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Dissertation

Ovarian aging in growth hormone receptor knockout mice supplemented with 17α-Estradiol

José Victor Vieira Isola

Pelotas, 2018

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José Victor Vieira Isola

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Examination Comitee:

Prof. Dr. Augusto Schneider (Advisor)
Doctor in Biotechnology by Universidade Federal de Pelotas

Dr. Jorgea Pradieé
Doctor in Veterinary Science by Universidade Federal de Pelotas

Prof. Dr. Paulo Cavalheiro Schenkel
Doctor in Phisiology by Universidade Federal do Rio Grande do Sul

Prof. Dr. Monique Tomazele Rovani
Doctor in Veterinary Science by Universidade Federal de Santa Maria

I dedicate this work to my grandparents, *in memoriam.*

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Thanks to destiny;
To my family, friends, professors, and each and every one who somehow helped me or taught me something;
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“Never forget what you are, for surely the world will not. Make it your strength. Then it can never be your weakness. Armour yourself in it, and it will never be used to hurt you.”

George R.R. Martin, A Game of Thrones

Abstract

ISOLA, José Victor Vieira. **Ovarian aging in growth hormone receptor knockout mice supplemented with 17α-Estradiol.** 2019. 40f. Dissertation (Master degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

Mice with growth hormone (GH) receptor gene disruption (GHRKO) have absent GH activity. These mice are the main model to access the effects of GH and are known for having extended lifespan and female reproductive longevity. 17α-estradiol (17α-E2) is a molecule reported to extend mice lifespan. Hence, the aim of this study was to evaluate the ovarian reserve, in ovaries of normal and GHRKO mice supplemented or not with 17α-estradiol. Our hypothesis was that GHR disruption and 17α-Estradiol treatment would decrease the activation of primordial follicles in a complementary way in mice. Six GHRKO mice and six wild-type (WT) mice were divided into four groups: GHRKO mice normal diet (GHRKO; n=3), GHRKO mice supplemented with 17α-E2 (GHRKO-E2; n=3), WT mice normal diet (WT; n=3) and WT mice supplemented with 17α-E2 (WT-E2; n=3). E2 was provided in the food for four months. Histological slides were prepared from the mice ovaries. Images of the ovarian sections were captured by a camera coupled to a microscope, and the follicles were counted and measured. The statistical analysis considered the effect of the genotype (GHRKO vs WT), treatment (E2 vs CONT) and its interaction. Both genotype (p=0.0117) and diet (p=0.0136) had effects on the number of primordial follicles count as well as the interaction between them (p=0.004). The number of primordial follicles was higher on the GHRKO-CONT group (2632±181) than in the other groups. 17α-E2 treatment reduced the number of primordial follicles in the GHRKO mice to a level similar to WT, but had no effect on WT mice. WT mice had a higher number of primary follicles than GHRKO mice (p=0.006). 17α-E2 diet exerted no effect on the number of primary follicles (p=0.785). In summary, non-treated GHRKO mice had an increased primordial follicle reserve than WT mice. However, the genotypes treated with 17α-E2 responded differently, GHRKO decreased the primordial follicle reserve and WT mice maintained the primordial follicles number, which indicates that 17α-E2 has a differential role on follicle activation when there is a lack of GH /IGF-I.

Keywords: follicles; ovarian reserve; reproductive lifespan

Resumo

ISOLA, José Victor Vieira. **Envelhecimento ovariano em camundongos com knockout no receptor do hormônio do crescimento suplementados com 17α-Estradiol.** 2018. 40f. Dissertation (Master's degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2018.

Camundongos knockout para o gene do receptor do hormônio de crescimento (GH) (GHRKO) têm atividade de GH ausente. Estes camundongos são o principal modelo para acessar os efeitos do GH e são conhecidos por terem maior longevidade e as fêmeas apresentarem vida reprodutiva prolongada. O 17α-estradiol (17α-E2) é um hormônio capaz de prolongar a longevidade dos camundongos. Assim, o objetivo deste estudo foi avaliar a reserva ovariana, em ovários de camundongos normais e GHRKO suplementados ou não com 17α-estradiol. Nossa hipótese era que o knok out do GHR e o tratamento com 17α-estradiol diminuiriam a ativação de folículos primordiais de forma complementar em camundongos.Seis camundongos GHRKO e seis camundongos wild-type (WT) foram divididos em quatro grupos: camundongos normais GHRKO (GHRKO; n = 3), camundongos GHRKO suplementados com 17α-E2 (GHRKO-E2; n = 3), wild-type (WT) camundongos com dieta normal (WT; n = 3) e camundongos WT suplementados com 17α-E2 (WT-E2; n = 3). E2 foi fornecido na comida durante quatro meses. Lâminas histológicas foram preparadas a partir dos ovários dos camundongos. Imagens das seções ovarianas foram capturadas por uma câmera acoplada a um microscópio, e os folículos foram contados e medidos. A análise estatística considerou o efeito do genótipo (GHRKO vs WT), tratamento (E2 vs CONT) e sua interação. Tanto o genótipo (p = 0,0117) quanto a dieta (p = 0,0136) tiveram efeito no número de folículos primordiais e na interação entre eles (p = 0,004). O número de folículos primordiais foi maior no grupo GHRKO-CONT (2632 ± 181) do que nos outros grupos. O tratamento com 17a-E2 reduziu o nï¿½ero de folï¿½ulos primordiais nos ratinhos GHRKO para um nï¿½el semelhante ao WT, mas nï¿½ teve efeito nos ratinhos WT. Camundongos WT tiveram um número maior de folículos primários do que ratos GHRKO (p = 0,006). A dieta 17α-E2 não exerceu efeito sobre o número de folículos primários (p = 0,785). Em síntese, camundongos GHRKO não tratados tiveram um aumento na reserva ovariana do que os camundongos WT. No entanto, os genótipos tratados com 17α-E2 responderam diferentemente. Quando tratado, grupo GHRKO diminuiu a reserva de folículos primordiais enquanto os camundongos WT mantiveram o número de folículos primordiais, o que indica que o 17α-E2 tem um papel na ativação folicular que não atua da mesma forma quando os níveis de GH/IGF são baixos.

