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ORIGINAL ARTICLE *Infertility*

Gonadotrophin-releasing hormone agonists for fertility preservation: unraveling the enigma?

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STUDY QUESTION: Can gonadotrophin-releasing hormone agonists (GnRH-a) preserve long-term fertility when administered prior to and concomitantly with chemotherapy?

SUMMARY ANSWER: GnRH-a display a differential protective effect on fertility, depending upon the specific chemotherapy-induced mechanism of ovarian injury.

WHAT IS KNOWN ALREADY: The role of GnRH-a in fertility preservation has been constantly debated and their use is considered experimental due to conflicting clinical evidence and paucity of data regarding their mechanism for ovarian protection.

STUDY DESIGN, SIZE, DURATION: *In vivo* model: 7–8 weeks old imprinting control region (ICR) mice were injected with GnRH-a (Leuprolide-acetate) or saline prior to and concomitantly with cyclophosphamide, doxorubicin or saline and sacrificed at various time-points on a longitudinal follow-up; 24 h ($n = 36$), 1 week ($n = 40$), 1 month ($n = 36$) and 9 months ($n = 66$) post chemotherapy treatment. Blood samples were drawn on Day 0 and on a monthly basis after chemotherapy treatment. On the day of sacrifice, blood samples were drawn and ovaries excised and processed for either immunohistochemistry (IHC), protein or RNA extraction. *In vitro* model: 21–23 days old Wistar-derived rats were sacrificed, their ovaries excised and primary granulosa cells (PGC) were either isolated for *in vitro* culture, or processed for immunofluorescence (IF) as well as for protein or RNA extraction.

MATERIALS, SETTING, METHODS: Ovarian reserve was estimated by serial measurements of serum anti-mullerian hormone (AMH), quantified by the AMH Gen II ELISA assay. Ovarian AMH and phosphorylated Akt (pAkt) were detected by immunoblotting. Vascular endothelial growth factor (VEGF) was measured by quantitative PCR. Ovarian GnRH receptor (GnRHR), AMH and CD34 were visualized by IHC, and apoptosis was evaluated using TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labeling (TUNEL).

MAIN RESULTS AND THE ROLE OF CHANCE: Cyclophosphamide-induced ovarian injury caused a prompt decrease in AMH level ($P < 0.01$) and a further long-term decline in serum AMH ($P = 0.017$), indicating damage to the ovarian reserve. Pretreatment with GnRH-a diminished AMH-decrease ($P < 0.05$) and maintained serum AMH level in the long run ($P < 0.05$). Doxorubicin-exerted ovarian-vascular-injury is also displayed by an acute increase in ovarian VEGF level ($P < 0.05$) and a sustained decrease in serum AMH level ($P < 0.001$). This was followed by ovarian recovery manifested by increased neovascularization. GnRH-a delayed the recovery in AMH level and decreased the level of VEGF ($P < 0.001$), thus interfering with the vascular recovery subsequent to doxorubicin-induced vascular damage.

LIMITATIONS, REASONS FOR CAUTION: To portray the differential mechanism of each chemotherapy, cyclophosphamide and doxorubicin were given separately, whereas most of the clinical protocols include several types of chemotherapies. Thus, future study should explore a prospective evaluation of various chemotherapies, as well as combined chemotherapeutic protocols.

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WIDER IMPLICATIONS OF THE FINDINGS: Our study demonstrates that different chemotherapy agents affect the ovary via diverse mechanisms and thus the administration of GnRH-a concomitantly, could be beneficial to a subpopulation of patients treated with cyclophosphamide-based protocols.

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Key words: GnRH-a / doxorubicin / cyclophosphamide / AMH / VEGF

Introduction

Novel treatment approaches have improved the long-term survival of cancer patients, posing chemotherapy-induced infertility a major health concern. Former studies indicate that chemotherapy-induced premature ovarian failure is reflected by depletion of primordial follicles (PMF) reserve and impaired follicular maturation (Lee *et al.*, 2006); nevertheless, the exact mechanism by which classes of chemotherapies exert ovarian demise, remains unclear.

The prerequisite for maintaining a long-term ovarian function is a large ovarian reserve (i.e. resting pool of PMF and small primary follicles). The ovarian reserve declines physiologically throughout reproductive age, as the PMFs undergo apoptosis or enter the growth phase. The mechanism regulating follicle dormancy is gonadotrophin-independent and relies on a delicate balance between local inhibitory and stimulatory factors (Adhikari and Liu, 2009). The fundamental role of phosphatidylinositol 3-kinase (PI3K)/PTEN/AKT signaling pathway in maintaining this balance has been well established in many studies (Reddy *et al.*, 2008; Adhikari and Liu, 2009) and phosphorylation of its downstream key-protein AKT was indicated in activation of follicle recruitment (Reddy *et al.*, 2005; Goto *et al.*, 2007; Brown *et al.*, 2010). Anti-Mullerian hormone (AMH), an inhibitor of PMF recruitment, acts in contrast to phosphorylated-AKT (pho-AKT), and was shown to maintain PMF dormancy in cultured neonatal mouse ovaries (Durlinger *et al.*, 2002); its absence in knock-out (KO) mice resulted in accelerated depletion of the PMF pool (Durlinger *et al.*, 1999). The level of AMH in the serum has been established in recent years as a valuable quantitative indicator of ovarian reserve, correlating with the number of PMF in humans (van Rooij et al., 2002) and in mice (Kevenaar *et al.*, 2006). AMH has been further acknowledged as a reflector of chemotherapy-induced ovarian toxicity (Anderson and Cameron, 2011; Broer *et al.*, 2011; Brougham *et al.*, 2012). Changes in serum AMH level could be detected prior to changes in the level of FSH and inhibin-B (Fanchin *et al.*, 2003), which are also markers of ovarian aging. Furthermore, in contrast to other serum markers, the level of AMH remains relatively constant during the menstrual cycle (van Rooij et al., 2002; van Beek et al., 2007).

Two mechanisms of chemotherapy-induced ovarian toxicity were suggested: one that involves direct apoptosis of PMF (Oktem and Oktay, 2007) or rapid PMF recruitment (Kalich-Philosoph *et al.*, 2013); and the other that involves impairment of ovarian stroma and vasculature that leads to indirect PMF loss (Meirow *et al.*, 2007).

It has been suggested that administration of GnRH agonists (GnRH-a) prior to and during chemotherapy treatment might reduce gonadotoxicity by down-regulating the secretion of FSH and LH from the pituitary; consequently creating a hypo-gonadotropic milieu, in which follicular recruitment is inhibited and fewer PMF attain the chemotherapy-sensitive stages of proliferation and follicle maturation (Blumenfeld and von Wolff, 2008). Whereas down-regulation of gonadotrophins by GnRH-a can take up to 2 weeks in humans (Blumenfeld and Dann, 2013); in rodents it takes only 4 days of GnRH-a administration, which is in accordance with their much shorter reproductive cycle (5 days) (Kitahara *et al.*, 2007; Matsuo *et al.*, 2007).

Former studies appraised the role of GnRH-a in reducing chemotherapy-induced ovarian toxicity mostly in the clinical setting, with no discrimination upon the type of chemotherapies (which were usually administered as multi-agent protocols). Since the mechanism underlying GnRH-a protective effect on the ovarian reserve has not been studied upon known patterns of toxicity caused by specific classes of chemotherapy, the different toxicity patterns may lie at the core of inconsistency observed in clinical studies. Our aim was, therefore, to characterize the long-term pattern of ovarian toxicity of two distinct chemotherapeutic agents widely used in oncology practice: Cyclophosphamide (Cyc), an alkylating agent that is considered a prototype for gonadotoxic chemotherapy, and Doxorubicin (Dxr), an anthracycline, which is considered less toxic to the ovary. Another aim was to study whether a concomitant administration of GnRH-a can reduce the long-term chemotherapeutic toxic effect, by following changes in ovarian reserve throughout the reproductive lifespan. The acute toxic phase exerted by Cyc, Dxr and the effect of GnRH-a were also studied; focusing on follicle recruitment and ovarian vascularization.

