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Differential effects of a high-fat diet on serum lipid parameters and ovarian gene expression in young and aged female mice

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Summary

The aim of this study was to compare serum lipid profiles and ovarian gene expression between aged and younger female mice fed a control or a high-fat diet for 2 months. For this 16 female mice (C57BL/6) of 4 months (Young, $n = 8$) or 13 months (Old, $n = 8$) of age were used. The females were divided into four groups: (i) young females fed a normal diet; (ii) young females fed a high-fat diet; (iii) old females fed a normal diet; and (iv) old females fed a high-fat diet. Food intake was reduced ($P < 0.05$) in mice fed with a high-fat (2.9 ± 0.1 g) diet in comparison with control mice (3.9 ± 0.1 g). Body weight was higher for old females on the high-fat diet (35.1 ± 0.3 g) than for young females on the same diet (23.3 ± 0.4 g; $P < 0.05$). PON1 activity was lower in the high-fat than control diet group (114.3 ± 5.8 vs. 78.1 ± 6.0 kU/L, respectively) and was higher in older than younger females (85.9 ± 6.4 vs. 106.5 ± 5.3; $P < 0.05$, respectively). Females fed a high-fat diet had lower expression of *Igf1* mRNA ($P = 0.04$). There was an interaction between age and diet for the expression of *Gdf9* and *Survivin*, with lower expression in older females in both diets and young females that received the high-fat diet ($P < 0.05$). Concluding, the high-fat diet reduced the expression of ovarian *Igf1* mRNA, and *Gdf9* and *Survivin* mRNA in younger females, which can indicate lower fertility rates. High-density lipoprotein concentration and PON1 activity were higher in aged female mice.

Keywords: AMH, FOXO3a, IGF-1, SIRT1

Introduction

Several studies indicate that ageing reduces female fertility due to a reduction of the ovarian oocyte reserve, represented by primordial quiescent follicles (te Velde *et al.*, 1998). The number of primordial follicles in women decreases from 700,000 at the beginning of life to 1000 at the beginning of menopause at the mean age of 51 years (Faddy *et al.*, 1992). The initial pool of primordial follicles gradually decreases as they are recruited to ovulate, a process strictly regulated to prevent premature exhaustion of reserves (Gougeon *et al.*, 1994). Current evidence points to a reduction in oocyte quality and embryonic abnormalities as the main cause of lower fertility observed in women after the age of 35 (van Noord-Zaadstra *et al.*, 1991). Therefore, prolonging reproductive lifespan in females will have a direct impact on the fertility window.

A metabolic pathway that has attracted interest in this regard is the phosphotidylinositol 3 kinase (PI3K)/protein kinase B (Akt1) pathway, an important regulator of cell proliferation and survival, which is involved in activation of primordial follicles (Brown *et al.*, 2010; Reddy *et al.*, 2008). It is known that activation of PI3K/Akt1 and forkhead boxO3a (FOXO3a) transcription factor by the oocyte is a key regulatory

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mechanism of the primordial follicle activation process (Castrillon *et al.*, 2003). Akt1 hyperactivation results in hyperphosphorylation of FOXO3a culminating in the global activation of primordial follicles and premature ovarian failure (John *et al.*, 2008). Thus FOXO3a has been reported as a potential determinant of the onset of menopause, as well as a potential target for intervention agonists to reduce the decline in fertility in females of advanced age (Pelosi *et al.*, 2013).

Phosphorylation and nuclear translocation of FOXO3a decreases the expression of the enzyme manganese-superoxide dismutase (MnSOD) (Li *et al.*, 2006). MnSOD is one of the major cellular antioxidant defense systems, and its expression decreases with ageing (Li *et al.*, 2006). It is hypothesized that oxidative ovarian protection is reduced with ageing and may be a cause of reduced oocyte quality and embryo survival in older females (Lim & Luderer, 2011). Diets rich in fat can have negative effects on embryo development (Leroy *et al.*, 2010; Wu *et al.*, 2011) and mice fed a high-fat diet have impaired ovarian activity and aberrant expression of several genes, including FOXO3a (Nteeba *et al.*, 2013). In addition, activation of the insulin intracellular signaling pathway (Pi3k/Akt/mTOR/FOXO) is a critical step in the pathophysiology of the polycystic ovarian syndrome (PCOS) and is associated with obesity, cardiovascular disease after menopause and reduced fertility in women (Rojas *et al.*, 2014). Therefore, we hypothesized that a high-fat diet can be detrimental to aged female mice and the aim of this study was to compare serum lipid profiles and ovarian gene expression between aged and younger females fed either a control or a high-fat diet.

