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Original Article

Modified Gengnianchun Formula Outperforms Androgens in Treating Chronic Stress-Induced Diminished Ovarian Reserve: An Animal Study

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Abstract

Background and objectives: Chronic stress-induced hypercortisolism causes diminished ovarian reserve (DOR), contributing to infertility and miscarriage. Androgen supplementation is an emerging therapeutic approach for DOR. The traditional Chinese herbal decoction modified Gengnianchun formula (MGNC) has shown clinical efficacy in treating DOR. This study aimed to compare the effectiveness of MGNC with that of androgens in a stress-induced DOR mouse model.

Methods: Sexually mature female C57 mice aged six weeks were randomly assigned to six groups (n = 10 per group, with 3 independent replicates per group), including the control, model, low-dose testosterone (LT), medium-dose testosterone (MT), high-dose testosterone (HT), and MGNC groups. This sample size and study design were determined based on preliminary experimental data. Chronic stress was induced in mice, except for the control group, by daily glucocorticoid injection, and the mice in the LT, MT, HT, and MGNC groups were treated at the same time with testosterone (low, medium, or high dose) or MGNC for six weeks. Body weight, estrous cycles, ovarian follicle counts, hormone profiles, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and testosterone, and *in vitro* preantral follicle growth rates (via MGNC-enriched or androgen-treated serum) were assessed.

Results: All groups presented stable body weights. MGNC ameliorated estrous cycle irregularities caused by stress, while testosterone exacerbated the abnormality. Moreover, MGNC outperformed LT in improving primordial/primary/antral follicle counts and corpus luteum formation, while MT and HT did not improve ovarian follicle reserve. LT was associated with the highest serum estradiol level, but none of the testosterone doses reduced FSH levels or the FSH/LH ratio, whereas MGNC lowered FSH and the FSH/LH ratio. Additionally, MGNC-enriched serum significantly enhanced the *in vitro* follicular growth rate in corticosterone-supplemented culture medium, and this effect was superior to that observed with testosterone-pretreated serum.

Conclusions: MGNC demonstrates superior efficacy over androgen therapy in treating chronic stress-induced DOR in mice, supporting further investigations into its clinical potential and mechanisms.

Keywords: Modified Gengnianchun formula; Diminished ovarian reserve; Androgen; Stress; Glucocorticoids; Ovarian follicle.

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Introduction

A diminished ovarian reserve (DOR) signifies a reduced number or quality of functional ovarian follicles, leading to complications such as infertility, recurrent pregnancy loss, irregular menstruation, and premature menopause. A 2016 prospective cohort study reported a 48% incidence of DOR in young women of reproductive age who experienced unexplained recurrent miscarriage.[1] The cause of DOR is associated with genetic factors, environmental pollution, iatrogenic ovarian injury, reproductive

toxicants, and psychological factors.[2] With the accelerating pace of modern life and the increase in women's social pressure, factors contributing to chronic stress have gradually attracted increasing attention.

Stress elicits a nonspecific bodily response when the internal homeostasis is threatened. Chronic stress, which predominantly activates the hypothalamic–pituitary–adrenal axis, results in sustained glucocorticoid release. These hormonal disturbances impair granulosa cell function and follicular development,[3,4] promote apoptosis,[5] and increase reactive oxygen species levels in the ovarian milieu,[6] thus influencing ovarian follicle development. Moreover, increased stress intensity and prolonged stress can further increase the adverse effects on follicular development and oocyte quality.[7]

In allopathic medicine, DOR management primarily involves hormone replacement therapy and gonadotropin-induced ovulation. Estrogen can inhibit the release of follicle-stimulating hormone (FSH) through a negative feedback mechanism, thereby reducing serum FSH levels, restoring the follicular function, and promoting ovulation. Antioxidants, including vitamin E, vitamin D, resveratrol, melatonin, and coenzyme Q10, have been shown to exhibit beneficial effects by counteracting mitochondrial ovarian aging and reducing embryonic aneuploidy.[8] Both treatments help support follicular growth without increasing the functional follicular reserve.[9,10] In recent years, studies have demonstrated that androgen supplementation is promising for enhancing follicular development through regulating the FSH receptor.[11] A balanced level of androgen is indispensable for early-stage ovarian follicle development.[12] Recent high-impact clinical research has demonstrated that androgen supplementation is associated with increased live birth rates and clinical pregnancy rates, making it one of the most promising interventions.[13] Our previous work revealed that a balanced level of testosterone can significantly improve early-stage follicle count through a synergistic effect of insulin-like growth factor-1 (IGF1)-FSH signaling in a stress-induced mouse model.[14] Nonetheless, there remains considerable debate in clinical application concerning the optimal androgen type, dosage, and treatment duration, with inconsistent clinical outcomes and potential adverse effects.[15]

TCM can exert multitarget regulatory effects on ovarian function. A variety of compound Chinese patent medicines, including Dingkun pills, Kuntai capsules, and Qilin pills, have been clinically applied, and their efficacy in treating DOR has been verified.[16,17] However, the efficacy of these traditional Chinese medicines in treating DOR caused by stress has not been further verified or explored. The Gengnianchun formula, a traditional Chinese medicinal compound comprising 12 herbs, has demonstrated effectiveness in alleviating menopausal syndromes and related conditions such as Alzheimer's disease caused by estrogen depletion.[18] Our team augmented the traditional Gengnianchun recipe with three herbs specifically aimed at chronic stress-related etiology, and the resulting modified Gengnianchun formula (MGNC) significantly increased follicle counts and improved serum hormone profiles in a chronic unpredictable stress-induced DOR murine model.[14] Hormone changes include elevated testosterone levels and decreased corticosterone (CORTN) levels. These findings indicate that improving serum testosterone secretion may be one of the mechanisms by which MGNC is effective in chronic stress-induced DOR. However, there is currently a lack of comparative studies on the efficacy of MGNC and direct androgen therapy. Whether MGNC can exert "androgen-like effects" while achieving superior efficacy compared with androgen alone, thereby providing more benefits to patients with DOR caused by chronic stress, remains unclear.

This study aimed to compare the effects of different doses of androgen and MGNC in a glucocorticoid-induced chronic stress animal model and an *in vitro* follicle growth model to clarify the potential therapeutic advantages of MGNC.

