

Dyslipidemia's influence on the secretion ovarian's steroids in female mice

Influência da Dislipidemia na Secreção de esteroides ovarianos em Camundongas

Influencia de la dislipidemia en la secreción de esteroides ováricos en ratones

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Abstract

Introduction: The synthesis ovarian's steroids is a process that depends on the supply of cholesterol. **Objective:** to evaluate the influence of dyslipidemia on the secretion ovarian's steroids. **Methodology:** wild female mice were used (C57BL6) and LDL (LDLR^{-/-}), which they were separated into 4 groups (n = 10): WTS: fed a standard diet; WTHL: fed a high-fat diet; KOS: fed a standard diet; KOHL: fed a high-fat diet. After 60 days, the estrous cycle was analyzed and after anesthetized, blood was collected for the to assess the lipid profile, glucose, plasma insulin level and HOMA index was calculated. In addition, plasma levels of C-reactive protein, estrogen and progesterone were determined. **Results:** The hyperlipidic diet in both the WTHL and the KOHL group generated hypercholesterolemia when compared to the WTS and KOS, respectively, with a decrease in HDLc, associated with an increase in CRP levels. Severe hypercholesterolemia in the KOHL group generated insulin resistance, marked by an increase in HOMA_{air}. Food hypercholesterolemia in the WTHL group, food and genetics in the KOHL group, compared to their WTS and KOS controls, was definitive in reducing plasma levels of estrogen and progesterone. The genetic hypercholesterolemia associated with insulin resistance observed in the KOS and KOHL groups reduced the levels of progesterone, this reduction being more severe in the KOHL group, which had the highest HOMA_{air}. **Conclusion:** dyslipidemia affected ovarian steroidogenesis in mice by means of oxidative stress, inflammation and insulin resistance and / or by decreasing HDL cholesterol levels.

Keywords: Hypercholesterolemia; Estrogen; Progesterone; Oxidative stress.

Resumo

Introdução: A produção de esteroides ovarianos é um processo dependente do suprimento de colesterol. **Objetivo:** avaliar a influência da dislipidemia na secreção dos esteroides ovarianos. **Metodologia:** Utilizou-se camundongas "wild type" (C57BL6) e knockout para o gene do receptor de LDL (LDLR^{-/-}). Foram separadas em 4 grupos (n=10): WTS: receberam ração padrão; WTHL: receberam ração hiperlipídica; KOS: LDLR^{-/-}, receberam ração padrão; KOHL: LDLR^{-/-}, receberam ração hiperlipídica. Após 60 dias, foi analisado o ciclo estral e o sangue foi coletado para a avaliar o perfil lipídico, glicose, nível plasmático da insulina, e o índice de HOMA foi calculado. Além disso, os níveis plasmáticos de proteína C reativa, estrógeno e progesterona foram determinados. **Resultados:** A dieta hiperlipídica tanto no grupo WTHL quanto KOHL gerou uma hipercolesterolemia quando comparadas aos WTS e KOS, respectivamente, com diminuição de HDLc, associada ao aumento dos níveis da PCR. A hipercolesterolemia severa no grupo KOHL gerou uma resistência insulínica, marcada por aumento do HOMA_{air}. A hipercolesterolemia alimentar no grupo WTHL, alimentar e genética no grupo KOHL, comparada com seus controles WTS e KOS, foi determinante para reduzir os níveis plasmáticos de estrógeno e progesterona. A hipercolesterolemia genética associada à resistência insulínica observada nos grupos KOS e KOHL reduziu os níveis de progesterona, sendo essa redução mais grave no grupo KOHL, que apresentou maior HOMA_{air}. **Conclusão:** a dislipidemia afetou a esteroidogênese ovariana em camundongas por vias que envolvem o estresse oxidativo, inflamação e resistência insulínica e/ou pela diminuição dos níveis de HDL colesterol.

Palavras-chave: Hipercolesterolemia; Estrógeno; Progesterona; Estresse oxidativo.

