Published in final edited form as:

Exp Gerontol. 2020 January ; 129: 110769. doi:10.1016/j.exger.2019.110769.

17 α -estradiol promotes ovarian aging in growth hormone receptor knockout mice, but not wild-type littermates

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Abstract

Growth hormone receptor knockout mice (GHRKO) have reduced body size and increased insulin sensitivity. These mice are known for having extended lifespan, healthspan and female reproductive longevity. Seventeen α -estradiol (17 α -E2) is reported to increase insulin sensitivity and extend lifespan in male mice, with less robust effects in female mice. The aim of this study was to evaluate the ovarian reserve in wild type and GHRKO mice treated with 17 α -E2. The mice were divided into four groups, GHRKO mice receiving a standard chow diet, GHRKO mice treated 17 α -E2, wild type mice receiving a standard chow diet and WT mice treated with 17 α -E2. 17 α -E2 was provided in the diet for four months. IGF1 plasma concentrations and changes in body weight were assessed. Histological slides were prepared from the ovaries and the number of follicles were counted. GHRKO mice receiving the control diet had a greater number of primordial follicles and lower numbers of primary follicles compared to the other groups ($p < 0.05$). 17 α -E2

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⁵-Conflicts of Interest

The authors declare no conflicts of interests.

treatment decreased the number of primordial follicles in GHRKO mice ($p < 0.05$), however had no effect in wild type mice. Treatment with 17 α -E2 had no significant effect on the change in body weight during the experiment ($p = 0.75$). Plasma IGF1 concentrations were significantly lower in GHRKO mice as compared to wild type. In conclusion, we found that GHRKO mice displayed lesser primordial follicle activation as compared to wild type mice, but this phenotype was reversed by 17 α -E2 administration, suggesting that ovarian aging is increased by 17 α -E2 in long-living mice with extended reproductive longevity.

Keywords

follicles; ovarian aging; ovarian reserve; reproductive lifespan

1. Introduction

Mice with a disruption of the growth hormone receptor (GHR)/GH binding protein gene (GHRKO) are characterized by a severe reduction in hepatic insulin-like growth factor (IGF1) production and reduced adult body size (Dominici et al., 2000). Interestingly, these mice exhibit an extended lifespan (Coschigano et al., 2003), resulting from lower morbidity and disease-related mortality (Ikeno et al., 2009). Growth hormone receptor knockout mice have increased percentage of body fat with a disproportionate amount of fat deposition in the subcutaneous white adipose tissue depot (Berryman et al., 2010, Masternak et al., 2012). GHRKO mice also have increased insulin sensitivity (Junnala et al., 2016) which is believed to be associated with their extended longevity phenotype (Masternak et al., 2009).

Many characteristics of GHRKO mice are similar to animals subjected to caloric restriction (CR), including reduced body mass, decreased IGF1 production, robust metabolic plasticity, and extended lifespan (Li et al., 2011). However, it seems unlikely that CR will ever become a long-term interventional strategy in humans due to adverse effects associated with thermoregulation, libido, and mood (Dirks and Leeuwenburgh, 2006). As such, pharmacological interventions that mitigate age-related morbidities may prove to be appropriate alternative strategies. Recent studies have shown that 17 α -estradiol (17 α -E2) extends lifespan in male mice similarly to CR (Harrison et al., 2014). 17 α -E2 is a naturally occurring enantiomer of 17 β -estradiol (17 β -E2), although it is considered a nonfeminizing estrogen due to its minimal activation of classical estrogen receptors (Anstead et al., 1997). The physiological functions of endogenous 17 α -E2 are unclear, even though it is found in several tissues from both sexes in mammals (Dyken et al., 2005, Toran-Allerand, 2004). Treatment with 17 α -E2 reduces body mass, visceral adiposity, and ectopic lipid deposition without decreasing lean mass only in males, suggesting a sexually dimorphic regulation (Stout et al., 2017, Miller et al., 2019, Garratt et al., 2019). These changes were associated with reductions in energy intake due to the activation of hypothalamic anorexigenic pathways, thereby reducing food intake (Steyn et al., 2018, Garratt et al., 2019).