Palavras-chave: folículos, longevidade reprodutiva, reserva ovariana.

Figure List

Table List

Abbreviations List

17ß-E2	17 beta-Estradiol
17α-E2	17 alpha-Estradiol
Akt1	Protein kinase B
AR	Androgen receptor
ArKO	Female aromatase knockout
CR	Caloric restriction
df/df	Ames dwarf genotype
E2	Estradiol
ER	Estrogen receptor
FoxO	Forkhead Box O
FoxO3a	Forkhead Box O 3a
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	Growth hormone releasing hormone
GHRKO	Growth hormone receptor knockout
IGFBP	Insulin-like growth factor binding protein
IGFBP3	Insulin-like growth factor binding protein 3
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
KO	Knockout
mRNA	Messenger RNA
Pi3k	Phosphoinositide 3-kinase

POMC	Pro-opiomelanocortin
T	Testosterone
WT	Wild-type

Symbol List

°C	Degree Celsius
%	Percentage
±	Plus-minus
®	Registered Trade Mark

Summary

1 Introduction

The term ‘ovarian reserve’ refers to the functional potential of the ovary and reflects the number and quality of oocytes (RICHARDSON et al., 2014). The ovarian reserve accounts for the preantral follicles (primordial follicles, which have not been recruited, as well as primary and secondary) and antral (tertiary) follicles (LI et al., 2015). The follicles are continuously recruited from the reserve, which is an irreversible process. Once the primordial follicle is activated it can either grow until ovulation or enter in atresia in any stage of development (BAKER, 1963). The primordial follicles pool is established during fetal development in most mammals, including humans (TE VELDE et al., 1998). In rodents, germinal vesicle breakdown and establishment of the primordial pool occurs right after birth (PETERS, 1969).

A primordial follicle is defined as an oocyte surrounded by a layer of flattened granulosa cells (LI et al., 2015). The activated primordial follicle becomes a primary follicle, which contains an oocyte surrounded by a single layer of granulosa cuboid cells instead of flattened ones (LI et al., 2015). When the follicle has already been activated but does not yet have all granulosa cells in the cuboid form, it is considered a primordial follicle in transition. Non-growing oocytes are surrounded by 2-8 follicular cells. The number of cells surrounding the oocyte increases as the follicle grows. As the number of cells increases, the transition from flattened to cuboidal cells occurs gradually. The point of initiation of oocyte growth is around 9 cells and at this point, most of the follicles have exclusively cuboidal cells (LINTERN-MOORE & MOORE, 1979). Progressively, the diameter of nuclear and oocyte areas increases, as does the number of cells, until a second layer of cells accumulates, and the follicle becomes a secondary follicle. Later, more cell layers are added, and the antrum starts to be formed. The follicle then becomes a tertiary or antral follicle (LI et al., 2015). The primordial follicle reserve is finite, since germ cells cannot proliferate itself, and constitutes the totality of oocytes the female will be able to ovulate during her reproductive life (TE VELDE et al., 1998). Hence, the reproductive lifespan of a female depends on the initial size of the primordial follicles pool and the depletion

rate of these follicles (FORTUNE et al. 2013). Therefore, the activation and recruitment of follicles, results eventually in a complete depletion of the reserve, resulting in natural sterility, which in humans is called menopause (RICHARDSON et al., 2014). However, even before complete depletion of the follicular reserve, the fertility starts to decline as the size of the follicular reserve is reduced and oocyte quality is diminished (RICHARDSON et al., 2014).

Several hormones are involved in the regulation of ovarian function. Growth hormone (GH), besides its role as an anabolic hormone with pleiotropic effect upon cell growth, differentiation and metabolism (MAHRAN et al., 2015), is also related to ovarian function. GH is produced primarily in the somatotrophs of the pituitary gland, and its secretion is stimulated by the GH Releasing Hormone (GHRH; DEVESA et al., 2016). Although GH receptors are found throughout the body, most of its effects are mediated by the Insulin-Like Growth Factor I (IGF-I; DEVESA et al., 2016). IGF-I expression is stimulated by GH mainly in the liver (LE ROITH et al., 2001). In organs other than the liver, IGF-I expression seems to be independent of GH control, as observed in the ovary (SCHNEIDER et al., 2014). GH negatively regulates its own secretion by decreasing hypothalamic GHRH and increasing somatostatin levels (DURAN-ORTIZ et al., 2017). IGF-I levels also act in a negative feedback loop at the hypothalamus and pituitary to downregulate GH secretion (VIJAYAKUMAR et al., 2010).

The lack of GH activity has great consequences to mammals' phenotypes. Several mouse lines with reduced activity of the GH/IGF-I axis were produced, and all of them showed improved lifespan (JUNNILA et al., 2016). Besides having improved lifespan, mice lacking GH activity have reduced body size, low levels of IGF-I, increased insulin sensitivity and are less prone to several age-associated disorders including cancer and diabetes (JUNNILA et al., 2016; IKENO et al., 2009). The GHR knockout (GHRKO) mice is the main experimental model for experiments to access the effects of GH. These mice have the GH receptor gene disrupted and therefore have absent GH activity. GHRKO mice have very low IGF-I levels, reduced adult body size, and are very sensitive to insulin (DOMINICI et al., 2000). These mice exhibit an extended life span by up to 50% (COSCHIGANO et al., 2003). This extended lifespan is associated with lower morbidity and disease-related mortality (LIST et al., 2013). Half of GHRKO mice die of old age without obvious lethal pathological lesions, which is observed in only 10% of the wild-type mice, who

usually die before (IKENO et al., 2009). GHRKO mice also have an increased percentage of body fat with a disproportionate amount of fat deposition in the subcutaneous white adipose tissue depot (LIST et al., 2013). GHRKO mice phenotypes are similar to those of people who suffer from the Ecuadorian Laron Syndrome. This population has enhanced insulin sensitivity, reduced IGF-I, IGF binding protein 3 (IGFBP3) and estradiol levels in serum (ZHOU et al., 1997b). Although no life extension is reported for this population, it is reported that they rarely develop diabetes and fatal neoplasms (LIST et al., 2013).