Materials and methods

Animals and diets

The Ethics Committee for Animal Experimentation at Universidade Federal de Pelotas approved this study under the number 9106. Sixteen female mice (C57BL/6) of 4 months (Young, $n = 8$) or 13 months (Old, $n = 8$) of age were used. The females were divided into four groups: (i) young females fed a normal diet; (ii) young females fed a high-fat diet; (iii) old females fed a normal diet; and (iv) old females fed a high-fat diet. The mice were maintained in a temperature (22 ± 2°C) and humidity (40–60%) controlled environment with 12 h light/dark cycles.

The diets provided to the mice were prepared according to AIN-93 instructions (Reeves *et al.*, 1993). For the high-fat diet 120 g of lard and 10 g of cholesterol were added replacing 191 g of cornstarch for every 1 kg of the AIN-93 control diet. In the control diet the main source of lipids was soy oil. Both diets had 14.1% crude protein in its composition, however the control diet was composed of 75.9% carbohydrates, 10.0% lipids and 3061 kcal of energy, while the high-fat diet was composed of 54.6% carbohydrates, 32.7% lipids and 4402 kcal of energy. The mice underwent an adaptation period of 1 week with the control diet prior to the beginning of the experiment and then the supplementation started. Young and old mice were fed control and high-fat diets for 56 days (8 weeks).

Tissue collection and biochemical assays

For ovarian tissue collection mice were subjected to an estrous synchronization protocol (Luo *et al.*, 2011). Briefly, mice received a single intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG; Novormon, Syntex S.A., Buenos Aires, Argentina) was followed by an injection of 5 IU of human chorionic gonadotropin (hCG; Vetecor, Hertape S.A., Juatuba, MG, Brazil) 48 h later. Ovaries were collected 72 h after the hCG injection. At the end of the experiment mice were fasted for 8 h overnight and were anesthetized, euthanized and dissected. Blood samples and pairs of ovaries were collected. The blood was centrifuged to recover the serum, which was stored at −20°C for later analysis. Ovaries were dissected and stored in a microtube, and 0.5 ml of Trizol (Life Technologies, Carlsbad, California, USA) was added, the tissue was homogenized and frozen at −80ºC until RNA extraction.

Serum samples were evaluated for total cholesterol, high-density lipoprotein (HDL), and paraoxonase 1 (PON1) activity. Assays for total cholesterol and HDL were based on commercial protocols (Labtest Diagnostica S.A., Lagoa Santa, MG, Brazil) through colorimetry in a spectrophotometer. The analysis for PON1 activity was based on Browne *et al.* (2007). First a 20 mM Tris/HCl buffer was prepared and 8 mM phenylacetate and 1 mM magnesium chloride were added. This solution was mixed with the sample and the increase in absorbance at 270 nm over 60 s was measured using a spectrophotometer. PON1 activity was expressed as kU/l.

Ovarian gene expression analysis

RNA extraction was performed using the Trizol methodology, following the manufacturer's instructions. Briefly, the homogenized sample was thawed at room temperature and centrifuged at 12,000 *g* for 10 min at 4°C, discarding possible cellular debris. After that, 0.2 ml of chloroform was added, the tube was manually agitated for 15 s and incubated for 2–3 min at room temperature and then centrifuged at 12,000 *g* for 10 min at 4°C. For RNA precipitation, 0.5 ml of 100% isopropanol was added for each sample, which was incubated at room temperature

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Table 1 Primer pairs used in the experiment