Materials and methods

Drugs and reagents

CORTN (Selleck, S4752, China) was initially dissolved in 1% v/v dimethylsulfoxide (Merck, D8418, Germany) and 4% v/v Tween-80 (Merck, P5188, Germany) before being diluted to concentrations of 2, 2.5, and 3 mg/mL with normal saline (NS). The mice received subcutaneous injections at 10 mL/kg. The testosterone dosage for mice was calculated on the basis of the human dosage from clinical trials.[19,20] Consequently, 700 μg/kg was categorized as a high dose, 350 μg/kg as a medium dose, and 175 μg/kg as a low dose. Testosterone (NatureStandard, 4049563, China) was first dissolved in 1% v/v absolute ethanol (China National Pharmaceutical Group, 10009218, China) and then brought to the desired concentration with sesame oil prior to subcutaneous administration to the mice at 5 mL/kg.

The MGNC decoction was formulated on the basis of an earlier study,[14] which confirmed its efficacy in a chronic unpredictable stress-induced DOR mouse model. Water extracts of 15 raw herbs from Tianjiang Pharmaceutical Limited Company (Jiangyin, China) were dissolved in water at 100 °C and subsequently diluted to 0.444 g/mL. The mice were given 2 mL/kg MGNC decoction via gavage. Table 1 lists the conversions between the water extract weight and the weight of the raw herbs provided by the manufacturer.

Animals and treatment

On the basis of the findings from previous animal model pilot experiments, a sample size of ten mice per group with three independent replicates was determined to ensure statistical power and reliability. In total, 180 sexually mature female C57 mice aged six weeks were used in this study. The mice were acclimatized in the SPF facility for one week. All the mice were weighed and divided into three body weight groups. The mice in each group were then numbered and randomly assigned to six groups via a random number table. This experimental design, including both sample size and number of replicates, was based on preliminary data. The experimental groups were as follows: the control, model, low-dose testosterone (LT), medium-dose testosterone (MT), high-dose testosterone (HT), and MGNC groups. The experimental procedures were conducted over a total duration of six weeks. All groups except the control group received chronic stress modeling via subcutaneous CORTN injections to induce DOR. The CORTN dosage was adjusted in three two-week intervals: 20 mg/kg for the first two weeks, 25 mg/kg for the next two weeks, and 30 mg/kg for the final two weeks. For the same six-week period, the control group received subcutaneous injections of an equal volume of CORTN solvent to eliminate confounding effects from the injection procedure itself. During the six-week modeling phase, each intervention group received targeted treatments, whereas the control and model groups received vehicle/solvent controls, as detailed below:

LT, MT, and HT groups: Subcutaneous injections of testosterone at low, medium, and high concentrations, respectively (dos-

Table 1. Information on the modified Gengnianchun formula

English name (Chinese name)	Latin name	Plant part	Crude herb (g)	Water extract(g)
Radix Rehmanniae (Shengdi)	*Rehmannia glutinosa* (Gaertn.) DC.	Root	15	4.5
Epimedium (Yinyanghuo)	*Epimedium brevicornum* Maxim.	Leaf	12	0.6
Radix Paeoniae Alba (Baishao)	*Paeonia lactiflora* Pall.	Root	12	1.2
Fructus Lycii (Gouqizi)	*Lycium barbarum* L.	Fruit	12	4.8
Plastri Testudinis (Guiban)	Carapax et plastrum Testudinis	Carapax	15	0.75
Rhizoma Anemarrhenae (Zhimu)	*Anemarrhena asphodeloides* Bunge	Root	15	3.75
Semen Cuscutae (Tusizi)	*Cuscuta australis* R.Br.	Seed	12	0.6
Radix Morindae Officinalis (Bajitian)	*Morinda officinalis* F.C.How	Root	12	3.6
Cistanche Salsa (Congrong)	*Cistanche deserticola* Y.C.Ma	Stem	12	3.6
Cortex Phellodendri Amurensis (Huangbai)	*Phellodendron chinense* Schneid	Bark	9	0.75
Rhizoma Coptidis (Huanglian)	*Coptis chinensis* Franch.	Rhizome	3	0.5
Poria (Fuling)	*Poria cocos* (Schw.) Wolf	Sclerotium	9	0.9
Radix Bupleuri (Chaihu)	*Bupleurum chinense* DC.	Root	9	1.5
Angelica Sinensis (Danggui)	*Angelica sinensis* (Oliv.) Diels	Root	12	4.8
Rhizoma Ligustici Wallichii Chuanxiong	*Ligusticum chuanxiong* Hort.	Rhizome	9	3

ages aligned with preexperimental optimization), in addition to CORTN injections for modeling.

The MGNC group received two concurrent treatments: 1) subcutaneous injections of CORTN (for modeling) and 2) oral gavage of the MGNC decoction (once daily, dosage on the basis of body weight).

Control group: Subcutaneous injections of CORTN solvent + oral gavage of an equal volume of NS.

Model group: Subcutaneous injections of CORTN (for modeling) + oral gavage of an equal volume of NS. Additionally, MGNC group mice were orally administered the MGNC decoction, and those in the remaining groups received equal volumes of NS via gavage.

Sample collection and monitoring

The weights of the mice were recorded weekly. Testosterone and MGNC dosages were adjusted dynamically on the basis of individual weight fluctuations to ensure consistent drug exposure. For the final three weeks of the experiment, daily vaginal smears were collected from each mouse at the same time every day to track the estrous cycle status. After the six-week period, the mice in the nonestrus phase were sacrificed. The mice in the estrus phase continued receiving their assigned treatments until they entered the non-estrus phase, at which point sacrifice was performed (to standardize the ovarian status at tissue collection). The entire animal experiment was repeated three times. Animal cage locations and the order of treatment administration were rearranged to minimize potential confounding effects from environmental factors and procedural order. The experimental protocol is illustrated in Figure 1.

Vaginal smear and estrous cycle determination

With the left hand, the skin on the nape of the mouse's neck was gently grasped, and with the right hand, the tip of a pipette containing 10 μL of NS was inserted into the vaginal canal, and the liquid was carefully expelled and aspirated several times. Saline containing vaginal epithelial cells was transferred onto a glass slide, which was subsequently allowed to dry. The slides were then stained with hematoxylin for 1.5 min, rinsed with tap water to remove excess stain, and briefly stained with eosin for 3 s. After a quick rinse, the prepared slide was ready for microscopic examination. The characteristics of each estrus stage are described in the standard protocol.[21]

Enzyme-linked immunosorbent assay (ELISA)

Following anesthesia with a 1% sodium pentobarbital solution administered intraperitoneally at a dosage of 80 mg/kg, mouse cardiac blood was collected. The blood was allowed to clot at 4 °C for 2 h prior to serum extraction via centrifugation at 3,000 × g for 15 min at the same temperature. The resulting serum was aliquoted and stored at −80 °C for subsequent ELISA analysis. Serum reproductive hormones, including estradiol (E2, LDN, FR E-2000, detection range: 10.6–2,000 pg/mL), testosterone (LDN, AR E-8000, minimum detectable level: 0.066 ng/mL), FSH (ImmunoWay, KE1425, detection range: 1.563–100 ng/mL), and luteinizing hormone (LH, ImmunoWay, KE1421, detection range: 0.469–30 ng/mL), were quantified following the manufacturer's guidelines.