The hormones of the somatotropic axis (GH/IGF-I) were shown to play essential roles in reproduction regulation. These hormones enhance steroidogenesis and follicular development by increasing the sensitivity of ovaries to gonadotropins stimulation (PORETSKY et al., 1999). GH action is mediated through enhancing serum and ovarian IGF-I levels and also by direct GH receptor-mediated effects in the ovaries (HERRINGTON & CARTER-SU, 2001). IGF-I ovarian actions are well established (BACHELOT et al., 2002). A study using IGF-I knockout (IGFIKO) mice demonstrated that IGF-I is required to the acquisition of follicle stimulating hormone (FSH) responsiveness, follicular development beyond the antral stage, and completion of oocyte growth and maturation (ZHOU et al., 1997a). Deletion of the IGF-I gene leads to failure of ovulation due to a block in folliculogenesis at a late preantral or early antral stages (BAKER et al., 1996) and IGF-I is shown to enhance FSH action on granulosa cells (GIUDICE, 1992). The fate of the follicle (growth or atresia) depends on the ability of IGF-I within the follicle to interact with its cognate receptor, the IGF type I receptor (IGF-IR; BACHELOT et al., 2002). Regarding the direct action of GH in ovarian function, little is known. GHR has been detected in granulosa, thecal, and luteal cells in many species (CHASE JR et al., 1998). Several studies suggest that GH plays a role in reproductive function, but it is unclear whether GH acts directly on the ovary or via an indirect endocrine route through stimulation of the release IGF-I by the liver (HULL & HARVEY, 2000). GH deficiency in female mice causes reduced litter size due to reduced ovulation rate, reduced number of antral follicles, and increased ratio of atretic/healthy follicles, whereas the implantation process occurs normally, since the number of implantation sites is consistent with the litter size (BACHELOT et al., 2002). These effects cannot be reversed by IGF-I treatment, suggesting that the main defect in GHR/GHBP KO mice

may be related to the absence of GHR itself and not to reduced IGF-I levels (BACHELOT et al., 2002).

Another mouse strain to study GH effects is the Ames dwarf (df/df) mice. These mice were first described by Schaible & Gowen (1961) and have a recessive spontaneous mutation on the Prop1 gene, resulting in abnormalities in the pituitary gland, causing them to have very low levels of GH and IGF-I. Many characteristics of GHRKO and df/df animals are similar to those experiencing caloric restriction (CR), such as reduced body weight, decreased GH and IGF-I levels, decreased plasma insulin and glucose levels, reduced fertility, delayed puberty and extended life span (LI et al., 2011). It is believed that these similarities between CR and low levels of GH/IGF-I are due to similar mechanisms of action (MCKEE ALDERMAN et al., 2010). That is why when GHRKO mice are subjected to a CR diet no improvements in lifespan and insulin sensitivity are observed (BONKOWSKI et al., 2006). Moreover, when GH is given to mice subjected to CR, its positive effects were reverted (GESING et al., 2014).

Preantral follicular development has generally been considered to be largely gonadotrophin-independent (SLOT, et al 2006). However, both GH and IGF-I affect numerous processes associated with the ovarian function, like gonadotrophin release and ovarian steroidogenesis, as well as follicular growth, development, and atresia (ZHOU et al. 1997a). GHR and IGF-I receptors are widely expressed in the ovary together with receptor-derived soluble binding proteins, GH-binding protein (GHBP) and IGF-binding proteins (IGFBPs), that regulate the bioavailability and action of GH and IGF-I, respectively (HULL & HARVEY, 2001). Mice experiencing CR and GHRKO mice, are capable to reproduce, despite having smaller litter size, due to a decreased number of antral follicles and consequently lower ovulation rate (LI et al., 2011; MAHRAN et al., 2015). Df/df GH-deficient females have normal cycles and can ovulate but are unable to maintain the pregnancy without exogenous prolactin supplementation (CHANDRASHEKAR et al., 2004). GHRKO females have more primordial follicles than normal mice, but when treated with IGF-I have a significant decrease in the number of primordial follicles, reaching numbers similar to those observed in wild-type mice (SLOT et al., 2006). Df/df female mice also have more primordial follicles than their normal (N/df) littermates (SACCON et al., 2016). When treated with exogenous GH, the number of primordial follicles decreases in df/df compared to non-treated mice (SACCON et al., 2016). As stated before, CR mice

have a similar phenotype to GH or GHR deficient mice. In mice subjected to CR, more primordial follicles are found than in control mice (LI et al., 2011; XIANG et al., 2012). Overall, this suggests that mice lacking GH or its receptor, as well as mice under CR, have a decreased activation of primordial follicles. One common observation in these mice models is the reduced systemic concentration of IGF-I and insulin, and reduced activation of its signaling pathways.

One of the most essential steps for the activation of the primordial ovarian reserve irreversible growth is the activation of the transcription factor Forkhead Box O 3a (FoxO3a; CASTRILLON et al., 2003). FoxO3a is a downstream effector of the phosphoinositide 3-kinase (Pi3k)/protein kinase B (Akt1) signaling pathway (JOHN et al., 2008). Hyperphosphorylation of FoxO3a results in its nuclear exclusion, culminating in the global activation of primordial follicles and premature ovarian failure (CASTRILLON et al., 2003). Oocytes enclosed in primordial/primary follicles from df/df mice have lower levels of pFoxO3a, which is associated with an increased primordial follicle reserve in older age (SCHNEIDER et al., 2014). The reduced activation of the FoxO pathway by both insulin and IGF-I seems to have a central role in the extended longevity phenotype observed in the df/df and GHRKO mice (BARTKE, 2003). Therefore, the reduced activation of FoxO3a seems a common point between different mice models and the main mechanism of preservation of the follicular reserve in its quiescent stage.