Resumen

Introducción: La producción de esteroides ováricos es un proceso dependiente del aporte de colesterol. **Objetivo:** evaluar la influencia de la dislipidemia en la secreción de esteroides ováricos. **Metodología:** Se utilizaron ratones de tipo salvaje (C57BL6) y knockout del gen del receptor de LDL (LDLR^{-/-}). Se dividieron en 4 grupos (n = 10): WTS: recibió comida estándar; WTHL: recibió una dieta alta en grasas; KOS: LDLR^{-/-}, recibió comida estándar; KOHL: LDLR^{-/-}, recibió una dieta alta en grasas. Después de 60 días, se analizó el ciclo estral y se extrajo sangre para evaluar el perfil de lípidos, la glucosa, el nivel de insulina en plasma y se calculó el índice HOMA. Además, se determinaron los niveles plasmáticos de proteína C reactiva, estrógeno y progesterona. **Resultados:** La dieta alta en grasas en los grupos WTHL y KOHL generó hipercolesterolemia en comparación con los grupos WTS y KOS, respectivamente, con una disminución de cHDL, asociada con un aumento en los niveles de PCR. La hipercolesterolemia severa en el grupo KOHL generó resistencia a la insulina, marcada por un aumento en HOMA_{air}. La hipercolesterolemia dietética en el grupo WTHL, la hipercolesterolemia dietética y genética en el grupo KOHL, en comparación con sus controles WTS y KOS, fue fundamental para reducir los niveles plasmáticos de estrógeno y progesterona. La hipercolesterolemia genética asociada con la resistencia a la insulina observada en los grupos KOS y KOHL redujo los niveles de progesterona, siendo esta reducción más severa en el grupo KOHL, que tenía mayor HOMA_{air}. **Conclusión:** la dislipidemia afectó la esteroidogénesis ovárica en ratones a través de vías que implican estrés oxidativo, inflamación y resistencia a la insulina y / o mediante la disminución de los niveles de colesterol HDL.

Palabras clave: Hipercolesterolemia; Estrógeno; Progesterona; Estrés oxidativo.

1. Introduction

The production of ovarian hormones – progesterone and estrogen – is a complex process that occurs in granulosa and theca cells (Miller & Auchus, 2011) and depends on the supply of cholesterol substrates (Dyer & Curtiss, 1988; Argov & Sklan, 2004). Evidence suggests that plasma lipoproteins are the major source of cholesterol for the production of steroids in the adrenal glands and ovaries (Azhar, Leers-Sucheta & Reaven, 2003). Henderson et al. (1981) stated that changes in the amount or type of cholesterol present in the blood may play an important role in the regulation of steroid hormone biosynthesis by the ovary.

Dyslipidemic animals, which have altered lipid levels, may have impaired fertility. According to Miettinen, Rayburn and Krieger (2001) dysfunctions in HDL metabolism affect female fertility, in addition, polycystic ovary syndrome (PCOS) may be associated with dyslipidemia (Premoli et al., 2000).

LDLR - / -, knockout (KO) mice for the LDL receptor were developed to understand the consequences of this deficiency, how to reverse it and assist in the development of gene therapy techniques for the expression of hepatic LDL receptors (Ishibashi et al., 1993). Cell surface low density lipoprotein (LDLR) receptors play a key role in regulating plasma cholesterol levels by mediating the absorption of LDL and IDL (intermediate density lipoprotein) (Carmeliet, Moons & Collen, 1998). Such receptors measure the output of lipoprotein from plasma, by recognizing B and E particles on its surface (Breslow, 1993).

Studies in our laboratory showed that LDLR - / - mice had increased blood levels of LDLc, VLDLc and triglyceride lipoproteins with or without high-fat diets, generating mixed dyslipidemia (Garcia et al. 2011; Sarto et al., 2018; Martins et al., 2020), exhibiting a scenario of moderate hypercholesterolemia similar to that described in humans (Ishibashi et al., 1993). Research has shown that genetic dyslipidemia associated with food increased inflammatory markers and generated insulin resistance in LDLR - / - mice, causing damage to the cardiovascular system (Garcia et al., 2011; Santos et al., 2017) and greater stress oxidative (Sarto et al., 2018; Martins et al., 2020).