Follicle counting

One ovary from each mouse was fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned at intervals of 50 μm above and 100 μm below the largest diameter of the ovary. The sections were subsequently stained with hematoxylin and eosin, dehydrated, and mounted with neutral gum to facilitate follicle enumeration. The number of follicles at various developmental stages and the corpora lutea were quantified across the five sections and summed to yield the total count for each ovary. Follicle staging was performed in accordance with the methodology outlined by Oktay.[22]

Preparation of MGNC-enriched serum

Following anesthesia with a 1% sodium pentobarbital solution, bilateral ovariectomies were performed on 20 Sprague–Dawley rats.

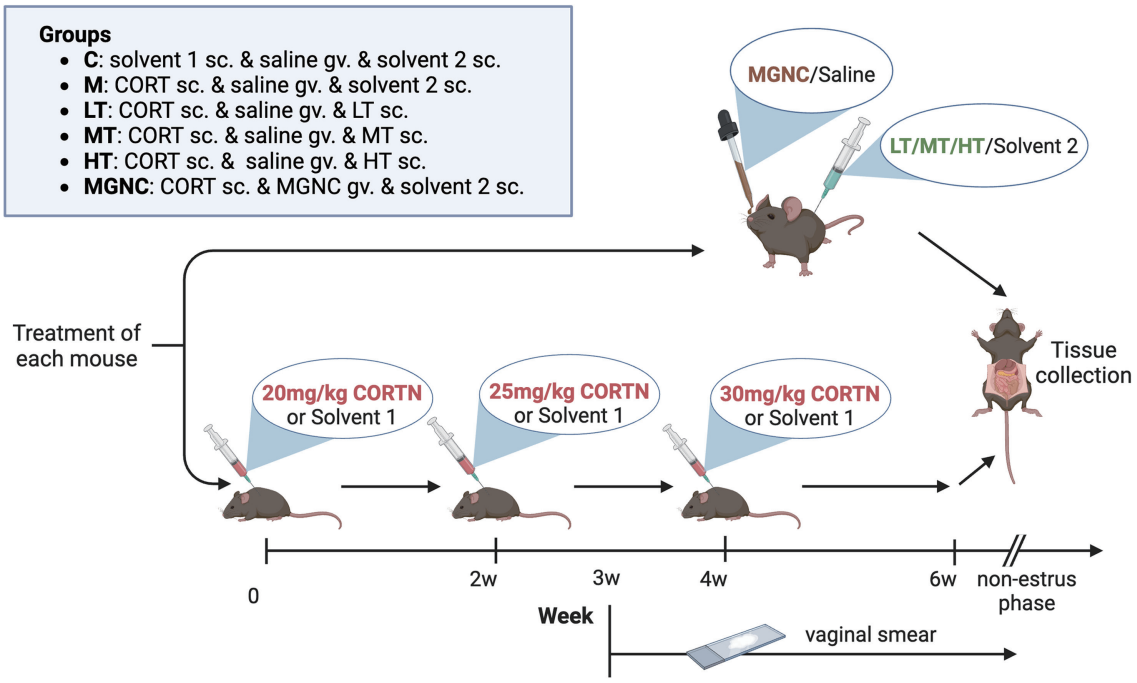

Fig. 1. Animal experimental protocol. C, control; CORTN, corticosterone; gv., gavage; HT, high-dose testosterone; LT, low-dose testosterone; M, model; MGNC, modified Gengnianchun formula; MT, medium-dose testosterone; sc., subcutaneous injection.

After the operation, the rats received intramuscular injections of penicillin at a dosage of 80,000 units per day for two days and were then allowed a seven-day recovery period. On the seventh postoperative day, daily vaginal smear tests were conducted for five consecutive days to ascertain the phase of each rat's estrous cycle. The qualifying rats were then randomly allocated into two groups: the MGNC group and the NS group. The rats in both groups were administered either MGNC or an equal volume of NS via gavage twice daily for five days. The administered dose of the MGNC decoction (0.25 g/mL) was set at 2.49 g/kg for the rats, which was calibrated from the standard human dosage.[21] One hour after the final gavage, the rats were anesthetized again via a 1% sodium pentobarbital solution; cardiac blood was drawn and filtered through a 0.22 μm filter for bacterial removal, and the serum was heat-inactivated at 56 °C. The serum was subsequently aliquoted and stored at −80 °C for future experimentation.

In vitro culture of mouse preantral follicles

In a modified version of our previously described technique,[23] the ovaries of female C57 mice aged 14–16 days were removed postmortem and used for follicle isolation. The secondary follicles, each with a diameter ranging from 100 to 130 μm, were excised under a stereomicroscope and immersed in 1% alginate solution droplets. These droplets were then solidified via the addition of 40 mM $CaCl_2$ with 150 mM NaCl solution to form follicle-containing alginate beads. In this study, the fetal bovine serum traditionally used in culture media was replaced with either 2.5% MGNC-enriched serum or 2.5% NS serum. For the *in vitro* cultures, which lasted three days, the follicle-containing beads were randomly sorted into five experimental groups: the control group, MGNC group, NS with a high concentration of CORTN (HC) group, NS plus HC and dihydrotestosterone (DHT) group, and HC+MGNC group. A high CORTN concentration was defined as 1 μM, with DHT at a concentration of 10 nM. Daily microscopic observations of the follicles were conducted, and three representative diameters of each follicle were measured via Image-Pro Plus 6.0 software; the mean diameter was calculated from these measurements. Data on follicle diameter for each experimental group were collated from three independent experiments, encompassing 10 follicles per group.

Data analysis

Statistical analyses and generation of the results were performed via GraphPad Prism software version 9.4.1. Comparisons of hormone levels and follicle counts between groups were conducted via one-way analysis of variance. Differences in the animal weight changes and mean follicle diameter growth during culture were compared via one-way repeated-measures analysis of variance. The Tukey method was used for multiple comparisons. A *P*-value of less than 0.05 was recognized as statistically significant.