CR without malnutrition is the most studied intervention in mammals to protect against age-related diseases, although its mechanisms of action are not fully understood (STOUT et al., 2017). It remains unclear whether CR is feasible or appropriate for non-obese adults and CR in humans is difficult to maintain in the long term as observed for experimental procedures (DIRKS & LEEUWENBURGH et al., 2006). Hence, pharmacological interventions may be alternatives to alleviate age-related dysfunctions.

Recent observations report that 17α-estradiol (17α-E2) is capable to extend adult mice lifespan (HARRISON et al., 2014). 17α-E2 is a naturally occurring enantiomer of 17β-estradiol (17β-E2), yet, it appears to be non-feminizing due to minimal activation of classical estrogen receptors (ANSTEAD et al., 1997). The physiological functions of endogenous 17α-E2 are unclear (STOUT et al., 2017), even though it is found in several mammalian tissues, urine, and plasma from both sexes (DYKENS et al., 2005; TORAN-ALLERAND, 2005). 17α-E2 has been

consistently found in the brain, so it is believed that it is likely generated there and may protect against neuronal death (PEREZ et al., 2005; IKEDA et al., 2015). When testing whether 17α-E2 could alleviate age-related metabolic dysfunction and inflammation, Stout et al. (2017) found that 17α-E2 reduces body mass, visceral adiposity, and ectopic lipid deposition without decreasing lean mass. These changes were associated with reductions in energy intake due to the activation of hypothalamic anorexigenic pathways, reducing food intake (STOUT et al., 2017). Pomc-expressing neurons constitute the dominant anorexigenic node of appetite-regulating neurons and are viewed as key regulators of energy homeostasis (STOUT et al., 2017). 17α-E2 treatment increased hypothalamic expression of members of the melanocortin system, which is why it is believed that 17a-E2 promotes satiety through Pomc-expressing neurons (STEYN et al., 2018). Since 17α-E2 is a molecule that diminishes feed intake, we may assume that it might have similar effects to CR when it comes to ovarian depletion. However, until now, to the best of our knowledge, no experiments have been conducted analyzing ovarian reserve in females treated with 17α-E2. Additionally, CR is not able to improve lifespan and health span of GHRKO mice. Hence, it is possible that 17α-E2 through a different pathway may improve survival and health of GHRKO mice and also ovarian reserve preservation.

2 Objective

The aim of this study was to evaluate the number of primordial, transition, primary, secondary and tertiary follicles, as well as the diameter these follicles, oocytes and its nucleus, and the number of granulosa cells in ovaries of normal and GHRKO mice supplemented or not with 17α-estradiol.

3 Hypothesis

GHR disruption and 17α-Estradiol decreases the activation of primordial follicles in a complementary way in mice.

4 Material and Methods

4.1 Animals and treatments

The in vivo part of the experiment was conducted in the University of Central Florida. Twelve female mice were used, six GHRKO mice and six wild-type (WT) mice. The mice were kept in individual ventilated shelves, under controlled temperature (22 ± 2ºC) and humidity (40-60%). All the procedures were approved by the Ethics Committee for Animal Experimentation from the University of Central Florida.

The mice were divided into four groups, GHRKO mice normal diet (GHRKO; n=3), GHRKO mice supplemented with 17α-E2 (GHRKO-E2; n=3), wild type (WT) mice normal diet (WT; n=3) and WT mice supplemented with 17α-E2 (WT-E2; n=3). The groups supplemented with 17α-E2, had it mixed with their chow, prepared externally, at a proportion of 14.4 milligrams per kilogram diet (STOUT et al., 2017). The supplementation started when mice had 6 months of age and lasted for 4 months. Mice weights were recorded at the start of the diet and before euthanasia.

Mice were anesthetized and euthanized after 12h fasting at 10 months of age. The ovaries were collected, placed in 10% buffered formaldehyde and sent to Federal University of Pelotas for evaluation.

4.2 Ovarian histological analysis

Ovaries were removed from formaldehyde, dehydrated in alcohol, cleared in xylol and included in Paraplast Plus® (Sigma Chemical Company®, St. Louis, MO, USA). The ovaries included in Paraplast Plus® were sequentially cut at 5µm on a microtome (Model RM2245, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK). One every six cuts were selected and placed on standard histological slides. All the ovary was serially cut and used. The slides, after drying in the oven at 56°C for 24 hours, were stained with hematoxylin-eosin and mounted with coverslips and synthetic resin (Sigma Chemical Company®, St. Louis, MO, USA). Images of the

ovarian sections were captured by a camera coupled to a microscope (Nikon Eclipse E200MV), using the software TCCapture with the 10 and 40x lenses. The counted follicles were those with clearly visible oocyte nuclei and their quantity was multiplied six times, to account for the sampling method, and by two, to account for the two ovaries of the female, as already published (SCHNEIDER et al., 2014; SACCON et al., 2016).

Follicles were classified as primordial when surrounded by a single layer of flattened granulosa cells, as primary when surrounded by a single layer of granulosa cuboid, as transition follicle when surrounded by both flattened and cuboid cells, as secondary follicle when surrounded by more than one layer of granulosa cuboid cells, with no visible antrum and when the follicle had a clearly defined antral space and a layer of cumulus granulosa cells around the oocyte, it was classified as tertiary follicles (LI et al., 2015). Examples of the histological images of the follicles are presented on Figure 1.

