GnRH agonist leuprolide acetate does not confer any protection against ovarian damage induced by chemotherapy and radiation *in vitro*

by

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A Thesis Submitted to the

Graduate School of Health Sciences

in Partial Fulfillment of the Requirements for

the Degree of

Master of Science

in

Reproductive Biology

Koc University

January 2016

Koc University

Graduate School of Health Sciences

This is to certify that I have examined this copy of a master's thesis by

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ABSTRACT

Recent approaches in anticancer therapy led to increased rate of young cancer survivors; hence it became extremely important to minimize the long-term side effects of cancer treatments. For female cancer patients of childbearing age, the major concern is gonadotoxicity of chemotherapy and radiation which eventually results in premature ovarian failure (POF) and infertility. Premature ovarian failure is also associated with other adverse health-related consequences, including osteoporosis, cardiovascular diseases, sleep disturbance, and sexual dysfunction, which can negatively impact on short- and long-term quality of life.

Three fertility preservation strategies are currently available for women prior to cytotoxic chemotherapy/radiation for cancer: cryopreservation of oocyte, embryo and ovarian tissue. But ovarian tissue cryopreservation is considered still experimental due to unknown success rate of this procedure. Even though oocyte or embryo freezing prior to chemotherapy can help women achieve pregnancy, these strategies cannot reverse menopause in the native ovaries. Furthermore, ovarian tissue transplantation carries the risk of re-introducing cancer cells especially in hematological malignancies. Therefore, any drug that preserves ovarian reserve during chemotherapy can potentially sustain normal reproductive life span and obviate the need for gamete freezing prior to chemotherapy.

Encouraged by the initial reports of animal studies and non-randomized human trials showing a beneficial effect of GnRH (gonadotropin-releasing hormone) agonists in the preservation of ovarian function during chemotherapy, GnRHas (GnRH agonists) have been proposed as a fourth potential fertility preservation strategy. But randomized controlled trials launched so far to assess the effectiveness of this method have shown contradictory results in cancer patients. Some of these trials demonstrated a protective effect of GnRH agonists in preserving ovarian function after chemotherapy, whereas the others could not. This fact, together with the lack of a proven molecular mechanism of action for ovarian protection with GnRHa places this approach under scrutiny as a fertility preservation strategy.

We therefore aimed in this thesis to provide in vitro evidence for-or-against the role of GnRHa in the prevention of chemotherapy induced damage in human ovary. Lack of molecular data in this under-studied issue led us to investigate in this study if GnRH agonist leuprolide acetate decreases DNA damage and follicular apoptosis through either activation of GnRH receptors and upregulation of intragonadal antiapoptotic genes during adjuvant chemotherapy. For this purpose we conducted several specific end-point assays in this study to provide a molecular evidence for-or-against the role of GnRHa in the preservation ovarian function and reserve after chemotherapy.

This thesis provides in vitro evidence that the co-administration of GnRH agonist leuprolide acetate with cytotoxic chemotherapy agents does not prevent or ameliorates ovarian damage and follicle loss in vitro. This data can be useful when consulting a young patient who opts to receive GnRH treatment with chemotherapy to protect her ovaries from chemotherapy induced damage. Our findings do not conclusively rule out the possibility that GnRHa may offer protection if any, through some other mechanisms in vivo.

ÖZET

Kanser tedavisindeki yeni yaklaşımlar kanserden kurtulan gençlerin oranında artış sağlamış, dolayısı ile kanser tedavilerinin uzun dönemde neden olduğu yan etkileri azaltmak büyük önem kazanmıştır. Kanser tedavisi gören doğurganlık yaşındaki kadın hastalar için en önemli tehlike, gonadotoksik kemoterapi ve radyoterapinin prematür overyan yetmezlik (POY) ve infertilite ile sonuçlanmasıdır. Prematür overyan yetmezlik, infertilitenin yanısıra osteoporoz, kardiyovasküler hastalıklar, uyku bozuklukları, cinsel işlev bozuklukları gibi yaşam kalitesini kısa ve uzun dönemde olumsuz etkileyen sağlık sorunlarına neden olur.

Kanser tedavisi için sitotoksik kemoterapi/radyoterapi alacak kadınlarda kullanılabilir üç fertilite koruma stratejisi mevcuttur: oosit, embriyo ve over dokusu dondurulması. Ancak başarı oranının bilinmemesi nedeniyle over dokusu dondurulması prosedürü hala deneysel olarak kabul edilmektedir. Her ne kadar kemoterapi öncesi oosit ve embriyo dondurulması ile gebelik elde edilebilirse de, bu stratejiler menopozu engelleyememektedir. Ayrıca, over dokusu transplantasyonu özellikle hematolojik malignansilerde kanser hücrelerinin vücuda yeniden yerleşmesi riskini taşımaktadır. Bu sebeplerden dolayı, kemoterapi süresince over rezervini koruyan herhangi bir ilaç potansiyel olarak normal üreme fonksiyonlarını sürdürebilecek ve kemoterapi öncesi gamet dondurma ihtiyacını ortadan kaldırabilecektir.

Hayvanlar üzerinde yapılan çalışmaların ve randomize olmayan insan çalışmalarının ilk raporlarının gonadotropin salgılatıcı hormon (GnRH) agonistlerinin kemoterapi sırasında overyan fonksiyonların korunmasında yararlı etkileri olduğunu göstermesiyle, GnRH agonistleri dördüncü potansiyel fertilite koruma stratejisi olarak önerilmiştir. Ancak bu yöntemin etkinliğini değerlendirmek için kanser hastalarında bugüne kadar yapılan randomize kontrollü çalışmalar çelişkili sonuçlar vermiştir. Bu çalışmaların bazıları GnRH agonistlerinin kemoterapi sonrası overyan fonksiyonunu koruyucu etkileri olduğunu gösterirken, diğerlerinde koruyucu bir etki gözlenmemiştir. Ek olarak, GnRH agonistlerinin over fonksiyonlarını koruduğunu gösteren kanıtlanmış bir moleküler mekanizmanın olmaması, bu strateji ile ilgili yapılacak araştırmalara ağırlık verilmesini gerektirmektedir.

Bu tezde, GnRH agonistlerinin kemoterapi kaynaklı over hasarını önlemede etkisi olup olmadığını gösteren in vitro düzeyde kanıtlar elde etmek hedeflenmiştir. Yeterli çalışma yapılmamış olması ve konu ile ilgili moleküler veri eksikliği, bizi GnRH agonisti löprolid asetatın GnRH reseptörü ya da intragonadal antiapoptotik genleri aktivasyonu aracılığı ile DNA hasarını ve foliküler apoptozisi azaltıp azaltmadığını araştırmaya yöneltmiştir. Çalışmamızda bu amaçla, GnRH agonistinin kemoterapi sonrası over fonksiyonunu ve rezervini korumadaki etkisine moleküler kanıt sağlamak için bu çalışmada çeşitli spesifik analizler düzenlenmiştir.

Bu tez, sitotoksik kemoterapi ajanları ile birlikte GnRH agonisti löprolid asetat uygulanmasının in vitro şartlarda over hasarını ve folikül kaybını önlemediği ve azaltmadığını gösteren moleküler kanıtlar sağlamaktadır. Bu veriler overlerini kemoterapi kaynaklı hasardan korumak amacıyla GnRH tedavisi kullanmayı seçen genç bir hastaya danışmanlık yapılırken faydalı olabilir. Bulgularımız GnRH agonistinin in vivo'da başka herhangi bir mekanizma ile koruyuculuğu olma ihtimalini elimine etmez.

ACKNOWLEDGEMENTS

Though only my name appears on the cover of this thesis, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible.

My deepest gratitude is to my advisor, Dr. Özgür Öktem. I have been amazingly fortunate to have an advisor who gave me the freedom to explore on my own, and at the same time the guidance to recover when my steps faltered.

I am grateful to Dr. Serçin Karahüseyinoğlu for her encouragement, practical advice and commenting on my views and helping me enrich my ideas. I would like to thank Dr. Ercan Baştu and Dr. Nezih Hekim for being in my thesis committee and sparing their precious time for me. In addition, special thanks to Dr. Ercan Baştu for introducing me to this field.

I would like to thank the Koç University School of Medicine and Koç University Graduate School of Health Sciences for their funding during my master studies.

I thank my labmates, Nazlı Akın and Tuğçe Ayık, for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had during this time. I am indebted to them for their help and continuous encouragement. In addition, I owe special thanks to Filiz Şenbabaoğlu for her help. I wish to thank to the current staff at American Hospital IVF laboratory, for their various forms of support during my graduate study. Without their precious support, it would not be possible to conduct this research.

My dear friends, Dolay Damla Çelik and Seda Ateş, have helped me stay sane through difficult times. Their support and care helped me overcome setbacks and stay focused on my graduate study.

Most importantly, my family to whom this thesis is dedicated to, has been a constant source of love, concern, support and strength all these years. None of this would have been possible without their love and patience.

Last but not the least, my special thanks to Burak Elcik, his patience and support helped me overcome many crisis situations and finish this thesis. I deeply appreciate his belief in me.

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NOMENCLATURE

POF	Premature ovarian failure
GnRH	Gonadotropin-releasing hormone
GnRHa	GnRH agonist
RCT	Randomized controlled trial
GnRH-r	GnRH receptor
HLGCs	Human luteal granulosa cells
4-HC	4-hydroperoxy cyclophosphamide
Cyc	Cyclophosphamide
5-FU	Fluorouracil
TAC	Docetaxel, doxorubicin, cyclophosphamide combination regimen
H&E	Hematoxylin-eosin
MT	Masson's trichome
VEGF	Vascular endothelial growth factor
PFA	Paraformaldehyde
LH	Luteinizing hormone
cAMP	Cyclic AMP
PKA	Protein kinase A
CI	Normalized cell index
RIPA	Radio-Immunoprecipitation Assay
AMH	Anti-mullerian hormone
E_2	Estradiol
P	Progesterone
BcL-2	B-cell lymphoma 2
Bcl-2L2	Bcl-2-like protein 2
BcL-xL	B-cell lymphoma-extra large
BIRC-2	Baculoviral IAP repeat-containing protein 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Mcl-1	Myeloid leukemia cell differentiation protein
RT	Radiotherapy
SAPK/JNK	Stress-activated protein kinases /c-Jun N-terminal kinases
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
p53	Tumor protein p53

Chapter 1

Review of Literature

1. Anticancer treatments and premature ovarian failure (POF)

Recent approaches in anticancer therapy led to increased rate of young cancer survivors; hence it became extremely important to minimize the long-term side effects of cancer treatments. Building on 40 years of progress in cancer detection and treatment, survival rates for childhood cancer have risen from 20% to almost 80% [1-3]. By 2020, 1 in 250 adults will be living with a history of childhood cancer [2, 3]. For female cancer patients of childbearing age, the major concern is gonadotoxicity of chemotherapy and radiation which eventually results in premature ovarian failure (POF) and infertility. Premature ovarian failure and other poor reproductive outcomes are important sequelae of previous exposure to chemo and/or radiotherapy in children and adults with cancer. Cytotoxic chemotherapy regimens and radiotherapy induce apoptotic death of the oocytes and surrounding granulosa cells in the ovary leading to early exhaustion of the follicle stockpile, infertility and premature ovarian failure [4, 5]. Young females diagnosed with breast cancer, lymphomas/leukemias as well as non-malignant diseases requiring the use of cytotoxic chemotherapy regimens (i.e.: alkylating regimens) are at the greatest risk of premature ovarian failure and infertility following adjuvant chemotherapy [6, 7]. Premature menopause not only results in reduced quality of life but also has associated risks including hot flashes and night sweats; mood swings and disrupted sleep; genitourinary disorders; skeletal abnormalities like osteoporosis with resultant fractures; cardiovascular diseases; and infertility [8]. The impact of anticancer treatments on female fertility depends on the woman's age at the time of treatment, the chemotherapy protocol, the duration and dosage [9].

1.1. Type of chemotherapy agents

Gonadotoxicity of a chemotherapy agent in women was first reported in 1956. Louis et al. [10] described gonadal toxicity in women treated with busulfan for chronic myelogenous leukemia. At the present time, it is well-known that anticancer therapies induce damage in ovary, yet it is still not clearly explained the molecular mechanisms behind this event.

1.1.1. Alkylating Agents

Alkylating agents are known to be the most toxic regimens to the ovary than other chemotherapy classes. These agents are not cell cycle spesific and do not require cell proliferation for their cytotoxic actions. Thus, they may affect cells that are not actively dividing, such as oocytes or primordial follicles. Cyclophosphamide is an alkylating agent that serves as a backbone in many

chemotherapeutic regimens and is considered a prototype for gonadotoxic chemotherapy. Destruction of follicles at all stages of development in a dose-dependent manner has been reported in pre-clinical studies and in human ovarian tissues exposed to cyclophosphamide. The risk of POF rates rise above the age of 35 years and reach >80% for women over 40 years old [11]. This cyclophosphamide-induced toxicity was documented in other studies as well [12, 13].

1.1.2. Platinium-Based Regimens

Platinium-based antineoplastics are frequently used in the treatments of many types of cancers. Cisplatin, which is the most investigated member of this class, acts by spesifically provoking various types of chromosomal damage and DNA cross-links, induces the cell to undergo apoptosis [14]. Cisplatin induced ovarian toxicity relates its “off-target” actions on normal cells such as oocytes, granulosa and theca cells besides rapidly growing cancer cells [15].

1.1.3. Anthracylins

Doxorubicin; an often used anthracylin to treat lymphomas, leukaemia, breast cancer and sarcomas; intercalates with DNA and prevent its replication and transcription partly through inhibition of topoisomerase II. Doxorubicin can also cause DNA double-strand breaks leading to activation of ataxia telangiectasia mutated protein kinase (ATM), a DNA-repair protein which may initiate apoptotic cell death in the presence of high levels of DNA damage [16, 17]. Although doxorubicin was once considered to be only weakly ovotoxic, recent findings reveal that this may not be the case and doxorubicin could affect the ovary by any, or indeed all, of the above mechanisms [18].

1.1.4. Antimetabolites

Fluoropyrimidines act primarily on cells that are actively synthesizing DNA (S-phase of the cell cycle) and are considered as the backbone of adjuvant treatment for colorectal cancer. The widely used member of this class is 5-fluorouracil (5-FU). Standard 5-FU-based chemotherapy is considered to have minimal effects on female fertility. The clinical data regarding the impact of fluoropyrimidines on fertility are limited mainly due to the older age of patients and paucity of premenopausal population in the trials [9].

1.1.5. Taxanes

Taxanes are widely used in various malignancies and have become a pivotal cornerstone in the adjuvant treatment of breast cancer. Paclitaxel and docetaxel act on the cytoskeleton: they stabilize microtubules and disrupt normal polymerization/depolymerization, leading to an arrest of the cells at the G2–M phase of the cell cycle. The evidence for the potential gonadotoxicity taxanes may confer limited and inconsistent. Few clinical studies have found no additional increase in amenorrhea rates in

women treated by taxane- containing regimens, or a mild increase in a reversible amenorrhea [19-21]. Nevertheless, several prospective studies have shown that the incidence of amenorrhea in taxane-based chemotherapy regimens was higher than in anthracycline-based chemotherapy regimens [22, 23].

1.2. Potential mechanism of chemotherapy-induced ovarian toxicity

At any one time, there are follicles at different stages of maturation within the ovary. It is possible that spesific stages are more vulnerable to chemotherapy induced damage than others. Oktem and Oktay found that ovarian biopsies from patients treated with chemotherapy had significantly lower primordial follicle counts than untreated controls [24]. More mature follicles are also susceptible to damage by chemotherapy agents. Diminution of growing follicles also accelerate the depletion of primordial follicle reserve due to the decreased level of inhibitory substances produced by growing follicles. Consequently, more primordials undergo growth initiation to replace damaged growing follicles and eventually ovarian reserve burn out (Figure 1).

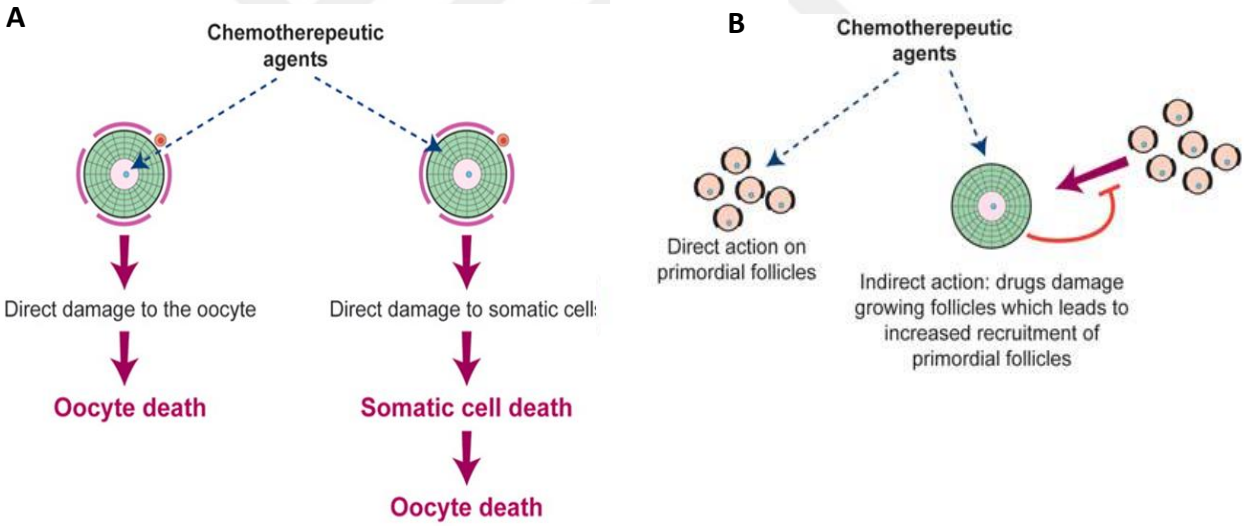


Figure 1. Potential targets of chemotherapeutic damage within the ovary. **(A)** Chemotherapeutic agents could be directly targeting the oocyte or the somatic cells. Oocyte death would result from death of the follicular somatic cells, as the oocyte is dependent on these for its survival. **(B)** Chemotherapeutic agents could be directly affecting the resting pool of primordial follicles or the growing follicle population. As growing follicles inhibit the recruitment of primordial follicles, the loss of this growing population will lead to increased activation of primordial follicles and so the loss of that reserve [5].

2. Protecting the ovary from damage

Three fertility preservation strategies are currently available for women prior to cytotoxic chemotherapy/radiation for cancer: cryopreservation of patient's oocyte, embryo and ovarian tissue. But ovarian tissue cryopreservation is considered still experimental due to unknown success rate of this procedure. Even though oocyte or embryo freezing prior to chemotherapy can help women achieve pregnancy, these strategies cannot reverse menopause in the native ovaries. Furthermore, ovarian tissue transplantation carries the risk of re-introducing cancer cells especially in hematological malignancies. Therefore, any drug that preserves ovarian reserve during chemotherapy can potentially sustain normal reproductive life span and obviate the need for gamete freezing prior to chemotherapy.

Encouraged by the initial reports of animal studies and non-randomized human trials showing a beneficial effect of GnRH (gonadotropin-releasing hormone) agonists in the preservation of ovarian function during chemotherapy, GnRHas (GnRH agonists) have been proposed as a fourth potential fertility preservation strategy. But randomized controlled trials launched so far to assess the effectiveness of this method have shown contradictory results in cancer patients. Some of these trials demonstrated a protective effect of GnRH agonists in preserving ovarian function after chemotherapy, whereas the others could not. This fact, together with the lack of a proven molecular mechanism of action for ovarian protection with GnRHa places this approach under scrutiny as a fertility preservation strategy.