Results

The impact of MGNC and androgen supplementation on body weight and the estrous cycle under chronic stress

Figure 2a shows that over a six-week period involving the modeling and administration of various concentrations of androgens and MGNC, the control group exhibited a consistently higher rate of weight gain than did the experimental groups. Specifically, the weight gain in the model group was the least substantial. Although the androgen and MGNC treatment groups presented slight improvements in weight gain trends relative to the model group, the differences were not statistically significant across the groups.

As shown in Figure 2b, the frequency of the diestrus phase in the model group and the androgen treatment groups was greater than that in the control group. Recovery of the estrous cycle in the

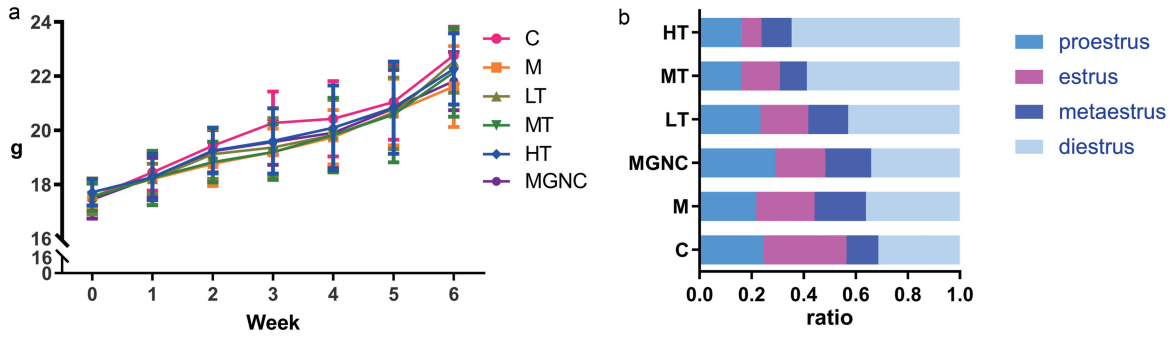

Fig. 2. The effects of MGNC and androgen treatment on body weight changes (a) and estrous cyclicity (b) in a chronic stress-induced diminished ovarian reserve model. The body weights of the animals in all the groups at the beginning of each experimental week were measured. Body weight data are presented as the means ± standard deviations. The estrous cycle is displayed as the ratio (%) of the days in each stage to the total observation days (21 days). C, control; HT, high-dose testosterone; LT, low-dose testosterone; M, model; MGNC, modified Gengnianchun formula; MT, medium-dose testosterone; N = 30. Statistical analysis was performed via repeated-measures one-way analysis of variance, and no statistically significant differences were found among all the groups.

MGNC-treated mice was noted, which aligns more closely with the pattern observed in the control group. Notably, the estrous cycle irregularities in the androgen treatment groups were more pronounced than those in the model group compared with those in the control group. The highest degree of abnormality was observed in the HT group, followed by the MT and LT testosterone groups, in decreasing order of severity.

Effects of MGNC and androgen supplementation on the ovarian reserve under chronic stress

The effects of MGNC and androgen supplementation on the ovarian reserve were assessed under conditions of chronic stress. Figure 3 shows the follicle counts at various stages, ranging from the primordial to the antral stages (Fig. 3a–d), and the number of corpora lutea (Fig. 3e). In the model group, there was a notable decrease in follicle counts across all stages compared with those in the control group, with significant reductions in primordial follicle counts (P = 0.0378). Moreover, the average follicle count in the MGNC group was greater than that in the model group at all stages. Compared with those in the model group, the follicle counts in the LT group across stages were greater and comparable to those in the MGNC group. However, the differences were not significant between the LT and MGNC groups across all follicle stages. In contrast, the MT group had lower mean follicle counts than the LT group did (P > 0.05 in all follicle stages), and the mean numbers of follicles in all stages were comparable to those in the model group (P > 0.05 in all follicle stages). Compared with the MT group, the HT group presented even lower follicle counts in all stages (P > 0.05 in all follicle stages). The mean primordial follicle count (P = 0.0121) and primary follicle count (P = 0.0111) were significantly lower in the HT group than in the MGNC group. The mean primary follicle count in the HT group was significantly lower than that in the LT group (P = 0.0001). Compared with that in the control group, the corpus luteum count in the model group was significantly lower. Although the MGNC group presented an increased corpus luteum count relative to that of the model group, a dose-dependent decrease was observed in the three androgen treatment groups as the androgen concentration increased. Compared with that in the model group, the corpus luteum count in the LT group tended to increase, yet it did not reach the mean level observed in the MGNC group. Moreover, the MT and HT groups presented significantly fewer corpora lutea than the model group did (MT vs. model, P = 0.0081; HT vs. model, P = 0.0090). Representative images of hematoxylin-eosin -stained slices of ovarian sections from different groups of mice are shown in Figure 3.

The impact of MGNC and androgen supplementation on serum reproductive hormones during chronic stress

We employed ELISA to measure the levels of reproductive hormones in each group, with the findings depicted in Figure 4. Compared with those in the control group, the serum testosterone and E2 levels in the model group tended to decrease (Fig. 4a). Compared with the model group, the MGNC group presented a greater level of serum testosterone, which was comparable to that of the control group. Compared with those in the control group, the serum testosterone levels in the androgen-treated groups tended to increase in a dose-dependent manner. The difference between the HT group and the model group was significant (P = 0.0148). Compared with those in the control group, serum E2 levels were lower in the model group (Fig. 4b), and both the MGNC and the LT groups presented elevated E2 levels compared with those in the model group (model vs. MGNC, P > 0.05; model vs. LT, P = 0.0009). However, as the androgen dose increased, the E2 level in the MT and HT groups decreased in a dose-dependent manner compared with that in the LT group (LT vs. HT, P = 0.0479). The serum FSH levels did not differ significantly among all the groups (Fig. 4c); however, there was an increasing trend in the model group compared with the control group in terms of the FSH level, and the FSH level in the MGNC group tended to decrease compared with that in the model group. Compared with those in the model group, the FSH levels in the LT group were similar, and the mean serum FSH level decreased as the androgen dose increased. The LH levels did not significantly differ among the groups (Fig. 4d). As shown in Figure 4e, the serum FSH/LH ratio was greater in the model group than in the control group (P > 0.05). The ratio was lower in the MGNC group than in the model group (P > 0.05). The FSH/LH ratio of the LT group increased significantly compared with that of the control group (P = 0.0078) and was greater than that of the model group (P > 0.05). A dose-dependently decreasing trend in the FSH/LH ratio was observed in the MT and HT groups compared with the model group.