Guo et al. (2015) demonstrated that LDLR - / - mice showed no change in progesterone levels, but had a decrease in estrogen concentration when compared to wild mice. The aim of this study was to evaluate and correlate the influence of food and genetic dyslipidemia on the secretion of ovarian steroid hormones in mice

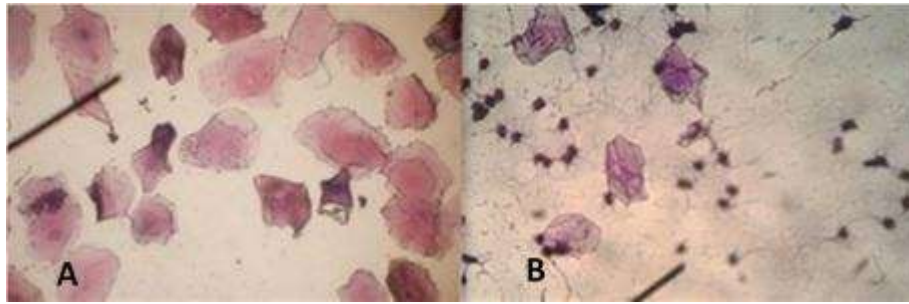
2. Methodology

The experiments were carried out in wild type mice (strain C57BL6) and in homozygous mice for the absence of the LDL receptor gene (LDLR - / -), generated in the background C57BL6 (knockout), females with 70 ± 5 days, weighing 22 ± 2 g. The animals came from the UNIFENAS Graduate School vivarium with temperature control and 12-hour control in the light/dark cycle. The use of animals and the experimental protocol were forwarded and approved by the Ethics Committee on the Use of Animals (CEUA, in portuguese), from José do Rosário Vellano University, process n° 23A/2017.

The animals were separated into 4 experimental groups, each containing 10 animals ($n = 10$), namely: WTS group: wild type mice, which received standard food (Nuvital®) for 60 days; WTHL group: wild type mice, which received a high fat diet with 20% total fat and 1.25% cholesterol, 0.5% cholic acid for 60 days; KOS group: LDLR - / - mice, which received standard food (Nuvital®) for 60 days; KOHL group: LDLR - / - mice, which received a high fat diet with 20% total fat and 1.25% cholesterol, 0.5% cholic acid for 60 days. All animals were fed the respective diets and received water ad libitum.

After 60 days of experiment, the stage of the estrous cycle of each female was analyzed and noted, through the collection of vaginal material and smear preparation, which was stained with hematoxylin and eosin. The slides were analyzed under an optical microscope and classified according to the estrous cycle stage in estrus (A) and diestrus (B) (Figure 1).

Figure 1 – Collection of vaginal secretion for the evaluation of the estrous cycle of mice, estrus (A) and Diestrus (B)



Source: Authors.

Once the estrous cycle phase was determined, the mice were anesthetized intraperitoneally (ip), using Xylazine/Ketamine (Bayer AS and Parke-Davis®) at a concentration of 6-40 mg/Kg, respectively.

After anesthesia, blood was collected intracardially. For the measurement of serum triglycerides, total cholesterol (TC) and high-density lipoprotein (HDLc), enzymatic assays were used as described by Hedrick et al. (2001). Low-density lipoprotein (LDLc) was determined using the formula by Friedewald, Levy and Fredrickson (1972) and very low-density lipoprotein (VLDLc), as described by Tian et al. (2006).

The determination of the circulating glucose concentration was performed by the colorimetric enzymatic method, using the PAP Liquiform glucose kit from Labtest Diagnóstica. The plasma level of insulin was measured with the specific commercial ELISA kit (Dako Ltd High Wycombe, Bucks, UK). The HOMA index (HOMA-ir) was calculated using the formula: $\{HOMA-ir = \text{fasting insulinemia [mU/L]} \times \text{fasting blood glucose [mmol/L]} / 22.5\}$ to determine insulin resistance. The quantitative determination of C-reactive protein (CRP) was done by means of turbidimetry and photometry (Humastar 300® Apparatus), expressing the results in mg/dL (Lima et al., 2000).