The reproductive lifespan of a female depends on the initial size of the ovarian primordial follicle reserve and the depletion rate of these follicles (te Velde et al., 1998). The continuous activation and recruitment of primordial follicles during the female lifespan

eventually leads to depletion of this reserve, resulting in a natural sterility, which in humans is known as menopause (Richardson et al., 2014). However, even before complete depletion of the follicular reserve, fertility starts to decline as the size of the follicular reserve is reduced and oocyte quality is diminished (Richardson et al., 2014). Compared to WT mice, young GHRKO females have a larger primordial follicle reserve, but when treated with exogenous IGF1 they demonstrate a significant decrease in the number of primordial follicles (Slot et al., 2006). Similarly, while GH-deficient Ames dwarf mice (df/df) have a larger ovarian primordial follicle reserve (Saccon et al., 2017), transgenic mice overexpressing GH have a smaller primordial follicle reserve (Saccon et al., 2017). Preservation of the primordial follicle reserve is also observed in rats undergoing CR (Li et al., 2011, Li et al., 2015, Xiang et al., 2012, Garcia et al., 2019). Since the lack of GH signaling and CR are related to both aging and ovarian reserve depletion, drugs that can increase longevity may also decrease primordial follicles activation. One of these drugs is rapamycin, which increases longevity in mice (Anisimov et al., 2011, Harrison et al., 2009) and also reduces depletion of primordial follicles (Zhou et al., 2017, Garcia et al., 2019). Collectively, these studies suggest that different lifespan-extending treatments also prolong fertility and decrease ovarian activation of primordial follicles.

In males, 17 α -E2 has similar effects to CR and GH signaling deficiency (Strong et al., 2016, Stout et al., 2017). Despite the fact that female mice appear less responsive to 17 α -E2 treatment (Garratt et al., 2017, Garratt et al., 2018, Garratt et al., 2019), it still seems reasonable to hypothesize that 17 α -E2 treatment could reduce ovarian primordial follicle depletion. Therefore, the aim of this study was to evaluate the ovarian reserve of normal and GHRKO mice treated with 17 α -E2.

2. Material and Methods

2.1. Animals and treatment

Six-month-old WT (n=10) and GHRKO (n=10) mice were individually housed at 24 $\pm$ 0.5°C on a 12:12-hour light-dark cycle. Unless otherwise noted, mice had ad libitum access to food and water throughout the experiment. LabDiet® 58YP (66.4% carbohydrate, 20.5% protein, 13.1% fat) was utilized for the control diet and was supplemented with 17 α -E2 (14.4ppm; Steraloids, Newport, RI) for the treatment diet (TestDiet; Richmond, IN, USA). The treatment was provided during four months.

At the end of treatment, when mice were 10-month-old, mice were anesthetized with isoflurane and euthanized by cervical dislocation prior to dissection. Blood was collected into EDTA-lined tubes by cardiac puncture, and plasma was collected and frozen for IGF1 assessment. Tissues were excised, weighed, flash frozen, and stored at -80°C unless otherwise noted. The ovaries were excised and placed in 10% buffered formaldehyde for histological analyses. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees at the Southern Illinois University.

2.2. Plasma Analytes

Plasma IGF1 concentrations were evaluated using ELISA kits from R&D Systems Inc. (Minneapolis, MN) as previously described (Chen et al., 2016).

2.3. Ovarian histological analysis

For histological analysis ovaries were removed from formaldehyde, dehydrated in alcohol, cleared in xylol and embedded in Paraplast Plus® (Sigma Chemical Company®, St. Louis, MO, USA). The ovaries embedded in Paraplast Plus® were sequentially cut at 5 µm in a microtome (Model RM2245, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK). One section every six cuts was placed on standard histological slides. The entire 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 using the software TC Capture (Tucson Photomics Co.) with the 10x and 40x lenses. The counted follicles were those with clearly visible oocyte nuclei and their quantity was multiplied by six, to account for the sampling method, and by two, to account for the two ovaries of the female, as previously published (Saccon et al., 2017, Schneider et al., 2014).

Follicles were classified as primordial when surrounded by a single layer of flattened granulosa cells, as primary when surrounded by a single layer of cuboid granulosa and as transition follicle when surrounded by both flattened and cuboid cells. Secondary follicles were surrounded by more than one layer of cuboid granulosa 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 a tertiary follicle (Li et al., 2015).

To access follicle and oocyte size, three follicles from each stage were randomly selected from each mouse and the widest cross section for nuclei, oocyte and follicle was measured by the software Motic Image Plus 2.0 (Motic®, Hong Kong, China). The number of granulosa cells surrounding primordial, transition and primary follicles were also counted.

2.4. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism 6. A Two-way ANOVA was performed to compare the number of follicles and percentage of follicles in each phase; nucleus, oocyte, and follicle size, IGF1 plasma concentrations and percentage of body weight gain. The analysis considered the effect of the genotype (GHRKO vs WT), treatment (E2 vs CONT) and their interaction. When the interaction was significant, means were compared among groups using the Tukey post-hoc test. A value of P lower or equal to 0.05 was considered significant.