3. Co-administration of GnRH agonist as a fertility preservation strategy

The administration of gonadotropin-releasing hormone agonists during chemotherapy has been proposed as a potential fertility preservation strategy to preserve ovarian reserve after emergence of the promising findings from anecdotal reports, primate models and non-randomized trials in human [25]. However, randomized controlled trials (RCTs) have shown inconsistent results in female patients with cancer (for review [25]), giving rise to a debate among the physicians and scientists in the fields of oncology and reproductive medicine over the actual role of GnRHa in the prevention of chemotherapy-induced ovarian failure. And a very recently, another RCT stirred the debate further by showing in breast cancer patients that the administration of GnRH agonist goserelin during adjuvant chemotherapy protected against ovarian failure and reduced the risk of early menopause [26]. The professional societies of oncology and reproductive medicine/fertility preservation currently emphasize lack of a proven molecular mechanism with gonadal protection with GnRHa during chemoterapy, and underscore the need for research in this under-studied issue [25, 27].

Based on current evidence, the role of GnRHa as ovarian protection agents remains controversial and unproven. The results are conflicting, and heterogeneity across trials further impacts on interpretability of the data. Patient age for eligibility varies considerably between trials. Only three studies limited age for eligibility to 40 years [28-30]. While prevention of premature menopause has important health consequences for women over 40 years, the proportional benefits in terms of fertility preservation are likely to be minimal, with very low ovarian reserve expected in most women over 40 [31].

Various mechanisms have been suggested, including GnRHa-induced decrease in the number of primordial follicles entering the differentiation stage, reduction of ovarian perfusion due to a GnRHa-induced hypoestrogenic state, and decreased ovarian cell apoptosis, through either activation of GnRH-R (GnRH receptors) or upregulation of intragonadal antiapoptotic molecules during adjuvant chemotherapy [32]. But none of these theories has been validated so far [4, 5, 25].

It has been shown that not only rodent but also primate and human gonads contain GnRH receptors. In an ovarian carcinoma cell line, Grundker and Emons have shown that GnRH-I and GnRH-II receptor activation may result in decreased apoptosis [33]. Whether the GnRH agonist effect is direct on the oocyte–cumulus complex or on the GC, or possibly on another ovarian compartment in addition to its possible hypogonadotropic effect, is an open question of significant interest. However, due to a lack of substantiating data, the validity of these theories has been questioned [34]. In preclinical animal studies, GnRHa administration was shown to reduce the loss of primordial follicles following cyclophosphamide treatment [35-37], although data in humans are limited.

Therefore we designed a translational research study to investigate if GnRHa administration during chemotherapy preserves ovarian reserve from chemotherapy through the activation of its cognate receptors and up-regulation of anti-apoptotic genes in human ovary.

Chapter 2

Materials and Methods

1. Patients

Ovarian cortical tissues were obtained from 15 patients (mean age ± SD: 27.8 ± 2.7, range: 14-37) undergoing laparoscopic surgery for the removal of the benign ovarian cysts between the years 2014-2015. One patient (age 14) had mature cystic teratoma. The remaining patients had ovarian endometrioma. All patients underwent operations at late follicular phase of the cycle (the mean ± SD of the cycle day: 8.2 ± 2.4).

Human luteal granulosa cells (HLGCs) were recovered from follicular fluid during oocyte retrieval procedure in 20 IVF patients (mean age ± SD: 32.6 ± 3.5). The etiologies for infertility were as follows: unexplained (n=12), diminished ovarian reserve (n=8). Informed consents were obtained in all patients and the study was approved by the institutional review board of Koc University.

2. Chemicals, chemotherapy drugs and GnRH agonist leuprolide acetate

Chemotherapy drugs and GnRH agonist leuprolide acetate were administered at their therapeutic blood concentrations. 4-hydroperoxy cyclophosphamide (4-HC), the active in vitro metabolite of the drug was used at 50 and 100 μM concentration [38] (Niomech (Bielefeld, Germany). Fluorouracil (5-FU) and cisplatin were used at 50 μg/mL and 40 μg/mL; respectively [39, 40] (Eli Lilly and Company (IN, USA). Paclitaxel was used at 2 μg/mL (Bristol-Myers Squibb Company (NY, USA) [41]. Docetaxel was from Rhone-Poulenc Rorer/Sanofi company [40]. Adriamycin was obtained from Sandoz, Novartis Inc. (Germany). TAC was administered at the following concentrations; cyclophosphamide 100 μM, adrimycin and docetaxel at 10 ng/mL [42, 43]. GnRH agonist leuprolide acetate (Abbott Pharmaceutical Products, USA), was given at 3 different concentrations (12,5, 25 and 50 ng/mL), which correspond to the serum and ovarian follicular fluid concentrations of the drug [44]. DMEM-F12 culture media, fetal bovine serum (FBS), Penicillin-Streptomycin Amphotericin B Solution, YO-PRO-1, Alexa probes were purchased from Life Technologies (Thermo Fisher Scientific Inc., MA, USA). xCelligence system® is a product of Roche Diagnostics (Mannheim, Germany). Hoechst 33342 (#4082), Anti-cleaved caspase-3 (mAb#9664), Anti-SAPK/JNK (#9252), Anti-Phospho-SAPK/JNK (Thr183/Tyr185, #9251), Anti-Phospho-Chk1 (Ser345, #2348), Anti-Phospho-Chk2 (Thr68, #2197) and Phospho-p53 (Ser15, #9286) antibodies were obtained from Cell Signaling Technology Inc., (MA, USA). Anti-phospho-Histone H2A.X antibody (Ser139, clone JBW301) was from Millipore (MA, USA). Anti-Vinculin Antibody (sc-25336) was purchased from Santa Cruz. COV434 cell line was purchased from

Sigma (St.Louis, MA, USA). HGrC1 was a gift from Dr. Ikara Iwase (Nogoya University, Japan). All western blotting buffers and reagents were purchased from BioRad.

3. Human Ovarian Tissue

3.1. Preparation and culture of ovarian cortical tissues

Ovarian cortices embedded in the cyst wall was removed under sterile conditions, minced into pieces of equal size (0.5x0.5 cm) and cultured for 24 hrs in 24 well format culture plate using 1 ml of DMEM-F12+10% FBS. Chemotherapy agents ± GnRHa were added to culture media at the indicated concentrations.

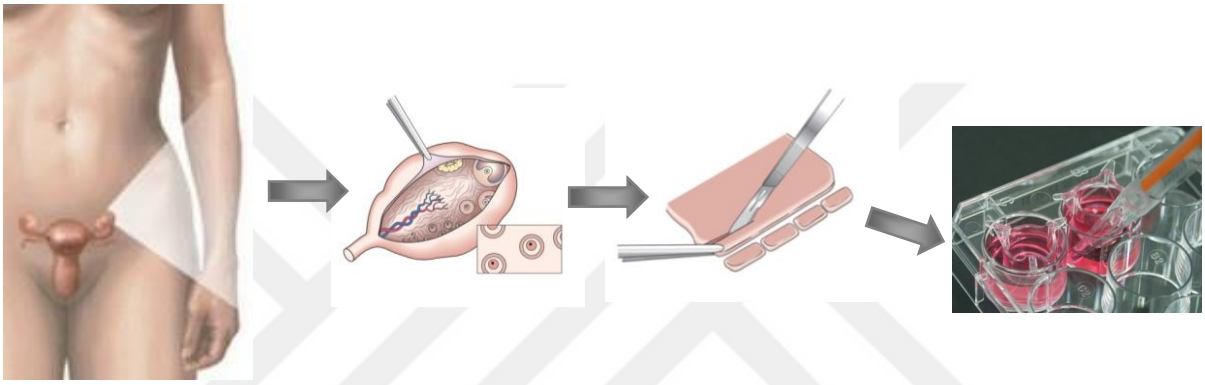


Figure 2. Schematic diagram demonstrates preparation and culture of human ovarian cortical tissues

3.2. Irradiation of ovarian cortical samples

Single fraction dose of 2 Gy (at a dose-rate of 600 MU/min) mimicking conventional daily fractionation in clinical use was prescribed as one piece in culture medium per Petri dish per beam. We used 2 Gy since the LD_{50} of human oocyte was reported to be < 4 Gy [45]. The ionizing irradiation in vitro was performed using a Varian Trilogy Linac capable of delivering X-ray beams with 6 MV energy. The X-ray field size aligned at the beam central axis with a source-to-cell layer distance of 100 cm perpendicular to the Petri dish was set to 10 cm x 10 cm. A 6–8 mm air layer was present above the medium within the Petri dish at set up. The water equivalent depth was adjusted to the depth of maximum dose and the build-up to maximum dose was provided by slabs of Plastic Water (RW3 water equivalent phantom, PTW, Freiburg) of appropriate thickness.

3.3. Histomorphometric assessment of the ovarian samples

Paraffin-embedded sections were serially sectioned at 5-µm thickness and primordial follicle density was determined from serial sections as the mean of follicle counts per square millimeter in every fifth section after staining with hematoxylin-eosin (H&E). Follicle density was expressed as follicle count/mm^2. Ovarian samples were also stained with Masson's trichome (MT), VEGF and

Hoechst 33342 for the examination of ovarian stroma and vasculature [46]. In brief, the ovarian samples were fixed in 4% PFA at 4°C for 24 hours followed by 20% (wt/vol) and 30 % (wt/vol) sucrose treatment until the tissues have sunk. Cryosections of 5 µm were obtained for MT and VEGF staining. For MT staining, Masson's Goldner staining kit (Merck Millipore, USA) was used according to manufacturer's instructions. The sections were treated with tap water followed respectively by Weigert's iron hematoxylin staining, azophloxine, tungstophosphoric acid orange G and light green SF solutions. One percent acetic acid was used after each step. The sections were then washed with distilled water and mounted with an aqueous mounting medium (Abcam, USA). For VEGF staining the sections were washed in PBS (Sigma, USA) followed by a 1:50 diluted anti-VEGF antibody (abcam, USA). An Alexa 594 conjugated goat anti mouse antibody (Abcam, USA) was used as the secondary antibody in 1:100 dilution. Both primary and secondary antibodies were incubated at 37 °C for 90 minutes in a humidified chamber at dark. Hoechst 33342 (1 µg/ml, Sigma, USA) was used in mounting medium (1:1, PBS/glycerol). Light microscopic images and fluorescent images were taken under a Zeiss Axioscope. Microvessel density was determined by averaging four microscopic fields in a defined area of each specimen.

4. Human Granulosa Cells

4.1. Cell culture

COV434 cells were purchased from American Tissue Type Culture Collection (USA). HGrC1 cells were kind gifts from Dr. Ikara Iwase (Nogoya University, Japan). HLGCs were recovered from follicular fluid during oocyte retrieval procedure in 20 IVF patients. All cells were cultured in 37^{o}C humidified incubator with 5% CO2 in DMEM-F12 medium with 10% fetal bovine serum and 1% Penicillin-Streptomycin Amphotericin B.

4.2. Mitotic non-luteinizing human granulosa cells

4.2.1. HGrC1

HGrC1 is a human non-luteinized granulosa cell line expressing enzymes related to steroidogenesis, such as steroidogenic acute regulatory protein, aromatase and gonadotropin receptors. They were granulosa cells obtained from a 35-yr-old female and immortalized by lentivirus-mediated transfer of several genes so as to establish a human nonluteinized granulosa cell line HGrC1 by Bayasula, et al. [47]. These cells are not capable of undergoing luteinization, resembling the characteristics of granulosa cells belonging to follicles in the early stage. HGrC1 might also be capable of displaying the growth transition from a gonadotropin-independent status to gonadotropin-dependent one [47].

4.2.2. COV434

COV434 is obtained from granulosa cell tumor. The biological characteristics of this cell line include the production of 17 beta-estradiol in response to follicle stimulating hormone (FSH); the absence of luteinizing hormone (LH) receptor; no lutenization capability, and the presence of specific molecular markers of apoptosis enabling the induction of follicular atresia [48].

4.3. Non-mitotic luteinized human granulosa cells

HLGCs were recovered from follicular fluid during oocyte retrieval procedure in 20 IVF patients. These cells are highly specialized primary luteinized granulosa cells, they do not proliferate either spontaneously; or after stimulation with a mitogenic agent. They produce large amounts of progesterone and estradiol hormones in vitro.

In IVF protocols, the developed follicles are punctured under transvaginal ultrasound guidance and the contents are aspirated. The cumulus–oocyte complexes are then visualized and manually collected from the aspirated fluids. After the first follicle has been punctured, localized bleeding occurs, so that subsequent follicular aspirates contain some blood. Therefore, once follicular aspirates have been pooled, the majority of cells in these preparations are erythrocytes. These red blood cells have a greater density than granulosa-luteal cells and settle to the bottom of culture dishes faster. If the culture surface is completely covered with these cells, the granulosa-luteal cells will not be able to attach. Therefore, it is necessary to remove these erythrocytes to maximize plating efficiency and obtain enriched cultures.

For the isolation of human luteal granulosa cells from follicular fluid, hypo-osmotic lysis technique was used described by Lobb et. al. [49]. The aspirates of follicular fluids were spun down at 500×*g* for 5 min and supernatant was discarded. 0.5 ml of the cell slurry was pipetted into a 15 ml conical bottomed polystyrene centrifuge tube. 9 ml of sterile distilled water added and the tube was capped and mixed. After 20 second, 1 ml of 10X concentrated PBS, pH 7.4 was added and the tube was capped and mixed. The tubes were then centrifuged at 150×*g* for 5 min and then decanted by inverting the tubes. The cell pellet was resuspended in culture media. Then recovered cells were plated in 24 well format culture plate in a density of 5000 cells per well.

5. cAMP-Glo™ Assay

cAMP-Glo™ Assay (Promega Corporation, Madison, WI) is a bioluminescent assay to measure cAMP levels in cells in response to the effects of an agonist or test compound on G protein-coupled receptors (GPCRs). The assay is based on the principle that cyclic AMP (cAMP) stimulates protein

kinase A (PKA) activity, decreasing available ATP and leading to decreased light production in a coupled luciferase reaction. Therefore, luminescence is inversely proportional to cAMP levels (Figure 3).

The cells were seeded in a poly-D-lysine-coated, white, clear bottom 96-well plate at the density of 10.000 cells per well and incubated overnight to allow attachment. After 24h, the medium was removed and the cells were treated with 50 ng/ml leuprolide acetate in serum-free medium for 10 minutes to initiate cAMP production. Subsequently, the cells are lysed by adding 20 µl lysis buffer to all wells to release intracellular cAMP. Then cAMP detection solution which contains PKA and reaction buffer was added and the plate was incubated at room temperature for 20 minutes. To terminate PKA reaction and detect the level of remaining ATP, the Kinase-Glo Reagent was added to all wells. Luminescence signal is measured with a plate reading luminometer. Luminescence is corrolated to the cAMP concentrations by using a cAMP standard curve.

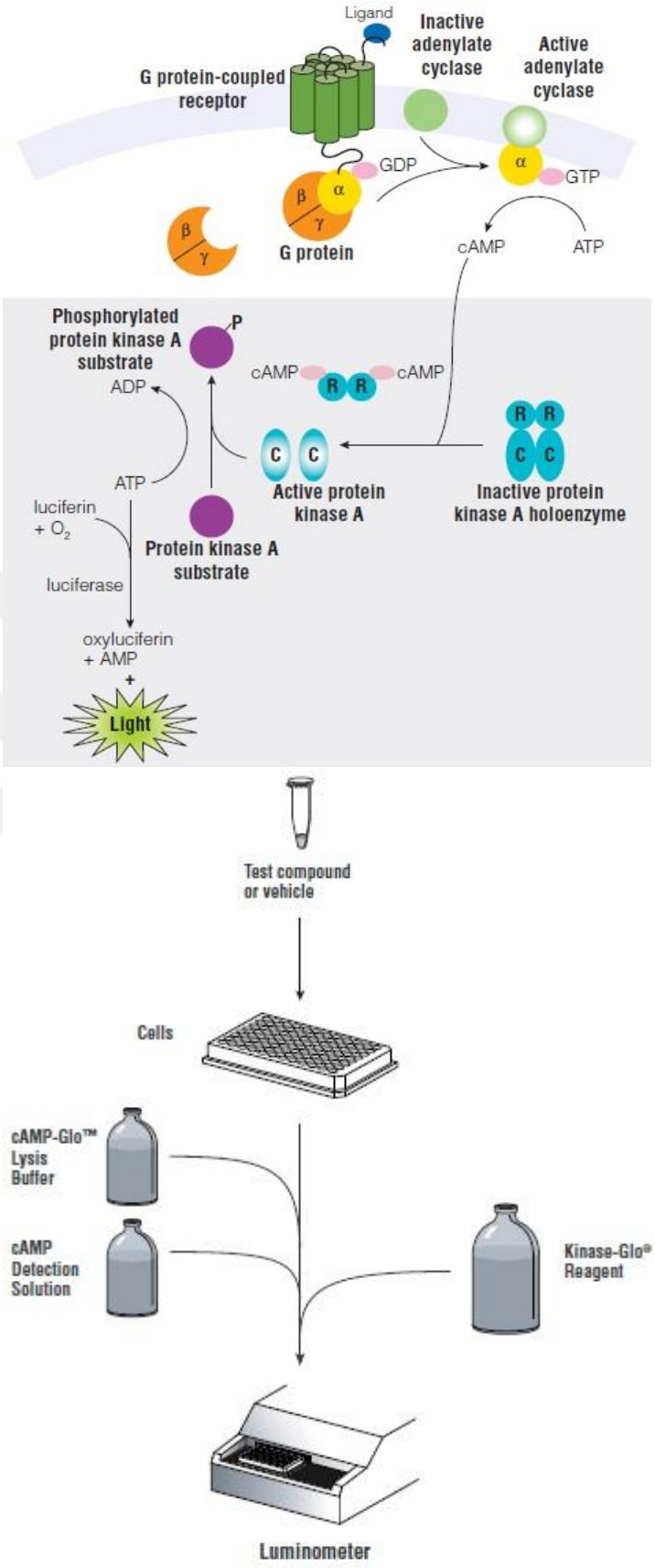


Figure 3. Schematic diagram of cAMP production in cells and the cAMP-Glo™ Assay protocol.

6. Real-time and quantitative assessment of cell proliferation and viability using xCelligence system

xCelligence is a cell culture system that allows real-time quantitative analysis of cell proliferation, viability, cytotoxicity, adhesion/invasion/receptor activity assays without using any compound and labeling agent. The system uses specially designed microtiter plates containing interdigitated gold microelectrodes to noninvasively monitor the viability of cultured cells using electrical impedance as the readout and generates real-time curves of cell viability and proliferation [50].

xCelligence RTCA system is composed of an impedance analyzer, a computer with RTCA software, a 96-well electronic microtiter plate (E-Plate) and the RTCA station, which is placed inside the cell culture incubator (Figure 4A). The application of a low voltage leads to generate an electric field between the electrodes, which can be impeded by cell presence. The electronic readout of cell–sensor impedance is displayed in real-time as cell index (CI), a value directly influenced by cell attachment, spreading, and/or cell proliferation (Figure 4B).