For the quantification of estrogen and progesterone, blood was collected with anticoagulant and, after centrifugation, the plasma from around 250 μ L was frozen and sent for analysis. The plasma estrogen concentration was quantified by the double antibody radioimmunoassay technique, using the iodine (125I) estradiol Radioimmunoassay kit (Jiuding Biological Engineering, Tianjin, China). The coefficients of variation (CVs) of the inter and intra test were 8.9% and 7.7%, respectively. The sensitivity of the test was 2.1 pg/mL. Plasma progesterone concentrations were detected using the iodine kit (125I) Progesterone Radioimmunoassay Kit (Jiuding Biological Engineering, Tianjin, China). The sensitivity of the test was 0.03 ng/mL. The inter and intra test interaction CVs were 8.9% and 7.2%, respectively.

The collected data were processed by the analysis of variance test (one-way ANOVA) to compare continuous data, followed by the Tukey test, used to compare the means between different groups. The differences were considered significant when the value is $p < 0.05$. Serum and plasma determinations were expressed as mean \pm standard error of mean (SEM).

3. Results and Discussion

In the analysis of the lipid profile, there was an increase in the serum levels of TC and the LDLc fraction in the KOS group in relation to the WTS group. However, the mice in the KOS group showed a serum HDLc level similar to the WTS group (Table 1). It was also found that the consumption of a high-fat diet, in wild type mice of the WTHL group, increased the serum levels of TC and the LDLc fraction, with a decrease in the HDLc fraction, without altering the levels of triglycerides, when compared with the mice of the WTS group (Table 1). When LDLR - / - mice received high fat diet (KOHL group), they presented severe hypercholesterolemic dyslipidemia, with a significant increase in TC, their LDLc fraction and a decrease in

their HDLc fraction, when compared with all experimental groups (Table 1). Regarding serum triglyceride levels, there were no differences between the groups studied (Table 1).

Table 1 - Comparison of serum total cholesterol levels and their fractions (LDLc, HDLc, VLDLc), triglycerides, insulin and glucose between wild type mice, fed a standard rodent diet (WTS) and a high fat diet (WTHL), and mice knockout for LDL receptor (LDLR - / -), fed a standard diet (KOS) and a high fat diet (KOHL).

Groups	WTS	WTHL	KOS	KOHL
N	9	9	9	9
Total cholesterol (TC) (mg/dL)	105,27±3,36 ^c	141,77±4,97 ^b	174,23±20,40 ^b	232,58±19,37 ^a
LDLc (mg/dL)	39,21±2,13 ^c	97,56±4,91 ^b	111,87±29,46 ^b	197,43±22,05 ^a
HDLc (mg/dL)	58,25±2,45 ^a	33,87±1,99 ^b	50,27±1,79 ^a	25,16±4,26 ^c
VLDLc (mg/dL)	8,19±0,61	10,05±1,13	12,45±1,21	10,16±2,66
Triglycerides (mg/dL)	40,98±3,03	54,15±7,5	62,33±6,07	50,93±13,35
Glucose (mMol/L)	4,5±0,3	4,5±0,2	4,5±0,2	4,6±0,3
Insulin (mU/L)	2,3±0,2 ^c	3,1±0,2 ^b	3,2±0,4 ^b	4,9 ±0,8 ^a
HOMA-ir	0,5±0,06 ^c	0,6±0,05 ^{b,c}	0,7±0,01 ^b	1,0±0,02 ^a
CRP mg/dL	4±0,5 ^d	5,8±0,3 ^c	8,8±0,7 ^b	13±0,8 ^a

Values were expressed as mean ± standard error of the mean. Different letters on the same line indicate significant differences between groups (p <0.05, Tukey test).

Source: Authors.

In the glyceimic profile, we did not observe significant differences between the groups studied. The hyperlipidic diet, in wild type mice (WTHL group), induced an increase in serum insulin levels when compared to mice in the WTS group. There was no difference in insulin levels between the WTHL and KOS groups. The hyper lipidic diet, in LDLR - / - mice (KOHL group) induced a significant increase in serum insulin levels and the HOMA index, when compared with the other experimental groups (Table 1), inducing insulin resistance in the mice of this group .

As for the CRP analysis, we observed that the levels of this protein increased in the mice of the WTHL and KOHL group, when the respective WTS and KOS controls were compared. However, the KOS group showed a higher serum level when compared to the WTHL group.

