The effect of gonadotropin-releasing hormone analogues on the preservation of ovarian function against cyclophosphamide-induced damage in adult mice

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Keywords: GnRH analogue, cyclophosphamide, ovarian toxicity, ovarian function

Abstract

Objective:

To assess the effect of gonadotropin-releasing hormone analogue (GnRHa) on the preservation of ovarian function against cyclophosphamide-induced gonadal toxicity.

Materials and Methods:

In a controlled, experimental study, 64 female mice were divided into four groups: control (C), triptorelin acetate (T), cyclophosphamide (CY), and triptorelin plus cyclophosphamide (T+CY) groups. Mice in the group (T) were subcutaneously injected with GnRHa (triptorelin acetate) in a dose of 0.5 mg/kg daily for 21 days. In contrast, mice in the (CY) group and (T+CY) group were injected intraperitoneally with 75 mg/kg of CY on day 15. After 21 days, half of the mice in each group were sacrificed, and their ovaries were removed. The rest of the mice in each group were left without any intervention for an additional 21 days, and the same procedures were repeated to assess the ovarian follicles.

Results:

There was significant depletion of ovarian follicles in the CY group compared to the control group ($p<0.05$). There were significant decreases in the number of secondary and antral follicles at late stage as compared to early stage in the CY group ($p<0.05$). There was also a significant increase in the number of primordial and primary follicles in the T+CY group as compared with the CY group early post-treatment, while the increase was significant in all follicles after 42 days ($p<0.05$).

Conclusion:

Cyclophosphamide destroys primordial and primary follicles at an early stage while damage in secondary and antral follicles was prominent after 42 days. Triptorelin acetate reduces the toxic effect of CY; it has early and late protective effects and preserves ovarian function in mice.

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Introduction

In recent years, cancer treatments have greatly improved, leading to a significant increase in the survival of patients of reproductive age. However, the use of chemotherapies has been concomitant with gonadotoxic effect and loss of ovarian function.[1] Young women with cancer should be aware of the potential effects of chemotherapy on gonads and counseled to preserve fertility and ovarian function.[2]

Cyclophosphamide (CY) is an alkylating cytotoxic agent that is used widely as an anti-neoplastic and immunosuppressive agent and is proved to lead to impaired fertility through the destruction of ovarian follicles and development of premature ovarian failure.[3] Non-cycling cells are less susceptible to fatal damage from cytotoxic agents, so hormonal suppression may participate in ovarian protection.[4]

Several modalities have been suggested to preserve ovarian function following chemotherapy. These include prior in-vitro fertilization cycles with embryo cryopreservation, ovarian tissue cryopreservation and less-costly, more convenient pre-treatment with gonadotropin-releasing hormone analogues (GnRHa).[5] However, although GnRHa have been utilized for at least two decades to preserve ovarian function during the administration of cytotoxic drugs in women with cancer, their effect is still debatable.[6-8] A recent meta-analysis observed a benefit from GnRH analogues in breast cancer,[9] although clinical evidence remains uncertain for other types of cancers.[7,10,11]

To this end, animal studies have been used to verify the value of GnRHa in protecting against chemotherapy-induced ovarian compromise. Several studies have proven efficacy.[12, 13] However, others failed to reveal an improvement in outcomes.[14,15] The mechanisms of fertility preservation by GnRH analogues are not clearly understood. Several hypotheses have been postulated, including interruption of FSH secretion, stimulation of intra-ovarian antiapoptotic molecules such as sphingosine-I-phosphate, decrease in utero-ovarian perfusion, activation of GnRH receptors or protection of undifferentiated germ-line stem cells.[8]

Most previous studies show the positive effect of GnRH analogues on the resumption of menstruation and in patients with lymphoma.[11,16,17] However, these assumptions need to be re-evaluated due to marked methodological variations among studies of this sort. For example, return to fertility has been only short-term for several patients included in such studies. In addition, long-term analyses fail to show a clear benefit from GnRH agonists use depending on the type of cancer treated, type of chemotherapeutic agent used, and the demographic characteristics of the subjects involved in the studies. This inconsistency opened a door for us to use an adult mouse model to re-evaluate both the early and late effects of GnRHa on the preservation of ovarian function in the presence of cyclophosphamide-induced gonadal toxicity.

Materials and methods

Sixty-four female mice (6 weeks old) were used throughout the study. They were obtained from the Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt). All procedures were in accordance with their *Guiding Principles for the Care of Animals.*[12] The institutional ethical review board approved the study protocol.

All mice were accommodated individually for a 2-week acclimatization period. Mice were served ad libitum with standard laboratory pellets and tap water. A diurnal cycle of 12 hours of light and 12-hr dark cycle was maintained. Room temperature was set at 23±2◦C with a relative humidity of 45-55%.

Mice were randomly divided equally into four groups: Each animal was assigned a unique random identification number. Then new numbers were drawn to randomly assign mice in a logical fashion to different groups.

- Group (1): Control group (C). Needs to note procedure for age-matching.

- Group (2): Triptorelin acetate (T) group: mice were injected daily for 21 days with 0.5 mg/kg of subcutaneous triptorelin acetate (Decapeptyl®, Ferring Pharmaceuticals, Germany).[18]

- Group (3): Cyclophosphamide (CY) treated group: mice were injected intraperitoneally once on day 15 with cyclophosphamide (Endoxan®, Baxter, Oncology, GmbH) at a dose of 75 mg/kg.[19]

- Group (4): Triptorelin acetate plus CY treated (T+CY) group: mice were injected with 0.5 mg/kg triptorelin acetate subcutaneously for 21 days. The animals were further injected intraperitoneally once on day 15 with CY at a dose of 75 mg/kg.[18]

On the 21st day, half of the mice in each group (n=8), including the age-matched controls, were randomly sacrificed. Their ovaries were removed immediately and processed for light microscopic examination. In all cases, including the age-matched controls, the mice were killed with an overdose of ether. Both ovaries were entirely removed from each mouse for histological processing.