3. Results

The number of primordial, transition, primary, secondary, tertiary and total follicles is presented on Figure 1. The number of primordial follicles was the highest in GHRKO mice (2479 ± 221) compared to other groups. No significant difference was detected among WT

(724 ± 117), WT-17 α E2 (1214 ± 179) and GHRKO-17 α E2 (1411 ± 239) mice for the number of primordial follicles (Fig. 1A). For transition follicles no effects of the genotype ($P=0.228$), diet ($P=0.332$) or interaction ($P=0.161$) were observed (Fig. 1B).

For primary follicles, the WT-17 α E2 group had the greatest number of primary follicles (1435 ± 136) compared to WT (787 ± 227), GHRKO (494 ± 104) and GHRKO-17 α E2 groups (424 ± 111) (Fig. 1C). For secondary follicles, no effects of genotype, diet or interaction were observed ($p>0.05$) (Fig. 1D). For tertiary follicles, only the diet had an effect ($p=0.035$), with more tertiary follicles observed for 17 α E2 treated groups (Fig. 1E). An effect of the diet by genotype interaction was found for total follicles ($p=0.012$), but no significant differences among individual groups were found (Fig. 1F).

Follicle, oocyte and nuclei size is presented in Table 1. GHRKO mice had slightly smaller primordial follicles when compared to WT mice ($p=0.005$). Regarding transition follicles, GHRKO mice also had smaller nuclei and oocytes ($p=0.012$ and 0.032 , respectively). GHRKO control mice had smaller transition follicles than the other groups ($p=0.005$). However, oocytes of primary follicles were smaller in WT-E2 mice.

Treatment with 17 α -E2 had no effect on the percentage of weight gain during the experiment ($p=0.752$). There was also no effect of genotype ($p=0.722$) or the diet by genotype interaction ($p=0.752$). Control WT and GHRKO females gained 5.91% and 5.50% of the initial weight during the experiment, while treated WT and GHRKO females lost 5.90 and 0.13% of their initial weight during the experiment. Plasma IGF1 concentrations were 3-fold lower in GHRKO mice ($p<0.0001$), as expected, however, treatment with 17 α -E2 did not affect IGF1 plasma concentrations (Figure 2).

4. Discussion

As previously reported, GHRKO mice have dramatically lower plasma IGF1 concentrations than WT littermates (Junnala et al., 2016, Duran-Ortiz et al., 2018). This condition was accompanied by decreased activation of primordial follicles as compared to WT mice. The reduced activation is suggested by the increased number of primordial and decreased number of primary follicles in ovaries from GHRKO mice. Others have previously reported an increased number of primordial follicles in GHRKO mice (Bachelot et al., 2002, Slot et al., 2006, Zaczek et al., 2002). We found an overall lower number of follicles than previously observed, which can be due the fact we used older mice (10 months old), while Slot et al. (2006) used young mice (9-weeks-old), and it is known that the primordial follicle reserve is depleted as the mouse ages (Kevenaar et al., 2006). The increased number of primordial follicles in GHRKO mice, even with advanced age, is further evidence of the interplay between somatic and gonadal aging. This was also evidenced by a previous report of a 24-month-old GHRKO female having different follicle categories and corpora lutea while WT mice had no evidence of ovarian structures (Sluczanowska-Glabowska et al., 2012). Our group has previously shown that GH deficient df/df mice have more primordial follicles than normal mice at 12 (Schneider et al., 2014) and 18 months of age (Saccon et al., 2017), further emphasizing the role of the GH/IGF1 axis in ovarian aging.

The GH/IGF1 axis is important for the activation of primordial follicles to start their growth, mostly through phosphorylation of FOXO3a protein (Castrillon et al., 2003, Saccon et al., 2017, Schneider et al., 2014). It is suggested that a local functional GHR may not be needed for ovarian IGF1 production, as there is ovarian expression of IGF1 mRNA in GHRKO mice, similar to the levels found in WT mice (Schneider et al., 2014). In addition, when GHRKO animals were treated with exogenous IGF1 for 14 days, the number of primordial follicles dropped drastically to a level similar to WT mice (Slot et al., 2006), suggesting a primary role of circulating IGF1 in follicle activation. Similarly, when exogenous GH was given to GH-deficient df/df mice the number of primordial follicles was reduced, followed by increased number of primary follicles (Saccon et al., 2017), suggesting increasing activation of the primordial follicle reserve stimulated by the GH/IGF1 axis.