Materials and Methods

Animals

Imprinting control region (ICR) female mice (7–8 weeks old) and Wistar-derived female rats (21–23 days old; Harlan Laboratories, Jerusalem, Israel) were housed in air conditioned, light controlled animal facilities of the Sackler faculty of Medicine in Tel-Aviv University. Animal care was in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committee.

Our experimental platform was comprised of *in vivo* mice model and *in vitro* granulosa cell model for a further study of the cellular mechanisms. We chose mice for the *in vivo* model because changes in the level of serum AMH in mice had already been well established as quantitative markers of the PMF reserve (Kevenaar *et al.*, 2006) and because they are measurable by the AMH Gen II ELISA assay. Rats were chosen as the source of primary granulosa cells (PGC) for culture because their ovaries yield significantly more granulosa cells than mice, and the PGC model had already been established and described in rats (Orly *et al.*, 1980).

Mouse in vivo *model*

Gonadotrophin down-regulation (confirmed by pituitary levels of LHβ and FSHβ mRNA) was achieved in mice after subcutaneous (S.C.) injection of GnRH-a, Leuprolide Acetate (LA; 0.35 μg/mouse; Sigma, St Louis, MO,

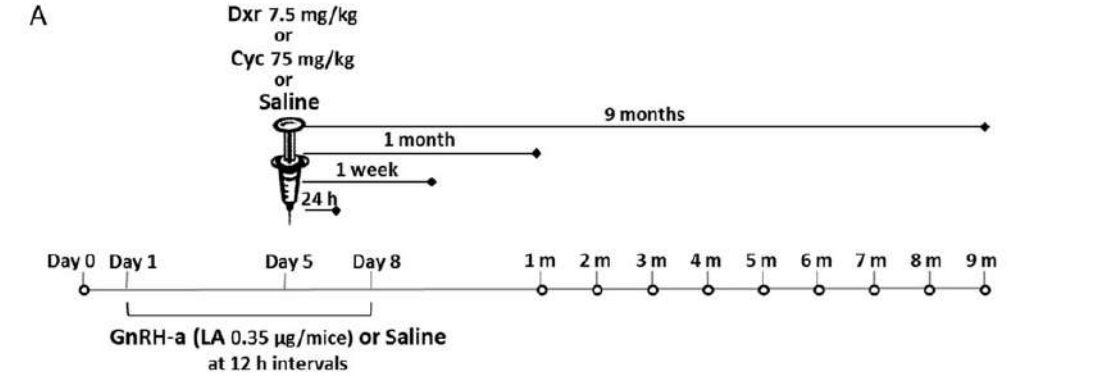
USA) or saline, given every 12 h for 5 consecutive days. They were treated for 3 additional days to cause sustained desensitization during chemotherapeutic treatment (Days 1–8, Fig. 1A). LA dosage was calculated using a conversion equation; Mouse Equivalent Dose = Rat Dose × Rat Km/Mouse Km (Rat Km = 6, Mouse Km = 3) (Gad, 2006) based on previous studies conducted in rats (Parborell *et al.*, 2002, 2008). Mice were randomly allocated into six experimental groups (Fig. 1B). On the fifth day they were injected intraperitoneally (i.p.) with Doxorubicine (Dxr; Teva, Petach-Tikvah, Israel; 7.5 mg/kg), Cyclophosphamide (Cyc; Baxter oncology, Halle, Germany; 75 mg/kg) or saline. Standard dosages of Cyc and Dxr were chosen based on former publications showing ovarian damage (Meirow *et al.*, 1999; Ben-Aharon *et al.*, 2010a). Mice were sacrificed 24 h ($n = 36$), 1 week ($n = 40$), 1 month ($n = 36$) or 9 months ($n = 66$) after chemotherapy treatment and their ovaries were excised (Fig. 1A). Pituitary glands of saline or LA-treated mice (groups 3,6) were excised after 5 consecutive days of treatment (Day 6).

Rat in vitro *model*

Rats were sacrificed, their ovaries excised and primary granulosa cells (PGC) isolated for either *in vitro* culture or immunofluorescence (IF).

Isolation of rat PGC

PGCs were isolated according to Orly *et al.* (1980) with some modifications. Ovaries of 21–23 days old immature rats were excised and transferred to Petri dishes containing serum-free Dulbecco's Modified Eagle's Medium ((DMEM)/F-12 (HAM) 1:1; Biological Industries, Beit-Ha'emek, Israel). The ovaries were then incubated in sucrose medium (2.5 ml DMEM/F12, 2.5 ml sucrose 1 M and 0.5 ml EGTA 0.1 M, pH = 7) for 45 min, followed by another 45 min incubation in serum-free DMEM/F12 containing 0.1% (v/v) indomethacin (10 μM dissolved in 100% ethanol; Sigma) to a final concentration of 10 nM. Follicles were then punctured with a 21G needle and the PGCs were gently squeezed out of the follicles into the culture medium. The PGCs were centrifuged (150*g* for five minutes) and the pellets were pooled and re-suspended in serum-free DMEM/F12 containing 10 nM indomethacin. Cells were then seeded according to Litichever *et al.* (2009) either in 1% (v/v) serum-coated 6-well plates (Nunc, Denmark), at an equivalent of 1.5 ovaries/well and cultured in a humidified incubator at 37°C and 5% CO_2 in air; or on glass coverslips (Marienfeld GmbH, Germany; for IF) within 24-well plates (Thermo Scientific, Denmark), at an equivalent of one ovary/3 wells. Each of both experiments was repeated three times.



B

	Pre-treatment S.C. (day 1–8)	Treatment I.P. (day 5)
1	Saline	Dxr 7.5 mg/kg
2	Saline	Cyc 75 mg/kg
3	Saline	Saline
4	GnRH-a	Dxr 7.5 mg/kg
5	GnRH-a	Cyc 75 mg/kg
6	GnRH-a	Saline

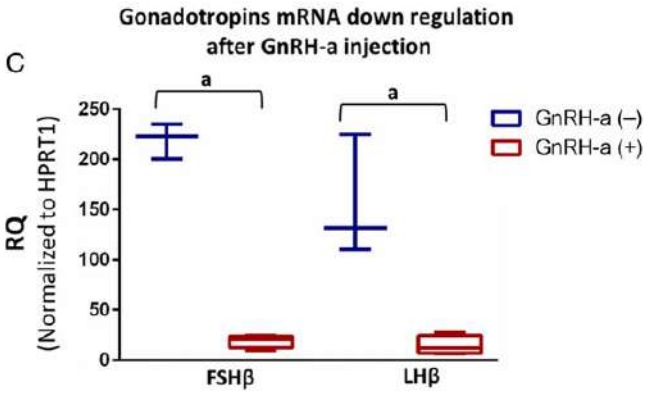


Figure 1 Graphic illustration of the mouse experimental schedule: (**A**) eight weeks old imprinting control region (ICR) mice were pre-treated with s.c., injections of saline or GnRH agonist (GnRH-a) (Leuprolide acetate; LA; 0.35 μg/mouse), twice daily (at 12 h intervals) for 5 consecutive days before achieving gonadotrophin down-regulation (confirmed by pituitary levels of LHβ and FSHβ mRNA), and for 3 additional days for sustained desensitization during chemotherapy treatment. On Day 5, mice were injected i.p., with saline, Doxorubicine (Dxr; 7.5 mg/kg) or Cyclophosphamide (Cyc; 75 mg/kg). Mice were sacrificed 24 h, 1 week, 1 month or 9 months after chemotherapy treatment. Blood samples for serum anti-Mullerian hormone (AMH) were collected on Day 0, on a monthly basis after chemotherapy treatment and at autopsy (o-represents blood sampling). (**B**) Mice were randomly divided into six treatment groups. (**C**) Pituitary desensitization prior to chemotherapy treatment (Day 5) was confirmed in mice injected twice daily for 5 consecutive days with either saline or GnRH-a (LA). Graph of real-time PCR exhibits diminished mRNA levels of FSHβ and LHβ subunits. The upper and lower limits of the boxes indicate the 75th and 25th percentiles, respectively; whereas the lines within the boxes indicate the median. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively ($n = 3–4$ for each group; $^{a}P = 0.002$ for LHβ, $^{a}P < 0.001$ for FSHβ); ^{a}GnRH-a (+) versus GnRH-a (−).