The rest of the mice in each group were left without any intervention for an additional 21 days and the same procedures were repeated to assess ovarian follicles. By the end of the experiments, four mice were dead; one each in the C and T+CY groups and two in the CY group. This number was an acceptable loss for the duration of the experiment and was anticipated for the mice receiving Cyclophosphamide. Histological examination showed no significant difference between the number of follicles in rats that died and those that survived until sacrifice.

After excision, the ovaries were fixed in a 4% formalin solution overnight and embedded in paraffin sections. Hematoxylin and eosin staining techniques were used for five micrometer serial sections, which were cut with a microtome. The primordial follicle (PMF) number was counted in every fifth section and then multiplied by five. The same

examiner was used for each section counted to diminish inter-observer error. The same procedure was used for primary, secondary, and antral follicles.

Morphometric Study

All images were captured using a calibrated standard digital microscope camera (Tucsen® ISH1000) with an Olympus® CX21 microscope, (Universal Infinity System, Olympus®, Japan). The numbers of primordial, primary, secondary and antral follicles were counted. Only the follicles that contained an oocyte were counted. A primordial follicle contains a partial or complete layer of flattened granulosa cells encircling the oocyte. In the primary follicle, the oocyte is surrounded by a single layer of cuboidal granulosa cells, and the secondary follicle includes multiple layers of cuboidal granulosa cells encircling the oocyte, whereas an antral follicle contains a single large antral space adjacent to the oocyte, as described by Myers et al.[20]

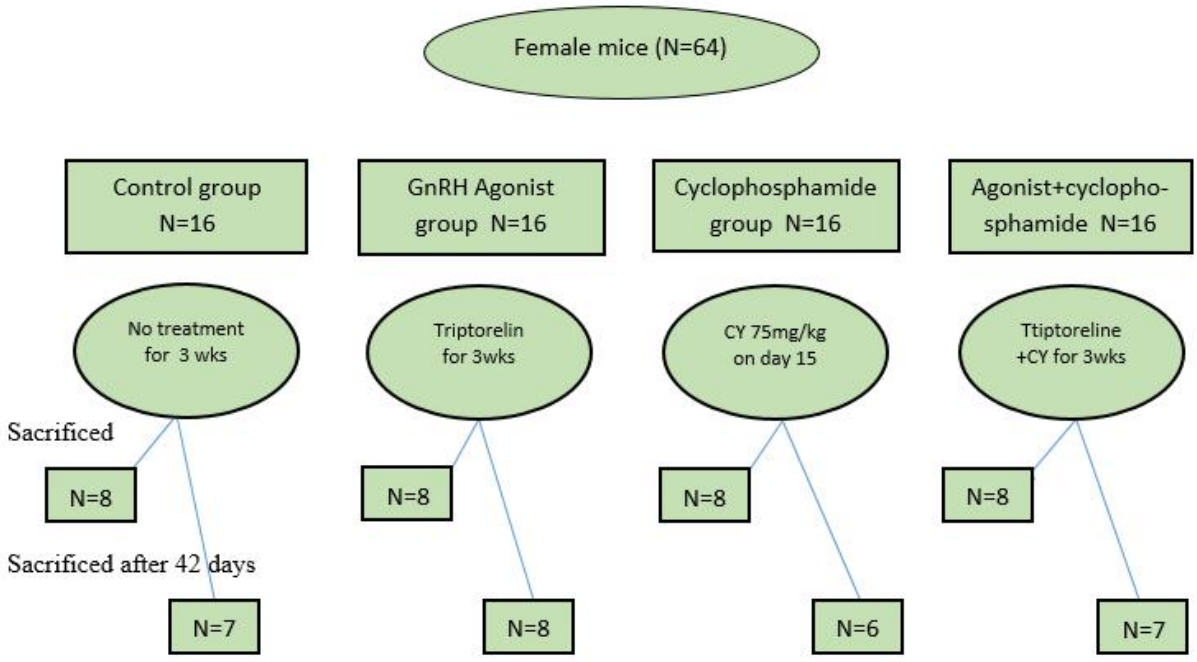

Figure 1: Flowchart of mouse allocations into groups

Statistical analysis

Data were analyzed using SPSS version 21 (SPSS Inc., Chicago, IL, USA).

Quantitative data were presented as mean ± SD. The ANOVA test was used to compare the four groups, while an independent t-test was used to compare subgroups. For example, subgroups in the CY group were compared at 21 and at 42 days. A p-value<0.05 was considered statistically significant.

Results

Sixty-four female mice were partitioned into four groups at random. By the end treatment course, each group was subdivided equally. Half of them were sacrificed, and the rest continued for 42 days (Figure 1).

Examination of the ovarian tissue in the control group showed that the cortex was occupied by follicles in various stages of development; classified into primordial, primary, secondary, and antral follicles (Figures 2a and 2b).