Interestingly, treatment with 17 α -E2 produced a diet by genotype interaction effect on the number of primordial follicles. 17 α -E2 reduced the number of primordial follicles in GHRKO mice, however had no effect on primordial follicle activation in WT mice. It is interesting to note that this was not followed by increased number of primary or secondary follicles suggesting that there was no increased activation of the primordial reserve. Treatment with 17 α -E2 had no effect on body weight in this study. Previous reports indicate that weight loss due to 17 α -E2 is predominantly sexually dimorphic, with more weight loss occurring in males, while little or no effects are observed in females (Harrison et al., 2014, Garratt et al., 2019), in agreement with our observations. Treatment with 17 α -E2 produced CR like effects in previous studies by stimulating the anorexigenic POMC neurons within the ARC to reduce feed intake (Steyn et al., 2018). 17 α -E2 treated mice had reduced body weight, fat mass and increased insulin sensitivity (Stout et al., 2017). These effects are very similar to the phenotype of GHRKO mice (List et al., 2011). Therefore, we speculated that 17 α -E2 treatment would elicit similar effects on ovarian primordial follicle activation. Our group and others have shown that CR decreases the activation of primordial follicles in the ovary (Liu et al., 2015, Xiang et al., 2012, Garcia et al., 2019). However, we observed that 17 α -E2 did not slow ovarian aging as observed for other life extension therapies. This is consistent with previous work showing that female mice longevity is less affected by 17 α -E2 treatment (Garratt et al., 2017, Garratt et al., 2018, Garratt et al., 2019). Therefore, additional studies that provide mechanistic insight into the interplay of germline and somatic aging are greatly needed. In the current study, despite no significant effects were observed, mice treated with 17 α -E2 did not increased body weight during the experiment, while control treated mice had a small increase. However, it should be taken into account the low number of mice used in this study.

As 17 α -E2 treatment reduced the primordial follicle reserve only in GHRKO mice, it is possible that it had a local, direct ovarian effect, increasing follicle activation. However, previous evidences suggest otherwise. The effects of estradiol (E2) on follicular development and oocyte maturation are mediated through interaction with specific estradiol receptors (ER) (Britt and Findlay, 2002). ER α , is expressed in cumulus cells, germinal epithelium, interstitial cells and thecal cells, while ER β , is expressed in oocytes, cumulus cells, and granulosa cells of primary, secondary, and mature follicles (Britt and Findlay, 2002, Couse et al., 2005, Drummond et al., 2002). 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), however a role for E2 in primordial follicle formation and activation in rodents is not yet well established. A previous study has shown that ovariectomized female mice receiving 17 α -E2 displayed increased uterine mass, similar to that of intact controls (Strong et al., 2016), suggesting 17 α -E2 may also have mild effects through classical estrogen receptors resulting in divergent effects in male and female aging.

Most models with ER deficiency have normal follicle development until the antral stage (Couse and Korach, 1999) but no quantitative measures of preantral follicle numbers have been reported in these models. In aromatase knockout (ArKO) mice, which are deficient in 17 β HSD, a decreased number of primordial and primary follicle number compared with WT mice was reported (Britt et al., 2004). Additionally, the oocyte diameter in primordial follicles was larger in ArKO mice (Britt et al., 2004), while we observed that 17 α -E2 increased oocyte diameter of transition follicles. Primordial oocyte diameter is believed to be associated to the activation and transition to primary stage (Lintern-Moore and Moore, 1979). This suggest that despite the beneficial metabolic effects of 17 α -E2, at the local ovarian level 17 α -E2 increased oocyte growth which may be related to increased follicle activation, especially in GHRKO mice, who possess inherent follicular reserve. Traditionally, ER β is considered the main ER in the ovaries (Lee et al., 2009). Disruption of the gene that codes for the ER β , however, caused no significant fertility problems (Schomberg, et al. 1999). Knockout of the ER α gene (Esr1) in mice, on the other hand, resulted in infertile females (Schomberg et al., 1999). In humans, defects in the Esr1 gene have been associated with premature depletion of ovarian reserve (Bretherick et al., 2008). Recent studies with disruption of the Esr1 gene in zebra fish reported enhanced fertility in the mutant females during young ages, with greater egg production (Chen et al., 2018). However, the fertility of these females decreased with age, and ovarian reserve depletion was reached much earlier than in WT females (Chen et al., 2018), suggesting that Esr1 deficiency is associated with increased ovarian primordial reserve activation.