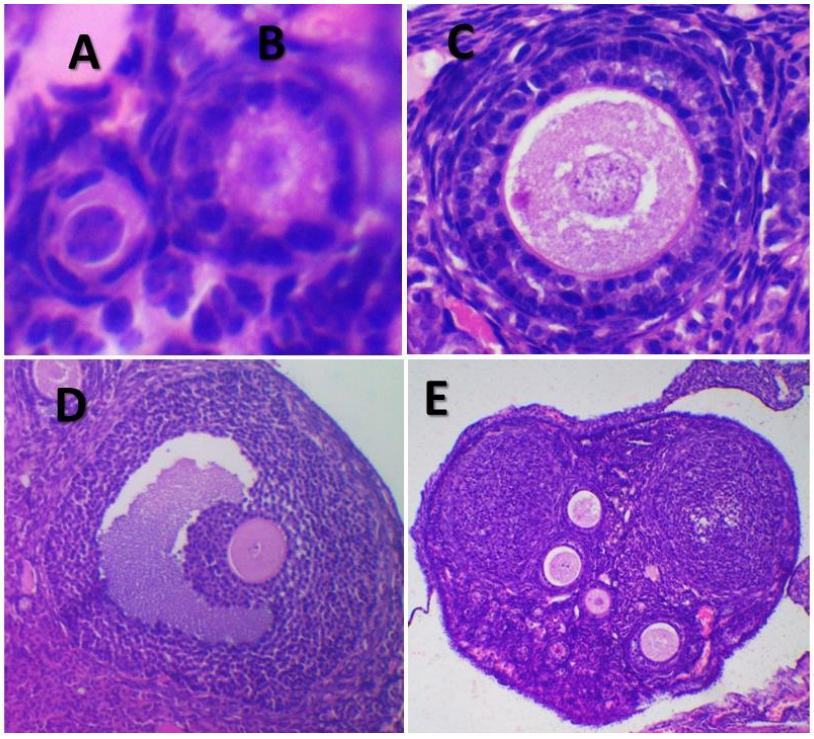

Figure 1 – Detailed histological images of primordial (A), primary (B), secondary (C), tertiary (D) follicles and whole ovary section (E).

To access follicle and oocyte size, three follicles from each stage were randomly selected for each animal and had its nuclei and oocytes measured by the

widest cross section in the software Motic Image Plus 2.0 (Motic®, Hong Kong, China). Granulosa cells surrounding primordial and primary follicles were also counted.

4.3 Statistical analysis

Statistical analysis was performed by the software Graphpad Prism 6. A Two-way ANOVA was performed to compare the number of follicles; nucleus, oocyte, and follicle size and percentual difference from initial to final body weight. The analysis considered the effect of the genotype (GHRKO vs WT), treatment (E2 vs CONT) and its interaction. Means were compared among groups using the Tukey post-hoc test. A value of P lower than 0.05 was considered significant.

5 Results

The number of primordial, transition, primary, secondary, tertiary and total follicles is presented on Figure 2. Regarding primordial follicles, both genotype (p=0.0117) and diet (p=0.013) had an effect, as well as the interaction between them (p=0.004). In the Tukey post-hoc test, the number of primordial follicles was higher on the GHRKO group (2632±181) than in the other groups. Among WT (936±51), WT-E2 (1080±411) and GHRKO-E2 (936±515) groups, no difference in primordial follicles count was found. For transition follicles no effects of genotype (P=0.228) or diet (P=0.332) was found.

Genotype effect evidenced that WT mice had a higher number of primary follicles than GHRKO mice (p=0.006). 17α-E2 diet however, exerted no effect on the number of primary follicles (p=0.785). In the Tukey post-hoc test, WT-E2 group had a greater number of primary follicles (1356±244; p=0.031), followed by WT (1064±387) and GHRKO (572±147), while the GHRKO-E2 group had the lowest number of primary follicles (392±291).

For secondary, tertiary and total follicles no effect from genotype or diet or difference between groups was found, as demonstrated on Figure 2.