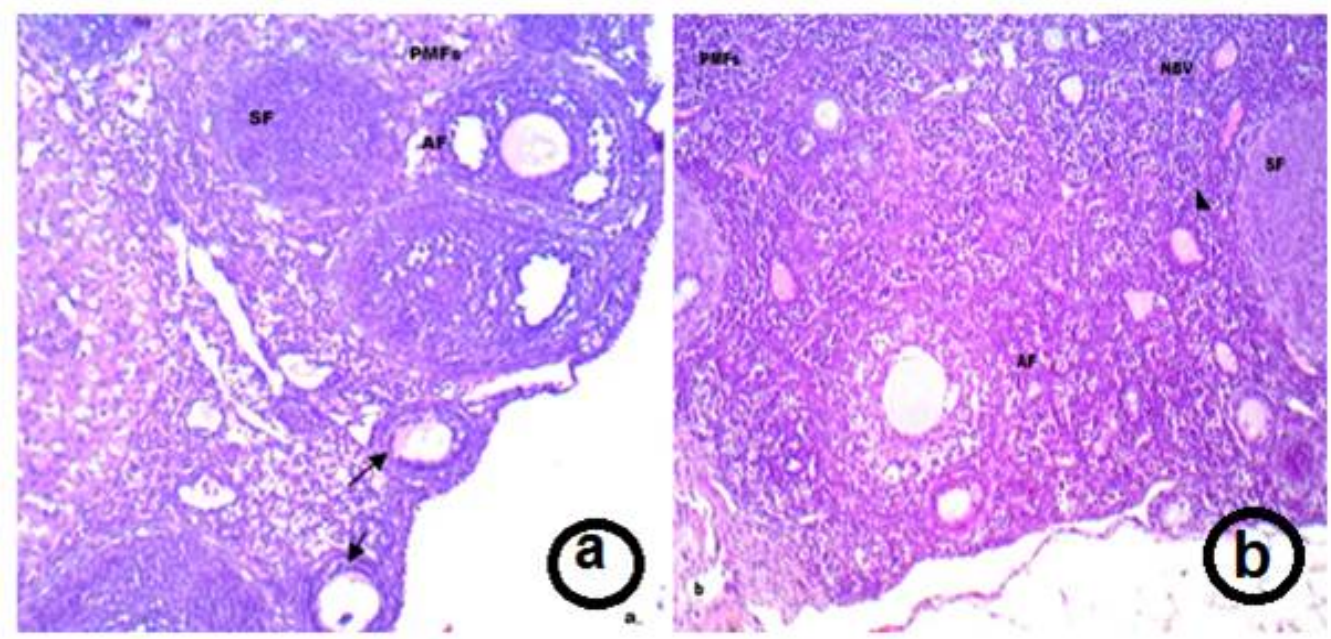

Figure 2a, 2b: A photomicrograph of a section in the ovary of a control mouse showing the cortex occupied by follicles (F) in various stages of development (PMFs; primordial follicle (Arrowheads), Primary follicles (Black arrows), SF; secondary follicle, NBV; normal blood vessels).

In the triptorelin acetate group, histological examination revealed normal ovarian tissue showing the cortex containing functional structures of the ovary (follicles) which showed well-developed follicles after 42 days (Figures 3a and 3b).

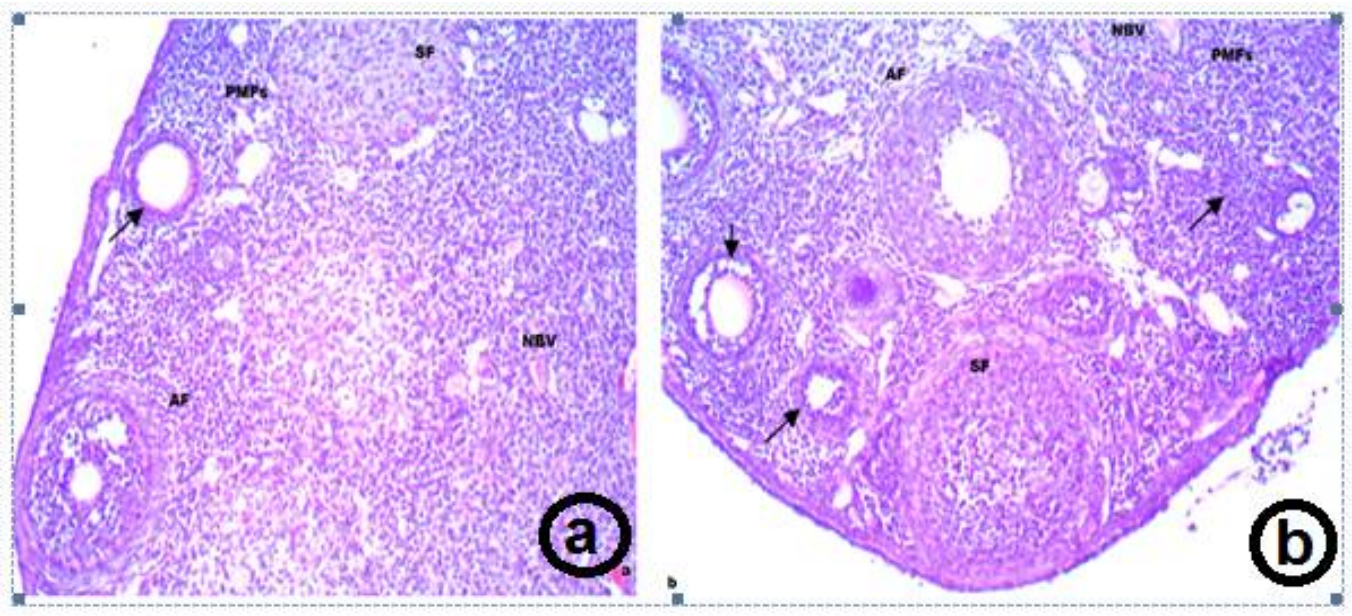

Figure 3a, 3b: A photomicrograph of a section in the ovary of a triptorelin group showing the cortex occupied by follicles in various stages of development (Primary follicles (Black arrows), SF; secondary follicle, NBV normal blood vessels) early (a) and after 42 days, which shows well-developed follicles (b).