The impact of MGNC and androgen supplementation on follicle development under chronic stress

Mouse preantral follicles were isolated and cultured *in vitro* with

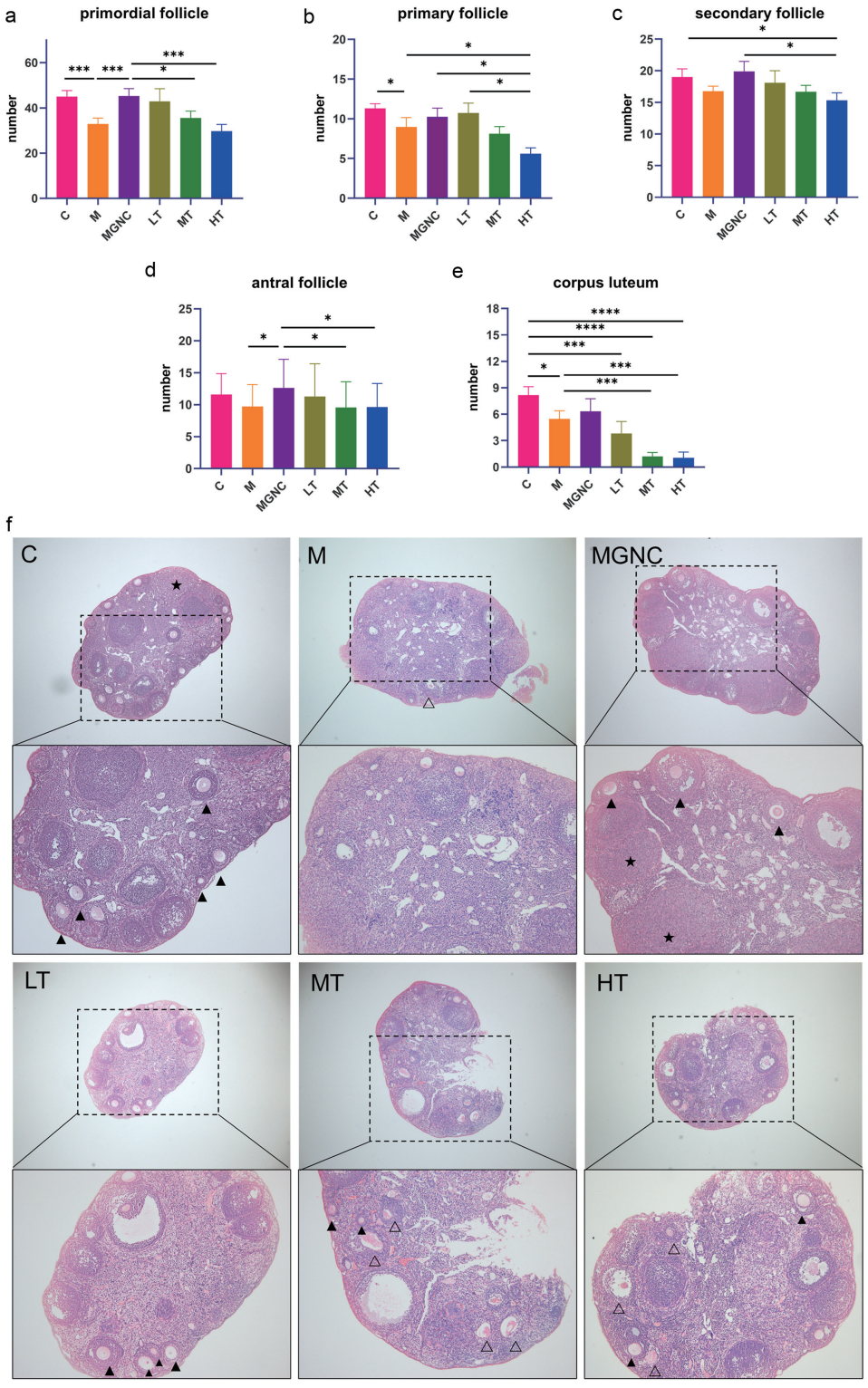

Fig. 3. The effects of MGNC and androgen treatment on the ovarian reserve in a chronic stress-induced diminished ovarian reserve model. The primordial follicle (a), primary follicle (b), secondary follicle (c), antral follicle (d), and corpus luteum (e) counts across various experimental groups were analyzed (n = 30). (f) Representative images of hematoxylin-eosin stained ovarian sections from different groups of mice. The follicle count data are presented as bar graphs of the mean values, with error bars representing the standard error of the mean. Comparisons among different groups were performed via one-way analysis of variance. **P* < 0.05; ****P* < 0.001; *****P* < 0.0001. ▲, healthy preantral and antral follicles; △, atretic follicles; ★, corpus luteum. C, control; HT, high-dose testosterone; LT, low-dose testosterone; M, model; MGNC, modified Gengnianchun formula; MT, medium-dose testosterone.