100 µL of complete media was added to the each well of 96-well E-Plate. Following 15 minute incubation at room temperature, the background impedance was measured. Cells were seeded in 96-well E-Plate at the density of 10,000 cells per well in a final volume of 200 µL, then incubated at 37°C with 5% CO2 and continuously monitored on the RTCA system at 30-minute time intervals. When the cells reached the log growth phase (4-6 hrs after plating), they were treated with the chemotherapy drugs ± GnRHa at the indicated concentrations. The proliferation and the viability of the cells were monitored in a real-time and quantitative manner every 30 min for up to 140 hrs. The results were expressed by normalized cell index (CI); which are derived from the ratio of CIs before and after the addition of the compounds. The normalization of CI arbitrarily sets CI to 1 at the indicated time points. Recording of CI and normalization CI was performed using the xCelligence RTCA Software 1.2.

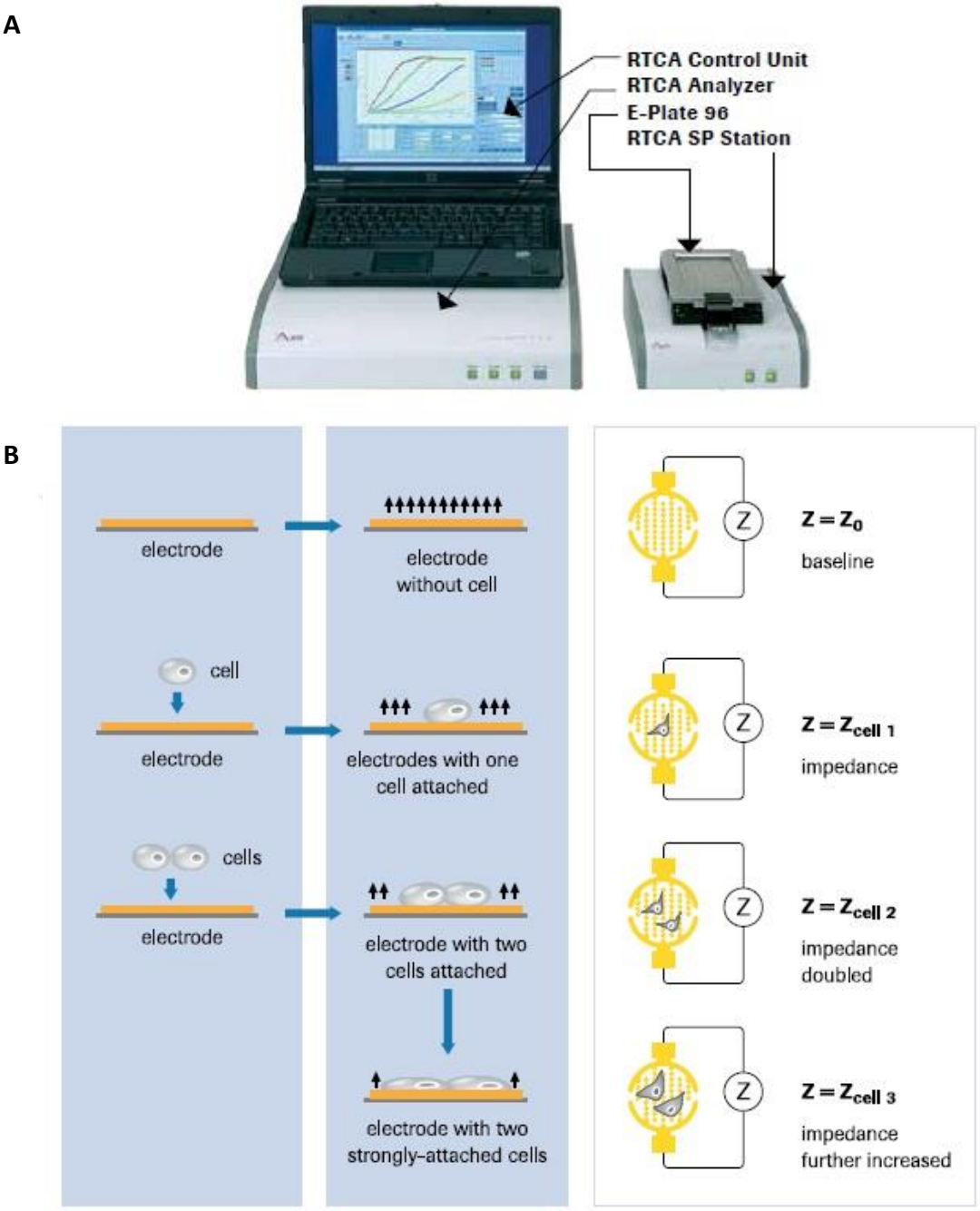


Figure 4. xCelligence RTCA SP system overview **(A)** xCELLigence RTCA SP Analyzer, a RTCA SP Station, and a RTCA Control Unit. **(B)** The presence of cells on top of the E-Plate 96 electrodes affects the local ionic environment at the electrode/solution interface, leading to an increase in electrode impedance. The more cells that are attached on the electrodes, the larger the increases in electrode impedance. When cells are not present or are not well-adhered on the electrodes, the CI is zero. Additionally, change in a cell status, such as cell morphology, cell adhesion, or cell viability will lead to a change in CI.

7. Live cell imaging with YO-PRO-1 staining for the assessment of cell viability

Apoptosis based changes occur in the permeability of cell membranes. Apoptotic cells become permeant to the green-fluorescent carbocyanine nucleic acid stain YO-PRO-1 (1μM) (absorbance 491nm, emission 509 nm), whereas live cells are impermeant to it. YO-PRO-1 nucleic acid stain selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence. Under fluorescent microscopy, YO-PRO-1-fluorescent cells demonstrate the morphological features of cells undergoing apoptosis such as nuclear shrinkage and fragmentation. An important characteristic of the dye that differs from all other nuclear dyes previously used for the detection of apoptosis is that it does not label living cells.

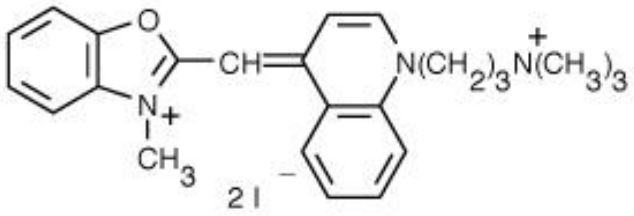


Figure 5. Chemical structure of YO-PRO®-1. Its relatively large size (630 Da) prevent it from penetrating the intact plasma membrabe of living cells.

Hoechst 33342 (1μg/mL) was added to the mounting medium for DNA staining. Live/dead cell imaging of the cells were undertaken under appropriate channels using IF microscope (Olympus IX71, Japan). Five hundred cells were counted at four different high magnification areas and the percentage of the cells expressing YO-PRO-1 was calculated.

8. Western blot analysis

Equal numbers of cells ($5x10^5$) were seeded to 6-well plates prior to chemotherapy agent with and without GnRHa treatment for obtaining equal amount of protein extraction. After 24 hours, medium was collected, cells were detached by trypsinization and cell pellet was obtained with centrifugation at 500g for 5 minutes. Pellets were then washed with ice cold PBS and then re-suspended in an appropriate volume of RIPA (Radio-Immunoprecipitation Assay) cell lysis buffer containing, 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, 1X phosphatase inhibitor cocktail and 1X protease inhibitor cocktail (Sigma, MA, USA). Following 20 minutes of incubation on ice, centrifugation at 14,000g for 20 min at 4^oC was performed to obtain supernatants containing total cell extract. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane by Trans-Blot® Turbo™ RTA Mini PVDF Transfer Kit (#170-4272, Biorad, USA). The membranes were blocked with 5% nonfat dry milk in TBS-T

(20 mM TrisHCl, pH 7.8, 150 mM NaCl, 0.1%, v/v Tween-20) at room temperature for 1 hour. Then the primary antibodies were added and incubated rocking overnight at 4^{o}C.

Anti-phospho-Histone H2A.X and Cleaved Caspase-3 antibodies were used at 1:1000 and 1:500 dilutions to assess DNA damage and apoptosis, respectively. Anti-SAPK/JNK and Anti-Phospho-SAPK/JNK antibodies were both performed at 1:1000 dilutions. Anti-Phospho-Chk1, Anti-Phospho-Chk2 and Anti-Phospho-p53 were used at 1:500 dilutions. Anti Anti-Vinculin (Sigma-Aldrich, Germany) at a dilution of 1:10000 is used as a loading control. Secondary antibodies conjugated to HRP, anti-rabbit and anti-mouse were used in 1:2000 (sc-2030 and sc-2005 respectively, Santa Cruz Biotechnology, USA). Quantification of protein within membranes was done by using Clarity™ Western ECL Substrate (#170-5061, Biorad, USA). Chemiluminescence detections were performed by ChemiDoc XRS+ Imaging Sytem (Biorad, USA).

9. Immunofluorescence staining

For immunofluorescence studies, cells were fixed with 10% neutral formalin for 20 min, washed twice with PBS, and then treated with blocking buffer (1X PBS / 5% normal goat serum / 0.3% Triton™ X-100) for 1hr. After rinsing with PBS, they were incubated overnight at 4 ^{0}C with cleaved caspase-3 antibody for detection of apoptosis and phospho-Histone H2A.X antibody for detection of DNA damage by immunofluorescence. Primary antibodies were diluted in antibody dilution buffer (1X PBS / 1% BSA / 0.3% Triton™ X-100) at 1:50 and 1:200 respectively. After rinsing with PBS, the cells were incubated with fluorochrome-conjugated secondary antibody (Alexa 486, Molecular Probes, USA) diluted in antibody dilution buffer for 1 hr. This step was followed by rinsing the coverslips slides and adding Hoechst 33342 (1µg/mL) for DNA staining. The images were taken under appropriate channels using IF microscope (Olympus IX71, Japan). The percentage of the caspase-3 positive cells was calculated after counting five hundred cells at four different high magnification areas.

10. Hormone assays

10.1. Anti-mullerian hormone (AMH)

AMH levels in the supernatants were determined using Active Mullerian Inhibiting Substance/ Anti-Mullerian Hormone (MIS/AMH) (Diagnostic Systems Laboratories, Inc., USA) ELISA kit. The analytical sensitivity of the kit was 0.006ng/mL. Intra-assay repeatability and the coefficient of variations were given as 4.6% (0.144 ng/mL), 2.4% (0.843 ng/mL), and 3.3% (4.408 ng/mL), respectively.

10.2. Estradiol and Progesterone

The levels of both hormones were determined using the electrochemiluminescence immunoassay "ECLIA", an immunoassay for the in vitro quantitative determination of estradiol and progesterone levels. ECLIA kits spesific for progesterone (Elecsys® Progesterone II, Cobas) and estradiol (Elecsys® Estradiol II, Cobas) were used according to manufacturers's instructions to measure the corresponding hormone levels in the culture media. All analyses were performed on Cobas® 6000 analyzer series (Roche Diagnostics, USA). Lower detection limits for estradiol and progesterone were 18.4 pmol/L (5.00 pg/mL) and 0.095 nmol/L (0.030 ng/mL), respectively.

11. Real-time reverse transcription PCR (qRT-PCR)

Ovarian tissue samples and granulosa cells treated with 100 µM cyclophosphamide, with and without 50 ng/ml leuprolide acetate for 12 hours. RNA isolation was performed by NucleoSpin® RNA Kit (Macharey-Nagel) following manufacturer's instructions. RNA quantification was performed by spectrophotometric read at 260 nm by Nanodrop (Thermo Scientific). 250 ng cDNA preparation was obtained by reverse transcription of RNA using M-MLV Reverse Transcriptase (Invitrogen). Relative mRNA expression levels of indicated anti-apoptotic genes were detected by using Light Cycler® 480 SYBR Green I Master (Roche, Germany).

Gene		Sequence
BcL-2	F	5'-CAGGGCGATGTTGTCCACC-3'
	R	5'-GGGGAGGATTGTGGCCTTC-3'
Bcl-2L2	F	5'-GCGGAGTTCACAGCTCTATAC-3'
	R	5'AAAAGGCCCCTACAGTTACCA-3'
BcL-xL	F	5'-GGTCGCATTGTGGCCTTTTTC-3'
	R	5'-TGCTGCATTGTTCCCATAGAG-3'
BIRC-2	F	5'-AGCACGATCTTGTCAGATTGG-3'
	R	5'-GGCGGGGAAAGTTGAATATGTA-3'
GAPDH	F	5'-AGCCACATCGCTCAGACAC-3'
	R	5'-GCCCAATACGACCAAATCC-3'
Mcl-1	F	5'-TGCTTCGGAAACTGGACATCA-3'
	R	5'-TAGCCACAAAGGCACCAAAAG-3'
XIAP	F	5'-TGTCCTGGCGCGAAAAG-3'
	R	5'-TGCCAGTGTTGATGCTGAAAC-3'

Table 1. List of primers and their sequences used in qRT-PCR. F: forward, R: reverse primer

12. Statistical analysis

Follicle counts were expressed as the median ± [SD]. Hormone levels and cell index readouts of xcelligence system were expressed as the mean ± [SD]. Statistical analyses were done using SPSS for windows 20.0 statistical package program. Friedman test and paired Wilcoxon test were used.

Data were first compared among the groups using ANOVA or Kruskal Wallis test where appropriate. Then the data were subjected to post-hoc analysis if multiple comparison test results return $p<0.05$. Paired t-test was conducted to compare the follicle counts and hormone levels between ovarian samples treated with a certain chemotherapy drug and its counterpart treated with the same drug+GnRHa. The percentages of viable and apoptotic cells were compared between the groups using Fisher' exact test. For correlation analysis two-tailed Spearman's correlation test was conducted. A p value <0.05 was considered significant for all statistical tests.

Chapter 3

RESULTS

1. Validation experiments

We conducted two different validation experiments to test if our experimental methodology is a suitable model to study the impact of chemotherapy with and without GnRHa on the ovarian tissue samples and granulosa cells. In the first experiment, the expression of GnRH receptor and its functionality in the ovarian tissue samples and granulosa cells were analyzed. We proved in the second validation experiment that chemotherapy induced cytotoxicity on the ovarian tissue samples and granulosa cells can be demonstrated at the level of follicle reserve, steroidogenic activity, DNA damage and apoptosis.

1.1. GnRH receptor assays

The presence of GnRH receptor in the samples was validated with quantitative RT-PCR analysis. The expression of the receptor was higher in the ovarian samples from younger donors compared to those of older ones (Figure 6). GnRH-r is not expressed by the granulosa cells of the follicles from primordial to early antral stage. Its expression begins in the late antral and ovulatory follicles and luteinized granulosa cells of the corpus luteum in human ovary [51]. Therefore higher GnRH-r expression in the younger ovarian samples is likely due to the higher number of growing follicles in these samples.

Stimulation of the receptor with GnRHa leuprolide acetate (50ng/mL) caused a marked increase in the intracellular cAMP level after ten minutes in the ovarian cortical samples and granulosa cells. The blockade of GnRH receptor with its specific receptor antagonist cetrorelix acetate (5ng/mL) given 1 hr before the administration of leuprolide acetate markedly blunted the response, leading to a slight increase in the cAMP level. The co-administration of GnRHa with cyclophosphamide did not interfere with the activation of the receptor (Figure 7).

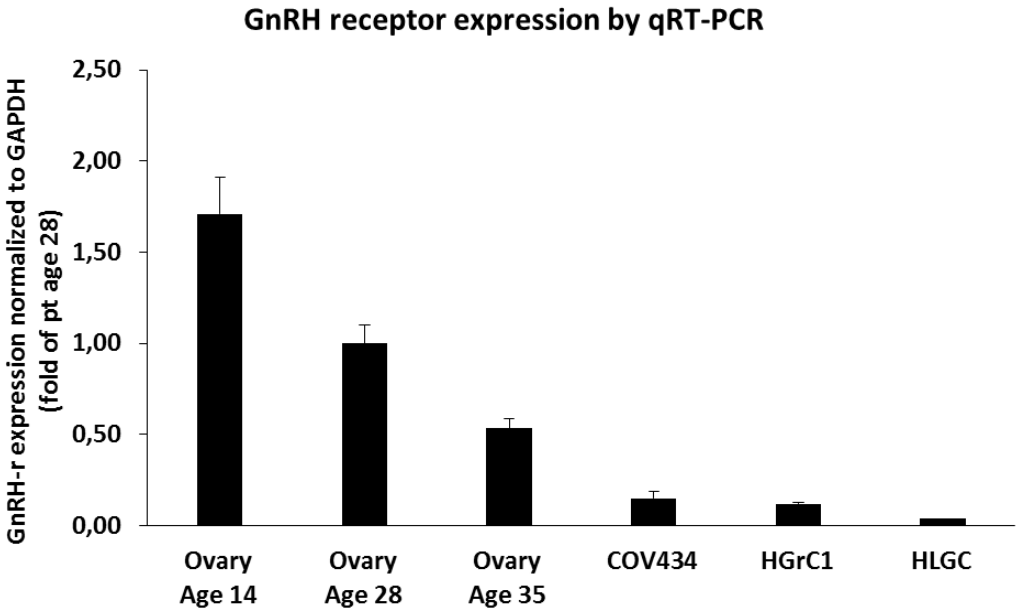


Figure 6. The expression of GnRH receptor in the ovarian samples and granulosa cells. The presence of GnRH receptor in the samples was validated with quantitative RT-PCR analysis.

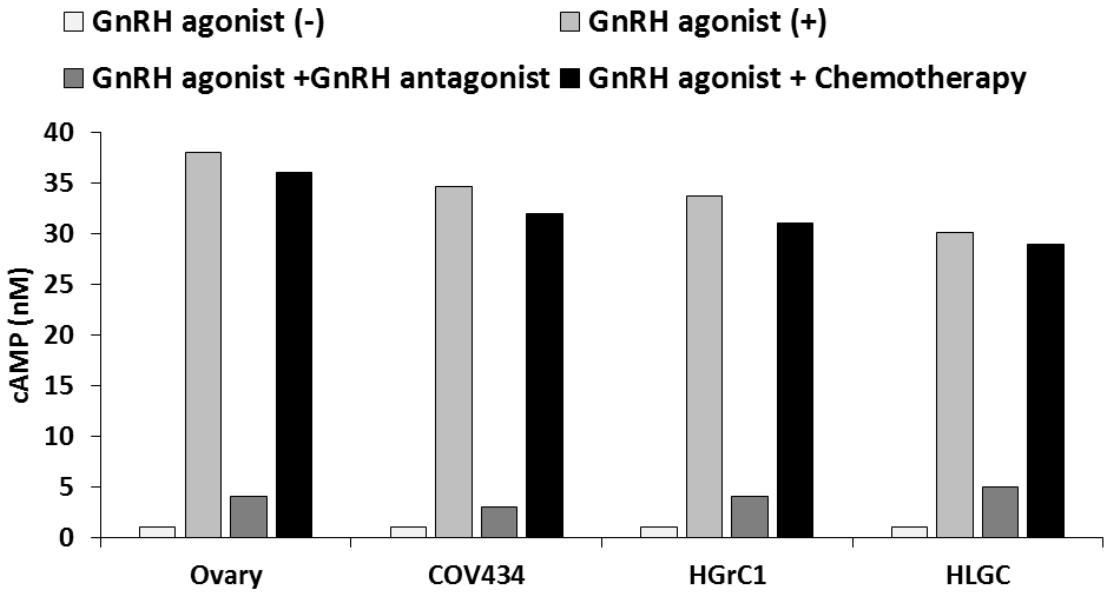
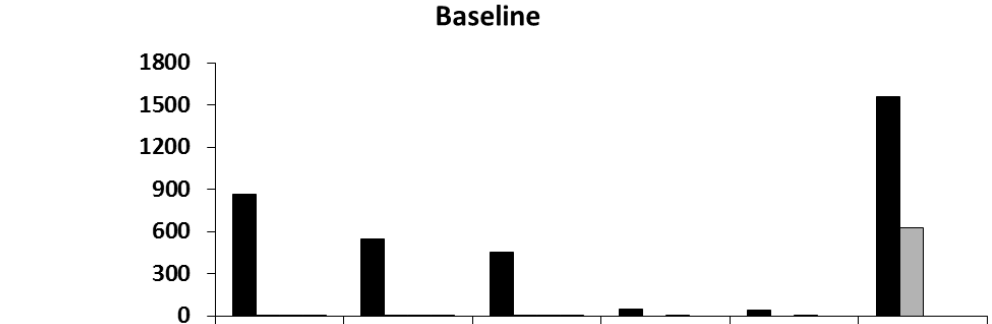


Figure 7. GnRH receptor activation by GnRHa. Stimulation of the receptor with GnRHa leuprolide acetate (50ng/mL) caused a marked increase in the intracellular cAMP level after ten minutes in both ovarian cortical samples and granulosa cells.