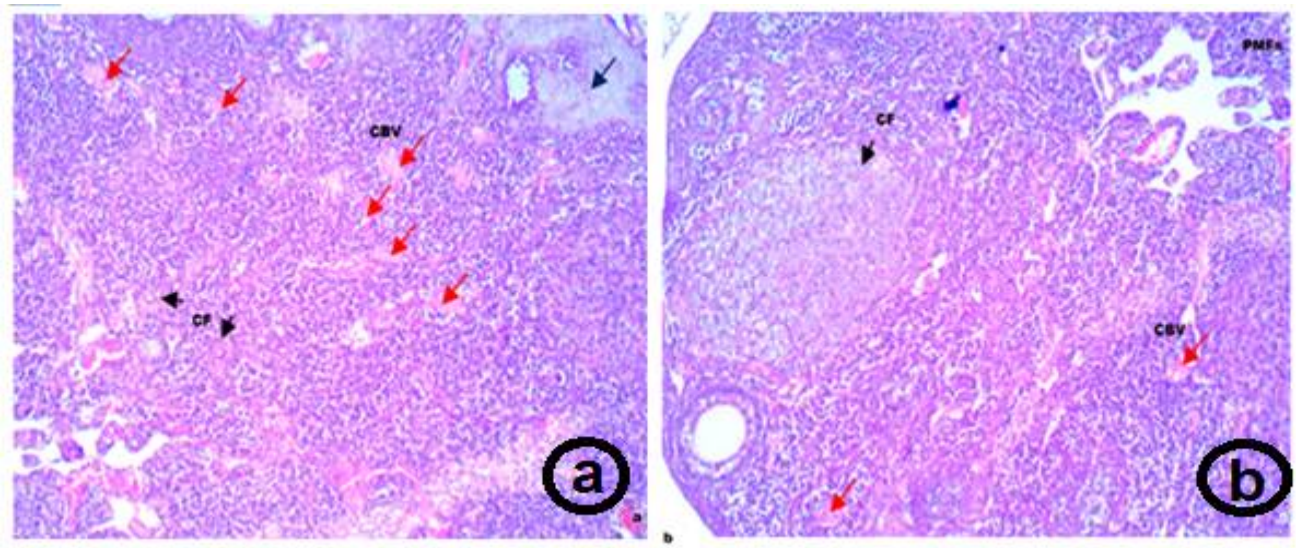

Figure 4a, 4b: A photomicrograph of a section in the ovary of a cyclophosphamide-treated mouse early and after 42 days showing collapsed follicles (Black arrows) with marked reduction of primordial (PMFs), primary, and secondary follicles at the surface of the ovary, and many congested blood vessels (Red arrows) and focal areas of necrosis (Blue arrow).

Examination of the ovarian tissue in the CY group revealed destruction of the ovarian structure, collapsed follicles, marked reduction in the number of primordial and primary follicles and congested blood vessels in acute stage (Figure 4a), extensive damage to all follicles was obvious with focal areas of necrosis after 42 days (Figure 4b).

In the (T+CY) group, the primordial, primary, secondary and antral follicles were preserved with decreased congestion early and after 42 days (Figures 5a and 5b).

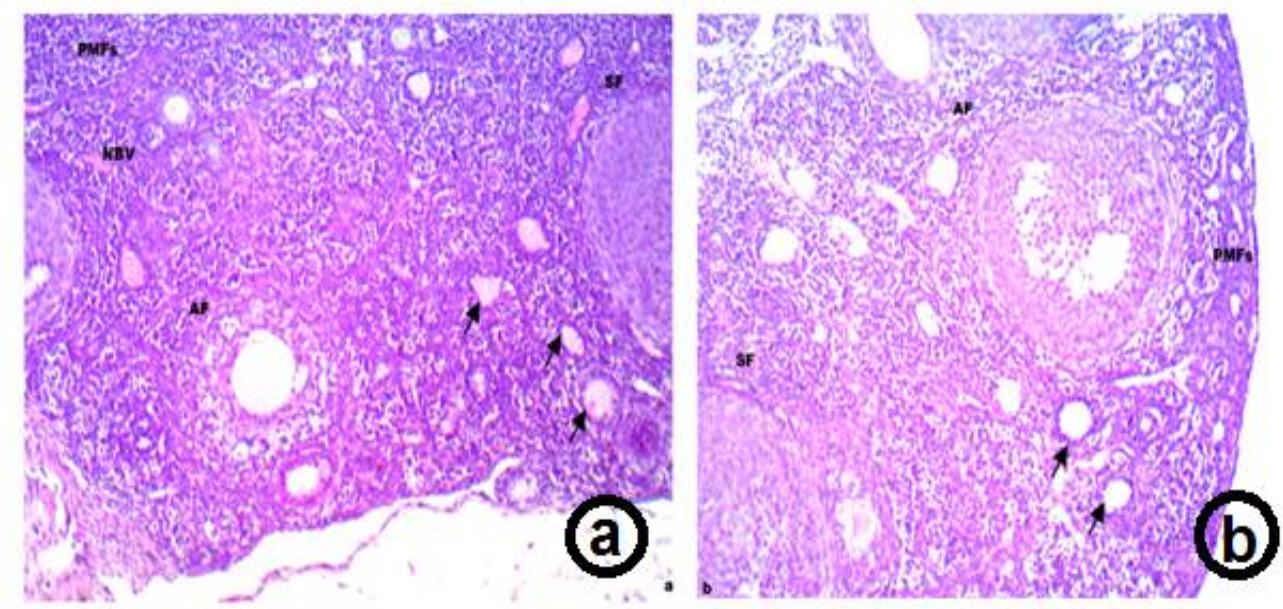

Figure 5a, 5b: A photomicrograph of a section in the ovary of triptorelin plus cyclophosphamide treated group early and after 42 days showing restoration of follicles in various stages of development, with significant reduction in the number of congested blood vessels and restoration of normal vasculature (PMFs; primordial follicles, Primary follicles (Black arrows), SF; secondary follicle, AF; antral follicle, NBV; normal blood vessels).