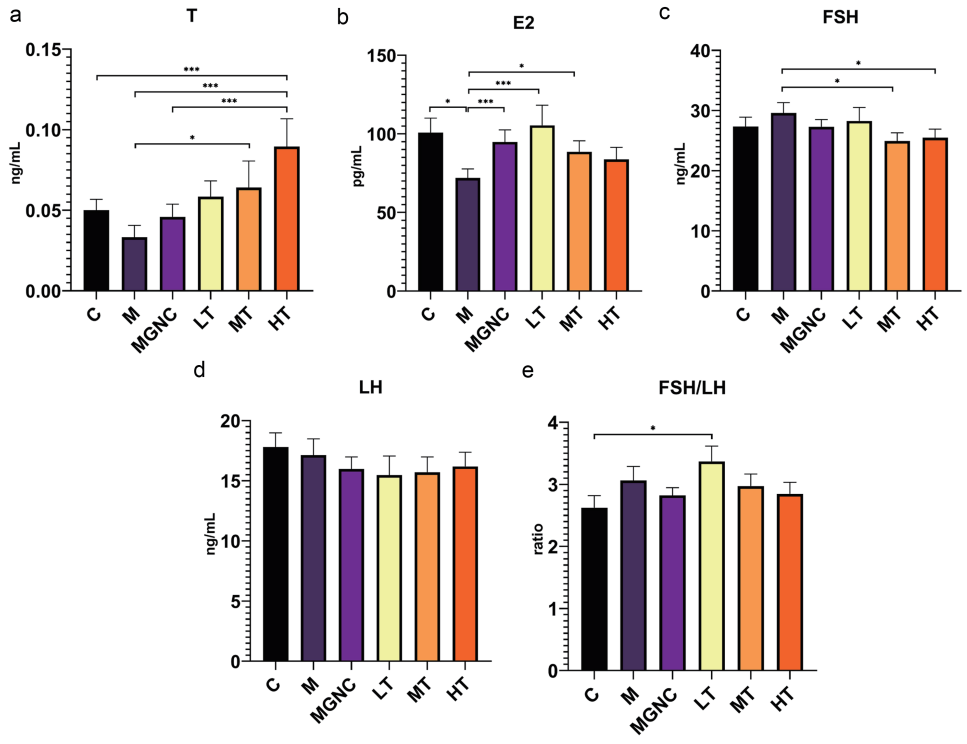

Fig. 4. The effects of MGNC and androgen treatment on reproductive hormone levels in a chronic stress-induced diminished ovarian reserve model. The levels of serum testosterone (a, n = 12), E2 (b, n = 21), FSH (c, n = 21), LH (d, n = 22), and FSH/LH (e, n = 20) among the various groups are shown. The data are presented as bar graphs of the mean values, with error bars representing the standard error of the mean. Comparisons among different groups were performed via one-way analysis of variance. The data are presented as the means ± standard errors of the mean. **P* < 0.05; ****P* < 0.001. C, control; FSH, follicle-stimulating hormone; HT, high-dose testosterone; LH, luteinizing hormone; LT, low-dose testosterone; M, model; MGNC, modified Gengnianchun formula; MT, medium-dose testosterone.

high concentrations of CORTN to simulate chronic stress. Figure 5a illustrates the process of early secondary follicle growth during three days of culture in the different groups. The follicle diameter became larger as the granulosa cell layers expanded. The follicle growth rate peaked in the MGNC group, whereas the follicles in the NS+HC group grew the slowest. The follicle diameter

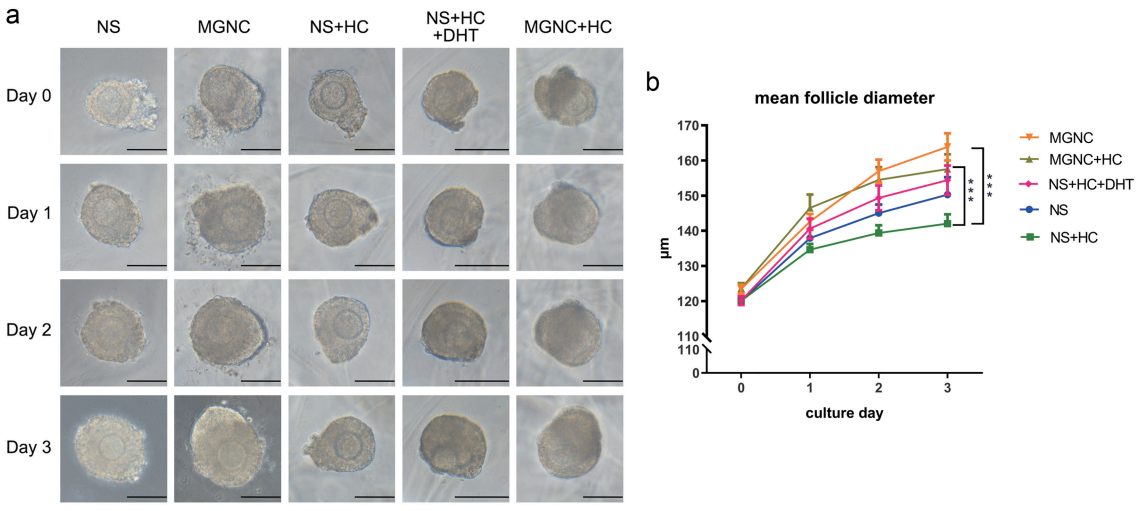

Fig. 5. Preantral follicle growth in MGNC-enriched serum treated and androgen-treated mice under conditions of chronic stress. (a) Representative images of follicles from days 0 to 3 across the various groups. Scale bar = 100 μm. (b) Mean follicle diameter variation among the groups during each culture day. The data are presented as the means ± standard deviations. Follicle diameter changes during culture were compared via one-way repeated-measures analysis of variance. ****P* < 0.001. HC, NS+1 μM CORTN; HC+DHT, NS+1 μM CORTN+10 nM DHT; HC+MGNC, MGNC-containing serum+1 μM CORTN; MGNC, modified Gengnianchun formula-enriched serum; NS, normal saline.

increased faster in the NS+HC+DHT group and the MGNC+HC group than in the NS+HC group. As shown in Figure 5b, a significantly greater follicle diameter in the MGNC group than in the HC group was observed from days 1 to 3 (day 1, $P = 0.0431$; day 2, $P = 0.0008$; and day 3, $P = 0.0004$). On culture days 2 and 3, the follicle diameter in the HC+MGNC group was significantly greater than that in the HC group (day 2, $P = 0.0114$; day 3, $P = 0.0298$). The follicle diameter increased faster in the HC+DHT group than in the HC group on culture days 2 and 3, but the differences were not significant.

Discussion

CORTN treatment as a way to establish a chronic stress-induced DOR animal model

Chronic stress is a critical factor that negatively affects female reproductive health, as manifested by elevated serum glucocorticoids. By administering high-dose glucocorticoids via subcutaneous injection to establish a hormonal environment that mimics chronic stress in mice, this study successfully induced the DOR phenotype. The existing animal models for stress-induced DOR are not widely accepted. Some studies have employed subcutaneous cortisol injections to assess their impact on the developmental potential of mouse oocytes,[3,24] but cortisol is predominantly secreted in response to acute stress in mice. CORTN is the primary hormone released during chronic stress,[25] and the durations of previous studies were too brief to properly emulate the persistent, high-glucocorticoid environment induced by chronic stress. Accordingly, this study adapted the CORTN dosage from research in which long-term CORTN administration produced a depression model in mice.[26] Drawing on our prior establishment of a chronic unpredictable stress-induced DOR model,[27] we incrementally increased the CORTN dose every two weeks during the experiment to limit habituation in the mice. Body weight gain is a straightforward indicator of chronic stress.[28] Figure 2a shows that after six weeks of treatment, the weight gain of the mice in the model group was less than that of the mice in the control group; however, this difference was not statistically significant, potentially because of the hyperglycemic effects of CORTN.[29] The negative impact of prolonged, high-dose CORTN on reproduction was evidenced by an aberrant estrous cycle (Fig. 2b) and a reduction in the number of follicles at various developmental stages (Figs. 3a–d). The abnormal estrous cyclicity suggested disrupted ovulatory activity, which was also indicated by a decreased number of corpora lutea (Fig. 3e). The serum E2 levels in the model group were lower than those in the control group, whereas the FSH level and the FSH/LH ratio were greater. These patterns align with the hormonal changes observed in DOR patients, suggesting that the CORTN dosage was effective in producing a mouse model of chronic stress-induced DOR.