1.2. Demonstration of chemotherapy induced cytotoxicity on the ovarian tissue samples and granulosa cells

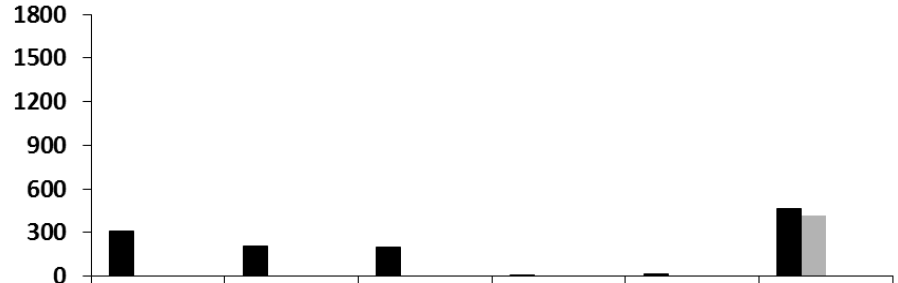
Ovarian cortical samples and granulosa cells (5000 cells/well) produced detectable amounts of E_2, P and AMH after 24 hr of culture (Figure 8A). Ovarian tissue samples from younger donors harbored more primordials and produced higher amounts of E_2 and AMH compared to those of older donors, indicative of higher ovarian reserve in the samples from younger donors (Figure 8A). HLGCs produced the highest amounts of E_2 and P in vitro. These luteinized granulosa cells typically do not produce AMH since this hormone is mainly produced by the granulosa cells of preantral and small antral follicles [52]. Incubation of the ovarian samples with active metabolite of cyclophosphamide (4-hydroperoxy cyclophosphamide, 100 μM) for 24 hrs resulted in a dramatic decline in follicle counts and hormone levels (Figure 8B). Similar cytotoxic effects were observed in the granulosa cells exposed to the same dose of the drug. They underwent apoptosis and their steroidogenic activity was substantially reduced. There was a strong expression of cleaved caspase-3 in the cells at 12 hr post-exposure. Then the intensity of the signal began to fade and nuclear fragmentation became evident at 24 hr (Figures 9A and 9B).

A



	Age 14	Age 28	Age 35	COV434	HGrC1	HLGC
E2 (pg/mL)	863	547	454	48	43	1560
P (ng/mL)	0,7	1,8	0,6	0	0	630
AMH (ng/mL)	2,49	1,47	1,04	0,6	0,5	0
Primordial/mm2	2,6	1,1	0,9			

B



	Age 14	Age 28	Age 35	COV434	HGrC1	HLGC
E2 (pg/mL)	312	207	198	11	15	465
P (ng/mL)	0,1	0,6	0,1	0	0	414
AMH (ng/mL)	0,67	0,13	0,1	0,06	0,05	0
Primordial/mm2	0,54	0,25	0,3			

Figure 8. Primordial follicle reserve and E_2, P and AMH productions of the samples at baseline and 24 hrs post-exposure to cyclophosphamide. **(A)** Ovarian tissue samples and granulosa cells (5000 cells/well) produced detectable amounts of E_2, P and AMH after 24 hr of culture. Ovarian samples from younger donors harbored more primordial follicles and produced higher amounts of E_2 and AMH than older donors. HLGCs produced the highest levels of estradiol and progesterone in vitro **(B)** Treatment of the ovarian samples with active metabolite of cyclophosphamide (4-hydroperoxy cyclophosphamide) at 100 µM concentration for 24 hrs resulted in a dramatic decline in the number of primordials with concurrent reduction in the production of AMH, E_2 and P.

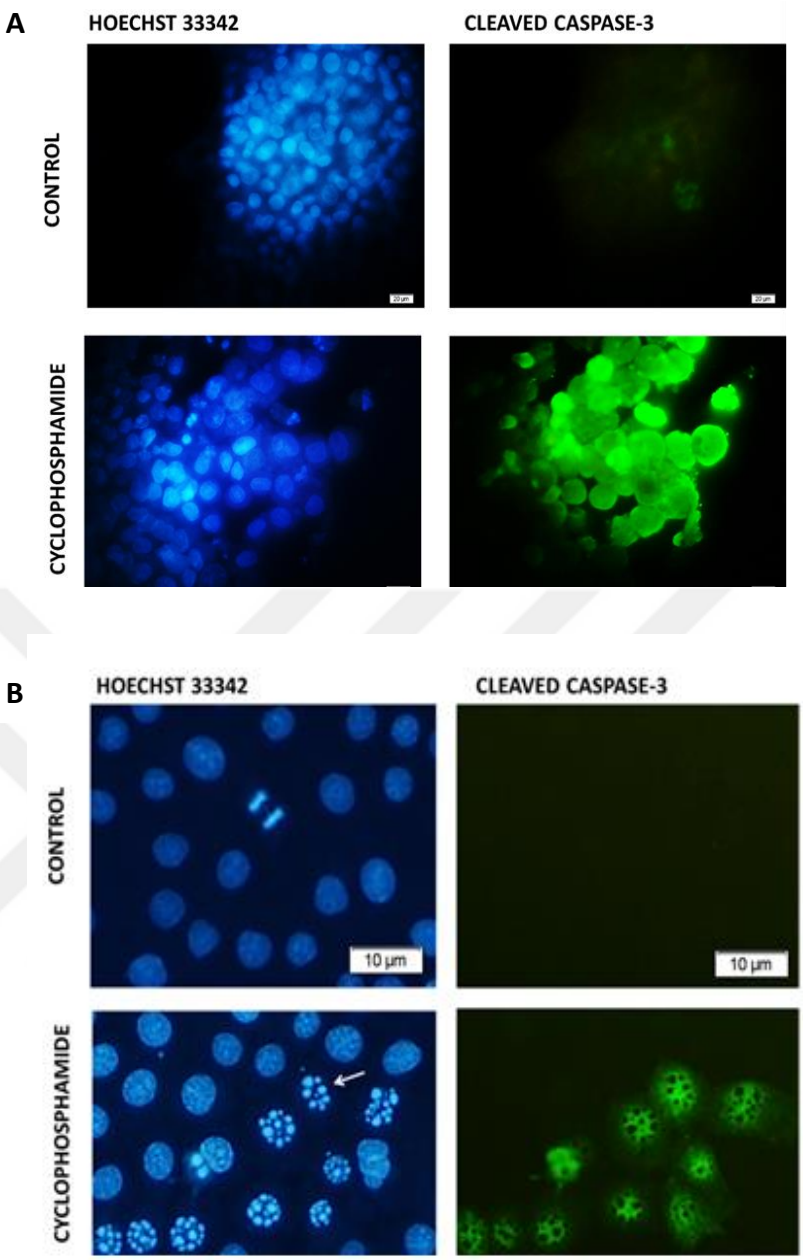


Figure 9. Demonstration of cyclophosphamide induced apoptosis in the granulosa cells. **(A)** There was a strong expression of cleaved caspase-3 in the cells at 12 hr post-exposure. **(B)** Then the intensity of the signal began to fade and nuclear fragmentation became evident at 24 hrs. Arrow indicates nuclear fragmentation.

Having obtained the promising results from these validation experiments, we treated ovarian tissue samples and granulosa cells with different chemotherapy agents and radiation with and without GnRHa and conducted the experiments described in the methods section and depicted in the figure of the experimental design (Figure 10).

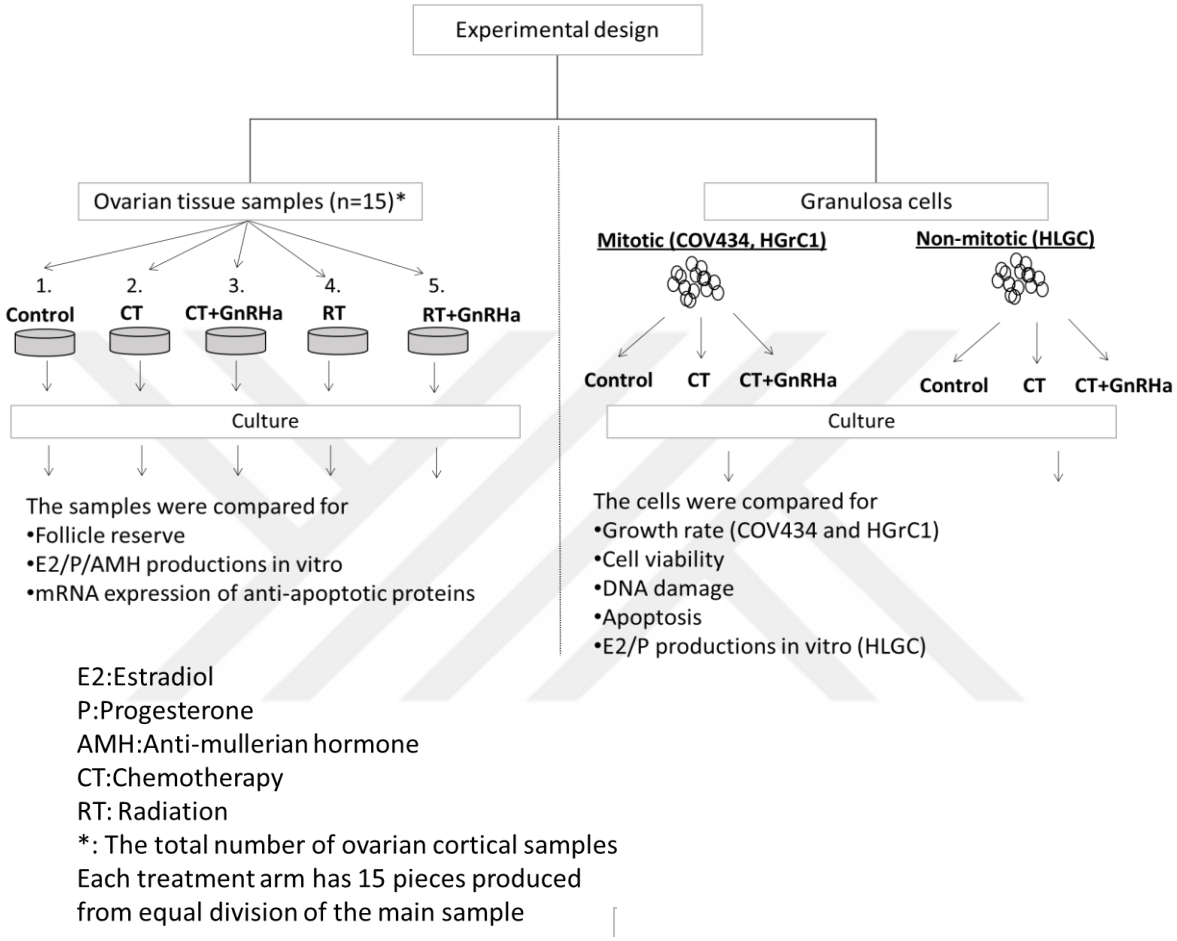


Figure 10. Experimental design of the study. Schematic overview shows segregation of the ovarian samples and granulose cells into treatment arms and the methods to assess and compare the cytotoxicity.

2. Ovarian cortical tissue samples

2.1. Comparison of follicle reserve and steroidogenic activity

Treatment of the ovarian samples with cyclophosphamide for 24 hrs resulted in a significant decline in follicle reserve and steroidogenic activity of the samples. The median numbers of primordials (0.33 ± 0.2 vs. 2.32 ± 0.5, p<0.01; respectively) and preantral/antral follicles (0.11 ± 0.01 vs. 0.57 ± 0.02, p<0.01; respectively) were significantly less than control samples (Figure 11). Also, these samples produced significantly lower amounts of E_2 (788 ± 98 vs. 185 ± 16 pg/mL, p<0.01; respectively), P (1.76 ± 0.3 vs. 0.33 ± .02 ng/mL, p<0.01; respectively) and AMH (1.2 ± 0.09 vs. 0.1 ± 0.03 ng/mL, p<0.01; respectively) than control ovarian samples (Figures 12A to 12C). The co-administration of GnRHa with cyclophosphamide did not prevent follicle loss in the samples. The mean number of primordial (0.33 ± 0.2 vs. 0.35 ± 0.2, p>0.05; respectively) and preantral/antral follicles (0.11 ± 0.01 vs. 0.13 ± 0.1, p>0.05; respectively) were comparable between cyclophosphamide and cyclophosphamide+GnRHa groups. The mean levels of E_2 (185 ± 16 vs. 148 ± 13 pg/mL, p>0.05; respectively), P (0.33 ± 0.2 vs. 0.31 ± 0.2 ng/mL, p>0.01; respectively) and AMH (0.1 ± 0.03 vs. 0.11 ± 0.02 ng/mL, p>0.05; respectively) were almost identical between cyclophosphamide and cyclophosphamide+GnRHa groups (Figures 12A to 12C). Similar cytotoxic effects were observed after cisplatin treatment. Both primordials and preantral/antral follicle counts were substantially reduced with a concurrent decline in E_2, P and AMH productions in the samples exposed to cisplatin. Somehow, paclitaxel appeared to be less gonadotoxic on the preantral/antral follicles than cyclophosphamide and cisplatin since the extent of follicle loss and reductions in hormone levels were less prominent in the samples exposed to this drug. Notably, primordial follicles seemed unaffected by paclitaxel because the mean number of primordials in the paclitaxel treated samples was comparable to control. On the contrary, preantral/antral follicles were significantly reduced after paclitaxel, albeit to a lesser extent than cyclophosphamide and cisplatin. The addition of GnRHa to cisplatin and paclitaxel did not prevent follicle loss (Figure 11).

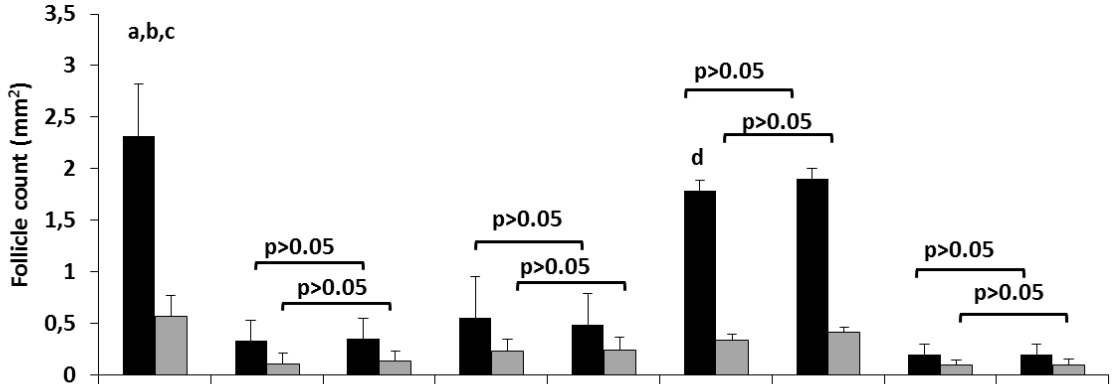
a: p<0.001 (Friedman test)
Primordial and preantral/antral follicle counts
(Wilcoxon paired analysis)
Control vs. Cyc, p<0.01
Control vs. Cyc±GnRHa, p<0.01
Cyc vs. Cyc+GnRHa p>0.05
Control vs. Cisplatin, p<0.05
Control vs. Cisplatin+GnRHa, p<0.05
Cisplatin vs. Cisplatin+GnRHa, p>0.05
Control vs. Radiation, p<0.01
Control vs. Radiation+GnRHa, p<0.01
Radiation vs. Radiation+GnRHa, p>0.05

b: p<0.05 Preantral/antral follicle counts
Control vs. Paclitaxel±GnRHa subgroups

c: p>0.05 Primordial follicle count
Control vs. Paclitaxel±GnRHa subgroups

d: p<0.05 Primordial follicle count
Paclitaxel vs. Cyclophosphamide

FOLLICLE RESERVE



	Control	Cyc	Cyc + GnRHa	Cisplatin	Cisplatin + GnRHa	Paclitaxel	Paclitaxel + GnRHa	Radiation	Radiation + GnRHa
■ Primordial	2.32±0.5	0.33±0.2	0.35±0.2	0.55±0.4	0.49±0.2	1.79±0.1	1.9±0.1	0.2±0.1	0.2±0.1
▒ Preantral-Antral	0.57±0.2	0.11±0.1	0.13±0.1	0.23±0.1	0.24±0.1	0.34±0.05	0.41±0.05	0.1±0.04	0.1±0.05

Figure 11. The impact of chemotherapy drugs and radiation administered ± GnRHa on the follicle reserve. Cyclophosphamide (Cyc), cisplatin and radiation exerted a similar degree of cytotoxicity on the dormant primordials and growing follicle fraction (preantral/antral follicles) whereas paclitaxel impacted only preantral/antral follicle cohorts in human ovary. Irradiation of the samples with 2 Gy radiation caused a massive follicle loss, more than 90 percent of primordials and growing follicles were lost 24hrs post-irradiation. The co-administration of GnRHa with these drugs or radiation did not prevent or attenuate follicle loss.