Stimulation of PGC with GnRH-a

Freshly isolated PGCs were incubated overnight; culture medium was replaced on the next day with fresh DMEM/F-12 with 10% (v/v) charcoal-stripped fetal calf serum (CS FCS; Biological Industries). Cells were then incubated with 1 μM LA for various periods of time (6 h, 24 h, 48 h) and harvested for determination of protein level. Each experiment was accompanied by its relevant control of untreated cells, cultured and harvested at the same time.

RNA isolation, reverse transcription, polymerase chain reaction and real-time quantitative PCR (qPCR)

Total RNA was isolated from mice pituitary glands, ovaries or rat PGC using Trizol reagent (Invitrogen, Grand Island, NY, USA) according to manufacturer's instructions, and quantified with Nano-Drop spectrophotometer (ND-1000; Thermo scientific, MA, USA). First-strand cDNA was created from 1 μg of total RNA by reverse transcriptase (RT; Fermentas, Burlington, ON, Canada; or Thermo Scientific). The expression of AMH and GnRH receptor (GnRHR) mRNA in PGC was detected by polymerase chain reaction (PCR) as previously described (Chuderland *et al.*, 2014). The specific primers used and the size of their amplified fragments are presented in Table I.

For qPCR: Changes in the expression level of mRNA were detected by SYBR green reagent (SYBR® Green PCR Master Mix, ABI, Carlsbad, CA, USA) on an ABI Prism 7900 Sequence PCR machine. The expression of LHβ, FSHβ and VEGF mRNA was normalized to Hypoxanthine Phosphoribosyltransferase (HPRT1); relative expression was calculated using the comparative Ct. Primers were designed using Roche Universal ProbeLibrary Assay Design Center and evaluated using IDT OligoAnalyzer tool. The specific primers and the size of their amplified fragments are presented in Table I.

Immunohistochemistry

Paraffin-embedded sections of mouse or rat ovaries were stained as described previously (Ben-Aharon *et al.*, 2010a), using either anti-AMH (1:200, SC-6886; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GnRHR (1:200, SC-13944; Santa Cruz Biotechnology) or anti-CD34 (1:200, CL8927AP; Cedarlane Laboratories, NC, USA) antibodies followed by donkey anti-goat Cy3-conjugated (1:100, Jackson Immunoresearch Laboratories, West Grove, PA, USA), goat anti-rabbit Alexa Flour488-conjugated (1:400, Cell signaling technology, MA, USA) or goat anti-rat Alexa Flour555-conjugated (1:400, Cell signaling technology) antibodies, respectively, together with a nuclear marker (Hoechst 33258, 1:1000, Sigma). Sections were visualized and photographed by a Leica laser confocal microscope (SP5 Wetzlar, Germany).

Immunofluorescence

Cells were fixed, stained and visualized as previously described (Ninio-Many *et al.*, 2014). The primary antibodies used were anti-AMH (1:50) and anti-GnRHR (1:20); followed by donkey anti-goat Cy3-conjugated (1:200) and goat anti-rabbit Alexa Flour488-conjugated (1:400) secondary antibodies together with Hoechst 33258.

Evaluation of apoptosis

DNA fragmentation was visualized *in situ* on paraffin-embedded sections of ovaries by terminal transferase-mediated dUTP nick-end labeling (TUNEL) staining (DeadEnd fluorometric TUNEL system, Promega, Madison, WI, USA) according to manufacturer's instructions. Labeled sections were visualized and photographed by a Leica laser confocal microscope (SP5 Wetzlar).

Immunoblotting

Cells were lysed and processed for immunoblotting as previously described (Ninio-Many *et al.*, 2014). Mice ovaries were homogenized in cold lysis buffer (20 mM HEPES, 150 mM NaCl and 0.2% (v/v) Igepal; sigma), centrifuged (15 000*g*, 15 min, 4°C) and subjected to western blot analysis. Membranes were incubated overnight with anti-AMH (1:200), anti-Actin (1:10 000, MAB1501; Millipore, Temecula, CA, USA), anti-GnRHR (1:200), anti-phosphorylated (ser473) AKT (1:1000, #9271; cell signaling technology) or anti-total AKT (1:10 000, P1601; Sigma) primary antibodies, followed by donkey anti-goat and goat anti-mouse IRDye 800-conjugated antibodies (1:10 000, LI-CORE, Lincoln, NE, USA) or goat anti-rabbit (1:5000, NA934V; GE health care, UK) and goat anti-mouse (1:5000, #115035166; Jackson Immunoresearch Laboratories) HRP-conjugated antibodies. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Thermo Scientific, IL, USA) according to manufacturer's guidelines or by the Odyssey infrared imaging system (LI-CORE).

Serum AMH measurements

Blood samples were drawn via the retro-orbital sinus at Day 0 and on a monthly basis after chemotherapy treatment, adding up to 10 samples per

Table I Primers used for reverse transcription and quantitative PCR.

GnRHR mouse (Control)	Forward-5′ GTC CTT CAT CAA GAC CCA C 3′ Reverse-5′ CGA ATG CGA CTG TCA TCT 3′	97 bp
GnRHR rat	Forward-5′ AGAGCAAGCTTGAACGGTCT 3′ Reverse-5′ CGGCTAGGTAGATCATCCTGAA 3′	99 bp
AMH mouse/rat	Forward-5′ GCA GTT GCT AGT CCT ACA TC 3′ Reverse-5′ TCA TCC GCG TGA AAC AGC G 3′	353 bp
LHbeta	Forward-5′ CTGAGCCCAAGTGTGGTGTG 3′ Reverse-5′ GACCATGCTAGGACAGTAG 3′	150 bp
FSHbeta	Forward-5′ CCATAGCTGTGAATTGACCAACA 3′ Reverse-5′ AGATCCCTAGTGTAGCAGTAGC 3′	111 bp
VEGF	Forward-5′ AGGCTGCTGTAACGATGAAGC 3′ Reverse-5′ AGGTTTGATCCGCATGATCTG 3′	82 bp
HPRT1	Forward-5′ CTCATGGACTGATTATGGACAGGAC 3′ Reverse-5′ GCAGGTCAGCAAAGAACTTATAGCC 3′	123 bp

GnRHR, GnRH receptor; AMH, anti-Mullerian hormone; VEGF, vascular endothelial growth factor; HPRT1, Hypoxanthine Phosphoribosyltransferase; bp, base pair.

mouse (baseline and months 1–9). At the day of sacrifice, blood samples were drawn via the inferior vena-cava. Sera were then separated by centrifugation (4000*g*, 10 min, 4°C) and frozen at −20°C until quantification with the AMH Gen II ELISA assay according to the manufacturer instructions (Beckman Coulter, Chaska, MN, USA). Calibrators for a standard-curve as well as low and high controls were included in duplicate wells to each Elisa plate. Inter-assay variation was 4.3% ($n = 10$) and intra-assay variation was 3.8% ($n = 80$).