Effective androgen treatment: Choice of time and dose

Our results revealed that androgen intervention at an appropriate concentration (the low dose in this study) for six weeks significantly increased the number of early follicle reserves in stressed DOR mice. Recent interest in androgen supplementation for poor ovarian responders or patients with DOR who exhibit low serum androgen levels has increased.[9,30] The administration of dehydroepiandrosterone or testosterone prior to ovarian stimulation in patients undergoing *in vitro* fertilization can significantly increase the oocyte retrieval rate.[13] However, consensus remains elusive regarding the optimal type, dosage, and duration of androgen administration.[31] To observe the concentration gradient effect of androgen, a suitable concentration range was applied to the mice. We referred to the testosterone skin patch dose employed in a Gleicher-led large-scale multicenter, double-blind, randomized controlled trial and converted it into an animal dose on the basis of pharmacological principles.[19,20] The extrapolated dose was defined as a 'high dose'. Half of the dose was subsequently classified as "medium", and further halving was classified as "low" (determined after previous validation). The duration of androgen treatment in clinical research also varies widely, ranging from 5–21 days. Gleicher *et al*.[32] indicated that androgens affect follicles from the primary to antral stages through androgen receptors, yet these follicles require weeks to months to develop into gonadotropin-sensitive follicles after androgen influence.[19] In mice, it takes at least 47 days for primary follicles to progress to the antral follicle stage.[33] This duration is consistent with our six-week intervention plan and was proven to be effective in achieving a favorable effect on ameliorating the stressed DOR phenotype in mice. In our previous work, the effect of a balanced level of androgen on follicle development in a stressed DOR mouse model was confirmed to involve upregulation of the synthesis of the FSH receptor and IGF1 receptor on granulosa cells,[23] which are essential signals for early-stage follicle growth.[34,35] In human cumulus cells, androgens have been found to downregulate AKT1 and possibly enhance oocyte reproductive potential,[36] suggesting that androgens can also play a role in improving the quality of follicles.

Background of MGNC in DOR treatment

Gengnianchun is known to be an effective compound for replenishing the kidney, tranquilizing the liver, purifying the heart, and eliminating excess fire. Our research group has previously demonstrated that Gengnianchun notably mitigates conditions stemming from reduced estrogen levels, such as perimenopausal syndrome and Alzheimer's disease.[18,37–39] The decline observed in the perimenopausal phase mirrors that of DOR, with both scenarios featuring a decreased follicle count and decreased ovarian endocrine function. In the prescription, Shengdi nourishes Yin and tonifies the kidney, cools blood, and clears heat; Xianlingpi tonifies the kidney and aids Yang. Together, these compounds serve as the principal herbs. The combination of these two drugs regulates Yin and Yang in the kidney. Baishao and Gouqizi soften the liver, calm the liver, clear the heart, and relieve urgency; Guiban, an animal-derived medicinal material traditionally considered nourishing in TCM theory, can replenish the marrow and fill the essence; Zhimu nourishes Yin, clears heat, moistens dryness, and calms the mind; and Tusizi, Bajitian, and Congrong tonify the kidney and benefit the thoroughfare and conception vessels. Together, these act as deputy herbs, assisting chief herbs in regulating the balance of Yin and Yang in Zang-Fu organs (according to TCM theory). Huangbai and Huanglian clear heat and purge fire from the heart and kidney; Fuling promotes diuresis through light infiltration, strengthens the spleen, and excretes dampness, provides tonic effects without causing excessive internal dampness (according to TCM theory), and harmonizes all the drugs. According to traditional Chinese medicine theory, chronic stress is often associated with "liver qi stagnation (a TCM concept)". The liver, which is considered crucial for blood storage and the foundation of the Chong meridian, is integral to female health and physiology. Unrelieved anger and resentment significantly harm liver Qi, leading to liver qi stagnation.[40] This stagnation can result in qi and blood stasis, which are implicated in causing menstrual disorders, various psychosomatic

illnesses, ovarian dysfunction, and acceleration of the aging process. To ameliorate these effects, we enhanced the original formulation, Gengnianchun, by incorporating Chaihu,[41] Danggui,[42] and Chuanxiong[43]—herbs revered for their liver-calming, blood-nourishing, and circulation-stimulating properties. The mechanism may involve relieving oxidative stress, regulating the HPA axis, and protecting neurons.[44] In a chronic unpredictable stress-induced DOR mouse model,[27] we observed substantially increased follicular counts across all developmental stages, notably increasing the quantity of early-stage ovarian follicles while concurrently increasing serum testosterone levels in model mice.[14] The FSH receptor was significantly upregulated after MGNC treatment, indicating a promising effect of increasing responsiveness in stressed DOR.

The effects of MGNC vs. androgen in vivo

In this study, *in vivo* experiments clearly demonstrated the differential therapeutic effects of MGNC and androgen supplementation on chronic stress-induced DOR in mice, with MGNC showing comprehensive superiority. The most notable difference between the two interventions lies in the regulation of the estrous cycle (Fig. 2b). MGNC significantly reversed the estrous cycle irregularities induced by chronic stress, making the cycle distribution closely resemble that of the control group; in contrast, androgen treatment not only failed to improve the cycle but also exacerbated the disorder. Specifically, the diestrus phase ratio of the LT, MT, and HT groups was greater than that of the model group, and the degree of disorder was positively correlated with the androgen dose, with the HT group showing the most severe abnormality. This phenomenon may be related to excessive androgen concentrations exceeding the physiological tolerance threshold of mice. As reported in studies on polycystic ovary syndrome,[45,46] hyperandrogenemia can disrupt ovulatory function by interfering with follicular development signals, which also suggests that the optimal androgen dose for DOR treatment may be lower than the low dose used in this study and requires further optimization.