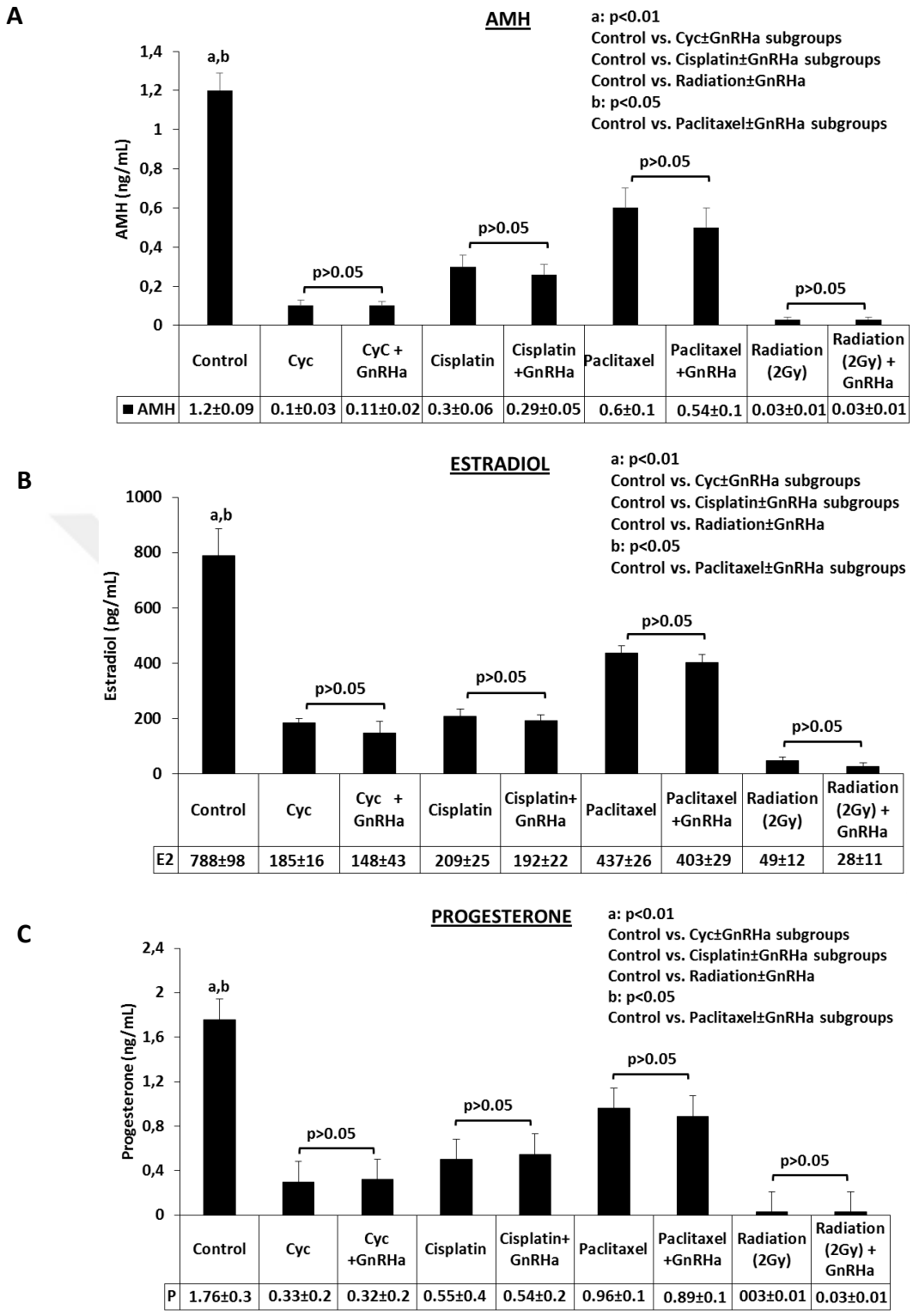


Figure 12. Comparison of the steroidogenic activity of the samples. Ovarian tissue samples produced significantly lower amounts of **(A)** anti-mullerian hormone, **(B)** estradiol, and **(C)** progesterone after exposure to cyclophosphamide, cisplatin, radiation, and to a lesser extent after paclitaxel. The addition of GnRHa did not cause any notable change in the steroidogenic activity of the samples.

2.2. Correlation analyses between follicle counts and in vitro levels of estradiol and AMH

AMH is mainly produced by the proliferating granulosa cells of the preantral and small antral follicles [52]. These cells are also the source of estrogen production. Serum AMH level is being used as a hormone marker of ovarian reserve and its levels correlate well with the number of antral follicles in the ovary on ultrasonography. We curiously investigated if such a correlation also exists in vitro. We found that there were significant positive correlations among estradiol, AMH and preantral/antral follicles in the control ovaries. The correlation co-efficients and the level of significance were as follows: follicle count-AMH (r=0.903, p=0.001); follicle count-estradiol (r=0.842, p=0.004); and AMH-estradiol (r=0.905, p=0.001). However, in the samples exposed to chemotherapy ± GnRHa, the correlation was either insignificant [AMH vs. estradiol (r=0.652, p=0.088)]; or less significant [follicle count vs. AMH (r=0.768, p=0.035); and follicle count vs. estradiol (r=0.831, p=0.015)] (Figure 13).

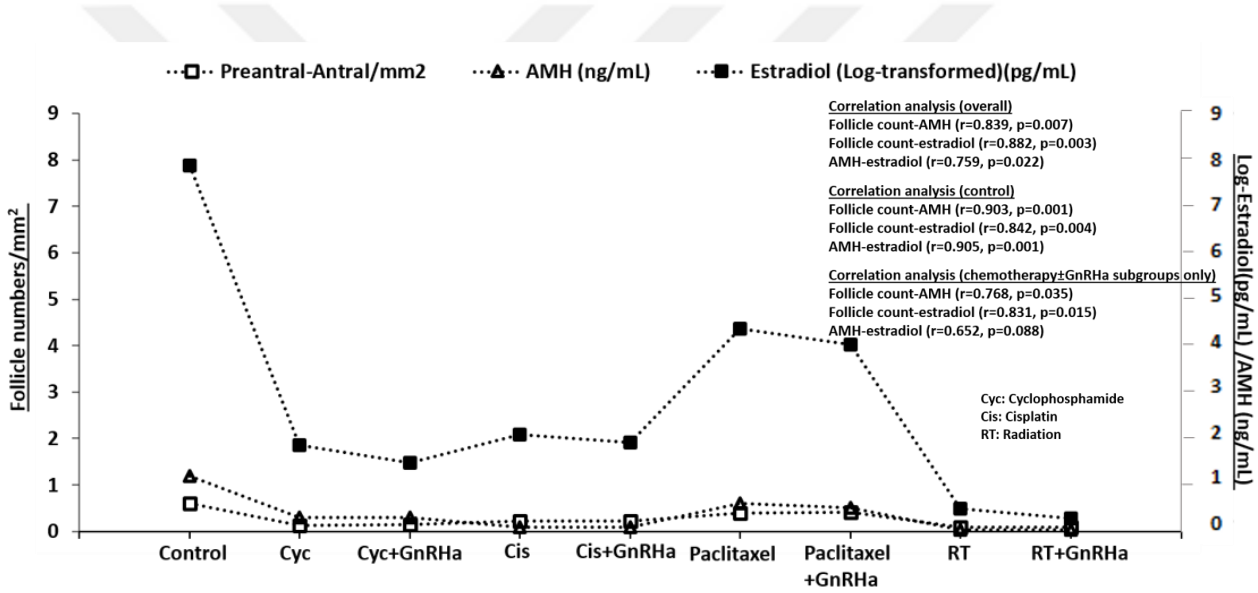


Figure 13. Correlation analysis between control and chemotherapy treated ovarian tissue samples. The number of growing follicles in the control ovarian samples was significantly correlated with the levels of AMH and E_2 they produced in vitro. There was also a significant correlation between AMH and E_2. However, the level of significance was either weak (follicle count vs. E_2; and follicle count vs. AMH) or absent (AMH vs. E_2) in the samples exposed to chemotherapy drugs compared to control samples. E_2 levels are log transformed (second Y axis).

2.3. The expression of anti-apoptotic genes in the control samples and those treated with chemotherapy agents ± GnRHa

In another set of experiments, we quantitatively compared the expressions of the anti-apoptotic genes (Bcl-2, Bcl-xL, Bcl-2L2, Mcl-1, BIRC2 and XIAP) among control, cyclophosphamide and cyclophosphamide+GnRHa groups to investigate if GnRHa activates anti-apoptotic genes in the ovary under the genotoxic stress of cyclophosphamide. Compared to their baseline levels, the expression of

Bcl-2, Bcl-2L2 and BIRC2 were significantly decreased after cyclophosphamide treatment whereas the levels of Bcl-xL, Mcl-1, and XIAP did not change. The co-administration of GnRHa with cyclophosphamide did not increase the expression of any of the genes studied. Furthermore, the expression of Mcl-1 and BIRC2 were further reduced after cyclophosphamide+GnRHa treatment compared to those ovarian samples treated cyclophosphamide only (Figure 14).

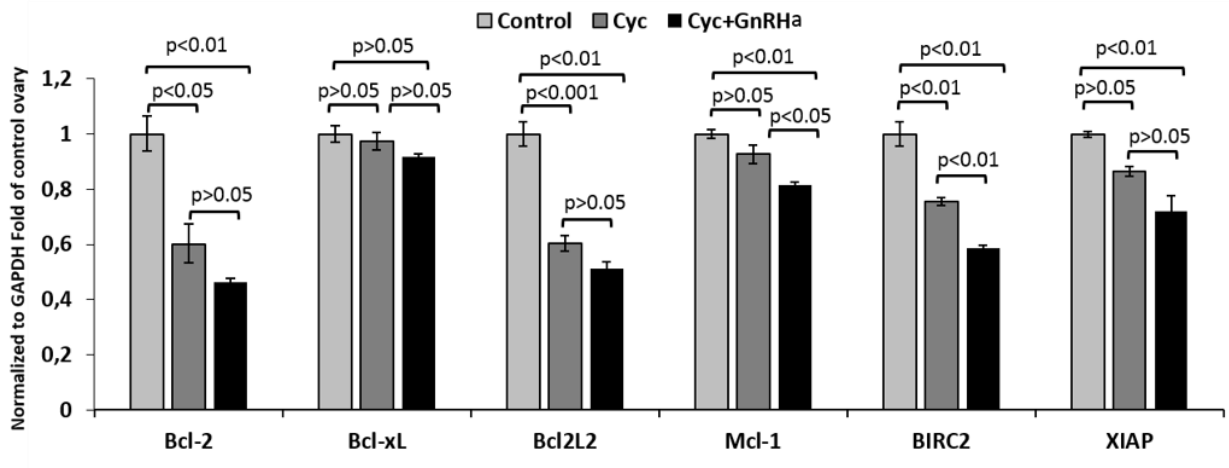


Figure 14. The mRNA expression of the anti-apoptotic proteins before and after exposure to cyclophosphamide with and without GnRHa. The co-administration of GnRHa with cyclophosphamide did not up-regulate the transcriptional activity of the anti-apoptotic genes compared to control and cyclophosphamide treated samples. There was a further reduction in the expression of the anti-apoptotic genes Mcl-1 and BIRC2 after treatment with cyclophosphamide+GnRHa.

2.4. Assessment of ovarian stroma and microvascular density

The follicles and stroma preserved well their structure after 24 hr culture period. Healthy follicles at primordial, transitional and preantral stages were easily identified. Ovarian stroma was stained uniformly HE and MT with many interstitial cells and easily identified microvascular structures. By contrast, the samples treated with cyclophosphamide and cisplatin were characterized by a less cellular, stroma with a marked disarray of the cells and extracellular matrix. Interstitial cells were sparser. Atretic follicles were visible within the surrounding stroma (Figure 15). Stromal damage was less prominent in paclitaxel treated samples. Similar structural changes were observed in the samples treated with chemotherapy+GnRHa groups, suggesting that GnRHa did not preserve ovarian stroma.

We also noticed a paucity of the vascular structures in the chemotherapy treated samples, particularly after cyclophosphamide and cisplatin. After staining with VEGF to assess microvascular structures we observed that there was a abundance of microvascular structures in the stroma of the control samples (88%) whereas the microvascular density was decreased to 18%, 22% and 56% after

treatment with cyclophosphamide, cisplatin and paclitaxel; respectively. Vascular structures were preserved to some point in paclitaxel-treated samples compared to cyclophosphamide and cisplatin. We did not observe any difference in the ovarian stroma and vascularity between chemotherapy and chemotherapy+GnRHa groups (Figure 16).

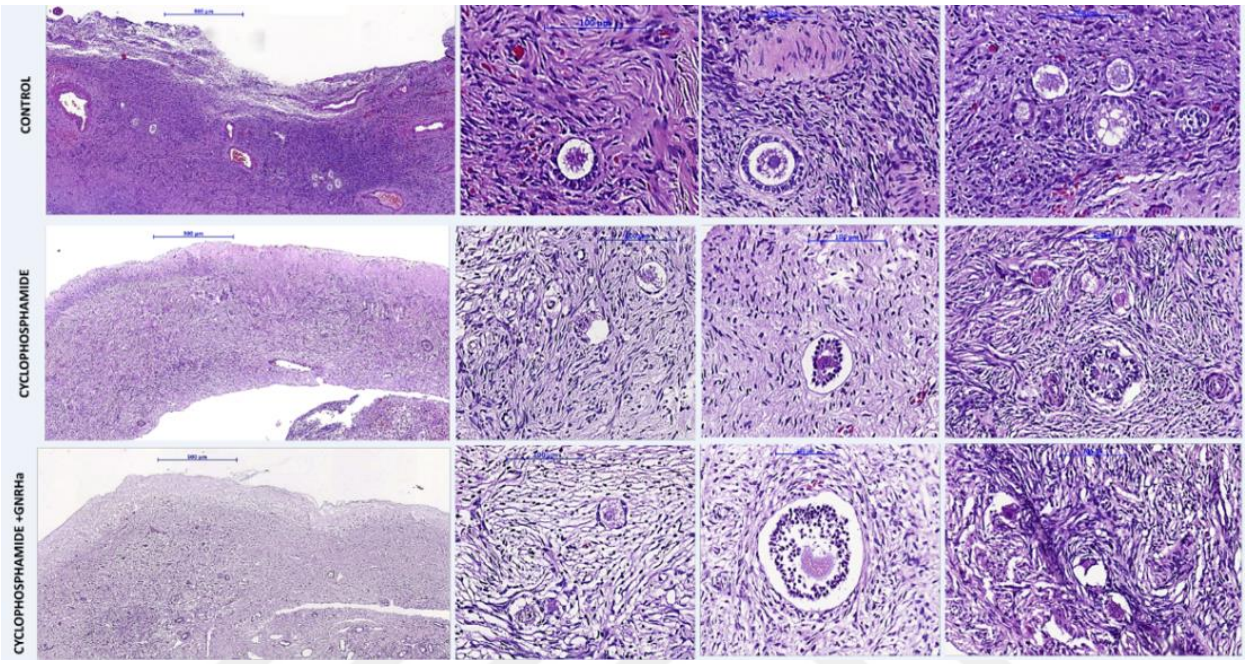


Figure 15. Histological examination of the ovarian samples after staining with hematoxylin-eosin (H&E). The sections of the ovarian samples before and after treatment with cyclophosphamide ± GnRHa, H&E staining. The follicles and stroma preserved well their structure after 24 hr culture period. Ovarian stroma was stained uniformly with H&E with many interstitial cells and easily identified microvascular structures. By contrast, the samples treated with cyclophosphamide were characterized by a less cellular, with a marked disarray of the cells and extracellular matrix. Interstitial cells were sparser. Atretic follicles with their pyknotic granulosa cells were visible within the surrounding stroma in cyclophosphamide treated samples. Similar structural alterations were observed in the samples treated with cyclophosphamide+GnRHa

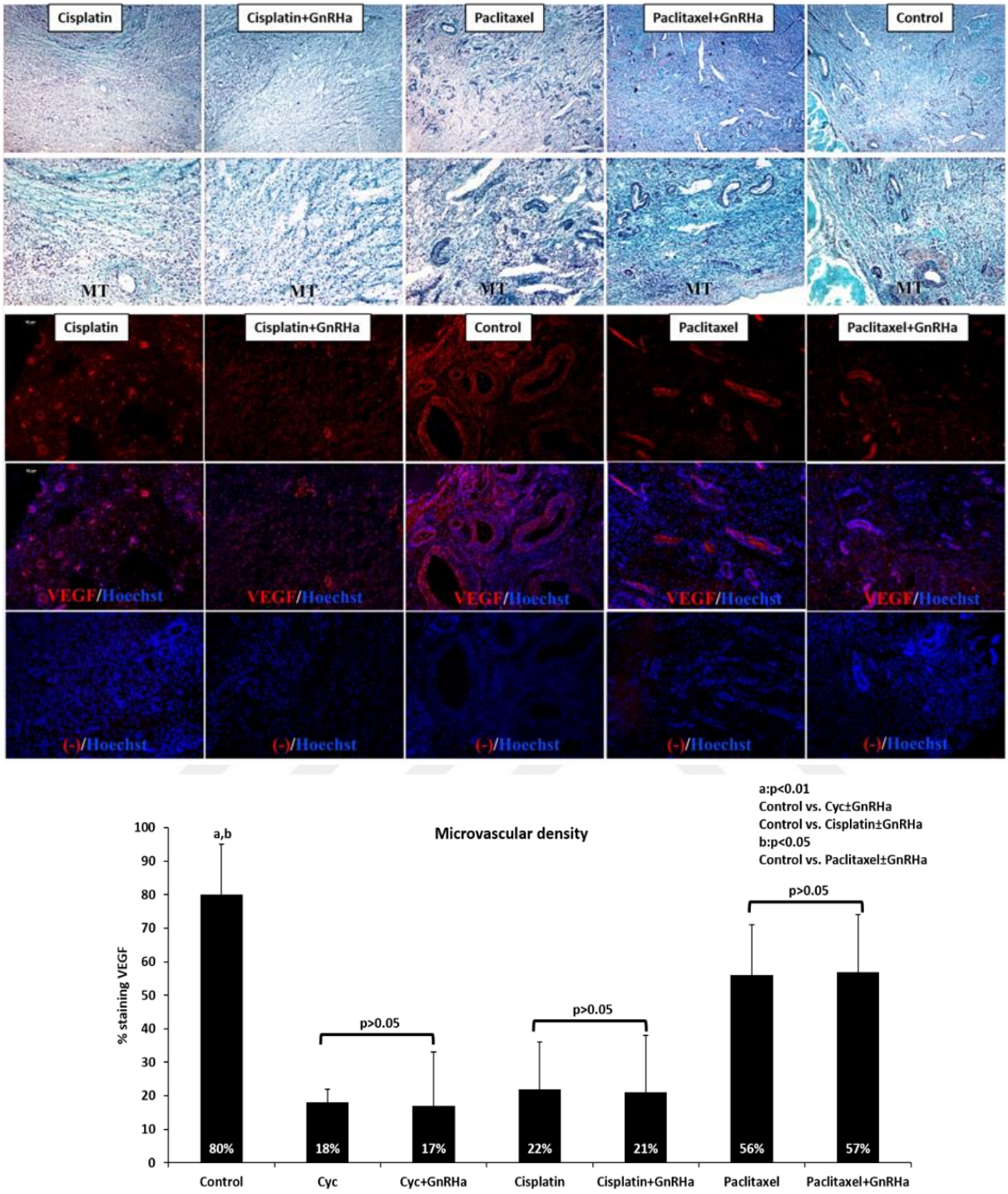


Figure 16. Histological examination of the ovarian samples after staining with Masson's trichome (MT) and vascular endothelial growth factor (VEGF). Stromal alteration and decrease in microvascular density were evident in the samples after staining with MT and VEFG; respectively. There is a marked paucity of vascular structures in the samples after treatment with cyclophosphamide and cisplatin compared to control and paclitaxel treated samples. The co-administration of GnRHa with chemotherapy did not preserve ovarian morphology and vascular structures from chemotherapy induced cytotoxicity.

2.5. Irradiation of the ovarian tissue samples

Irradiation with 2 Gy of the ovarian tissue samples produced a similar degree of cytotoxicity to cyclophosphamide. In addition to massive follicle loss, there was a marked decrease in the cellularity of the stroma. 92% and 83% of the primordials and preantral/antral follicles were lost after 2 Gy irradiation, challenging the previous notion that LD50 level of human oocyte is 2 Gy [45]. Co-treatment with GnRHa did not rescue follicles from radiation induced apoptosis. The mean levels of E_2, P and AMH produced by irradiated ovarian samples were significantly lower than controls. Follicle reserve and hormone productions of the samples irradiated with GnRHa were not different from those exposed to radiation alone (Figure 11 and 12).