The two groups studied were compared with a focus on the number of follicles at two points in time, post-treatment. Marked destruction of the ovarian structure and a significant reduction of primordial, primary, secondary and antral follicles were observed in the CY group as compared with the control group, confirming the gonadotoxic effect of cyclophosphamide ($p<0.05$). There was a significant decrease in primordial and primary follicular destruction in the T+CY group compared with CY group early post-treatment (190.8±21.5 and 70.3±8.8 vs. 82.4±12.8 and 35.8±6.1, respectively), while the decrease was significant in all follicles after 42 days ($p<0.05$).

This two-pronged comparison showed that there was a significant reduction in secondary and antral follicle numbers

after 42 days compared to early evaluation of the CY group (12.0±5.2 and 6.4±2.6 vs. 24.6±6.5 and 10.8±4.2, respectively) suggesting that the toxic effect of CY is rapid in the primordial and primary follicles while growing follicles were not affected as quickly ($p<0.05$) (Table1).

Discussion

In this research, we studied the gonadotoxic effect of CY on the ovaries of mature mice. We did this through the evaluation of ovarian follicles early and late in the post-treatment course. We also studied the possible protective effect of triptorelin acetate on CY-exposed mouse ovaries through the examination of the ovaries of mice treated with both CY and triptorelin.

Cyclophosphamide is a chemotherapeutic and immunosuppressive agent that has long been approved to treat different forms of cancer, including lymphomas, multiple myelomas, leukemias and breast cancer.[3] It is similar to the alkylating agent and nitrogen mustard group of medications and exerts its action through its metabolite phosphoramide mustard, which forms DNA strand crosslinks at the Guanine N-7 position.[1]

Cyclophosphamide was shown to have a destructive effect on the ovaries. As an alkylating agent, it is non-cycle specific, so resting follicles may be affected.[21] A study by Oktem and Oktay in 2007 has demonstrated marked atrophy of primordial follicles and oocytes following CY therapy.[22] This atrophy was shown to be more obvious in primordial follicles than in growing ones.[23] The mechanism of destruction is believed to be through an apoptotic pathway.[24]

This study has shown a destructive effect of CY on ovarian follicles at two different time points. Moreover, growing follicles were shown to be more affected after 42 days denoting a continuous destructive process. Possible explanations that could explain the late growing follicle damage include CY-induced activation of the PI3K pathway, leading to a triggering of follicle activation,[25,26] CY-induced blood vessel injury and focal ovarian cortical fibrosis.[27]

Our results showed both early and late protective effects of the GnRHa triptorelin acetate against CY induced follicular damage. Follicles at all stages of maturation appeared to be significantly higher in number in mice administered triptorelin acetate and CY than in those treated with CY alone. The effect was evident both at the early and the late stages of examination. Our results agreed with previous animal studies confirming the protective effect of GnRHa against chemotherapy-induced gonadal damage in mice[28] and female rats.[29] On the other hand; our results disagreed with others who failed to demonstrate any ovarian protection from the administration of GnRHa during chemotherapy.[15]

Table 1: Distribution of the ovarian follicles among the studied groups at two different time points.

Groups	Control		Triptorelin (T)		Cyclophosphamide (CY)		T+ CY	
	Early	**At 42 days**	**Early**	**At 42 days**	**Early**	**At 42 days**	**Early**	**At 42 days**
Primordial follicles	280.6±15.3	272.8±22.4	231.8±20.6	240.2±17.6	82.4±12.8*	75.9±10.2*	190.8±21.5$^{\#}$	175.3±18.6$^{\#}$
Primary follicles	110.2±18.2	98.6±15.4	102.6±22.1	114.3±19.6	35.8±6.1*	28.4±7.5*	70.3±8.8$^{\#}$	63.6±10.9$^{\#}$
Secondary follicles	48.8±12.4	55.3±14.1	25.3±7.7	40.2±8.5	24.6±6.5*	12.0±5.2$^{*\$}$	28.9±7.1	21.4±6.4$^{\#}$
Antral follicles	28.4±6.9	31.2±7.6	14.3±5.4	26.5±6.1	10.8±4.2*	6.4±2.6$^{*\$}$	12.8±5.1	14.3±6.6$^{\#}$

$^{*\#\$}$Statistically significant difference at $p < 0.05$
*CY subgroups vs. control subgroups
$^{\#}$T+CY subgroups vs. CY subgroups
$^{\$}$CY subgroup in early stage vs. CY subgroup after 42 days

In humans, prospective randomized trials have also demonstrated controversial data.[7,11,30-32] In human studies, the prospective trials evaluating the efficiency of GnRHa in ovarian protection against chemotherapy were flawed by methodological problems including a small study sample size, a short follow-up period and a lack of randomization.17 While the majority of animal studies likewise evaluated only the short-term effects of GnRHa, a strength in our study was our evaluation of both the long- and short-term effects of the GnRHa.