Statistical analysis

The SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data normality was assessed using Kolmogorov–Smirnov tests. In all

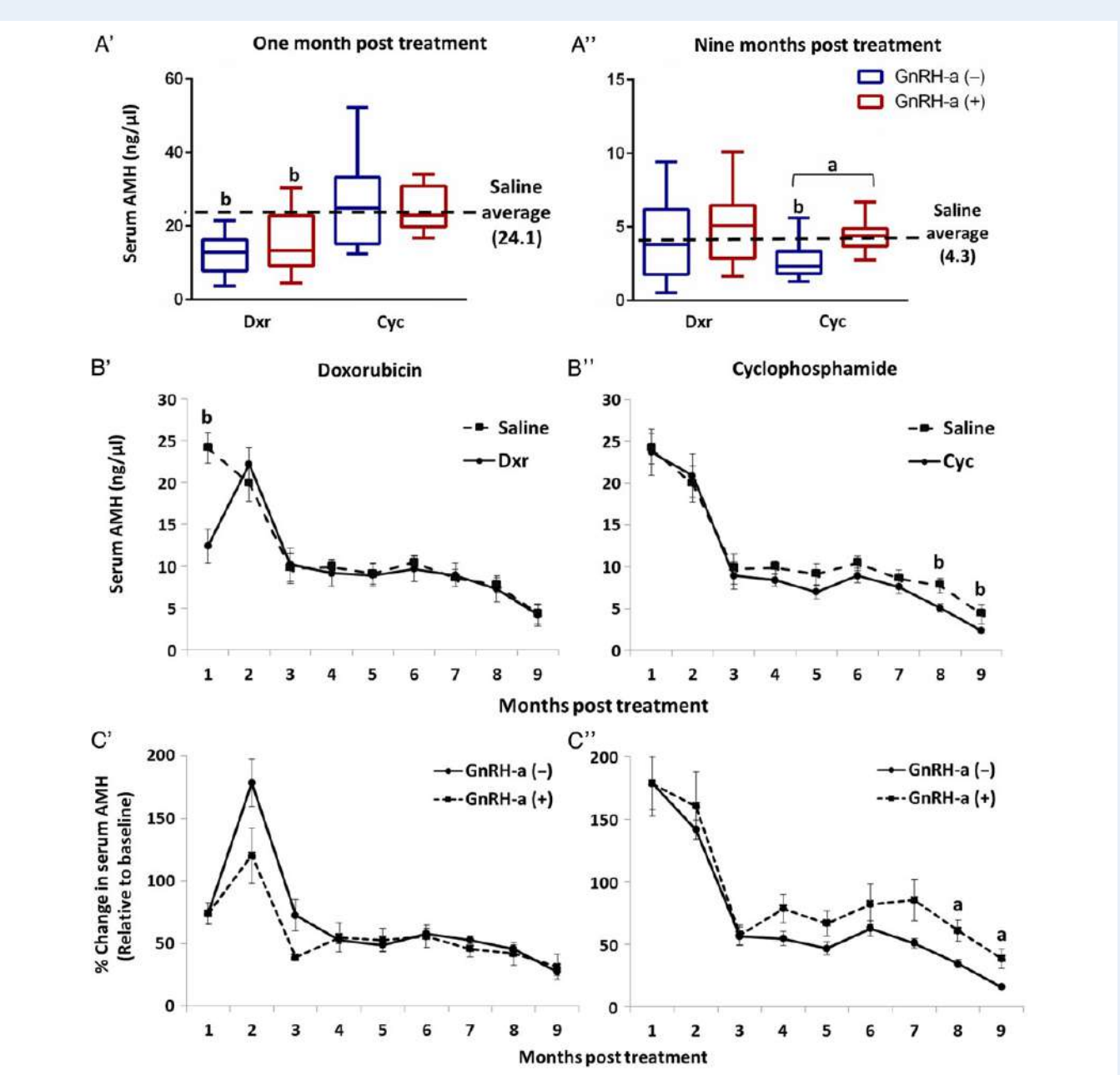


Figure 2 Long-term changes in serum anti-Mullerian hormone (AMH) level after Doxorubicin (Dxr) and Cyclophosphamide (Cyc) administration and the effect of GnRH agonist (GnRH-a) pretreatment. Monthly follow-up of serum AMH level. (**A**) Level of AMH in serum of mice treated with Dxr and Cyc either with or without GnRH-a compared with the level in serum of saline-treated control mice (dashed line), 1 month (**A′**) and 9 months (**A″**) post-treatment. The upper and lower limits of the boxes indicate the 75th and 25th percentiles, respectively; whereas the lines within the boxes indicate the median. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. $^{a,b}P < 0.05$; ^{a}GnRH-a (+) versus GnRH-a (−); ^{b}Dxr or Cyc versus saline. (**B**) Level of AMH in serum of mice treated with either Dxr (**B′**) or Cyc (**B″**) (continuous lines) compared with the level in serum of saline-treated control mice (dashed line). (**C**) Level of AMH in serum of mice treated with either Dxr (**C′**) or Cyc (**C″**) (continuous lines) compared with the level in serum of their matching GnRH-a pre-treated mice (dashed line). AMH is presented as percent of the baseline value of each mouse. Data is mean ± SEM ($n = 11$ for each group; $^{a,b}P < 0.05$); ^{a}GnRH-a (+) versus GnRH-a (−); ^{b}Dxr or Cyc versus saline.

instances where raw data were not normally distributed, square root or log transformation was applied to normalize data. Normally distributed data were then analyzed using two-way analysis of variance (ANOVA) test with a pretreatment factor (GnRH-a (+)/GnRH-a (−)) and a chemotherapy treatment factor (Sal/Dxr/Cyc). This was followed by Bonferroni *post hoc* analysis to specify significant changes among the various chemotherapies (Sal/Dxr/Cyc) and T-TESTs to specify significant changes within each chemotherapy (GnRH-a (+)/GnRH-a (−)), at a specific time point. When analyzing the serial serum AMH cohort (Fig. 2B and C), 2-way ANOVA with a time variable and a treatment or pretreatment variable was applied, followed by repeated *T*-TESTs for assessing differences in serum AMH level starting from 4 months post-treatment; Bonferroni correction (for 6 repeated tests) was applied where statistical significance was found. *T*-TEST with two-tailed distribution and two-sample unequal variance were also employed for assessing changes in the *in vitro* experiment. The *in vitro* experiment was repeated three times and changes in the level of AMH protein were expressed as percent of untreated, control cells value that was arbitrarily set as 100%. When statistical significance was recorded ($\alpha = 0.05$), power analyses were carried out to assess the usefulness of findings and shown as percent. Results of *in vivo* experiments are expressed as median (box plots with interquartile range). Results of *in vitro* experiments are expressed as mean ± standard error (SEM); $P < 0.05$ was considered statistically significant.

Results

GnRH-a down-regulates gonadotrophin transcription after 5 days of administration

In order to validate down-regulation of the pituitary-gonadal axis by GnRH-a, gonadotrophin transcription was measured following GnRH-a administration. The mRNA level of both FSHβ and LHβsubunits was significantly reduced after 5 consecutive days of GnRH-a administration ($P < 0.001$, power = 99.5 and $P = 0.002$, power = 99.5 respectively; Fig. 1C).

The temporary decrease in serum AMH level, induced by Dxr, can be prolonged by GnRH-a pretreatment

The level of AMH in the serum of saline and Dxr-treated mice (without GnRH-a pretreatment, Fig. 2B′) was significantly changed over time, regardless of treatment ($P < 0.001$, power = 99.8), with no interaction between groups. The level of AMH in the serum of Dxr-treated mice was decreased 1 month post-treatment ($P < 0.001$, power = 99.6; Fig. 2A′ and B′), but returned to control values 1 month later (Fig. 2B′). GnRH-a pretreatment appeared to delay the recovery in AMH level for 2 additional months; however, this effect was not statistically significant (Fig. 2C′).

GnRH-a pretreatment protected against Cyc-induced depletion of ovarian reserve

The level of AMH in the serum of saline and Cyc-treated mice (without GnRH-a pretreatment, Fig. 2B″) was significantly changed over time, regardless of treatment ($P < 0.001$, power = >99.9), with significant interaction between groups; meaning that, over time, AMH level in the serum of Cyc-treated mice was significantly lower than that of saline-treated mice ($P = 0.017$, power = 87.2). The level of AMH in the serum of Cyc-treated mice was preserved throughout the first 4 months post-treatment and resembled control values (Fig. 2A″ and B″). From this time point onwards, AMH level in the serum of Cyc-treated mice constantly declined and became significantly lower than that of saline-treated mice from 8 months onwards ($P < 0.05$, Fig. 2B″). The level of AMH in the serum of Cyc-treated mice, either co-treated with GnRH-a or not, was significantly changed over time ($P < 0.001$, power = 100) with a significant interaction between groups ($P = 0.001$, power = 99.3). The level of AMH in the serum of mice treated with Cyc and GnRH-a was significantly higher than that of mice treated with Cyc-alone and resembled the level of saline-treated mice ($P < 0.05$, Fig. 2A″ and C″).