3. Non-mitotic luteinized granulosa cells (HLGCs)

3.1. Comparison of the cytotoxic effects of cyclophosphamide and cisplatin w/o GnRHa

3.1.1. DNA damage and Apoptosis

Treatment with cyclophosphamide and cisplatin caused a significant degree of cytotoxicity on these cells. There was a tremendous increase in apoptosis along with a drastic decline in the steroidogenic activity of the cells exposed to these drugs. Ony 3% of the control cells were stained positive for the apoptosis marker YO-PRO-1 whereas 89% and 71% of the cells underwent apoptosis after treatment with cyclophosphamide and cisplatin, respectively ($p<0.001$). The co-administration of GnRHa with these drugs did not reduce apoptosis or DNA damage in the samples. Quantitative immunoblot analysis confirmed the occurrence of DNA damage and apoptosis after treatment with these drugs. The average density of the signal for cleaved caspase-3 in the quantitative immunoblot significantly increased from 1 [3] to 2.76 and 2.56 for the cells exposed to cyclophosphamide and cisplatin, respectively ($p<0.01$) and to 2.74 and 2.54 for cyclophosphamide+GnRHa and cisplatin+GnRHa, respectively ($p<0.01$). The co-administration of GnRHa with cyclophosphamide and cisplatin did not attenuate the intensity of the signal, confirming that GnRHa did not rescue the cells from DNA damage/apoptosis (Figure 17 and 18).

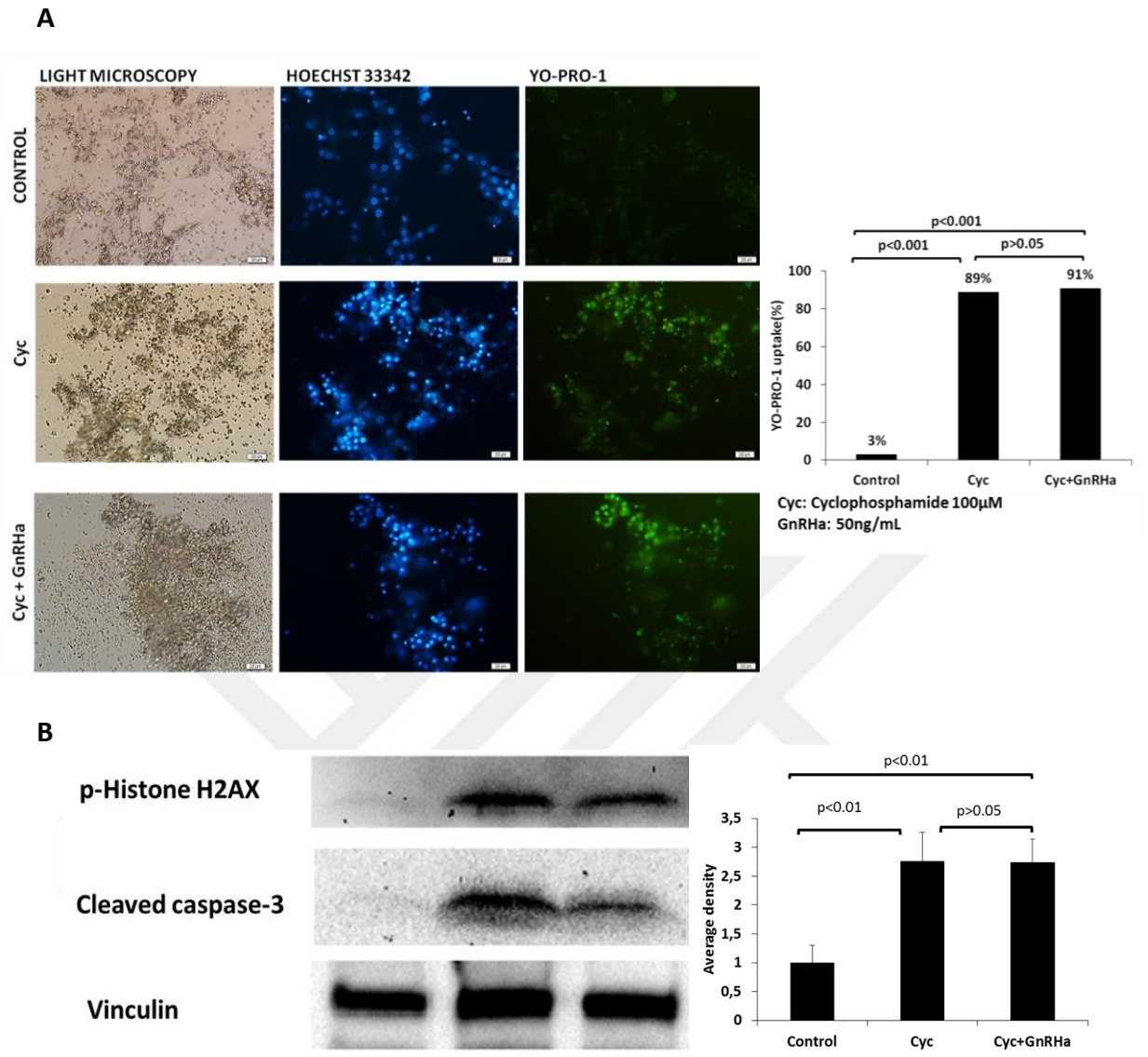


Figure 17. Cytotoxic effects of cyclophosphamide ± GnRHa on human luteinized granulosa cells. **(A)** Yo-Pro-1 staining showed that cyclophosphamide caused a significant degree of cytotoxicity on these cells. There was a more than 80% increase in apoptosis. **(B)** Quantitative immunoblot analysis shown as a graph bar did not show any difference in the intensity of the signal for cleaved caspase-3 in chemotherapy vs. chemotherapy+GnRHa groups. The addition of GnRHa did not prevent or attenuate DNA damage and apoptosis induced by cyclophosphamide.

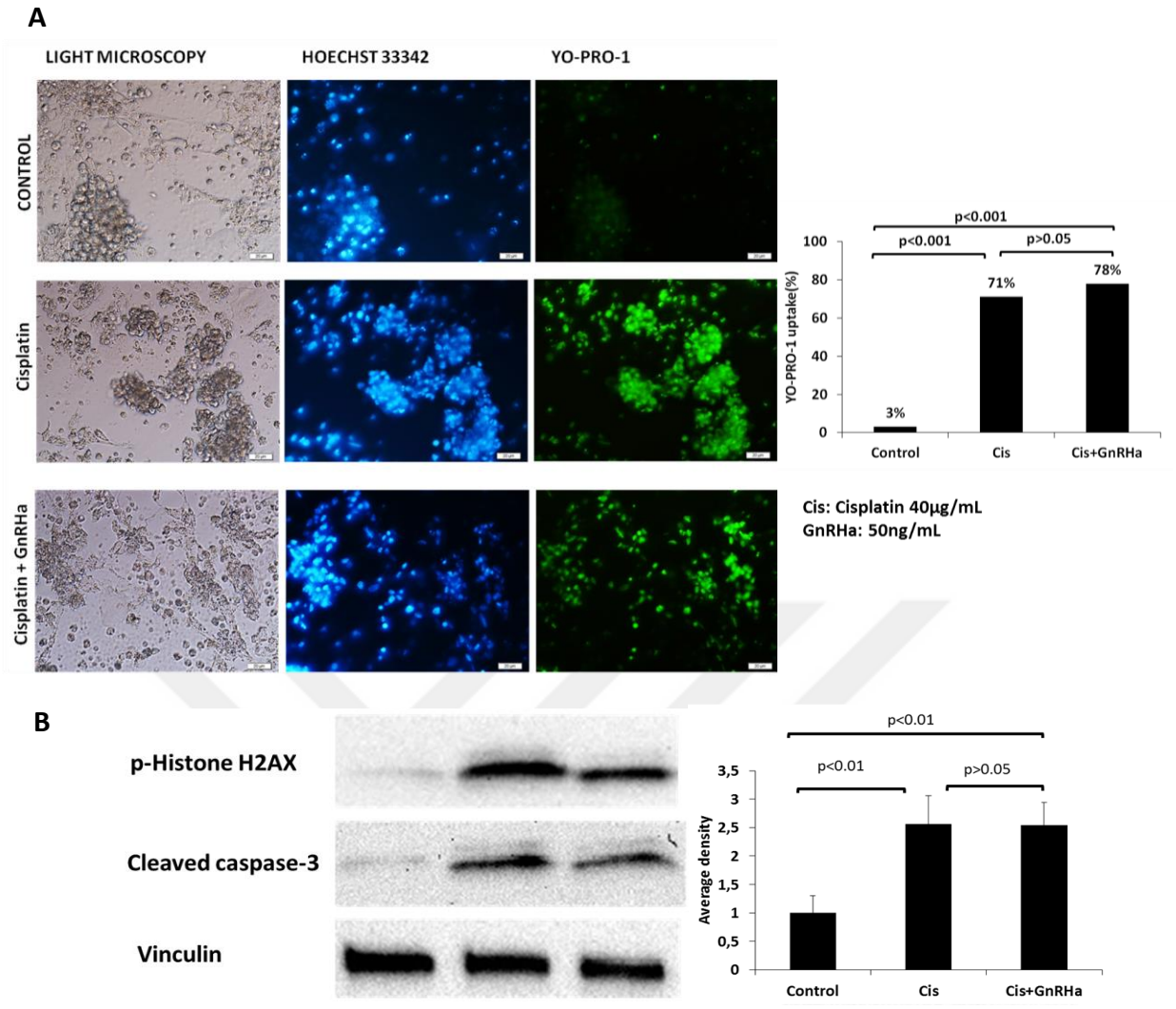
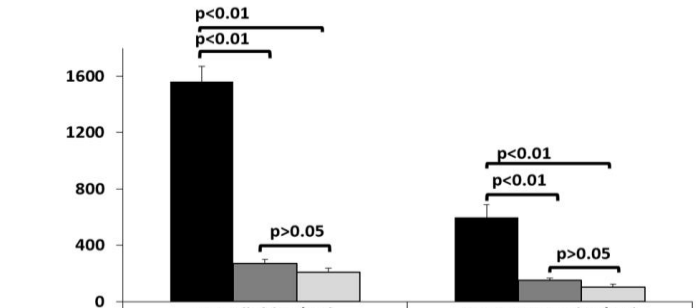


Figure 18. Cytotoxic effects of cisplatin ± GnRHa on human luteinized granulosa cells. **(A)** Yo-Pro-1 staining results of cisplatin treated luteinized granulosa cells demonstrated similar degree of cytotoxicity as cyclophosphamide treated ones. There was a more than 70% increase in apoptosis. **(B)** Immunoblot analysis of apoptosis and DNA damage markers shown as a graph bar did not show any difference in the intensity of the signal for cleaved caspase-3 in chemotherapy vs. chemotherapy + GnRHa groups. Co-administration of GnRHa with chemotherapy did not prevent DNA damage or prevent/attenuate apoptosis induced by cisplatin.

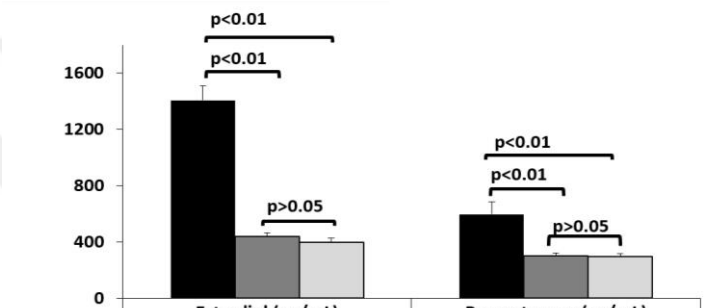
3.1.2. Stereogenic Activity

After demonstration of remarkable increase in apoptosis and DNA damage in the samples treated with cyclophosphamide and cisplatin ± GnRH, drastic decline in the steroidogenic activity was observed in the cells exposed to these drugs. Increased apoptosis was associated with a concurrent decrease in the steroidogenic activity of these cells. The co-administration of GnRHa with these drugs did not improve the steroidogenic activity of the samples (Figure 19 and 20).



	Estradiol (pg/mL)	Progesterone (ng/mL)
Control	1560±112	596±95
Cyc	271±28	150±18
Cyc+GnRHa	207±31	105±16

Figure 19. Steroidogenic activity of HLGCs treated with cyclophosphamide ± GnRHa. E_2 and P production of the cells declined after treatment with these drugs for 24 hrs. The addition of GnRHa did not improved their ability to produce E_2 and P.



	Estradiol (pg/mL)	Progesterone (ng/mL)
Control	1406±102	587±87
Cis	438±27	302±20
Cis+GnRHa	398±29	298±17

Figure 20. E_2 and P production in HLGCs treated with cisplatin ± GnRHa. The addition of GnRHa did not improved their ability to produce E_2 and P.

3.2. Comparison of the cytotoxic effects of other chemotherapy agents ± GnRHa

We also tested other chemotherapy drugs on HLGCs; paclitaxel alone, paclitaxel+cisplatin, 5-FU and TAC combination regimen. Of these drugs, paclitaxel and 5-FU were apparently devoid of any cytotoxic effects since the apoptotic fractions and E_2 and P productions of the cells treated with these drugs were comparable to untreated control cells (Figure 21 and Figure 22). However, when paclixatel was combined with cisplatin, the cells underwent apoptosis and their E_2 and P productions were decreased, resembling the cytotoxicity of cisplatin. The apoptosis rate and hormone productions of the cells treated with cisplatin+GnRHA and cisplatin+paclitaxel+GnRHa were not any better than their counterparts treated without GnRHa. TAC combination exerted the highest magnitude of cytotoxicity on the HLGCs among the drugs tested. The cells exposed to this combination regimen had the highest number of apoptotic cells and produced the lowest amounts of E_2 and P compared to control and other drugs. GnRHa co-administered with TAC did not alleviate the cytotoxic actions of this combination regimen (Figure 21 and Figure 22).

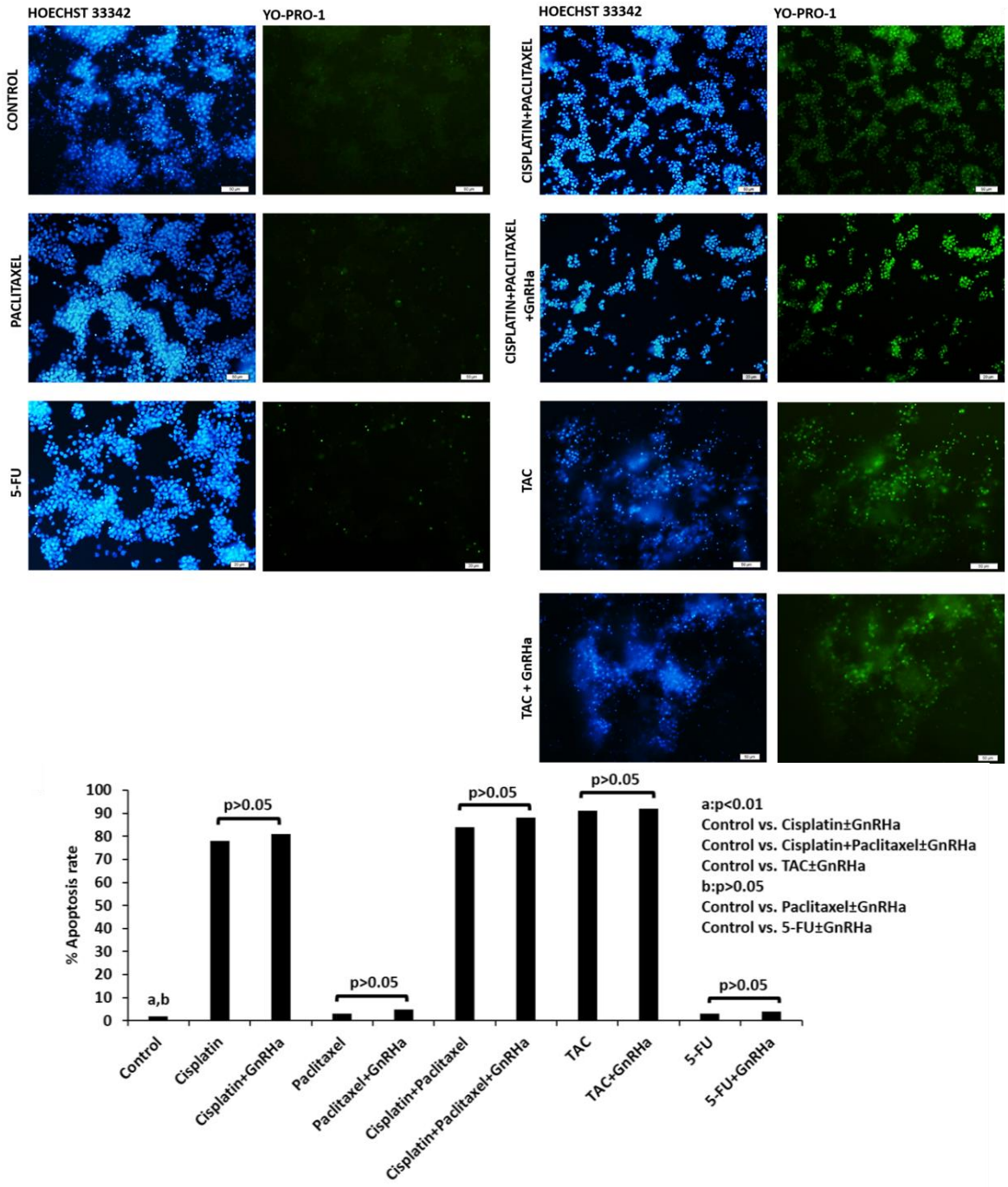


Figure 21. Comparison of the cytotoxic effects of different chemotherapy drugs on HLGCs as assessed by viability/apoptosis of the cells. Paclitaxel and 5-FU were devoid of any cytotoxic effects on HLGCs. The rate of apoptosis of the cells treated with these drugs were similar to control cells. When paclitaxel was combined with cisplatin the cells underwent apoptosis resembling the cells treated with cisplatin. The cells exposed to TAC combination regimen had the highest rate of apoptosis. The co-administration of GnRHa with cisplatin, cisplatin+paclitaxel and TAC did not prevent or alleviate the cytotoxic actions of these chemotherapy agents on these cells.