Recently, the *Prevention of Early Menopause Study* concluded that the use of GnRH agonists during chemotherapy protected ovarian function in patients with breast cancer through two years of follow up.[6] An updated analysis of the PROMISE-GIM6 trial assessing the 5-year cumulative occurrence of menstrual resumption also confirmed the benefit of GnRHa administration.[9] On the other hand, a long-term study by Demeestere and her colleagues (2016) failed to confirm the beneficial effect of GnRHa in preventing chemotherapy-induced premature ovarian failure in lymphoma through five years of follow up.[17]

Several mechanisms explaining the protective role of GnRHa against chemotherapy have been mentioned, including suppression of gonadotrophin levels in the ovary as a direct effect of GnRHa. According to this explanation, GnRHa preserves only growing follicles since GnRHa receptors in humans are found only in preovulatory follicles and corpus luteum.[33] Additionally, GnRHa induces a decline in ovarian blood flow, which subsequently decreases the dose of chemotherapy that reaches the ovaries, therefore limiting ovarian damage. This effect is still controversial as some studies show a decline in ovarian blood flow after the administration of GnRHa in rats,[34] while other studies, which measure blood flow of the ovarian stroma using three-dimensional power Doppler ultrasound, show a lack of change in blood flow.[35] Finally, GnRHa agonists may up-regulate extragonadal anti-apoptotic molecules such as sphingosine-1-phosphate (S1P).

Limitations of this study include that it does not assess anti-mullerian hormones, that it focuses on the histological examination of ovarian tissue and that it provides long-term evaluation mainly of primordial follicles after treatment to reflect the actual ovarian reserve and its fertility potential.

Conclusion

This study follows both late and early phase ovarian damage in mice after exposure to cyclophosphamide. The effects were considered for both resting and growing follicles. Results found that triptorelin acetate reduced the toxic effect of CY and that it had both early and late protective effects. GnRHa are simple and effective drugs. Therefore, they could be suitable for fertility preservation during chemotherapy. Long and large clinical trials, preferably randomized controlled trials, are required to confirm these findings.

References

1. Maltaris T, Seufert R, Fischl F, Schaffrath M, Pollow K, Koelbl H, Dittrich R. The effect of cancer treatment on female fertility and strategies for preserving fertility. Eur J Obstet Gynecol Reprod Biol. 2007 Feb;130(2):148-55. https://doi.org/10.1016/j.ejogrb.2006.08.006 Epub 2006 Sep 15. PubMed PMID: 16979280.

2. Marhhom E, Cohen I. Fertility preservation options for women with malignancies. Obstet Gynecol Surv. 2007 Jan;62(1):58-72. https://doi.org/10.1097/01.ogx.0000251029.93792.5d PubMed PMID: 17176489.

3. Behringer K, Mueller H, Goergen H, Thielen I, Eibl AD, Stumpf V, Wessels C, Wiehlpütz M, Rosenbrock J, Halbsguth T, Reiners KS, Schober T, Renno JH, von Wolff M, van der Ven K, Kuehr M, Fuchs M, Diehl V, Engert A, Borchmann P. Gonadal function and fertility in survivors after Hodgkin lymphoma treatment within the German Hodgkin Study Group HD13 to HD15 trials. J Clin Oncol. 2013 Jan 10;31(2):231-9. https://doi.org/10.1200/JCO.2012.44.3721 Epub 2012 Nov 13. PubMed PMID: 23150709.

4. Blumenfeld Z. Preservation of fertility and ovarian function and minimalization of chemotherapy associated gonadotoxicity and premature ovarian failure: the role of inhibin-A and -B as markers. Mol Cell Endocrinol. 2002 Feb 22;187(1-2):93-105. https://doi.org/10.1016/S0303-7207(01)00712-2 PubMed PMID: 11988316.

5. Turner NH, Partridge A, Sanna G, Di Leo A, Biganzoli L. Utility of gonadotropin-releasing hormone agonists for fertility preservation in young breast cancer patients: the benefit remains uncertain. Ann Oncol. 2013 Sep;24(9):2224-35. https://doi.org/10.1093/annonc/mdt196 Epub 2013 May 24. PubMed PMID: 23709175.

6. Peccatori F, Demeestere I. GnRH analogue for chemotherapy-induced ovarian damage: too early to say? Fertil Steril. 2009 Aug;92(2):e33; author reply e34. https://doi.org/10.1016/j.fertnstert.2009.06.002 PubMed PMID: 19646600.

7. Moore HC, Unger JM, Phillips KA, Boyle F, Hitre E, Porter D, Francis PA, Goldstein LJ, Gomez HL, Vallejos CS, Partridge AH, Dakhil SR, Garcia AA, Gralow J, Lombard JM, Forbes JF, Martino S, Barlow WE, Fabian CJ, Minasian L, Meyskens FL Jr, Gelber RD, Hortobagyi GN, Albain KS; POEMS/S0230 Investigators. Goserelin for ovarian protection during breast-cancer adjuvant chemotherapy. N Engl J Med. 2015 Mar 5;372(10):923-32. https://doi.org/10.1056/NEJMoa1413204 PubMed PMID: 25738668; PubMed Central PMCID: PMC4405231.

8. Del Mastro L, Boni L, Michelotti A, Gamucci T, Olmeo N, Gori S, Giordano M, Garrone O, Pronzato P, Bighin C, Levaggi A, Giraudi S, Cresti N, Magnolfi E, Scotto T, Vecchio C, Venturini M. Effect of the gonadotropin-releasing hormone analogue triptorelin on the occurrence of chemotherapy-induced early menopause in premenopausal women with breast cancer: a randomized trial. JAMA. 2011 Jul 20;306(3):269-76. https://doi.org/10.1001/jama.2011.991 PubMed PMID: 21771987.