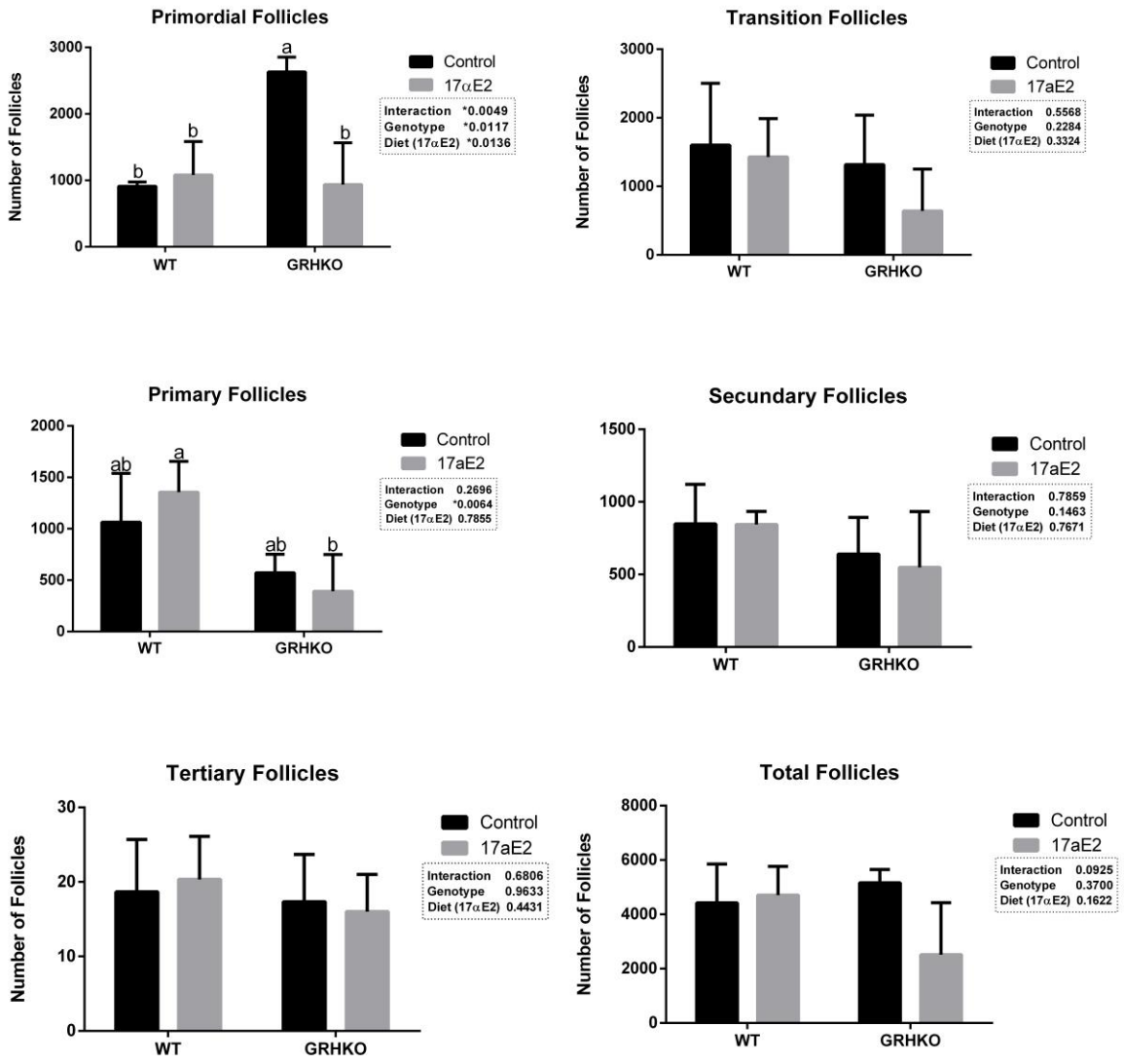

Figure 2 - Number of Primordial, Transition, Primary, Secondary, Tertiary and Total Follicles from Wild-type and GNRKO mice treated or not with 17αE2. *Different letters indicate statistical difference ($p<0.05$).

No difference was found for the majority of follicle parameters measured (Table 1). Diet effect was found on transition follicle nucleus (P=0.039) suggesting that it was bigger for 17α-E2 treated mice. Also, genotype effect was found for transition follicle oocyte diameter (P=0.042), suggesting it was smaller on the GHRKO mice. Diet effect was also found for the number of granulosa cells surrounding primary follicles (P=0.034).

Table 1 - Diameter of nucleus, oocyte and total follicle and number of granulosa cells from Wild-type and GNRKO mice treated or not with 17αE2.

	Wild-Type		GHRKO		P value		
	Control	**17αE2**	**Control**	**17αE2**	**Genotype**	**Diet (17αE2)**	**Interaction**
Primordial Follicle							
Nucleus diameter	6.66±0.6	6.97±0.6	6.42±0.7	6.86±0.9	0.460	0.116	0.793
Oocyte diameter	11.60±2.1	11.21±1.6	10.37±1.4	11.38±1.5	0.350	0.584	0.222
Total diameter	16.77±3.1	16.37±1.1	14.39±1.6	16.02±1.2	0.056	0.376	0.149
Granulosa Cells	6.00±1.5	7.22±1.6	6.67±2.1	6.00±1.1	0.610	0.610	0.089
Transition Follicle							
Nucleus diameter	6.86±0.7	7.56±1.3	6.21±0.7	6.93±1.1	0.064	*0.039	0.973
Oocyte diameter	12.10±1.3	12.37±3.8	10.14±1.1	11.18±1.5	*0.042	0.387	0.608
Total diameter	20.22±2.9	19.67±3.5	15.36±2.9	18.51±2.4	0.498	0.268	0.245
Granulosa Cells	7.67±1.6	6.89±1.2	7.22±1.9	7.22±2.0	0.922	0.496	0.496
Primary Follicle							
Nucleus diameter	9.14±1.8	9.97±2.4	10.17±1.8	9.23±1.9	0.829	0.195	0.934
Oocyte diameter	18.97±6.4	23.21±7.0	22.49±4.8	22.61±5.8	0.475	0.316	0.289
Total diameter	33.92±9.4	37.87±8.2	35.76±6.8	35.50±7.6	0.922	0.440	0.497
Granulosa Cells	15.00±7.4	19.11±5.2	18.89±4.0	14.89±4.8	0.928	*0.034	0.976

Groups treated with 17α-E2 had lower weight gain (p=0.045; Figure 3). GHRKO 17α-E2 group gained 3.04%, while WT 17α-E2 mice lost 9.59% of the initial weight. Control WT and GHRKO gained 9.61% and 10.77% of the initial weight, respectively. There was no effect of genotype (p=0.260) or diet/genotype interaction (p=0.343).

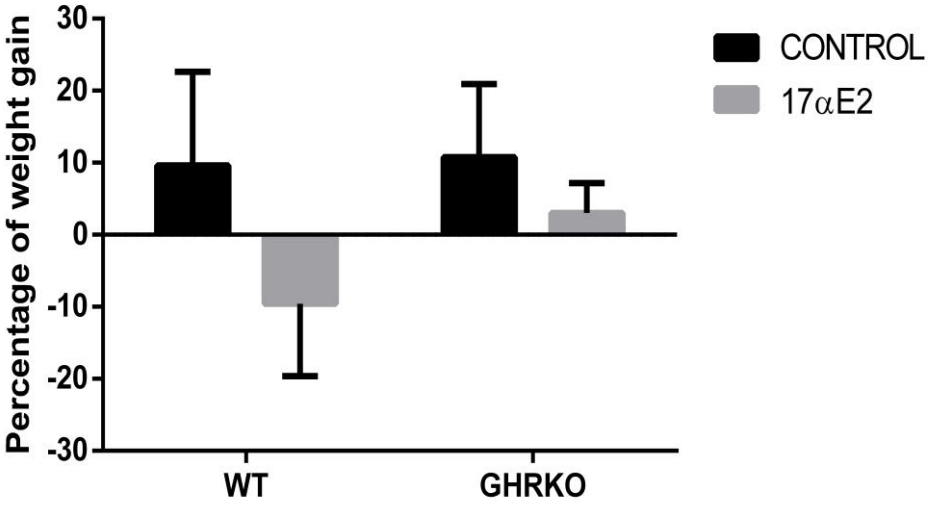

Figure 3 – Percentage to weight gain relative to initial weight in Wild-type and GNRKO mice treated or not with 17αE2 for 4 months.