Dxr and Cyc reduce AMH levels and GnRH-a pretreatment alters this effect 1 week post-treatment

Both chemotherapies caused a significant acute reduction of AMH level 24 h post-treatment, in ovaries (Dxr: $P < 0.001$, power = 97.2; Cyc: $P < 0.001$, power = 99.9; Fig. 3A′) and serum (Dxr: $P = 0.013$, power = 74.2; Cyc: $P = 0.009$, power = 77.9; Fig. 3B′). Furthermore, Dxr caused an increase in ovarian pho-AKT level (Supplementary Fig. S2A). Pretreatment with GnRH-a affected the follicle population, resulting in fewer multi-layered secondary to antral follicles, and more primary to small secondary follicles (Fig. 3C′-b,d,f). Nevertheless, the quantitative assessment of both ovarian and serum AMH showed only a non-significant increase in AMH level caused by GnRH-a at the acute phase (Fig. 3A′ and B′).

At the sub-acute phase, 1 week post-treatment, ovarian AMH level was still diminished in Cyc-treated mice ($P = 0.006$, power = 86.4; Fig. 3A″) and serum AMH level was diminished in both Dxr- as well as Cyc-treated mice (Dxr: $P < 0.001$, power = 99.7; Cyc: $P = 0.003$, power = 89.7; Fig. 3B"). GnRH-a pretreatment further reduced serum AMH level in Dxr-treated mice ($P = 0.043$, power = 54.9; Fig. 3B″ and C″), corresponding to the increase in apoptosis level (Supplementary Fig. S3B-c and d). GnRH-a pretreatment resulted in elevated AMH level in Cyc-treated mice ($P < 0.05$, power = 54.3; Fig. 3A″, B″ and C″), corresponding to the significant increase in ovarian weight ($P < 0.01$, Supplementary Fig. S1B″) and decrease in apoptosis level (Supplementary Fig. S3B-e,f).

GnRH-a directly increased the level of AMH protein in rat PGC expressing both GnRHR and AMH

AMH was found to be co-expressed with GnRHR in our *in vitro* model at both mRNA (Fig. 4A) and protein levels (Fig. 4B). Co-localization of the two proteins was further visualized in isolated PGC and in ovaries of prepubertal rats (Fig. 4C). A significant 30% increase in the level of AMH protein was detected after 6 h of stimulation with GnRH-a.

GnRH-a pretreatment decreases the level of ovarian VEGF mRNA and interferes with the vascular recovery subsequent to Dxr-induced vascular damage

Due to former evidence that Dxr-induced ovarian toxicity is partially mediated by vascular injury (Bar-Joseph *et al.*, 2011; Soleimani *et al.*, 2011), we evaluated vascular parameters as VEGF dynamics and pattern of ovarian vascularity in response to Dxr treatment and to

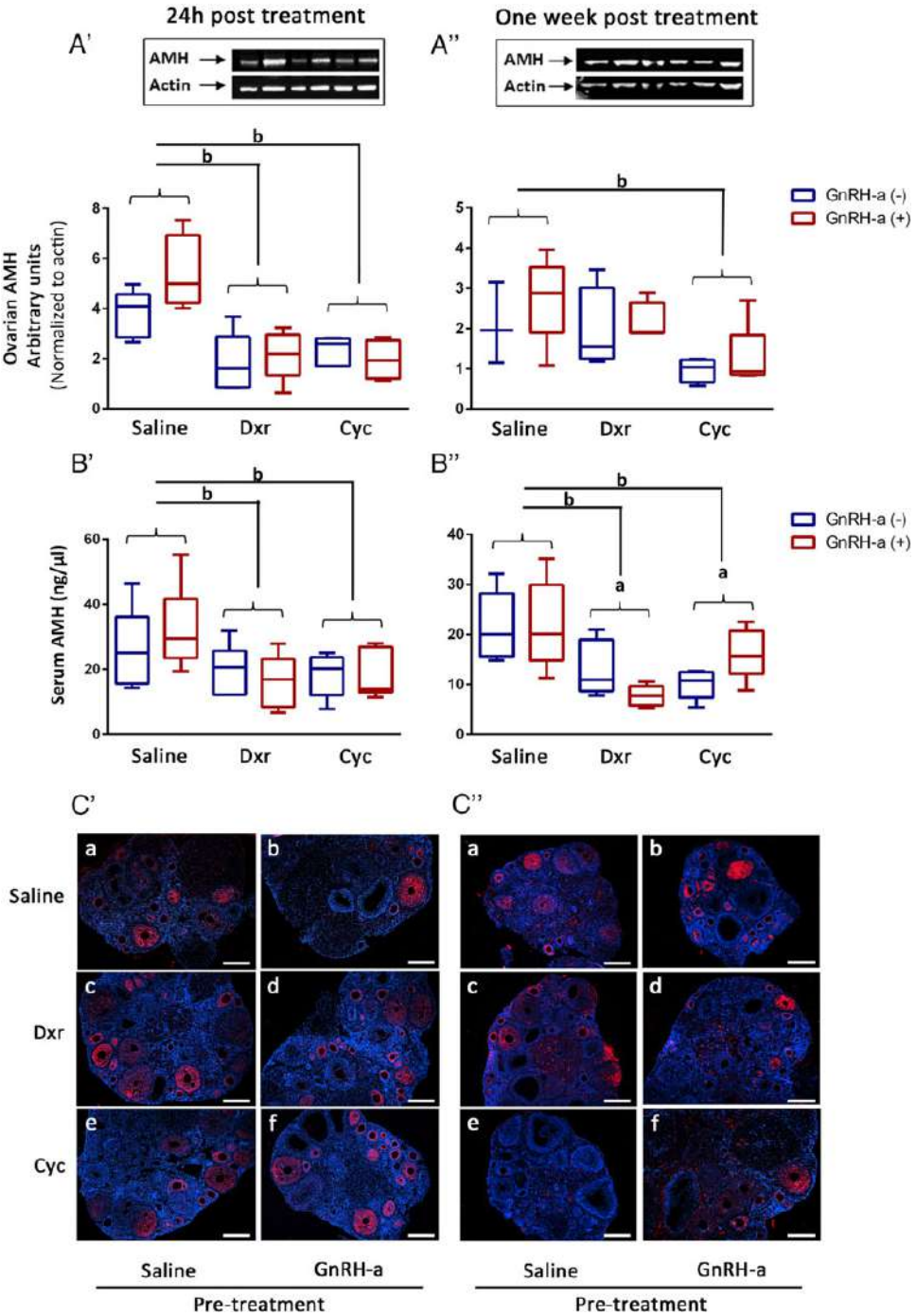


Figure 3 Changes in anti-Mullerian hormone (AMH) level after Doxorubicin (Dxr) and Cyclophosphamide (Cyc) administration and the effect of GnRH agonist (GnRH-a) pretreatment. (**A**) Representative blots and graphic quantification of ovarian AMH intensity. Proteins extracted from excised ovaries of mice 24 h (**A**′) or 1 week (**A**″) post saline/Dxr/Cyc injection were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-AMH and anti-Actin. Blots were quantified by the Image-J software ($n = 3–6$ for each group; $^{b}P < 0.05$); ^{b}Dxr or Cyc versus saline. (**B**) Serum AMH (ng/μl) was measured in mice 24 h (**B**′) or 1 week (**B**″) post saline/Dxr/Cyc injection ($n = 5–9$ for each group; $^{a,b}P < 0.05$); ^{a}GnRH-a (+) versus GnRH-a (−); ^{b}Dxr or Cyc versus saline. The upper and lower limits of the boxes indicate the 75th and 25th percentiles, respectively; whereas the lines within the boxes indicate the median. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. (**C**) Representative images of ovaries excised 24 h (**C**′) or 1 week (**C**″) following saline (a and b), Dxr (c and d), or Cyc (e and f) injections. Mice were pre-treated with either saline (a, c, e) or GnRH-a (LA-b, d, f). Ovaries were fixed, processed for histology, sectioned and labeled with anti-AMH antibody (red) for evaluation of changes in follicular population and Hoechst (blue) as a nuclear marker. Labeled sections were visualized and photographed by a Leica Laser microscope. Scale bar-200 μm.