Plasma levels of estrogen and progesterone evaluated in estrus and diestrus, respectively, showed that there was a decrease in both in the KOS group when compared to the wild group WTS. It was also demonstrated that the high-fat diet in the mice of the WTHL and KOHL group reduced both estrogen and progesterone when compared with their respective WTS and KOS groups (Table 2). An interesting result observed regarding estrogen levels is that there was no difference between the WTHL and KOS groups.

Table 2 – Comparison of plasma levels of estrogen (estrus phase) and progesterone (diestrus phase) between wild type mice fed a standard rodent diet (WTS) and a high-fat diet (WTHL), and knockout mice for the LDL receptor (LDLr - / -) fed a standard diet (KOS) and a high-fat diet (KOHL)

	WTS	WTHL	KOS	KOHL
N	6	6	6	6
Plasma estrogen levels in the estrus phase (pg/mL)	10±0,5 ^a	7±0,4 ^b	6±0,6 ^b	4±0,3 ^c
Plasma levels of progesterone in the diestrus phase (ng/mL)	41±1,5 ^a	32±2,0 ^b	20±1,1 ^c	13±1,0 ^d

Values are expressed as mean ± standard error of the mean. Different letters on the same line indicate significant differences between groups ($p < 0.05$, Tukey test) (n = 6 animals per group). Source: Authors.

3.1 Discussion

In the present study, it was observed that the hyper lipidic diet, in both mice of the WTHL and KOHL group generated hypercholesterolemic dyslipidemia when compared to their controls (WTS and KOS groups), with a decrease in HDLc levels. Hypercholesterolemic dyslipidemia, characterized by increased serum levels of total cholesterol and LDLc, caused by the ingestion of a hyper lipidic diet, both in the WT (WTHL group) and KO (KOHL) mice was expected as this type of dyslipidemia was widely registered in several studies (Yokode et al., 1990; Ishibashi et al., 1994; Martins et al., 2020). The main effect of this diet on plasma lipids is an increase in the number of apoB-containing lipoproteins (VLDL, remnants and LDL) in relation to HDL (Fazio & Liton, 2001).

The constant increase in CRP levels and hypercholesterolemia in mice from the WTHL, KOS and KOHL groups indicated an increase in the inflammatory process. In addition, studies have shown that a small exposure to high cholesterol diets can induce hypercholesterolemia and, consequently, oxidative stress (Sarto et al., 2018; Tomofuji et al., 2006; Aleisa et al., 2013). Hypercholesterolemia may be the factor that leads to inflammation and the induction of oxidative stress in mice (Garcia et al., 2008).

Egnatchik et al. (2014) stated that, in cases of a diet rich in carbohydrate, fat, or high fatty-acid supply, there will be more substrate for the mitochondria to synthesize ATP. In this way, the mitochondria is more active and produces more of its natural by-product known as reactive oxygen species (ROS) (Henriksen, Diamond-Stanic & Marchionne, 2011). When the levels of reactive species are increased in relation to the antioxidant defense capacity, they can cause significant cellular damage (Souza, 2018), which is known as oxidative stress. Studies reported that reactive oxygen species induced the release of pro-inflammatory cytokines (Shoelson, Herrero & Naaz, 2007; Souza, 2018), with the production of free radicals being stimulated by the inflammatory process (Souza, 2018), generating a vicious cycle.

Oxidative stress increases the lipid peroxidation of the HDL molecule and, consequently, its hepatic removal (Christison et al., 1996), leading to a decrease in its serum levels, as observed in the mice of the WTHL and KOHL groups. HDL has antioxidant and anti-inflammatory functions (Nofer et al., 2002; Chapman, 2004; Assmann & Gotto, 2004; Von Eckardstein & Asmann, 2000, Alsheikh-Ali, Kuvin & Karas, 2005), and its reduction may also contribute to the elevation of oxidative stress and consequent tissue inflammation in the mice in the present study.