Gene	Primer sequence
Beta 2 microglobulin (β2m)	*For* AAGTATACTCACGCCACCCA; *Rev* CAGGCGTATGTATCAGTCTC
Insulin-like growth factor 1 (Igf1)	*For* CTGAGCTGGTGGATGCTCTT; *Rev* CACTCATCCACAATGCCT
Protein kinase B (Akt1)	*For* CCG GTT CTT TGC CAA CAT CG; *Rev* ACACACTCCATGCTGTCATCTT
Phosphoinositide 3-kinase (Pi3k)	*For* TAGCTGCATTGGAGCTCCTT; *Rev* TACGAACTGTGGGAGCAGAT
Mammalian target of rapamycin (Mtor)	*For* CGGCAACTTGACCATCCTCT; *Rev* TGCTGGAAGGCGTCAATCTT
Forkhead Box O3a (Foxo3a)	*For* TCCCAGATCTACGAGTGGATGG; *Rev* CCTTCATTCTGAACGCGCAT
Sirtuin 1 (Sirt1)	*For* GCAACAGCATCTTGCCTGAT; *Rev* GTGCTACTGGTCTCACTT
Catalase (Cat)	*For* AGGAGGCAGAAACTTTCCCAT; *Rev* TTTGCCAACTGGTATAAGAGGGTA
Bone morphogenetic factor 15 (Bmp15)	*For* GAGCGAAAATGGTGAGGCTG; *Rev* GGCGAAGAACACTCCGTCC
Growth and differentiation factor 9 (Gdf9)	*For* AATACCGTCCGGCTCTTCAG; *Rev* GGTTAAACAGCAGGTCCACCAT
Anti-müllerian hormone (Amh)	*For* TCCTACATCTGGCTGAAGTGATATG; *Rev* CAGGTGGAGGCTCTTGGAACT
Survivin	*For* ATCGCCACCTTCAAGAACTG; *Rev* TGACTGACGGGTAGTCTTTGC

for 5 min and centrifuged again at 12,000 *g* for 10 min at 4°C. Finally, the supernatant was removed from the tube, the pellet washed twice with 1 ml of 75% ethanol followed by centrifugation and 5 min drying at room temperature. The RNA was then dissolved in 30 μl of ribonuclease-free water and its concentration estimated by spectrophotometry (NanoDrop, Thermo Scientific, Waltham, USA). The samples were diluted to 200 ng/μl and stored at −80°C until use. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA with reverse transcriptase (iScript, Biorad, Hercules, CA, USA) using random nucleotide hexamer primers in a 10 μl reaction volume. The final cDNA solution was diluted to 10 ng/μl before use.

Real-time PCR using SYBR Green dye was used to evaluate gene expression. β_2-Microglobulin expression was used as an internal control. The primer sequences are listed in Table 1. The PCR reactions were performed in duplicate in a 20-μl volume using 5 μl of SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA), 0.4 μl of each primer (10 μM stock) and 20 ng (2 μl) of cDNA. Fluorescence was quantified with the ECO Real-Time PCR System (iLLumina®, San Diego, CA, USA). For each assay, 45 PCR cycles were run (95°C for 3 s and 60°C for 30 s), and a dissociation curve was included at the end of the reaction to verify the amplification of a single PCR product. Each assay plate included a negative control. The coefficient of variation was below 5% for all the primer pairs used. Relative expression was calculated from the equation $2^{A-B}/2^{C-D}$ (where A is the cycle threshold [Ct] number for the gene of interest in the first control sample, B is the Ct number for the gene of interest in the analyzed sample, C is the Ct number for β_2-microglobulin in the first control sample, and D is the Ct number for β_2-microglobulin in the analyzed sample). The first control sample was expressed as 1.00 by this equation, and all other samples were calculated in relation to this value. Afterward, the results in the control group (young mice fed with control diet) were averaged, and all other outputs were divided by the mean value of the relative expression in the control group to yield the fold change of the genes of interest expression compared with the control group (Masternak *et al.*, 2005).

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Two-way analysis of variance (ANOVA) was applied to test the effect of age, diet and its interaction. When the interaction was significant a *t*-test was performed for comparison between groups. A *P*-value lower than 0.05 was considered statistically significant and as a tendency between 0.05 and 0.10.