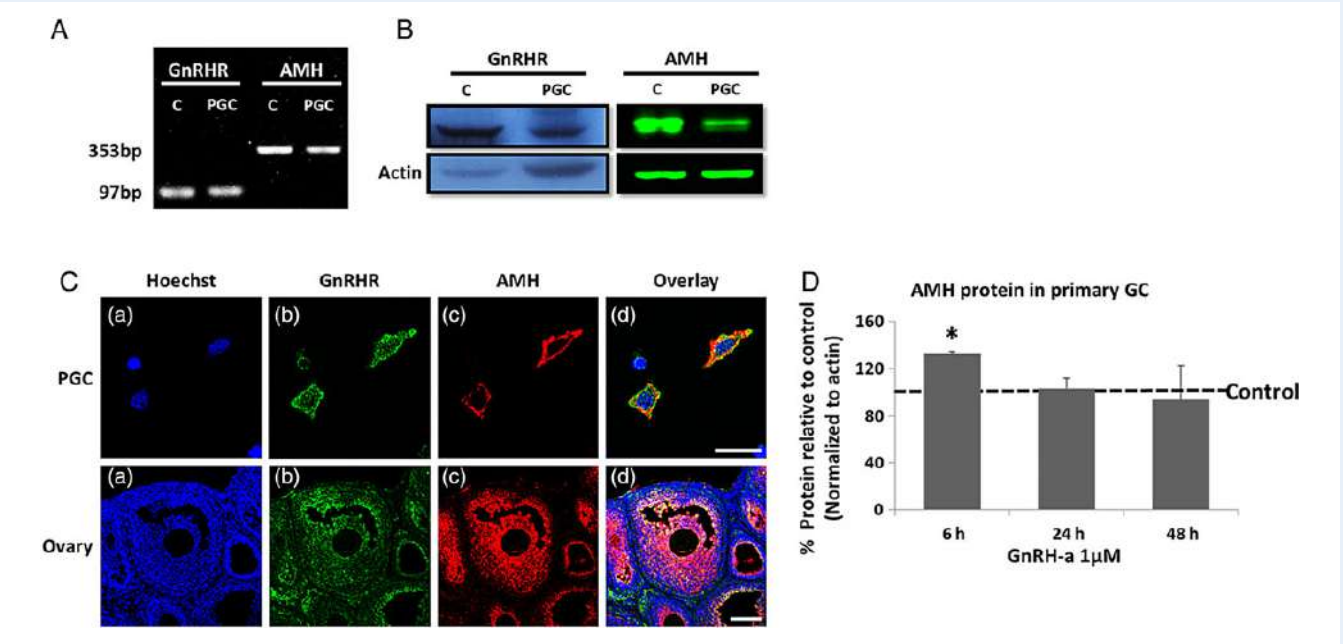


Figure 4 GnRH agonist (GnRH-a) increases anti-Mullerian hormone (AMH) level in primary granulosa cells (PGC) of a rat *in vitro* model. PGCs were extracted either for RNA, reverse transcribed and screened by PCR for GnRH receptor (GnRHR) and AMH mRNA (**A**), or for protein and analyzed by immunoblotting (**B**). LβT2 cells (mouse pituitary cell-line) served as positive control for GnRHR; testicular cells from 6 days old mice served as positive control for AMH. PGC and intact ovaries excised from pre-pubertal rats were stained for immunofluorescence (**C**); (a) DNA labeling (Hoechst 33258; blue), (b) anti-GnRHR (green), (c) anti-AMH (red), (d) overlay of b and c showing co-localization (yellow). Immunostaining by secondary antibody only served as negative control. PGC, Bar = 15 μm, Ovary, Bar = 75 μm. Graphic presentation of western blot of AMH in cultured PGC (**D**); densitometric analysis of three independent experiments. Results are mean ± SE, analyzed by one-sample Student's *t*-test. $^*P < 0.05$ compared with control.

combined Dxr-GnRH-a treatment. The level of VEGF mRNA was significantly elevated by Dxr during the acute phase of vascular injury ($P = 0.039$, power = 56.6; Fig. 5A′). Furthermore, increased neovascularization was observed in Dxr-treated mice 1 month post-treatment (Fig. 5C). GnRH-a pretreatment, resulted in a significantly decreased level of ovarian VEGF mRNA 24 h and 1 week post-treatment ($P = 0.001$, power = 99.3 and $P < 0.001$, power = 99.9, respectively; Fig. 5A) and reduced vascularity (Fig. 5B and C). VEGF level remained high in Dxr-treated mice 1 week post-treatment, but dropped far below this level in mice treated with both Dxr and GnRH-a ($P = 0.013$, Fig. 5A″). Cyc, had no effect on VEGF mRNA level, though a concomitant administration of Cyc and GnRH-a caused a significant decrease in VEGF mRNA level both 24 h and 1 week post-treatment ($P = 0.039$ and $P = 0.021$, respectively).

Discussion

The novelty of our work lies in studying the differential effects of two classes of chemotherapies on ovarian follicular reserve and function, as well as in the distinction that the potential effect of GnRH-a, in preserving ovarian follicular reserve, may be drug-specific and not an 'all or none' phenomenon.

The clinical evidence for the utility of GnRH-a in fertility preservation in cancer patients has been evaluated in several studies, randomized controlled and non-randomized trials, yielding conflicting results (Ben-Aharon *et al.*, 2010b; Turner *et al.*, 2013). The heterogeneity across the studies affects the interpretability of the data: the patients' ages range between early 20s to early 40s; age being a key contributor to ovarian reserve. The treatment regimens vary among trials, and most importantly, the measures used to determine ovarian reserve and function rely mainly on menstruation assessment. This is an inaccurate indicator of ovarian function that may be influenced by various factors other than ovarian reserve and does not meticulously reflect the state of the follicular pool (Beck-Fruchter *et al.*, 2008; Ben-Aharon *et al.*, 2010b; Turner *et al.*, 2013). AMH has gained much interest in the past decade and has been gradually recognized as a putative indicator of chemotherapy-induced ovarian toxicity (Anders et al., n.d.; Partridge *et al.*, 2010; Su *et al.*, 2010; Anderson and Cameron, 2011; Brougham *et al.*, 2012). The rationale for using AMH as a marker of ovarian reserve and later on as a reflector of chemotherapy-induced ovarian toxicity, was based upon the fact that it is secreted by a specific population of small follicles, not including apoptotic follicles, that correlates with the PMF reserve (van Rooij *et al.*, 2002; Kevenaar *et al.*, 2006). AMH is more sensitive than other hormonal markers (Fanchin *et al.*, 2003) and is not menstrual-cycle dependent (La Marca *et al.*, 2006).

Most former pre-clinical studies of GnRH-a for fertility preservation counted the PMF population in histological sections of ovaries retrieved from mice treated either with chemotherapy or with chemotherapy combined with GnRH-a (Ataya *et al.*, 1985, 1995; Bokser *et al.*, 1990; Yüce *et al.*, 2004; Matsuo *et al.*, 2007; Ozcelik *et al.*, 2010; Lin *et al.*, 2012; Kishk and Mohammed Ali, 2013; Li *et al.*, 2013). The major drawback of this design is that an evaluation performed at a single random time point does not reflect the dynamic process of ovarian toxicity in the same way as continuous surveillance of changes in PMF pool size.