6 Discussion

GHRKO mice primordial reserve was almost 3 times higher than in WT mice. Several studies have reported differences in ovarian follicles count in GH receptor deficient mouse lines compared to wild-type mice (BACHELOT et al., 2002; ZACZEK et al., 2002; SLOT, et al., 2006). Slot et al. (2006) were the first to find out that GHRKO mice have increased number of primordial follicles than WT mice, approximately 40% more primordial follicles. We found an overall lower number of follicles than previously observed. This lower number of follicles can be due the fact we used older mice (11 months old), while Slot et al. (2006) used very young mice (9-weeks-old). This is evidenced by a previous report of a 24 month-old GHRKO female having several ovarian structures while WT mice had none (SŁUCZANOWSKA-GŁĄBOWSKA et al., 2012). The greater number of primordial follicles in GHRKO mice even in advanced age is further evidence of the GH effects on follicle activation, allowing greater reproductive longevity in GHRKO mice.

WT mice had a higher number of primary follicles than GHRKO mice, even though no difference was found for secondary, tertiary and total follicles. This greater number of primary follicles indicates that there was a greater activation on the primordial follicles, which was expected. These results differ from those found by Slot et al. (2006) who found a greater amount of both primary and preantral follicles in GHRKO mice, and Bachelot et al. (2002) who found more total follicles in GHRKO mice. Again, this discrepancy might be due to the age difference between the experimental animals. The GH/IGF-I axis increases the sensitivity of the ovaries to gonadotrophins, thereby increasing the stimulation and follicular development (MAHRAN et al., 2015). It is not clear whether GH acts directly in the ovaries or only through IGF-I (HULL & HARVEY, 2000; BACHELOT et al., 2002; SLOT, et al., 2006). According to Schneider et al. (2014) a local functional GHR may not be needed for ovarian IGF-I production, since there is ovarian expression of IGF-I mRNA in GHRKO mice, similar to the levels found in WT mice. However, when GHRKO animals where treated with exogenous IGF-I for 14 days, the number of

primordial follicles dropped drastically to a level similar to WT mice (SLOT et al., 2006), which suggest that GH effect on follicles activation is mainly due to circulating IGF-I. The number of growing follicles is also reduced in IGFI-null mice (BAKER et al., 1996). Additionally, when exogenous GH treatment was given to GH-deficient df/df mice the number of primordial follicles was reduced, followed by increased primary follicles (SACCON et al., 2016), suggesting increasing activation stimulated by the GH/IGF-I axis.

Stimulation of other regulatory factors, such as insulin may also be involved on the process of primordial follicle activation (SLOT et al., 2006). Similar to GH-deficient mice and GHRKO mice, mice under CR also have a reduced systemic concentration of IGF-I and insulin and a reduced activation of its signaling pathways, which is also reflected in a decreased activation of primordial follicles (LI et al., 2011). Xiang et al. (2012) also evaluated the effects of CR in rats and reported a 47% increase on the number of primordial follicles of the rats submitted to CR compared to control ones, which is similar to a previous report by our group (GARCIA, 2018). GH is an important modulator of insulin actions in different organs (BARTKE, 2008). The greater sensibility to insulin is another anti-aging characteristic all these models (GHRKO, df/df and CR mice) have in common. The reduced GH secretion contributes to many symptoms of aging, including loss of muscle mass, increased adiposity, decreased bone mineral density, and declining energy levels along with changes in quality of life (BARTKE et al., 2003). Insulin sensitivity typically decreases during aging, leading to a compensatory increase in plasma insulin levels and reduced ability to eliminate glucose and limit postprandial elevations of glucose levels (FACCHINI et al., 2001).

One of the regulatory factors that needs to be activated in order for the oocyte and the follicle to be activated is the protein FoxO3a. When this protein is in its non-phosphorylated form, the follicles remain in resting stage, but the hyperphosphorylation of FoxO3a results in its nuclear exclusion, culminating in the global activation of primordial follicles (CASTRILLON et al., 2003). Both Schneider et al. (2014) and Saccon et at. (2016) found a lower amount of phosphorylated FOXO3a in GH-deficient df/df mice primordial/primary follicles compared to WT. This suggests that FOXO3a is less activated when there is lack of GH/IGF-I, even though df/df mice also have deficiencies in other hormones, since they have a less active hypophysis (SCHAIBLE & GOWEN, 1961). In the same study, GH-deficient mice

receiving exogenous GH had increased phosphorylated FOXO3a, again suggesting the central role of GH in primordial follicle activation.

Since lack of GH signaling and CR are related to both aging and ovarian reserve depletion, we may assume that drugs that can affect the overall aging process can also be related to primordial follicles activation. One of this drugs is rapamycin, which can increase longevity in mice (ANISIMOV et al., 2011). Recently, our group found that rapamycin reduces ovarian aging in a similar rate to CR (GARCIA, 2018). Like rapamycin, 17α-estradiol (17α-E2) is a compound able to extend adult mice lifespan (HARRISON et al., 2014). 17α-E2 reduces body mass, visceral adiposity, and ectopic lipid deposition without decreasing lean mass by reductions in energy intake due to the activation of hypothalamic anorexigenic pathways, reducing food intake (STOUT et al., 2017), resembling the mentioned effects of CR and GH-deficiency. In this study we found that the WT females treated with 17α-E2 lost body weight while the non-treated females gained weight. The treated GHRKO females gained just a little weight, however, approximately 3 times less than the non-treated. Similar results were reported by Harrison (2014) who found a 5% of weight loss on WT females treated with 17α-E2. These authors found greater weight loss on males (22%).