Oxidative stress and tissue inflammation in the WTHL, KOS and KOHL mice induced a constant increase in insulin and HOMA-ir levels, indicating insulin resistance. Studies have shown that exposure to oxidative stress in the soleus muscle, induced by hydrogen peroxide, caused a decrease in the activity of molecules involved in insulin signal transduction, such as: IRS-1 (insulin receptor substrates I), IRS-2 and reduction in phosphorylation of Akt (serine/threonine kinase) (36). The

inflammatory process, induced by oxidative stress, can also contribute to the increase in insulin resistance, observed in the mice in the present study. Souza (2018) stated that inflammatory mediators interfere in specific sites of the transduction of insulin signaling pathways, such as, for example, TNF α , which is a cytokine capable of modulating the peripheral action of insulin (Wensveen et al., 2015) e and inhibit insulin receptor signaling, preventing the spread of its signal (Yamasaki et al., 2018).

Insulin resistance has also been linked to a decrease in high density lipoproteins (HDL) (Premoli et al., 2000). Insulin has an anti-inflammatory profile (Dandona et al., 2013) and the increase in its levels in the mice of the group (KOHL) may have occurred by a compensatory effect, in which the body seeks to reduce the inflammation generated by oxidative stress and decreased HDLc.

The LDLR - / - mice which received the high-fat diet (group KOHL) showed insulin resistance and reduced levels of progesterone and estrogen. Insulin receptors are widely distributed in all parts of the ovaries, including granulosa, theca, stroma and oocyte cells (Myers et al., 1991; Sirotkin et al., 1998; Louhio et al., 2000), and stimulate a signaling pathway for the transport of glucose, or an alternative signaling for the stimulation or inhibition of steroidogenesis, which may involve the generation of inositol glycan. Chaves et al. (2011) reported that the inositol glycan signaling system can mediate insulin modulation in steroidogenic enzymes, such as P450 ssc, P450 c17 or aromatase P450.

In cultured ovarian cells, insulin acts specifically on non-luteinized granulosa cells, increasing the stimulation of follicle stimulating hormone (FSH) to produce ovarian steroids (Bhatia & Price, 2001). Van den Hurk Zhao (2005) stated that systemic insulin and luteinizing hormone (LH) activate the production of thecal androgens in secondary follicles, which induce the synthesis of FSH receptors in the granulosa. Thus, through the receptors, insulin acts as a co-gonadotropin to modulate ovarian steroidogenesis (Diamanti-Kandarakis & Dunaif, 2012). Thus, in the present study, inflammation and oxidative stress triggered by food and genetic dyslipidemia induced insulin resistance, probably through damage to insulin signaling pathways, including those responsible for the production of steroid hormones, FSH and LH, reducing serum levels of estrogen and progesterone.

On the other hand, the decrease in HDL levels, seen in the WTHL and KOHL group, may also have contributed to the reduction of progesterone and estrogen levels. The culture of luteinized cells of the granulosa of rats preferably uses HDL for steroidogenesis, even in the presence of LDL receptors (Andersen & Dietschy, 1978; Hwang & Menon, 1983; Paavola et al., 1985). Studies have shown that HDL lipoproteins have been associated with female reproduction because in several mammalian species these are the only class of lipoproteins detected, substantially, in the follicular fluid (Brantmeier; Grummer & Ax, 1987; Simpson et al., 1980). Therefore, it can be inferred that a drop in HDLc levels can lead to decreased secretion of steroid hormones, as observed in the present study.

Dietary hypercholesterolemia, observed in the WTHL group, and dietary and genetic hypercholesterolemia, observed in the KOHL group, compared to their respective WTS and KOS controls, were determinant to reduce plasma levels of estrogen and progesterone. Genetic hypercholesterolemia associated with insulin resistance observed in mice in the KOS and KOHL group reduced progesterone levels, with this reduction being more severe in the KOHL group, which had a higher HOMA-ir. However, no differences were observed between food hypercholesterolemia (WTHL group) and genetic hypercholesterolemia (KOS group), with no differences between these two groups observed in HOMA-ir and in serum estrogen levels.

4. Conclusion

In conclusion, both dietary and genetics dyslipidemia and the association of the two affected ovarian steroidogenesis in mice by means of oxidative stress, inflammation and insulin resistance and/or decreased HDL cholesterol levels.

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