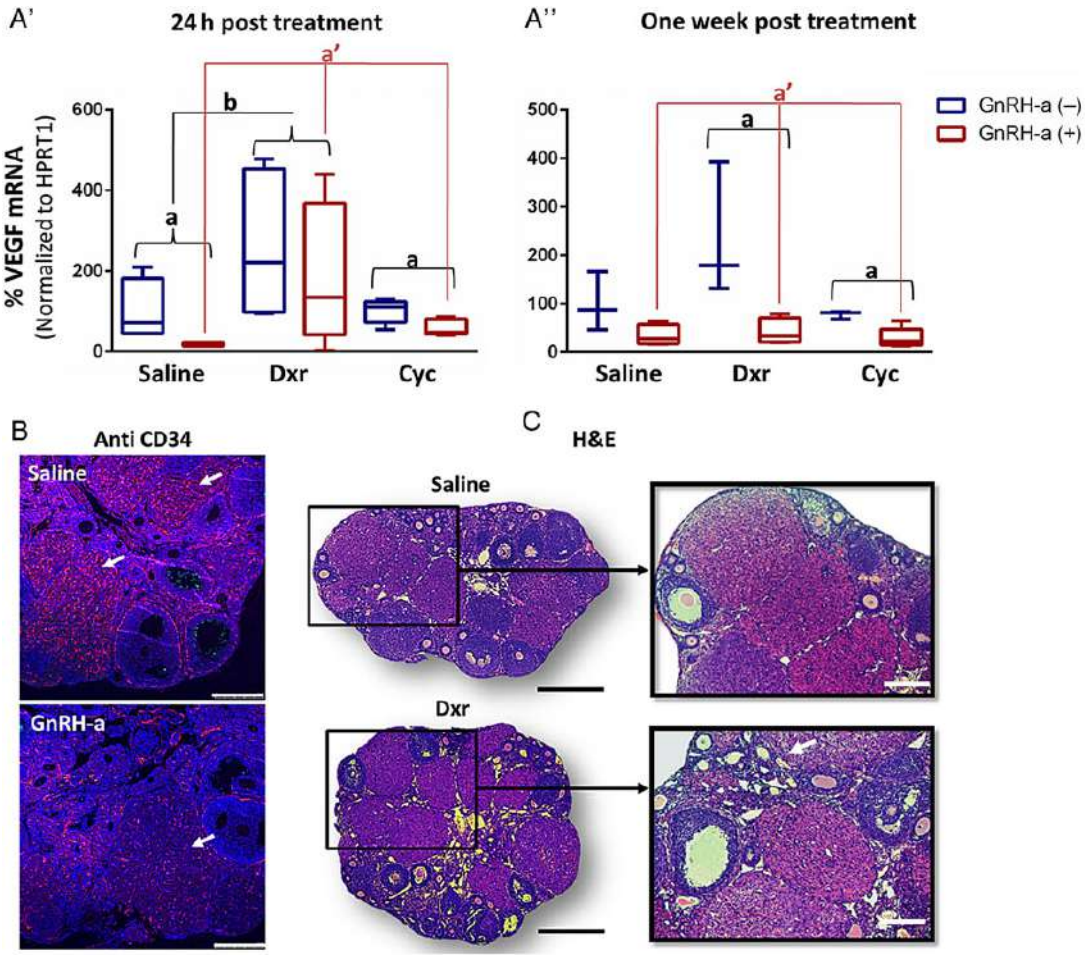


Figure 5 GnRH agonist (GnRH-a) decreases the level of ovarian vascular endothelial growth factor (VEGF) mRNA and interferes with Doxorubicin (Dxr)-induced vascular recovery. (**A**) Graphic representation of quantitative PCR (qPCR) VEGF analyses; calibrated with Hypoxanthine Phosphoribosyltransferase (HPRT1). mRNA was extracted from excised ovaries of mice 24 h (**A**′) or 1 week (**A**″) post saline/Dxr/Cyclophosphamide (Cyc) injection. The upper and lower limits of the boxes indicate the 75th and 25th percentiles, respectively; whereas the lines within the boxes indicate the median. The upper and lower horizontal bars denote the 90th and 10th percentiles, respectively. ($n = 3–5$ for each group; $^{a,a',b}P < 0.05$); ^{a}GnRH-a (+) versus GnRH-a (−); $^{a'}$all GnRH-a (+) versus all GnRH-a (−), regardless of chemotherapeutic treatment; ^{b}Dxr or Cyc versus saline. (**B**) Representative images of ovaries excised after 5 days of consecutive injections of saline (top) or GnRH-a (LA; bottom). Ovaries were fixed, processed for histology, sectioned and labeled with anti-CD34 antibody (red) and Hoechst (blue) as a nuclear marker. White arrows indicate highly vascular corpora lutea. Scale bar-250 μm. (**C**) Representative histological images of ovaries excised 1 month after an i.p. injection of saline (top) or Dxr (bottom), fixed and stained with Hematoxylin and Eosin (H&E). Black scale bar-500 μm, white scale bar-200 μm. Labeled sections were visualized and photographed by a Leica Laser confocal microscope.

Numerous clinical and pre-clinical studies have documented that Cyc exerted extensive loss of PMF (Warne *et al.*, 1973; Koyama *et al.*, 1977; Desmeules and Devine, 2006). Among the mechanisms of Cyc-induced ovarian toxicity are apoptosis of PMF (Marcello *et al.*, 1990), indirect PMF loss due to damaged ovarian stroma (Meirow *et al.*, 2007) and accelerated PMF recruitment (Letterie, 2004; Kalich-Philosoph *et al.*, 2013); all resulting in depletion of ovarian reserve. Our study is unique in its long-term follow-up with serial monitoring of ovarian response to Cyc administration. We demonstrated that Cyc shifted the balance maintaining PMF dormancy toward accelerated recruitment, thus possibly depleting the ovarian reserve (Fig. 6C′). The pattern of Cyc-induced ovarian toxicity is comprised of two phases: the early phase, manifested as an acute ovarian injury 24 h post-treatment, followed by a delayed insult, reflected by a decline in serum AMH level starting 4 months post-treatment, when the ovarian reserve crossed the limit of compensation due to accelerated follicular recruitment. Co-administration of GnRH-a preserved AMH levels 1 week following Cyc administration, possibly restoring the inhibition of PMF recruitment and preventing the accelerated depletion of the ovarian reserve (Fig. 6C″). Our results imply that VEGF does not play a key role in mediating Cyc-induced ovarian toxicity.

There is a paucity of data regarding the effect of GnRH analogues on AMH cellular level; most of the studies were performed *in vivo* and hence an indirect pituitary-gonadotrophin effect cannot be excluded (Thomas *et al.*, 2007; Jayaprakasan *et al.*, 2008; Lee *et al.*, 2008, 2010). Nevertheless, *in vitro* studies were performed on luteinized human GC collected during *in vitro* fertilization (IVF), representing a specific population of hormone-treated ovulated GC that differ from the AMH-secreting GC from early follicles (Winkler *et al.*, 2010; Dong *et al.*,