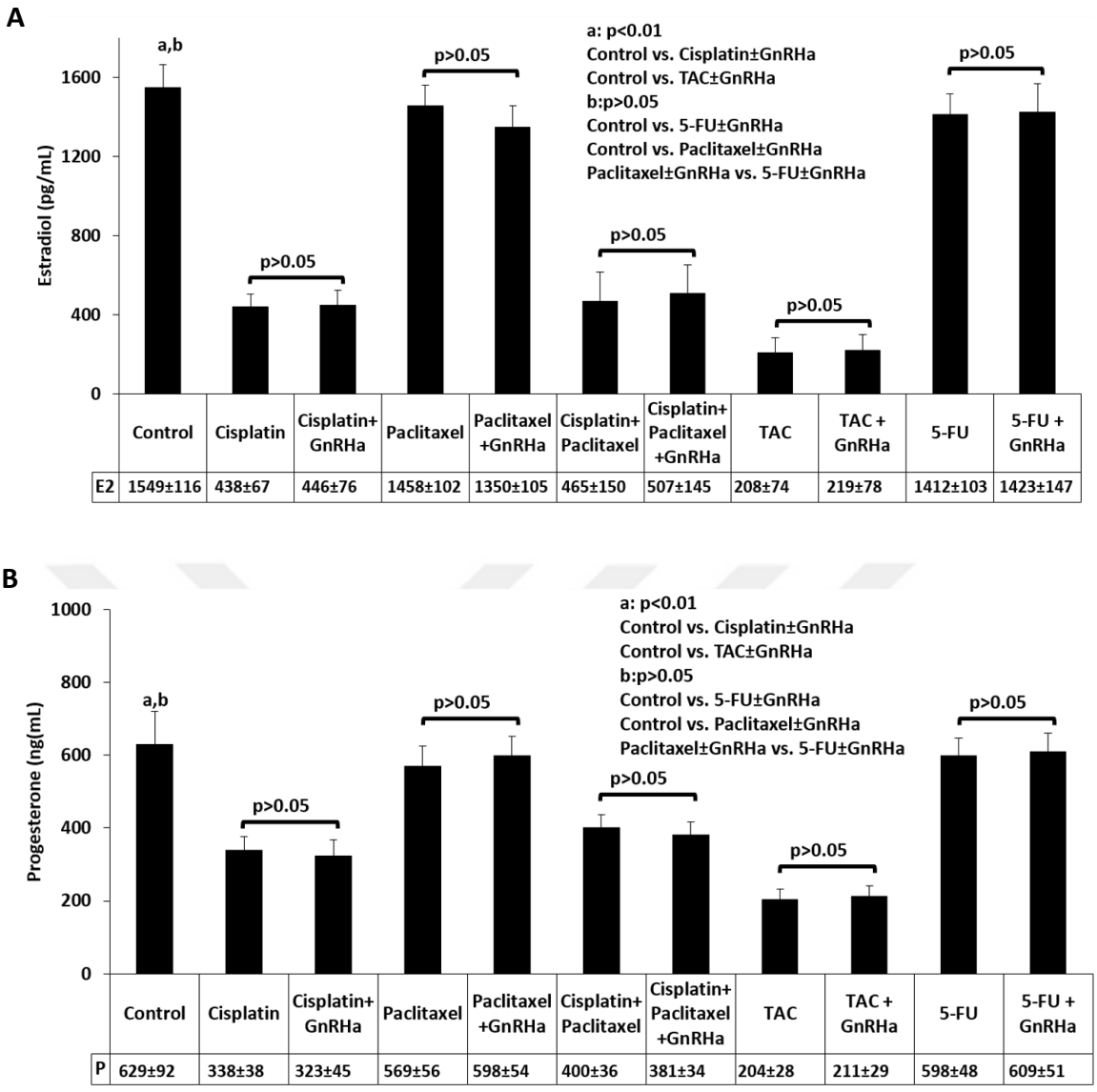
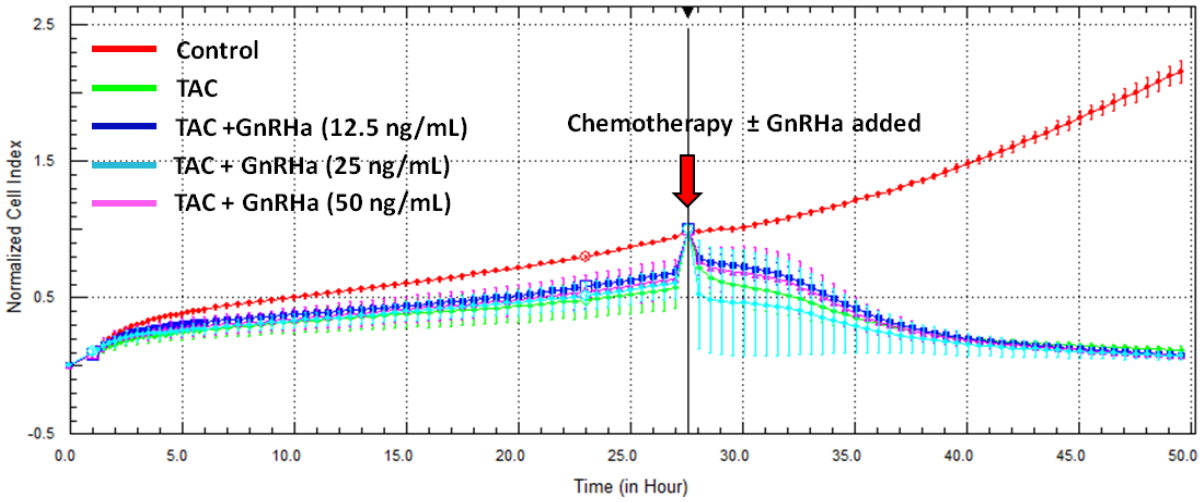


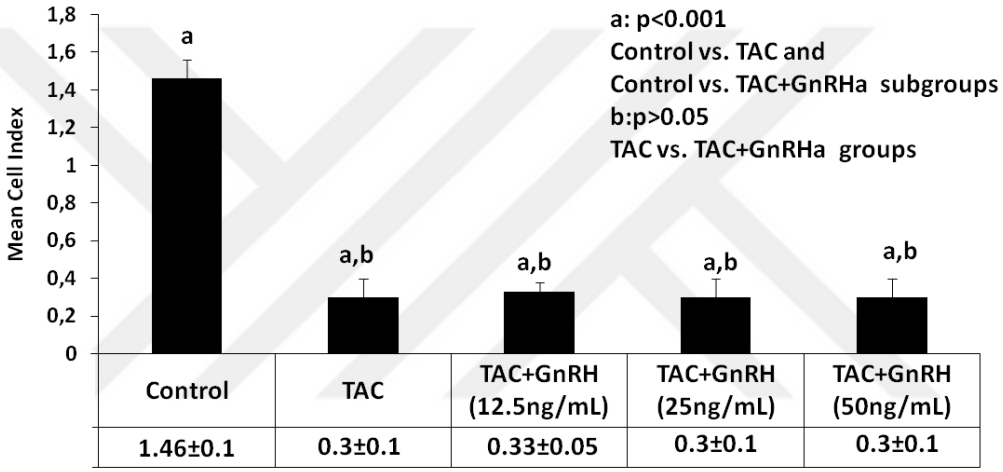
Figure 22. Comparison of the cytotoxic effects of different chemotherapy drugs on HLGCs as assessed by the steroidogenic activity of the cells. Paclitaxel and 5-FU were devoid of any cytotoxic effects on HLGCs. Steroidogenic activity of the cells treated with these drugs were similar to control cells. When paclitaxel was combined with cisplatin the cells underwent apoptosis and their hormone productions were reduced, resembling the cells treated with cisplatin. The cells exposed to TAC combination regimen had the highest rate of apoptosis and lowest hormone levels. The co-administration of GnRHa with cisplatin, cisplatin+paclitaxel and TAC did not prevent or alleviate the cytotoxic actions of these chemotherapy agents on these cells.

4. Mitotic non-luteinizing granulosa cells (COV434 AND HGrC1)

4.1. Comparison of real-time proliferation rate and apoptosis among different chemotheapy agents ± GnRHa

Cyclophosphamide, cisplatin and TAC combination were cytotoxic to HLGCs whereas paclitaxel and 5-FU were not. We also tested these drugs at the same doses on the mitotic granulosa cells COV434 and HGrC1 to investigate if there is a difference in chemosensitivity between mitotic and non-mitotic granulosa cells. Cyclophosphamide, cisplatin and TAC markedly halted the proliferation and induced apoptosis of mitotic granulosa cells in hours. In several hours post-exposure the real-time growth curves of the cells exhibited a downward shift, indicative of rapidly induced apoptosis by these drugs. Similar toxic effects were observed after paclitaxel but it was milder than cyclophosphamide, cisplatin and TAC. 5-FU neither inhibited the proliferation nor induced apoptosis of these cells. The co-administration of GnRHa with cyclophosphamide, cisplatin, TAC and paclitaxel did not prevent the cytotoxicity of these drugs (Figure 23 and 24).





Control	TAC	TAC+GnRH (12.5ng/mL)	TAC+GnRH (25ng/mL)	TAC+GnRH (50ng/mL)
1.46±0.1	0.3±0.1	0.33±0.05	0.3±0.1	0.3±0.1

Figure 23. Real-time growth curves of the proliferating non-luteinized granulosa cells treated with different dose combinations of TAC and GnRHa. The ordinate shows normalized cell index (CI) as a measure of viable cell mass, which are derived from the ratio of CIs before and after the addition of the compounds. The absciss denotes culture period time in hours. A fixed dose of TAC regimen (10ng/mL) is co-administered with GnRHa at three different concentrations (12.5, 25 and 50ng/mL). Note the prominent downward shift in the growth curve of the cells exposed to TAC. GnRHa did not rescue the cells from apoptosis induced by TAC. The rate of apoptosis in the cells treated with TAC+GnRHa was not different from those treated with TAC alone.

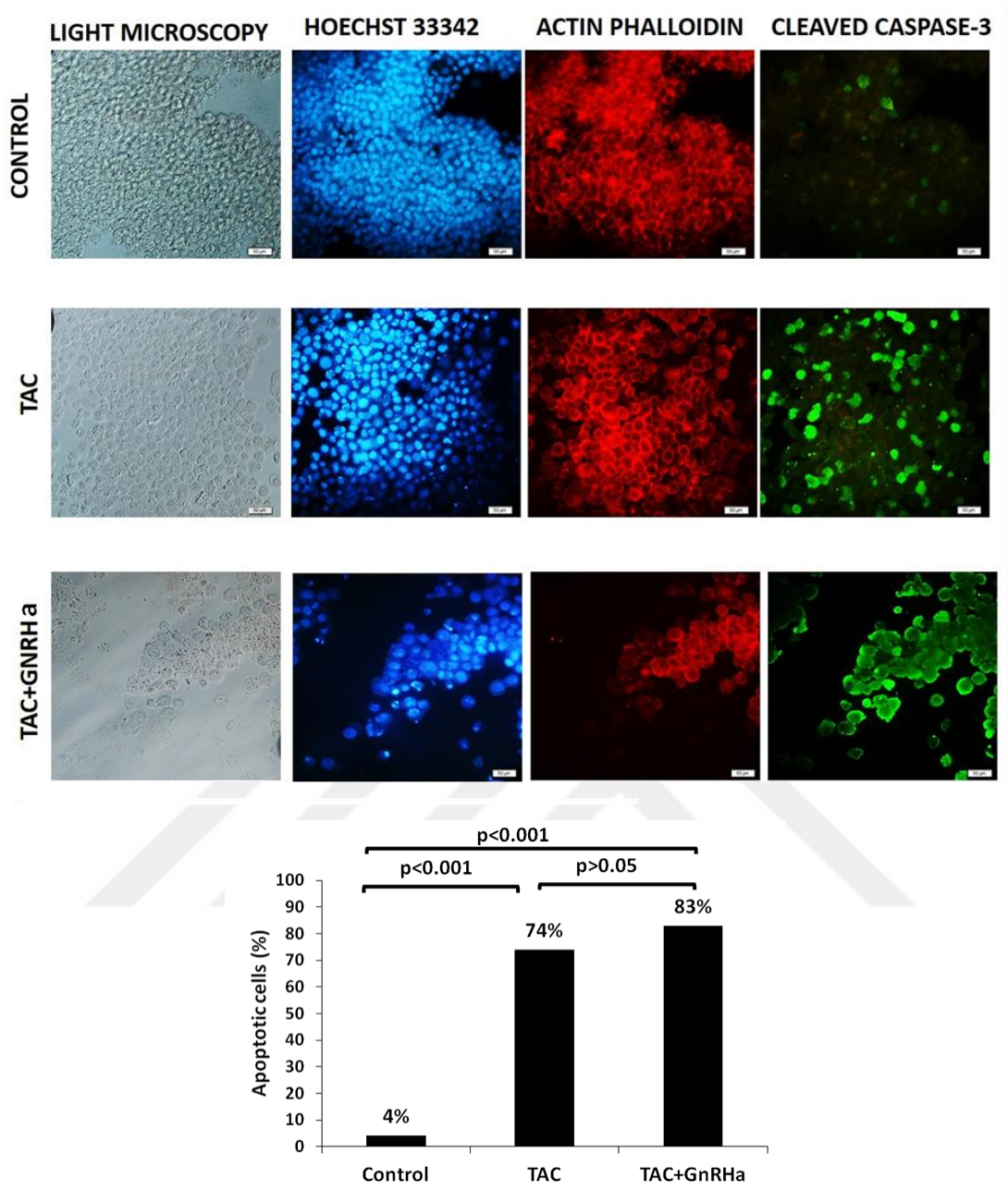


Figure 24. GnRHa did not rescue the cells from apoptosis induced by TAC. The rate of apoptosis in the cells treated with TAC+GnRHa was not different from those treated with TAC alone.

Cyclophosphamide, cisplatin, and to a lesser extent paclitaxel inhibited the proliferation and induced apoptosis of the mitotic granulosa cells. Exposure of the cells to cyclophosphamide and cisplatin induced caused a downward shift in their growth curves in hours. The anti-proliferative and pro-apoptotic effects of paclitaxel were less prominent than cyclophosphamide and cisplatin. 5-FU did not show any toxic effect on these cells. The co-administration of GnRHa with these chemotherapy agents did not sustain growth (Figure 25 and Figure 26) or reduce apoptosis of the cells (Figure 27).

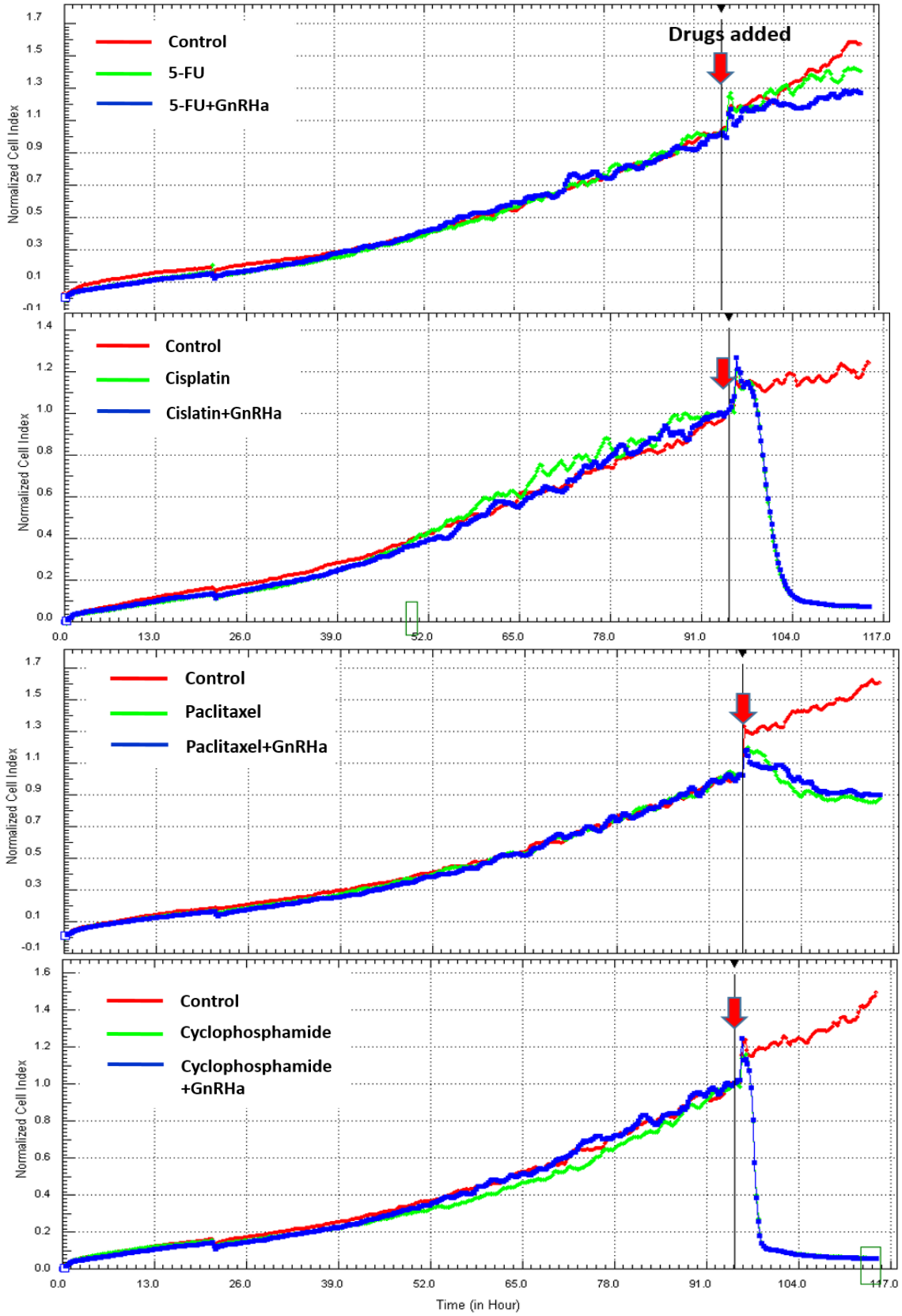


Figure 25. Real-time growth curves and of the proliferating granulosa cells treated with 5-FU, paclitaxel, cisplatin and cyclophosphamide.

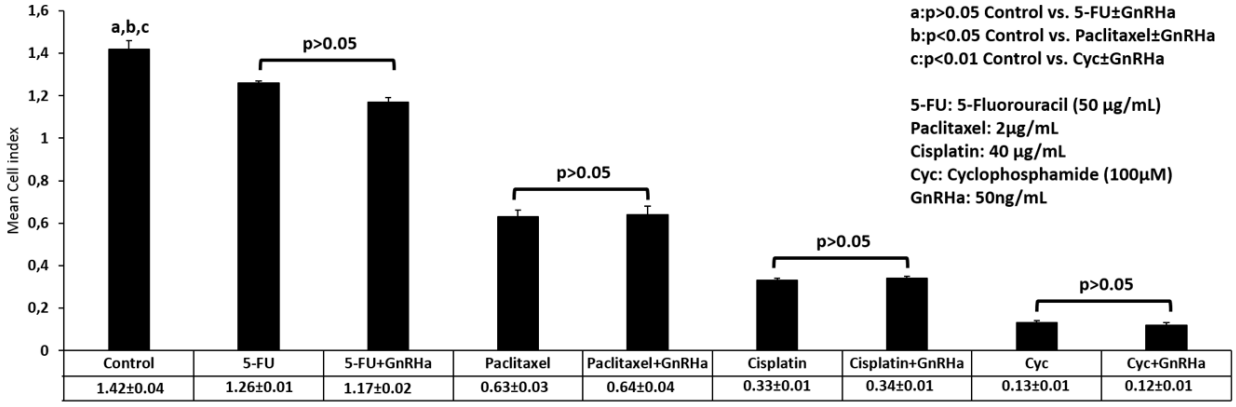


Figure 26. Mean cell indice of the proliferating granulosa cells treated with 5-FU, paclitaxel, cisplatin and cyclophosphamide.

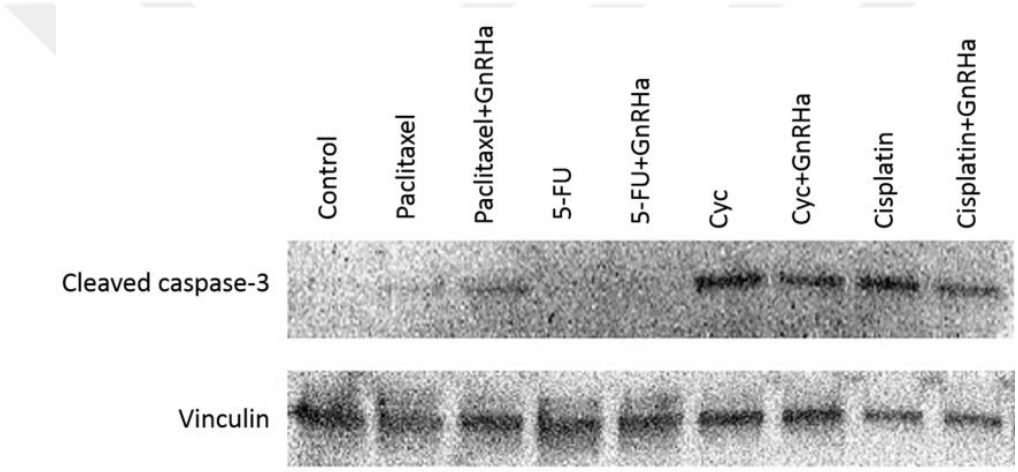


Figure 27. Cleaved caspase-3 expression of proliferating granulosa cells treated with different chemotherapeutics ± GnRH. Western blot analysis of cleaved caspase-3 showed that proliferating granulosa cells have different chemosensitivity and co-administration of GnRHa with any of these drugs did not provide any reduction in apoptosis signal.