Results

Food intake was reduced ($P < 0.05$) in mice fed with high-fat diet in comparison with the control group, and was higher in old than in young female mice ($P < 0.05$; Table 2). There was no interaction between diet and age for food intake ($P > 0.05$). Females fed a high-fat diet had higher body weight than the females fed the control diet ($P < 0.05$). Also, old females had a higher body weight gain than young females ($P < 0.05$; Table 2). There was an interaction between diet and age for body weight gain, in which old females on the high-fat diet had a higher body weight than young females on the same diet ($P < 0.05$).

The concentration of total cholesterol was higher in the high-fat diet group ($P < 0.05$), although it was not affected by age (Table 2; $P > 0.05$). Conversely, the

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Table 2 Average intake, body weight, cholesterol, HDL and PON1 in young and aged female mice fed a control or a high-fat diet

	Young		Old		*P*-value		
	Control	High-fat	Control	High-fat	Diet	Age	Interaction
Intake (g/day)	3.4 ± 0.2	2.6 ± 0.2	4.5 ± 0.2	3.3 ± 0.2	<0.0001	<0.0001	0.18
Body weight (g)	21.1 ± 0.4	23.3 ± 0.4	30.1 ± 0.3	35.1 ± 0.3	<0.0001	<0.0001	<0.0001
Cholesterol (mg/dl)	179.3 ± 20.6	262.8 ± 23	175.7 ± 18.8	225.3 ± 17.4	0.004	0.32	0.41
HDL (mg/dl)	58.0 ± 4.5	60.5 ± 5.0	72.2 ± 4.1	81.7 ± 3.8	0.07	0.008	0.44
PON1 (kU/l)	98.9 ± 8.6	72.9 ± 9.6	129.7 ± 7.9	83.4 ± 7.3	0.003	0.02	0.24

HDL fraction was higher in older mice ($P < 0.05$) and tended to be higher in mice fed with the high-fat diet ($P = 0.06$; Table 2). Finally, PON1 activity was lower in the high-fat diet group and was higher in older females ($P < 0.05$; Table 2), but no age to diet interaction was found ($P > 0.05$).

There was no significant effect of the diet, age or diet/age for the expression of most of the genes studied ($P > 0.05$; Fig. 1). However, older females had lower *Amh* expression than younger female mice ($P = 0.01$; Fig. 2) and there was an interaction between age and diet for *Gdf9* and *Survivin* expression, with lower expression in older females and those receiving the high-fat diet ($P < 0.05$, Fig. 1). In addition, females fed the high-fat diet had lower expression of *Igf1* mRNA ($P = 0.04$, Fig. 1).

Discussion

In the present study we observed that the high-fat diet had differential effects in older and younger female mice. Older females had a greater body weight gain on the high-fat diet compared with younger females on the same diet. The higher food intake observed for aged females could be explained by the higher baseline body weight. Generally, mice regulate food intake according to energy demands (Wolfgang & Lane, 2006), as it is seen for the food intake relative to body weight, which was 13% for the young female mice and 12% for older female mice. In addition, mice fed the high-fat diet had lower intake, which can be due to the fact that the high-fat diet was 26% more caloric than the control diet and had a high lipid content, especially in saturated and monounsaturated fatty acids and cholesterol, which could result in reduced food intake (Wolfgang & Lane, 2006). Interestingly, food intake was reduced on average by 25% when comparing high-fat and control diets, which could explain the difference found in our study. Nevertheless, body weight increased in females fed the high-fat diet, and increased even more in aged females fed the high-fat diet.

Despite the marked effects of the diet on body weight gain and serum lipid profiles, there was no effect of diet or age in the ovarian expression for most of the genes studied. A previous study observed that female mice fed a high-fat diet had decreased ovarian expression of *Irs1* and *Foxo3a* mRNA, while increased expression of *Akt1* mRNA (Nteeba *et al.*, 2013). In our study we did not find any change in the expression of *Foxo3a* mRNA. However, it should be considered that, in the Nteeba *et al.* (2013) study, mice were fed a high-fat diet for 7 months, while in our study mice were fed high-fat for only 2 months. Therefore, the differences in ovarian gene expression could be related to the duration of exposure to the high energy and fat diet as in the Nteeba *et al.* (2013) study body weight of mice on the high-fat diet was twice as high as those on the control diet, while we observed only a 10% increase in body weight. Nevertheless, we observed a reduced expression of *Igf1* mRNA in mice fed the high-fat diet. Previous studies have indicated that ovarian *Igf1* expression reduces with age, and this can be a local factor involved in preservation of the primordial follicle pool (Schneider *et al.*, 2014a,b). Despite that, ovarian *Igf1* mRNA expression was reported to also be involved in the development of larger follicles (Wandji *et al.*, 1998). Others have shown that a calorie-restricted diet has a negative impact on the activation of the ovarian mTOR signaling pathway (Rocha *et al.*, 2007; Li *et al.*, 2015), although no effects of the high-fat diet were observed (Liu *et al.*, 2015). Therefore, it is suggested that the chronicity of feeding has to be considered and longer exposure is more likely to modulate ovarian gene expression of the Pi3k/Akt pathway.

