeks of age there is an increase in the expression of IR and IRS-2 but a decrease in IRS-1 (p=x), PKC (P=x), PI3K, Akt (p) and GLUT4 (p) in EPT individuals.

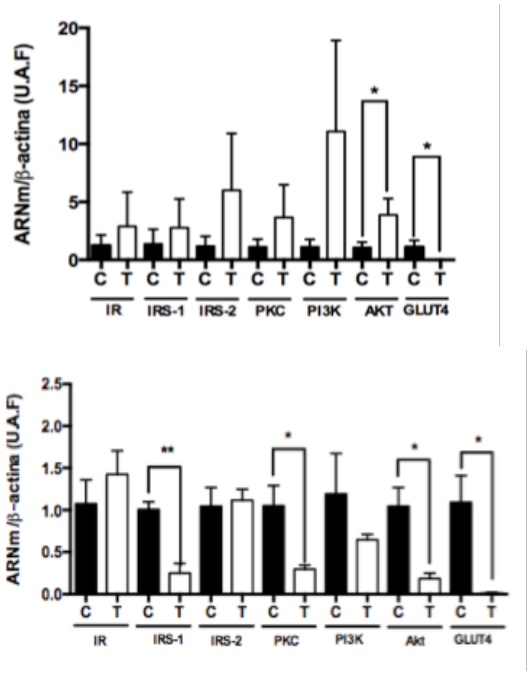

Figure 10. Gene expression profile of insulin signaling pathway in subcutaneous grease. A. Control (C) and treated (T) fetuses. B. Control (C) and treated (T) 38-week adults.* Statistically significant differences.

In the case of visceral fat, it is observed that at the fetal level there is an increase in the expression of IR and IRS-1 and a decrease in IRS-2 (p=), PKC, PI3K, AKT and GLUT4 in EPT individuals. At 38 weeks of age, the mayor differs from expression in the increase in PI3K and AKT (p).

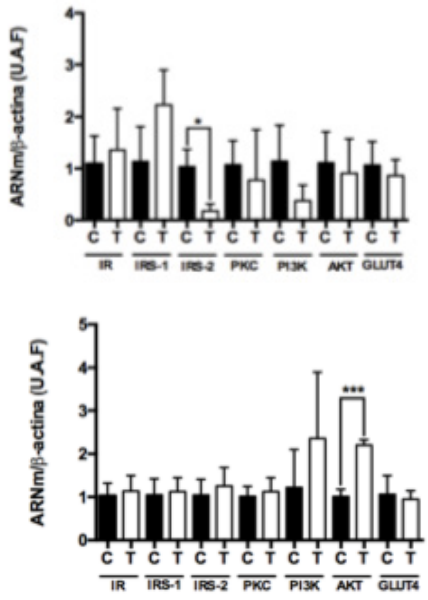

Figure 11. Gene expression profile of insulin signaling pathway in visceral fat. A. Control (C) and treated (T) fetuses. B. Control (C) and treated (T) 38-week adults.

*Statistically significant differences.

DISCUSSION

We evaluated the effect of prenatal exposure to testosterone on the adipose tissue of female sheep at different stages of life. During the fetal stage, the morphological parameters evaluated; Density, area, perimeter and diameter - of the adipose cells in the subcutaneous and visceral fat showed changes between the prenatally exposed testosterone group and the control group. The adipose tissue cells eat the lipid accumulation around the 70th day of gestation until the 30 days after birth [9, 26], so these results could suggest that at the 120th day of gestation, the moment of extraction of the samples, the adipose tissue is still in development and prenatal exposure to testosterone would not be a factor that influences the morphology during this period. As for the results of the insulin receptor function at this stage, an overexpression of mRNA of all the factors of the signaling pathway, especially Akt, is

observed, but the final decrease of the GLUT4 transporter in subcutaneous grease. It is a determining point in the signaling route [4], and in this case, this effect is manifested to produce a marked negative effect on GLUT4. On the other hand, the results of visceral fat in fetuses revealed a pronounced decrease in IRS-2 and a tendency to go down in the rest of the signaling route, which would be a critical point. In previous studies, it has been established that IRS-2 plays an important role in the transport of glucose at the preadipocyte level, and this could be linked to an immature tissue [17]. Subsequently, sexually mature females were evaluated at 38 weeks of age to rule out hormonal and physiological changes related to puberty. The results show that there is an increase in all the values of the evaluated morphometric parameters of the adipocytes, with a slight tendency in subcutaneous fat but very significant differences in visceral fat ($p < 0.05$). This last tissue increases both in area, perimeter and diameter of cells, decreasing the density by field, which suggests that the reprogramming effect of testosterone could exist over a long period of time. These results concur with the increase of these measurements in adipocytes of rats prenatally exposed to testosterone [2]. In the same way, studies of women with PCOS present aberrant adipocytes in size [7, 16]. In terms of the gene expression of these adipocytes, the results showed a significant decrease in IRS-1 and the use of all components of the route in subcutaneous grease. There is an IRS-1 deficit highly correlated with insulin resistance [5]. On the other hand, studies have shown that the in vitro culture of adipose tissue from women with PCOS does not present alterations in this protein, and it is proposed that it would be the effect of testosterone in vivo that could produce the alteration of the expression of IRS- 1[6] In view of the morphometric and gene expression differences between both types of fat, it can be inferred that there are changes in their metabolism, since the most significant increase is reflected in the visceral adipose tissue. This is due to the fact that visceral fat has a greater blood flow, metabolic rate and insulin response in comparison with subcutaneous fat [28, 13]. In women with PCOS, there is an increase in abdominal fat and greater risks are present as a result of metabolic dysfunctions, associated with hyperglycemia and hyperinsulinemia [25]. It is worth noting that both in GSC and GV it reduces the expression of the final mRNA of GLUT4. A low level of GLUT4 is a premature cellular indicator of insulin resistance [5], so this study could establish a trend towards insulin resistance. Also, the effect is more pronounced in GSC, which would be contradictory with the morphometric parameters evaluated, which shows a greater effect in GV due to the fact that the response in the size of adipose cells is inversely proportional to the expression of GLUT4. Based on the data obtained in this study, it is possible to suggest that the results are a consequence of prenatal exposure to testosterone with an effect on both morphometry and energy metabolism of adipose tissue.

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