9. Lambertini M, Boni L, Michelotti A, Gamucci T, Scotto T, Gori S, Giordano M, Garrone O, Levaggi A, Poggio F, Giraudi S, Bighin C, Vecchio C, Sertoli MR, Pronzato P, Del Mastro L; GIM Study Group. Ovarian Suppression With Triptorelin During Adjuvant Breast Cancer Chemotherapy and Long-term Ovarian Function, Pregnancies, and Disease-Free Survival: A Randomized Clinical Trial. JAMA. 2015 Dec 22-29;314(24):2632-40. https://doi.org/10.1001/jama.2015.17291 PubMed PMID: 26720025.

10. Lambertini M, Poggio F, Levaggi A, Del Mastro L. Protecting Ovaries During Chemotherapy Through Gonad Suppression: A Systematic Review and Meta-analysis. Obstet Gynecol. 2015 Oct;126(4):901. https://doi.org/10.1097/AOG.0000000000001076 PubMed PMID: 26393449.

11. Oktay K, Turan V. Failure of Ovarian Suppression With Gonadotropin-Releasing Hormone Analogs to Preserve Fertility: An Assessment Based on the Quality of Evidence. JAMA Oncol. 2016 Jan;2(1):74-5. https://doi.org/10.1001/jamaoncol.2015.3252 PubMed PMID: 26426406.

12. Ataya K, Ramahi-Ataya A. Reproductive performance of female rats treated with cyclophosphamide and/or LHRH agonist. Reprod Toxicol. 1993 May-Jun;7(3):229-35. https://doi.org/10.1016/0890-6238(93)90229-Z PubMed PMID: 8318754.

13. Bokser L, Szende B, Schally AV. Protective effects of D-Trp6-luteinising hormone-releasing hormone microcapsules against cyclophosphamide-induced gonadotoxicity in female rats. Br J Cancer. 1990 Jun;61(6):861-5. P https://doi.org/10.1038/bjc.1990.192 ubMed PMID: 2142603; PubMed Central PMCID: PMC1971695.

14. Montz FJ, Wolff AJ, Gambone JC. Gonadal protection and fecundity rates in cyclophosphamide-treated rats. Cancer Res. 1991 Apr 15;51(8):2124-6. PubMed PMID: 1901240.

15. Letterie GS. Anovulation in the prevention of cytotoxic-induced follicular attrition and ovarian failure. Hum Reprod. 2004 Apr;19(4):831-7. https://doi.org/10.1093/humrep/deh120 Epub 2004 Feb 12. PubMed PMID: 15033951.

16. Kalantaridou SN, Nelson LM. Premature ovarian failure is not premature menopause. Ann N Y Acad Sci. 2000;900:393-402. https://doi.org/10.1111/j.1749-6632.2000.tb06251.x PubMed PMID: 10818427.

17. Demeestere I, Brice P, Peccatori FA, Kentos A, Dupuis J, Zachee P, Casasnovas O, Van Den Neste E, Dechene J, De Maertelaer V, Bron D, Englert Y. No Evidence for the Benefit of Gonadotropin-Releasing Hormone Agonist in Preserving Ovarian Function and Fertility in Lymphoma Survivors Treated With Chemotherapy: Final Long-Term Report of a Prospective Randomized Trial. J Clin Oncol. 2016 Aug 1;34(22):2568-74. https://doi.org/10.1200/JCO.2015.65.8864 Epub 2016 May 23. PubMed PMID: 27217453.

18. Horicks F, Van Den Steen G, Houben S, Englert Y, Demeestere I. Folliculogenesis Is Not Fully Inhibited during GnRH Analogues Treatment in Mice Challenging Their Efficiency to Preserve the Ovarian Reserve during Chemotherapy in This Model. PLoS One. 2015 Sep 1;10(9):e0137164. https://doi.org/10.1371/journal.pone.0137164 eCollection 2015. PubMed PMID: 26325271; PubMed Central PMCID: PMC4556658.

19. Meirow D, Assad G, Dor J, Rabinovici J. The GnRH antagonist cetrorelix reduces cyclophosphamide-induced ovarian follicular destruction in mice. Hum Reprod. 2004 Jun;19(6):1294-9. https://doi.org/10.1093/humrep/deh257 Epub 2004 Apr 29. PubMed PMID: 15117898.

20. Myers M, Britt KL, Wreford NG, Ebling FJ, Kerr JB. Methods for quantifying follicular numbers within the mouse ovary. Reproduction. 2004 May;127(5):569-80. https://doi.org/10.1530/rep.1.00095 PubMed PMID: 15129012.

21. Lutchman Singh K, Davies M, Chatterjee R. Fertility in female cancer survivors: pathophysiology, preservation and the role of ovarian reserve testing. Hum Reprod Update. 2005 Jan-Feb;11(1):69-89. https://doi.org/10.1093/humupd/dmh052 Epub 2004 Nov 29. PubMed PMID: 15569700.

22. Oktem O, Oktay K. Quantitative assessment of the impact of chemotherapy on ovarian follicle reserve and stromal function. Cancer. 2007 Nov 15;110(10):2222-9. https://doi.org/10.1002/cncr.23071 PubMed PMID: 17932880.