The ovarian expression of *Amh* and *Gdf9* was reduced in older female mice, independent of the diet. AMH, BMP15 and GDF9 are members of the transforming growth factor β (TGFβ) family produced by developing preantral and small antral follicles (Jeppesen *et al.*, 2013). In the GDF9 null mice folliculogenesis does not progress beyond the primary stage (Dong *et al.*, 1996). Therefore, these are markers of ovarian activity and fertility, expected to reduce its activity during ageing of the ovaries, as

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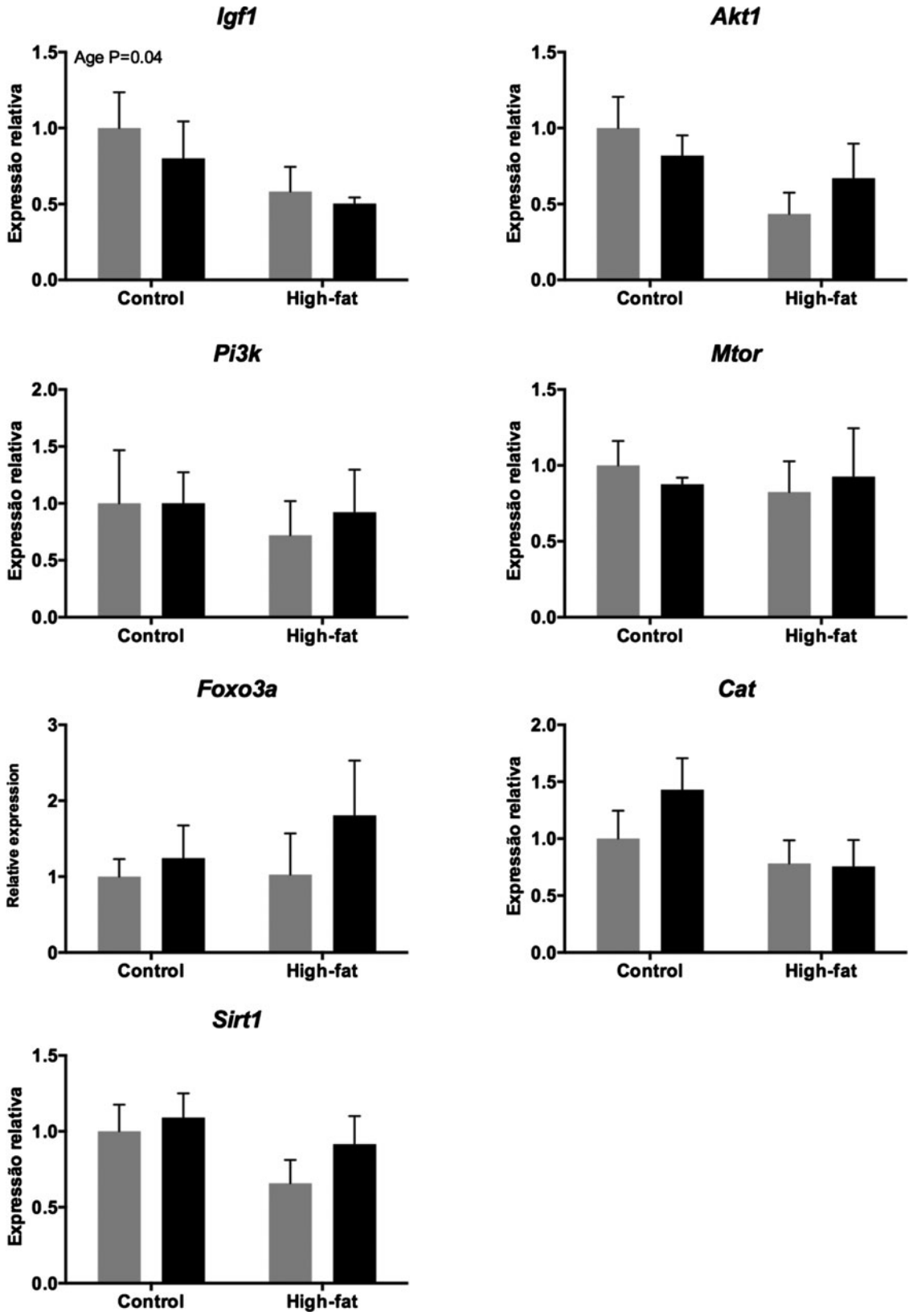