4.2. Evaluation of SAPK/JNK pathway activity

Jun N-terminal kinases or JNKs play a critical role in death receptor-initiated extrinsic as well as mitochondrial intrinsic apoptotic pathways. JNKs activate apoptotic signaling by the upregulation pro-apoptotic genes via the transactivation of specific transcription factors or by directly modulating the activities of mitochondrial pro- and anti-apoptotic proteins through distinct phosphorylation events [53]. All the three JNKs have been shown to involved in stimulating apoptotic signaling. The primary evidence that the activation of JNK-1 can be correlated with apoptosis came from the initial studies investigating γ-ray induced apoptosis [54].

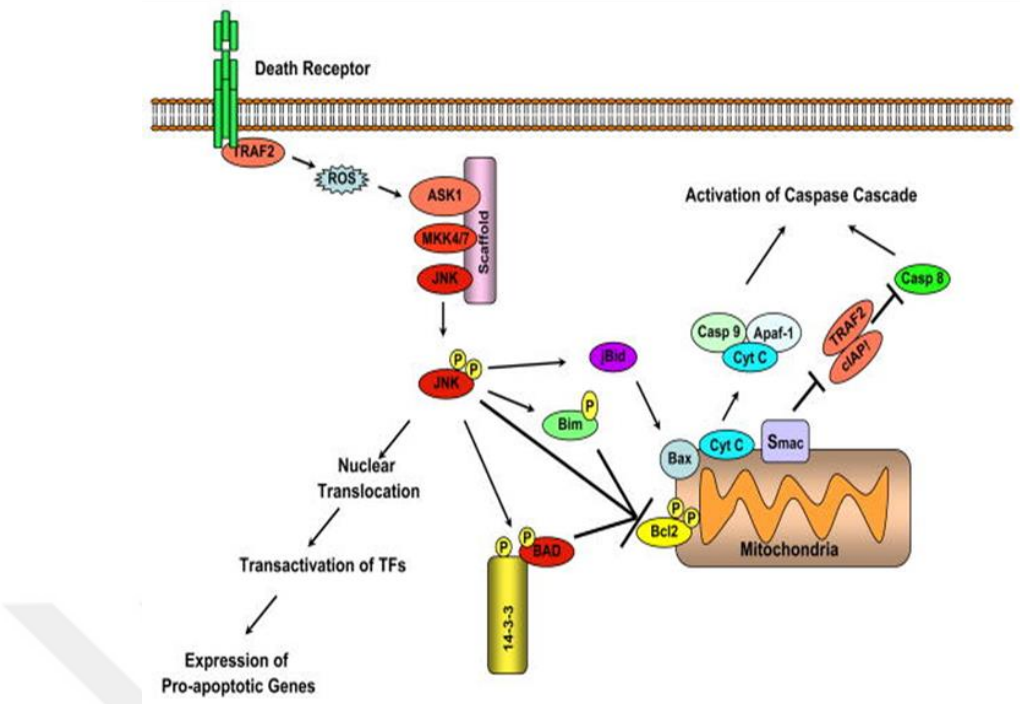


Figure 28. JNK signaling in apoptosis. [53]

It is a known fact that genotoxic agents induces the activation of the stress-activated protein kinase by phosphorilation and initiate a cascade reaction which results with apoptosis. Stimulation of JNKs has been implicated in cisplatin induced apoptosis of ovarian carcinoma cells [55]. Therefore, after showing no difference among chemotherapy ± GnRHa groups by means of apoptosis, DNA damage and expression of anti-apoptotic genes, we further investigate if co-administration of GnRHa makes any changes on the stress stimuli response in the SAPK/JNK pathway. In reference to this fact, if GnRHa had any protective effects on granulosa cells, chemotherapy ± GnRHa groups must differ by means of JNK activation.

JNK levels were measured by western blot analysis and equal amount of JNKs, both p46 and p54 kDa isoforms, was observed in control, cisplatin and cisplatin ± GnRH groups. However, when we compared phospho-JNK levels, activated form of JNK by phosporylation on Thr183/Tyr185 residues, both cisplatin and cisplatin+GnRHa groups showed a significant degree of increase compared to control group. Such observations are consistent with the notion that chemotherapy with and without GnRHa both induced the stress activated JNK pathway.

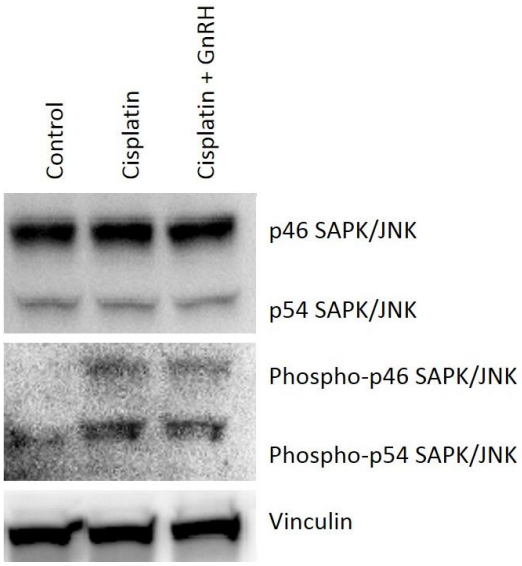


Figure 29. Comparison of stress-activated protein kinase SAPK/KNK pathway activation in proliferating non-luteinized granulosa cells treated with cisplatin with and without GnRHa.

4.3. Assessment of DNA Damage Response

Following their activation, Chk1 and Chk2 phosphorylate downstream effectors that further propagate the checkpoint signaling. Depending on the type of stress, velocity of DNA damage, and cellular context; activation of Chk1, Chk2 and p53 lead to switch to the stress-induced transcription program, direct or indirect initiation of DNA repair, and apoptosis.

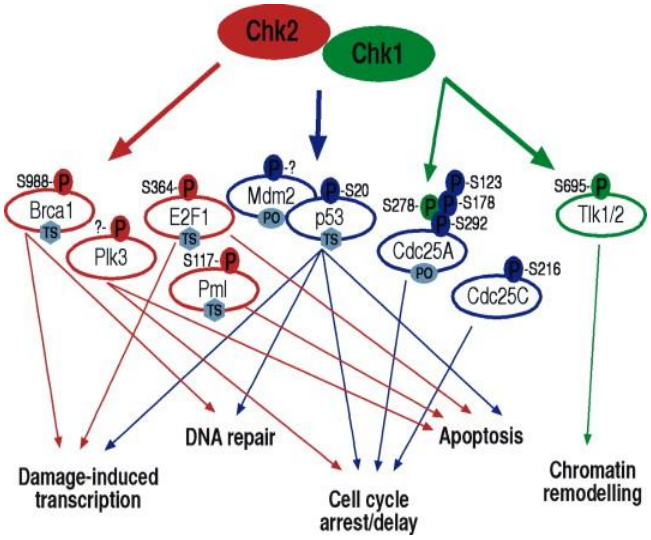


Figure 30. Chk1 and Chk2 as mediators of the checkpoint signaling network. [56]

We curiously investigated if co-administration of GnRHa with adjuvant chemotherapy prevent or decrease the activation of cell cycle checkpoint regulators Chk1, Chk2 and p53. The results demonstrated that cisplatin with GnRHa group did not respond differently to DNA damage caused by cisplatin. This observation is consistent with the perception that chemotherapy with and without GnRHa both led the same degree of DNA damage and apoptosis demonstrated

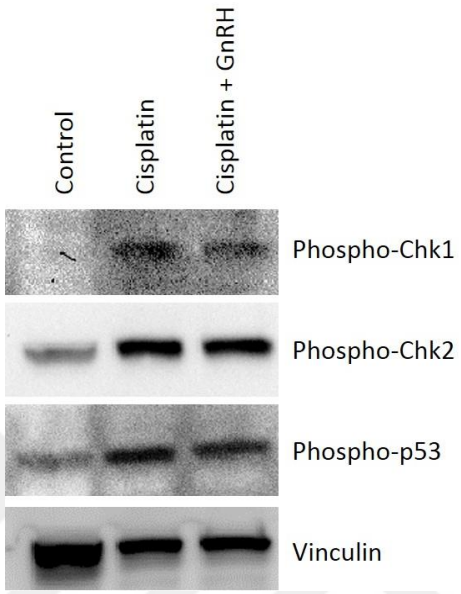


Figure 31. Comparison of DNA damage response in proliferating non-luteinized granulosa cells treated with cisplatin with and without GnRH.

Chapter 4

DISCUSSION

Infertility and premature ovarian failure are reproductive sequelas of exposure to cytotoxic chemotherapy regimens in young females with cancer. Premature ovarian failure is also associated with other adverse health-related consequences, including osteoporosis, hot flashes, sleep disturbance, and sexual dysfunction, which can negatively impact on short- and long-term quality of life. Three fertility preservation strategies are currently available for women prior to cytotoxic chemotherapy/radiation for cancer. Of these, oocyte and embryo freezing are the established methods of fertility preservation. But ovarian tissue cryopreservation is considered still experimental due to unknown success rate of this procedure and limited reports of pregnancies and live births achieved with this strategy [57, 58]. Even though oocyte or embryo freezing prior to chemotherapy can help women achieve pregnancy and live birth after chemotherapy induced premature ovarian failure, these strategies cannot reverse menopause in the native ovaries. Similarly, grafting of frozen-thawed ovarian tissue appears to be remote from restoring ovarian function due to poor survival of the grafts and postmenopausal levels of sex hormones and AMH produced by these graft after transplantation [59, 60]. Furthermore, ovarian tissue transplantation carries the risk of re-introducing cancer cells especially in hematological malignancies [61]. Therefore, any drug that preserves ovarian reserve during chemotherapy can potentially sustain normal reproductive life span and obviate the need for gamete freezing prior to chemotherapy.

Encouraged by the initial reports of animal studies and non-randomized human trials showing a beneficial effect of GnRH agonists in the preservation of ovarian function during chemotherapy, gonadotropin-releasing hormone agonists have been proposed as a fourth potential fertility preservation strategy. But randomized controlled trials launched so far to assess the effectiveness of this method have shown contradictory results in cancer patients. Some of these trials demonstrated a protective effect of GnRH agonists in preserving ovarian function after chemotherapy [26, 29, 62, 63], whereas the others could not [28, 64, 65]. Lack of molecular data in this under-studied issue led us to investigate in this study if GnRH agonist leuprolide acetate decreases DNA damage and follicular apoptosis through either activation of GnRH receptors and upregulation of intragonadal antiapoptotic genes during adjuvant chemotherapy. For this purpose we conducted several specific end-point assays in this study to provide a molecular evidence for-or-against the role of GnRHa in the preservation ovarian function and reserve after chemotherapy.

Our experiments on the ovarian cortical samples showed that cyclophosphamide and cisplatin impacted both primordials and growing follicle fraction whereas paclitaxel was detrimental to the

growing follicles only. The co-administration of GnRHa with these drugs did not preserve follicle stockpile or improve their steroidogenic activity. Apart from follicular structures, ovarian stroma and microvessels were also destructed by chemotherapy agents, particularly after cyclophosphamide and cisplatin. GnRHa did not preserve stroma and vessels from chemotherapy induced damage. Furthermore, GnRHa when co-administered with cyclophosphamide did not up-regulate the mRNA expression of antiapoptotic genes in the ovarian samples. Taken together, these results at least indicate that GnRHa does not confer any ovarian protection against the cytotoxic effects of chemotherapy drugs in vitro. Removal of ovarian tissue from its blood supply may change its sensitivity/resistance to cytotoxic stimuli and may impede with antiapoptotic mechanism in the ovary. Therefore in vitro environment may not actually represent the real in vivo environment in which the ovaries are exposed to cytotoxic drugs and GnRHa. This issue is particularly important when evaluating the cytotoxic effects of chemotherapy drugs and the protective actions of GnRHa on the long-term cultures of ovarian samples. In our study the samples were treated with chemotherapy agents ±GnRHa with no delay after removal and the culture period was restricted to 24 hr to minimize the changes in ovarian physiology related to in vitro conditions. Limited availability of ovarian cortical pieces precluded us from conducting this experiment with a larger sample size and adequate power. It should also be remembered that there is no an ideal model to harvest ovarian tissue samples from human donors containing a reasonable number of eggs [66]. Further, ovarian tissue fragments and granulosa cells obtained from ovarian cyst walls might have different sensitivity or expression of apoptotic pathways that can potentially change their sensitivity to toxic agents [67].

We also demonstrated in this study that the number of growing follicles in the control ovarian samples was significantly correlated with the levels of AMH and E_2 produced by these samples in vitro. However, in chemotherapy treated samples the correlations among these variables were either less significant or absent. Curiously, this finding at least arises a question as to whether the insult to granulosa cells may interefere with steroidogenetic activity of the surviving preantral/antral follicles.

Since GnRH receptors are mainly expressed by proliferating and luteinized granulosa cells of the growing follicles and corpus luteum respectively [51], representing types of granulosa cells were intentionally included in the study for a detailed analysis of the impact of chemotherapy+GnRHa. We demonstrated, as another finding of this study that chemosensitivity of proliferative granulosa cells seems to be different from non-proliferative luteinized ones. While cyclophosphamide, cisplatin and TAC were cytotoxic to both types of granulosa cells, paclitaxel selectively impacted proliferative granulosa cells with no apparent toxicity on the luteinized non-proliferative ones. 5-FU had the least or no toxic effects on either type of the cells regardless of their ability to proliferate. These results

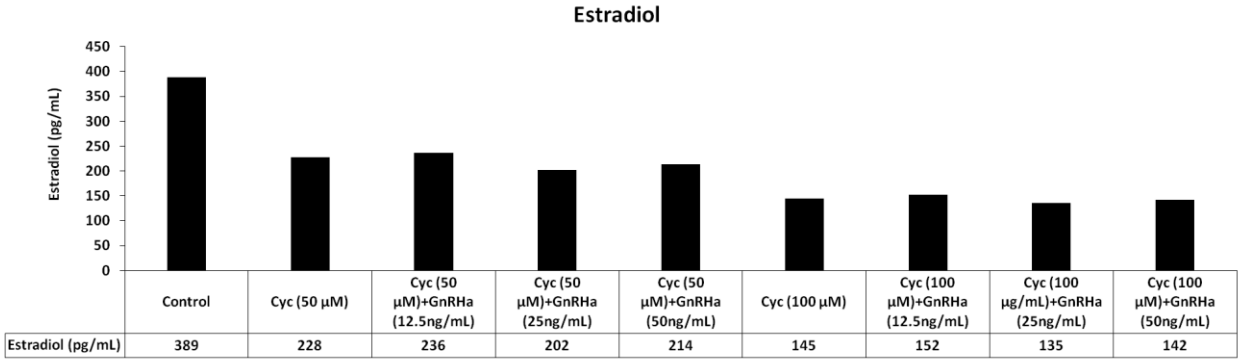
suggest that the inhibition of depolymerization of microtubules by paclitaxel has a more profound anti-proliferative effect than antimetabolite drug 5-FU. When this data was collectively analyzed with the impact of these drugs on ovarian follicles, paclitaxel ranks behind cyclophosphamide and cisplatin in terms of gonadotoxic potential. This data is particularly important for paclitaxel for which human data is limited and inconsistent so far [21, 68].

CONCLUSION

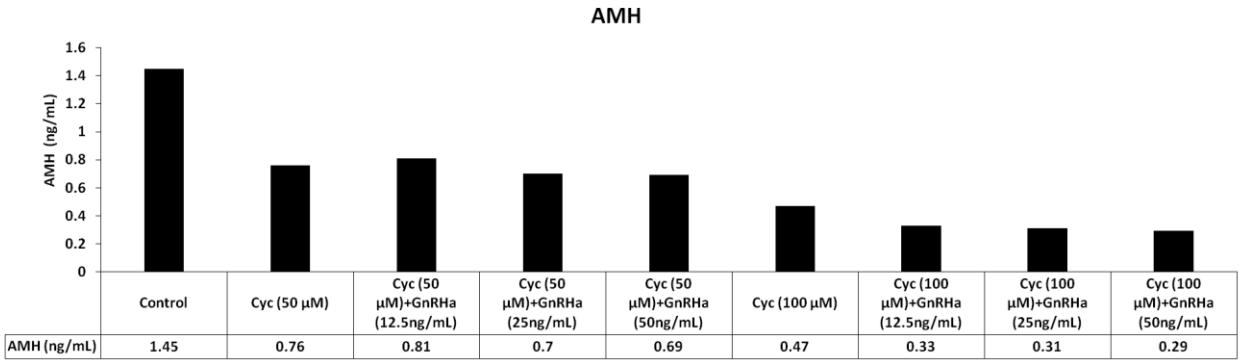
GnRH agonist leuprolide acetate did not protect human ovarian samples and granulosa cells from cytotoxic effects of chemotherapy agents and radiation in vitro If GnRHa had any protective effects in vivo through its intraovarian actions such as the inhibition of apoptosis and up-regulating anti-apoptotic genes, we could have reproduced at least some of these effects in vitro, either at the level of apoptosis and anti-apoptotic genes, or follicle reserve and steroidogenic activity in the ovarian samples and granulosa cells. On the other hand, our findings do not conclusively rule out the possibility that GnRHa may offer protection if any, through some other mechanisms in vivo.

APPENDIX

In order to rule out the possibility that the doses of GnRH and cyclophosphamide, timing of GnRHa administration, exposure time and culture condition might potentially hinder the protective actions of GnRHa, we repeated the experiments with a total of six different dose combinations of cyclophosphamide and GnRHa, extended culture period up to 96 hrs, administered GnRHa 1-2 hrs prior to cyclophosphamide, and used a serum-free defined culture media. None of these modification revealed any protective effect of GnRHa against the cytotoxicity of cyclophosphamide (Figure 31).



	Control	Cyc (50 µM)	Cyc (50 µM)+GnRHa (12.5ng/mL)	Cyc (50 µM)+GnRHa (25ng/mL)	Cyc (50 µM)+GnRHa (50ng/mL)	Cyc (100 µM)	Cyc (100 µM)+GnRHa (12.5ng/mL)	Cyc (100 µg/mL)+GnRHa (25ng/mL)	Cyc (100 µM)+GnRHa (50ng/mL)
Estradiol (pg/mL)	389	228	236	202	214	145	152	135	142



	Control	Cyc (50 µM)	Cyc (50 µM)+GnRHa (12.5ng/mL)	Cyc (50 µM)+GnRHa (25ng/mL)	Cyc (50 µM)+GnRHa (50ng/mL)	Cyc (100 µM)	Cyc (100 µM)+GnRHa (12.5ng/mL)	Cyc (100 µM)+GnRHa (25ng/mL)	Cyc (100 µM)+GnRHa (50ng/mL)
AMH (ng/mL)	1.45	0.76	0.81	0.7	0.69	0.47	0.33	0.31	0.29

Figure 32. Different dose combinations of cyclophosphamide and GnRHa. Ovarian samples were treated with 50 and 100 µM cyclophosphamide. GnRHa was administered at 12.5, 25 and 50 ng/mL concentrations for each dose of cyclophosphamide, making a total of six different dose combinations. There was a dose-dependent decrease in the production of E_2 and AMH in the samples with incremental doses of cyclophosphamide, GnRHa in none of these combinations provided a protection against follicle loss or improved steroidogenic activity of the samples.

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Vita

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