23. Plowchalk DR, Mattison DR. Reproductive toxicity of cyclophosphamide in the C57BL/6N mouse: 1. Effects on ovarian structure and function. Reprod Toxicol. 1992;6(5):411-21. https://doi.org/10.1016/0890-6238(92)90004-D PubMed PMID: 1463921.

24. Meirow D, Nugent D. The effects of radiotherapy and chemotherapy on female reproduction. Hum Reprod Update. 2001 Nov-Dec;7(6):535-43. https://doi.org/10.1093/humupd/7.6.535 PubMed PMID: 11727861.

25. Sobinoff AP, Nixon B, Roman SD, McLaughlin EA. Staying alive: PI3K pathway promotes primordial follicle activation and survival in response to 3MC-induced ovotoxicity. Toxicol Sci. 2012 Jul;128(1):258-71. https://doi.org/10.1093/toxsci/kfs137 Epub 2012 Apr 12. PubMed PMID: 22505044.

26. Kalich-Philosoph L, Roness H, Carmely A, Fishel-Bartal M, Ligumsky H, Paglin S, Wolf I, Kanety H, Sredni B, Meirow D. Cyclophosphamide triggers follicle activation and "burnout"; AS101 prevents follicle loss and preserves fertility. Sci Transl Med. 2013 May 15;5(185):185ra62. https://doi.org/10.1126/scitranslmed.3005402 PubMed PMID: 23677591.

27. Meirow D, Dor J, Kaufman B, Shrim A, Rabinovici J, Schiff E, Raanani H, Levron J, Fridman E. Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury. Hum Reprod. 2007 Jun;22(6):1626-33. Epub 2007 Feb 26. https://doi.org/10.1093/humrep/dem027 PubMed PMID: 17324957.

28. Tan SJ, Yeh YC, Shang WJ, Wu GJ, Liu JY, Chen CH. Protective effect of a gonadotropin-releasing hormone analogue on chemotherapeutic agent-induced ovarian gonadotoxicity: a mouse model. Eur J Obstet Gynecol Reprod Biol. 2010 Apr;149(2):182-5. https://doi.org/10.1016/j.ejogrb.2009.12.028 Epub 2010 Jan 13. PubMed PMID: 20074846.

29. Matsuo G, Ushijima K, Shinagawa A, Takahashi S, Fujiyoshi N, Takemoto S, Terada A, Fukui A, Kamura T. GnRH agonist acts as ovarian protection in chemotherapy induced gonadotoxicity: an experiment using a rat model. Kurume Med J. 2007;54(1-2):25-9. https://doi.org/10.2739/kurumemedj.54.25 PubMed PMID: 18332593.

30. Del Mastro L, Lambertini M. Temporary Ovarian Suppression With Gonadotropin-Releasing Hormone Agonist During Chemotherapy for Fertility Preservation: Toward the End of the Debate? Oncologist. 2015 Nov;20(11):1233-5. https://doi.org/10.1634/theoncologist.2015-0373 Epub 2015 Oct 13. PubMed PMID: 26463868; PubMed Central PMCID: PMC4718439.

31. Gerber B, Ortmann O. Prevention of Early Menopause Study (POEMS): is it possible to preserve ovarian function by gonadotropin releasing hormone analogs(GnRHa)? Arch Gynecol Obstet. 2014 Dec;290(6):1051-3. https://doi.org/10.1007/s00404-014-3493-0 PubMed PMID: 25322976.

32. Lambertini M, Ceppi M, Poggio F, Peccatori FA, Azim HA Jr, Ugolini D, Pronzato P, Loibl S, Moore HC, Partridge AH, Bruzzi P, Del Mastro L. Ovarian suppression using luteinizing hormone-releasing hormone agonists during chemotherapy to preserve ovarian function and fertility of breast cancer patients: a meta-analysis of randomized studies. Ann Oncol. 2015 Dec;26(12):2408-19. https://doi.org/10.1093/annonc/mdv374 Epub 2015 Sep 7. PubMed PMID: 26347105.

33. Choi JH, Gilks CB, Auersperg N, Leung PC. Immunolocalization of gonadotropin-releasing hormone (GnRH)-I, GnRH-II, and type I GnRH receptor during follicular development in the human ovary. J Clin Endocrinol Metab. 2006 Nov;91(11):4562-70. https://doi.org/10.1210/jc.2006-1147 Epub 2006 Sep 5. PubMed PMID: 16954155.

34. Kitajima Y, Endo T, Nagasawa K, Manase K, Honnma H, Baba T, Hayashi T, Chiba H, Sawada N, Saito T. Hyperstimulation and a gonadotropin-releasing hormone agonist modulate ovarian vascular permeability by altering expression of the tight junction protein claudin-5. Endocrinology. 2006 Feb;147(2):694-9. https://doi.org/10.1210/en.2005-0700 Epub 2005 Nov 3. PubMed PMID: 16269461.

35. Yu Ng EH, Chi Wai Chan C, Tang OS, Shu Biu Yeung W, Chung Ho P. Effect of pituitary downregulation on antral follicle count, ovarian volume and stromal blood flow measured by three-dimensional ultrasound with power Doppler prior to ovarian stimulation. Hum Reprod. 2004 Dec;19(12):2811-5. https://doi.org/10.1093/humrep/deh500 Epub 2004 Sep 9. PubMed PMID: 15358718.

36. Blumenfeld Z. How to preserve fertility in young women exposed to chemotherapy? The role of GnRH agonist cotreatment in addition to cryopreservation of embrya, oocytes, or ovaries. Oncologist. 2007 Sep;12(9):1044-54. https://doi.org/10.1634/theoncologist.12-9-1044 PubMed PMID: 17914074.