Figure 1 Gene expression of *Igf1*, *Akt1*, *Pi3k*, *Mtor*, *Foxo3a*, *Cat* and *Sirt1* in the ovarian tissue of young (grey bar) or old (black bar) mice fed a control or a high-fat diet for 2 months.

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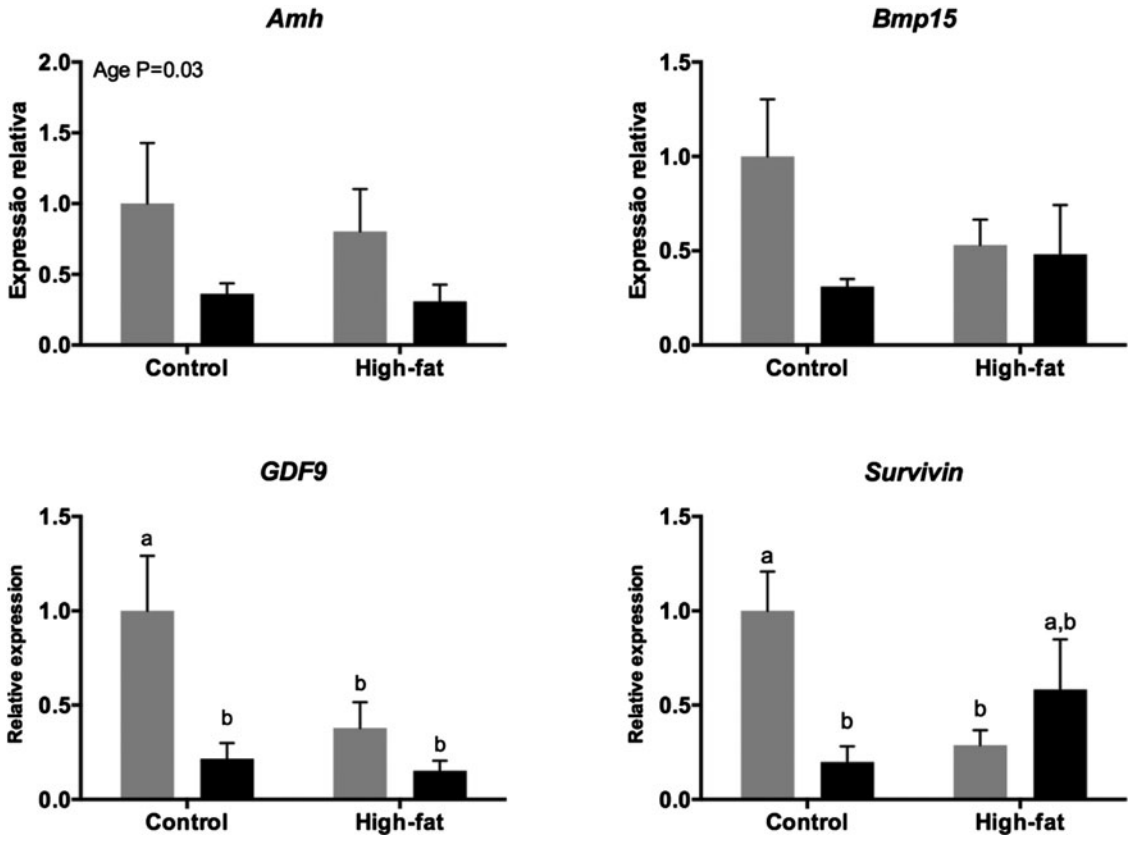