To summarize, our results do not confirm our hypothesis that 17 α -E2 would preserve ovarian primordial follicle reserve. CR-like effects and direct effects of 17 α -E2 on the ovaries appear to be not additive. In this sense, previous studies suggest that 17 α -E2 is able to increase median lifespan in males, but not in females (Harrison et al., 2014). Furthermore, evidence suggests 17 α -E2 has direct effects on reproductive tissues of females (Strong et al., 2016), which could explain the lack of effect of 17 α -E2 on longevity in females. The effects of 17 α -E2 treatment on metabolic profile are stronger in males and decrease upon castration, while in females the effects were milder and in general did not change after ovariectomy (Garratt et al., 2018). Further studies addressing 17 α -E2 effects on gonadal activity must be undertaken to better understand the association between somatic and germline aging and how the lack of effect on the ovaries can contribute to the lack of effect of 17 α -E2 on somatic aging.

In conclusion, we observed that GHRKO mice had lower activation of primordial follicles, preserving the primordial reserve. However, 17 α -E2 had no effect on the ovarian reserve of WT mice and decreased the number of primordial follicles in GHRKO mice.

Funding

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), The National Institutes of Health (R00 AG51661 to M.B.S., R15 AG059190 to M.M.M., R03 AG059846 to M.M.M., and R21 AG051869 to A.B.).

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Highlights

- GHRKO mice have increased reserve of ovarian primordial follicles
- 17 α E2 increased activation of primordial follicles in GHRKO mice but did not affect wild-type mice
- 17 α E2 did not affect weight gain or IGF-I concentration for GHRKO and wild-type female mice

Figure 1 -
Number of primordial (A), transition (B), primary (C), secondary (D), tertiary (E) and total (F) follicles from wild-type (WT) and growth hormone receptor knockout (GHRKO) mice treated with 17α estradiol (17αE2) or not (Control). *Different letters indicate statistical difference ($p < 0.05$)

Figure 2 - IGF1 plasma concentration wild-type (WT) and GHRKO mice treated or not with 17α estradiol (17αE2) for 4 months. *Different letters indicate statistical difference on the multiple comparisons test ($p < 0.05$).

Table 1 -

Diameter of the nucleus, oocyte and follicle and number of granulosa cells from wild-type (WT) and growth hormone receptor knockout (GHRKO) mice treated with 17 α estradiol (17 α E2) or not (Control).

-	Wild-Type		GHRKO		P value		
	Control	17 α E2	Control	17 α E2	Genotype	17 α E2	Interact.
Primordial							
Nucleus	6.5 $\pm$ 0.1	6.6 $\pm$ 0.1	6.39 $\pm$ 0.1	6.82 $\pm$ 0.2	0.141	0.323	0.323
Oocyte	11.0 $\pm$ 0.4	11.0 $\pm$ 0.4	10.18 $\pm$ 0.3	11.54 $\pm$ 0.3	0.095	0.701	0.089
Follicle	15.6 $\pm$ 0.7	16.2 $\pm$ 0.3	13.92 $\pm$ 0.4	16.31 $\pm$ 0.4	0.005	0.127	0.089
Cells	6.0 $\pm$ 0.3	6.8 $\pm$ 0.3	6.06 $\pm$ 0.4	5.73 $\pm$ 0.3	0.489	0.169	0.123
Transition							
Nucleus	6.8 $\pm$ 0.1	7.2 $\pm$ 0.3	6.41 $\pm$ 0.1	7.09 $\pm$ 0.2	0.012	0.223	0.596
Oocyte	11.7 $\pm$ 0.3	12.4 $\pm$ 0.7	10.26 $\pm$ 0.3	11.71 $\pm$ 0.3	0.032	0.025	0.440
Follicle	20.3 $\pm$ 0.5 ^a	19.9 $\pm$ 0.7 ^a	15.66 $\pm$ 0.6 ^b	18.96 $\pm$ 0.5 ^a	0.023	<0.0001	0.005
Cells	7.8 $\pm$ 0.4	7.2 $\pm$ 0.5	7.13 $\pm$ 0.4	7.2 $\pm$ 0.4	0.531	0.444	0.444
Primary							
Nucleus	9.3 $\pm$ 0.4	9.1 $\pm$ 0.5	9.49 $\pm$ 0.4	8.99 $\pm$ 0.4	0.768	0.972	0.768
Oocyte	22.9 $\pm$ 1.4 ^a	18.3 $\pm$ 1.4 ^b	21.58 $\pm$ 1.4 ^a	22.05 $\pm$ 1.5 ^a	0.0006	<0.0001	0.0018
Follicle	36.3 $\pm$ 2.0	32.2 $\pm$ 1.8	34.30 $\pm$ 2.1	34.53 $\pm$ 1.9	0.306	0.954	0.253
Cells	18.5 $\pm$ 1.5	14.4 $\pm$ 1.1	17.33 $\pm$ 1.5	15.20 $\pm$ 1.0	0.014	0.872	0.423