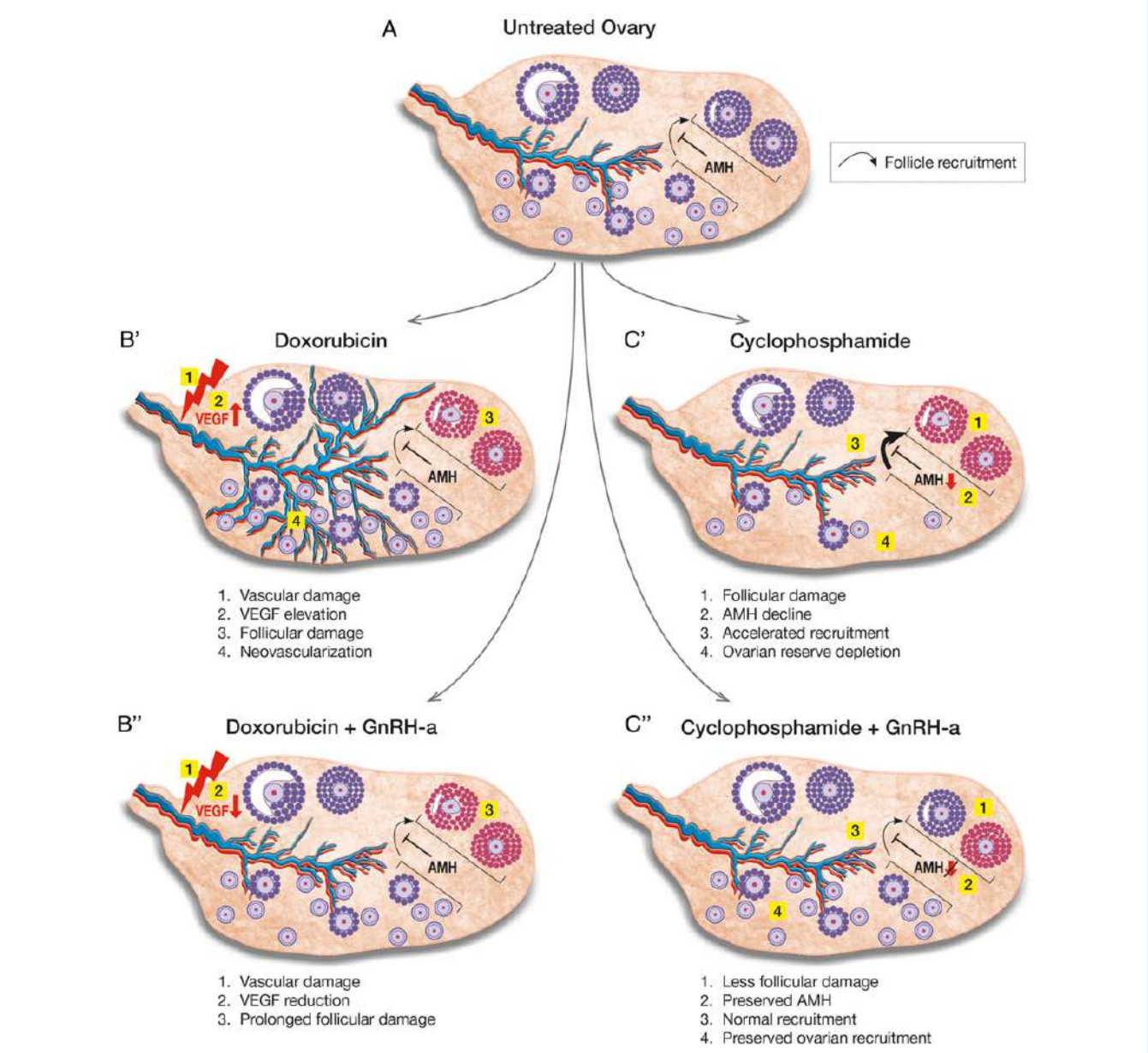


Figure 6 The potential of GnRH agonist (GnRH-a) for fertility preservation is chemotherapy-specific. (**A**) In the untreated ovary, the secretion of anti-Mullerian hormone (AMH) by growing follicles inhibits primordial follicle (PMF) recruitment thus maintaining a physiological state of PMF dormancy that is crucial for a long lasting ovarian reserve. Blood vessels are predominantly localized to the ovarian hilum. (**B′**) Doxorubicin (Dxr)-induced vascular damage causes transient inhibition of follicular growth and results in vascular endothelial growth factor (VEGF) elevation as part of the ovarian recovery mechanism, manifesting later on as cortical neovascularization. (**B″**) Co-administration of GnRH-a with Dxr, blunts VEGF elevation, thus compromising the vascular ovarian recovery process and prolonging follicular impairment. (**C′**) Cyclophosphamide (Cyc)-direct follicular damage results in AMH decline that diverts the paracrine balance maintaining PMF dormancy toward accelerated recruitment, thus depleting the ovarian reserve. (**C″**) Co-administration of GnRH-a with Cyc results in less follicular apoptosis and preserved AMH that maintains PMF dormancy and protects the ovarian reserve.

2011). Herein, we used GC from pre-pubertal rats unaffected by endogenous GnRH, to determine co-expression of AMH and GnRHR in the same cell, a major prerequisite for postulating a direct effect. Ultimately, we depicted a directly GnRH-a-induced increase in AMH protein as a potential underlying mechanism of protection from excess recruitment of PMF.

Dxr exerts its effect on the ovary by various mechanisms. We had previously suggested that Dxr exerts a unique pattern of gonadotoxicity mediated by a direct vascular injury as observed in mice and humans (Bar-Joseph *et al.*, 2011; Soleimani *et al.*, 2011). It has been also shown that Dxr treatment resulted in reduced ovulation rate, ovarian size and small-follicles population (Ben-Aharon *et al.*, 2010a). Dxr crosses the blood-follicle barrier and induces apoptosis via a cytotoxic effect exerted primarily upon dividing granulosa cells and consequently upon oocytes (Bar-Joseph *et al.*, 2010; Ben-Aharon *et al.*, 2010a; Soleimani *et al.*, 2011; Morgan *et al.*, 2013), as opposed to other mechanisms of

follicular toxicity where apoptosis commences in the oocyte (Morgan *et al.*, 2013). In the current study, Dxr induced enhanced follicular apoptosis, followed by a transient inhibition of follicular growth that lasted up to a month post-treatment. In contrast to the early direct injurious effect on the follicles, the sustained state of follicular growth inhibition may derive from impaired ovarian environment as most of the follicles directly affected by Dxr-insult have already ovulated or undergone atresia as this point. This mode of ovarian insult, mediated by vascular toxicity, was further implied by the sharp elevation in VEGF level after Dxr administration, resulting in increased ovarian neovascularization observed 1 month post-treatment (Fig. 6B′). It should be noted that compared with other groups, the PMF reserve in the Dxr-treated group was not compromised at the long-run, which is in accordance with clinical trials (Lee *et al.*, 2006). This supports the proposition that the mechanism of Dxr-toxicity is directed toward proliferating granulosa cells, as growing follicles sustained most of the damage while the quiescent PMF remained less affected.

The administration of GnRH-a concomitantly with Dxr failed to protect against Dxr-induced inhibition of follicular growth; furthermore, it altered ovarian recovery process in response to Dxr-induced vascular toxicity (Fig. 6B″). Although GnRH-a was previously shown to protect granulosa cells from Dxr-induced toxicity (Imai *et al.*, 2007), the research was conducted in a cell-culture model without assessing the indirect influence of ovarian vascularization. Our *in vivo* model led us to conclude that co-administration of GnRH-a with Dxr blunted Dxr-induced VEGF elevation, thus possibly impairing the vascular recovery that is exerted in the ovaries in response to Dxr treatment.

In conclusion, our study sheds light on the enigmatic role of GnRH-a in fertility preservation during chemotherapy, indicating that its potential impact is dependent upon the unique mechanism of chemotherapy-induced ovarian injury. GnRH-a reduced the rate of follicular loss in response to Cyc and hence preserved fertility, whereas administration of GnRH-a concomitantly with Dxr had an adverse effect on vascular recovery, resulting in a sustained state of impaired follicular growth (Fig. 6). Nevertheless, our findings are based on a mouse model in which down-regulation of gonadotrophins is established in a short period of only a few days; thus the clinical implication remains to be determined. Future research should entail conducting randomized controlled trials of homogenous patient populations treated with Cyc-based regimens with a long follow-up using surrogate biomarkers and establishing a registration of pregnancies in these cohorts. In clinical practice, GnRH-a may be considered as a treatment in selected patient populations treated with alkylating agents for protecting their ovarian reserve and there is no indication for its use in patients treated with Dxr-based protocols.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

N.H., I.B.-A. and R.S. conceived the concept and designed the experiments. N.H. carried out the experiments, organized the data and performed the statistical analyses. N.H. and I.B.-A. wrote the manuscript. R.S supervised the study and helped draft the manuscript. S.U. and K.G. participated in the experiments. I.M. and H.G. participated in the statistical analyses and designing of figures. S.M.S. participated in the study design and discussed the results and the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

None declared.

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