In terms of improvement in the ovarian reserve (Fig. 3), both MGNC and LT increased the follicle count at all stages compared with the model group, with MGNC exerting a more comprehensive and significant effect. MGNC significantly elevated the counts of primordial, primary, secondary, and antral follicles, particularly primary and antral follicle counts, which were significantly greater than those in all the androgen groups. In contrast, the follicle-protective effect of androgens was highly dose-dependent: LT resulted in partial protection of early follicles, MT resulted in no significant difference from the model group, and HT even reduced primordial and primary follicle counts compared with the model group, indicating direct ovarian follicle toxicity of high-dose androgens. This finding aligns with our previous finding that balanced androgens promote follicle growth via the synergistic effect of IGF1–FSH,[23] whereas excessive androgens may inhibit granulosa cell function by downregulating IGF1R expression. Although none of the treatments restored the normal corpus luteum counts, the MGNC group still presented higher counts than all the androgen groups, whereas the MT and HT groups presented significantly fewer corpora lutea than the model group—further confirming the adverse effects of medium and high doses of androgens on ovulation and luteinization.

Additionally, our findings, illustrated in Figure 4, revealed that androgen supplementation increased E2 secretion in chronically stressed DOR models. LT was more effective at increasing serum E2 levels than were MGNC or MT or HT. In addition, compared with MT and HT, only optimal androgen (LT) levels contribute positively to follicular development. This change may be attributed to the fact that androgens at an appropriate concentration (low concentration) can better promote the development of dominant follicles, thereby producing more E2. In contrast, excessive androgens may inhibit the production of mature follicles, similar to the role of hyperandrogenism in polycystic ovary syndrome.[47] The higher E2 level in the LT group than in the MGNC group may be due to the additional conversion of a portion of androgens into E2. Moreover, MGNC outperformed the MT and HT treatments in increasing E2 secretion and reducing FSH levels and the FSH/LH ratio. Collectively, these results demonstrate that MGNC is more efficacious than androgen treatments in improving ovarian follicle reserves and normalizing aberrant serum reproductive hormone levels in mice with chronic stress-induced DOR.

Effects of MGNC vs. androgen in vitro

In the follicle culture study, compared with normal NS serum, MGNC-enriched serum significantly promoted follicle growth, irrespective of whether it was applied alone or alongside high concentrations of CORTN. Enhancement of follicle growth was also noted when androgens were added to NS serum; however, the performance of the MGNC+HC group remained superior in the high-CORTN context. Although this study did not use gradient concentrations of androgens to intervene in the follicles and thereby compare the effects of such interventions on follicle development under stress conditions, previous studies have reported that prolonged exposure to androgen excess leads to aberrant follicle development.[48] During the dominant follicle development stage and within a certain threshold range,[49] the degree of follicle development is positively correlated with the maturity of oocytes and the quality of blastocysts.[50] Our results suggest that Chinese herbal compounds have greater effects than androgen therapy during the initial stage of follicle development.

The careful selection of follicles for culture was a critical aspect of this research. According to investigations using a mouse granulosa cell-specific androgen receptor knockout model, androgen receptors are present in follicles across all stages of development, from the primary follicle to the corpus luteum. However, the highest expression levels were detected in follicles transitioning from the secondary to the early antral stage.[12] Elevated concentrations of FSH diminish the influence of androgens on the development of follicles with initial diameters exceeding 130 μm.[51] The growth of larger follicles is predominantly FSH-driven rather than androgen-dependent.[52] Conversely, androgens play a pivotal role in the maturation of smaller follicles that express FSH receptors but do not depend on FSH.[53] Consistently, our research demonstrated that the detrimental impact of chronic stress on follicle reserves in mice was particularly pronounced during the primordial, primary, and secondary follicle stages.[27] Therefore, we chose to culture secondary follicles featuring 2–3 granulosa cell layers with diameters ranging from 110–130 μm.[52]

To determine the optimal androgen dosage for treating ovarian follicles, we consulted Gervásio *et al*.,[54] who investigated the effects of various androgens on follicle growth. This research indicated that a high concentration of androgens hinders follicle development and oocyte maturation, in contrast to low concentrations, which promote follicle development and oocyte maturation *in vitro*. The primary effective concentrations of DHT and testosterone are both within the range of 10–20 nM,[55] which aligns with the ideal intervention concentration identified in our previous work regarding the influence of androgens on E2 secretion by granulosa cells.[23] Consequently, 10 nM DHT was used to treat the ovarian

follicles in this study.

These findings further substantiate the potential of androgens in promoting early-stage follicle growth under chronic stress-induced DOR conditions. Nevertheless, compared with androgen, MGNC has superior efficacy.

Limitations

This study has certain limitations that should be acknowledged. First, the research was conducted exclusively on animal models to simulate chronic stress-induced DOR, and no clinical trials involving human participants were performed; thus, the therapeutic effects of MGNC in human DOR patients cannot be directly inferred, and further clinical validation is needed. Second, while MGNC has been confirmed to be superior to androgens in improving ovarian reserve and follicle development, its multitarget regulatory mechanisms remain insufficiently explored; specifically, how MGNC interacts with key signaling pathways (e.g., IGF1–FSH) or modulates molecular targets, as well as the identification of its key bioactive components, still needs in-depth investigation to support its clinical application. Third, the study lacked a blinding procedure for the researchers conducting outcome assessments (e.g., follicle counting, estrous cycle staging via vaginal smears). This may have introduced observer bias, as subjective judgments during data collection could skew the results and affect the objectivity of the study's findings.

Conclusions

Our findings demonstrate that MGNC and appropriately dosed androgens (LT) ameliorate CORTN-mediated chronic stress–induced DOR phenotypes; however, androgen effects were strongly dose-dependent, with MT and HT exacerbating abnormalities. In contrast, MGNC showed consistent efficacy across all outcomes and outperformed all androgen regimens, underscoring its translational potential for DOR treatment, particularly in patients with contraindications to hormone therapy. Nevertheless, the underlying molecular mechanism of MGNC and clinical efficacy validation through randomized clinical trials require further study.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Study conception and design, experiment performance, data analysis, draft of the manuscript (LG), assistance for most of the experimental work (YR, HG), support for data analysis (JL), support for part of the follicle culture experiment (JH), funding acquisition, and assistance for the revision of the final manuscript (WW). All the authors read and approved the final manuscript.

Ethical statement

This study was carried out in accordance with the Guidelines of the Ministry of Science and Technology of China on Treating Laboratory Animals with Respect. The protocol was approved by the Laboratory Animal Welfare and Ethics Committee of Fudan University (Authorization Number: 202405003S). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Data sharing statement

The datasets used in support of the findings of this study are included within the article.

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