Since 17α-E2 is a compound that diminishes feed intake, we assumed that it would have similar effects to CR and rapamycin regarding ovarian depletion, and would increase the number of primordial factors. However, the significant interaction between diet and genotype we found revels that that WT mice were not affected by the 17α-E2 treatment, but GHRKO were. The GHRKO group, controversially, had the primordial follicles number reduced to a level similar to WT mice. Despite of what was expected 17α-E2 decreased the primordial follicle reserve in GHRKO mice which suggests that 17α-E2 has a role on follicle activation, and this role may be differentially modulated when the levels of GH/IGF-I are low.

Also, the number of primary follicles, wasn’t increased in the GHRKO group treated with 17α-E2, which would be expected by the lower number of primordial follicles. Since many of the follicles aren’t found on the primordial phase it would be expected that they were found on transition and primordial phases, which was not the case. This discrepancy on the primordial:primary follicles ratio may indicate that a greater number of the primordial follicles in the GHRKO group treated with 17α-E2 is probably undergoing atresia right after being activated.

The effects of E2 on follicular development and oocyte maturation are mediated through interaction with specific receptors (ER; BRITT & FINDLAY, 2002). One of the estrogen (E2) receptors, ERα, is expressed in cumulus cells, germinal epithelium, interstitial cells and thecal cells, while other, ERß, is expressed in oocytes, cumulus cells, and granulosa cells in primary, secondary, and mature follicles (DRUMMOND et al., 2002; BRITT & FINDLAY, 2002; COUSE et al., 2005). A role for E2 in primordial follicle formation and activation in rodents is not yet well stablished. The presence of ER within developing ovaries, which possess only primordial and primary follicles, suggests that E2 may have a role in follicle activation (MONTANO et al., 1995). Most models with estrogen receptor (ER) deficiency have normal follicle development until the antral stage (COUSE & KORACH, 1999) but no quantitative measures of pre-antral follicle numbers have been reported in these models. In female aromatase knockout (ArKO) mice, which are deficient in E2, and considered an excellent model for E2 deficiency, the lack of E2 was associated with a decrease in primordial and primary follicle number compared with wild-type mice at 10 weeks of age (BRITT et al., 2004). Evidence also shows that when *in vitro* E2 treatment of mice ovary culture can inhibit primordial to primary follicle transition (KEZELE & SKINNER, 2003). Also, when added to *in vitro* culture of oocytes, E2 and E2 derived from androstenedione that was aromatized in the granulosa cells did not affect follicle growth but did act on follicular somatic cells and oocytes to induce morphologic and functional abnormalities (TARUMI et al., 2014). Despite this evidence, little is known about the role of E2 on primordial oocyte and primordial follicle granulosa cells. Our current work points to a role of 17α-E2 on follicle activation.

Testosterone (T) also have receptors (AR) distributed broadly over the ovaries, although its effects on females remain controversial (GILL et al., 2004). Apparently, increased levels of androgens accelerate the progression of follicle development and increase the number of preantral follicles. *In vitro* T treated mice ovaries had a higher primary follicle count and a higher primary to primordial follicles rate than control mice ovaries, suggesting T induced primordial follicle activation. T also increased FoxO3a phosphorylation, activating the PI3-K/Akt pathway, which may explain why T enhanced follicle activation (YANG et al., 2010). Androgens most likely enhance primordial follicle transition through its own receptor and not by

transformation in to estradiol, since E2 has been shown to inhibit primordial follicle development (KEZELE & SKINNER, 2003).

Due to the stereochemistry of the carbon atom 17, 17α-E2 has a much weaker binding affinity to the classical estrogen receptors and has a greater binding affinity for other estrogen receptors, such as the brain ER receptor (TORAN-ALLERAND et al., 2005). The effects of 17α-E2 on lifespan and metabolic health are strongly sex-specific. Females accrue no detectable metabolic benefit of 17α-E2 treatment (HARRISON et al., 2014). Evidences show modulation of sex-specific responsiveness to 17α-E2 with male castration, but not with female ovariectomy, which suggests that the sex-specificity in responsiveness to 17α-E2, may be caused by an interaction with male gonadal hormones (GARRAT et al., 2018).

17α-E2 has an ability to inhibit the activity of 5-alpha reductase enzymes (SCHRIEFERS et al., 1991), which convert T to dihydrotestosterone, a more potent activator of the androgen receptor. Since T, as stated before, has been reported to enhance primordial follicles activation, it is possible that the higher activation of primordial follicles we found on GHRKO mice treated with 17α-E2 occurred through this pathway. Also, 17α-E2 reduces the hepatic abundance of several metabolites (GARRAT et al., 2018). These changes in female metabolites after 17α-E2 treatment may also play an indirect role in follicle activation and deserves further attention.

7 Final Considerations

Non-treated GHRKO mice had an increased primordial follicle reserve than WT mice. However, the genotypes treated with 17α-E2 responded differently, GHRKO decreased the primordial follicle reserve and WT mice maintained the primordial follicles number, which indicates that 17α-E2 has a differential role on follicle activation when there is a lack of GH /IGF-I.

Our previous studies have shown that deficiency in GH, GHR, CR and rapamycin cause a reduction on activation of primordial follicles. 17α-E2, different from the other treatments, increases lifespan but not reproductive longevity. This may have implications, since ovarian physiology is important to longevity and determination of the menopause age, which may compromise the use of this treatment in females. More studies about 17α-E2 effects on ovarian follicle activation are needed in order to reinforce the current results and provide further insights on the subject.

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