Figure 2 Gene expression of *Amh*, *Bmp15*, *Gdf9* and *Survivin* for ovarian tissue of young (grey bar) or old (black bar) mice fed a control or a high-fat diet for 2 months. a,b indicate a difference ($P < 0.05$) between individual groups when a Diet vs Age interaction ($P < 0.05$) was found.

we demonstrated before (Schneider *et al.*, 2014a,b). Interestingly, young female mice fed the high-fat diet had reduced expression of *Gdf9* mRNA, similar to that observed for aged females, which can indicate a reduced fertility and can block primordial follicle development (Dong *et al.*, 1996). Survivin is an inhibitor of apoptosis and is involved in cell growth and follicle development (Gao *et al.*, 2015). *Survivin* mRNA was reduced in older mice, also indicating the reduced fertility and increased follicle apoptosis observed with ageing in females (Schneider *et al.*, 2014a,b). In accordance with the pattern observed for *Gdf9* mRNA expression, *Survivin* mRNA was reduced in young female mice fed the high-fat diet, indicating increased ovarian apoptosis and pointing to decreased fertility in these mice. Collectively, these evidence indicate that the high-fat diet can reduce fertility, especially in young female mice, as the older females already had a compromised fertility, as indicated by the gene expression profiles, at the beginning of the feeding period.

Aged female mice in our study had a higher HDL concentration and increased PON1 activity compared with younger females. The high-fat diet increased total cholesterol concentration and decreased PON1 activity independently of age. It is well known that PON1 provides protection against the risk of atherosclerosis, as it prevents LDL oxidation (Durrington *et al.*, 2001). Lower serum PON1 activity is often observed in female patients with cardiovascular diseases (Suehiro *et al.*, 2000). Although, atherosclerotic plaque formation is slower in women than men, after menopause plaque formation is faster and highly dependent on pre-menopausal risk factors (Sutton-Tyrrell *et al.*, 1998). Among the risk factors, PCOS is associated with decreased serum PON1 activity and increased incidence of cardiovascular disease (Wild *et al.*, 2000; Dursun *et al.*, 2006). However, the relationship between age and lipid profile is not yet fully understood. Nevertheless, some studies have reported an increase in HDL and decrease in total cholesterol and LDL concentration with advancing age in human populations (Abbott *et al.*, 1997). According to the reviewed literature, it was expected that PON1 activity would decrease in the presence of a diet rich in fatty acids and cholesterol and with ageing (Kim *et al.*, 2012). Therefore, although the high-fat diet leads to reduced PON1 activity as demonstrated before, a

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well known risk factor for cardiovascular disease, it can be concluded that it is no more harmful in aged than younger mice. In fact, it seems less harmful, as aged females had higher levels of PON1 activity even with the control diet. PCOS is associated with changes in the expression of several genes at the ovarian level, between them *Amh* and mediators of the activation of the insulin intracellular signalling pathway (*Pi3k/Akt/Mtor/Foxo*) are critical steps in the pathophysiology of the disease (Rojas *et al.*, 2014). In the current study PON1, an important marker of PCOS, was affected by diet in both young and aged females and, although not associated with changes in the pattern of expression of gene markers of insulin signalling (*Pi3k*, *Akt*, *Mtor*, *Foxo*), it was associated with changes in others marker of fertility and PCOS (*Igf1*, *Gdf9* and *Survivin*).

Concluding, the high-fat diet reduced the expression of ovarian *Igf1* mRNA in young and aged mice, and reduced *Gdf9* and *Survivin* in old mice and young mice fed the high-fat diet. In addition, female mice on either diet and regardless of age regulated feed intake according to energy demand, although weight gain was higher in aged females. HDL concentration and PON1 activity were higher in aged female mice and reduced by the ingestion of a high-fat diet.

Conflict of interests

None of the authors of this study has any conflict of interest.

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