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Research Article

MODULATION OF GENES AND PROTEIN LOCALIZATION FOR ESTROGEN AND PROGESTERONE RECEPTORS IN CYCLOPHOSPHAMIDE-EFFECTED OVARY BY TOCOTRIENOLS SUPPLEMENTATION

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ABSTRACT

Objective: The objective of the study was to investigate the role of tocotrienols (T3) in alteration and regulation of estrogen receptor (ER) and progesterone receptor (PR) genes and proteins expression that was hoped to explain the involvement of ER and PR signaling pathway as biomarker in maintaining fertility during chemotherapy treatment.

Methods: Fifty female ICR mice (10–12) weeks were equally divided into five groups: CPA, CPA and T3, normal saline, T3 only, and corn oil only. The treatment was given for 4 weeks and followed by super ovulation protocol application. Both ovaries were removed, RNA extraction and real-time quantitative polymerase chain reaction (PCR) were performed using the super green probe real-time PCR. Concentration of the forward, reverse primers involvement ER, PR, glyceraldehyde-3-phosphate dehydrogenase and α-actin genes. Histological processing was performed to apply IHC reactions using peroxidase for mouse biotinylated primary antibody.

Result: It was founded that significant downregulation of ERα gene was evident in the coadministration of CPA with T3 compared to CPA group ($p<0.01$). Meanwhile, highly significant expression of ERα gene in CPA-exposed ovary compared to the control group, which was by 5.9-fold ($p<0.01$). Moreover, the study revealed that CPA+ T3 is significantly reduced the protein localization of ER, PR in the ovary of CPA–exposed mice ($p<0.05$).

Conclusion: The results indicated that coadministration of T3 with CPA confers protection against altered genes and protein expression in ovaries. T3 may consider as a promising candidate for ovarian preservation due to chemotherapy-associated ER, PR genes, and protein overexpression.

Keywords: Estrogen receptor alpha, Progesterone receptor, Cyclophosphamide, Infertility, Tocotrienol.

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INTRODUCTION

Infertility has become a global problem affecting approximately 15% of both men and women of reproductive age. Anticancer therapy is one of the factors leading to decreased fertility [1]. The survival of adolescents and young women treated for cancer and autoimmune diseases resulted in an increased focus on the effects of chemotherapy on ovarian function and its preservation. Female reproductive function is largely influenced by estrogen; the function of estrogen depends on the presence of receptor, known as estrogen receptor (ER). Receptor-mediated effects of estrogen are found to contribute into most ovarian pathology [2-4]. Previously, the modulation of ER levels is important in determining cellular growth potential [5] and reviewed the mechanisms of ER expression and control in human cancer. Hence, these findings result in reproductive function impairment.

Chang and Mcdonnell [6] explained that ERα plays an important role in fertility as any alteration to its expression seems to influence fertility trend. Evidence of ER-α playing a key role in fertility control was provided by several transgenic experimental studies using different animal models [2,7,8]. A study has been noted that methoxychlor (MXC) ovarian toxicity was mediated in ER-α over-expression pathways. Atresia was also shown to be enhanced in antral follicles of ovarian toxicity compounds induced in ER-over expressing in experimental mice [9,10]. Classically, the effects of progesterone are mediated by the nuclear progesterone receptor (PR), the intracellular PR in the mammalian ovary is a part of the physiological pathway that facilitates ovulation. PR has been identified as an important regulator of gene transcription during the peri-ovulatory period, specifically of genes found to be necessary for successful oocyte release from the preovulatory follicle, reviewed by Russell and Robker [11]. The previous findings emphasize the important role of progesterone and its receptor in the ovulatory process. The expression of PR in granulosa cells in tertiary follicles is induced by the luteinizing hormone surge [12,13] Moreover, changes have been observed in the expression of PR isoform (A) mRNA in many tissues during the reproductive cycle and in malignant human breast tissues. This is consistent with the important physiological roles played by the receptor in a wide variety of reproductive functions and its potential involvement in tumorgenesis [14,15]. Thus, ER and PR expressions during the estrous cycle stages indicate the importance of ER and PR in the regulation of the ovarian activity cycle [16,17].

Although, recent study has focus on potential chemopreventive agent in mitigating cardiotoxicity by giving Geraniol against CPA so that this study considered T3 as possible preventive agent that would maintain reproductive system function (62).

So far, fertility data concerning changes in the ER and PR expression in CPA-exposed ovary *in vivo* have not been fully identified. Therefore, this study was undertaken to obtain a better insight into the role of gene and protein expression of ER and PR in the ovarian tissue cells. Tocotrienol (T3) is a component of Vitamin E along with tocopherol; chemically composed of chromanol ring with an attached side-chain. Both of them exists in four isomers (alpha, beta, gamma, and delta) named a tocotrienole (T3) is a component of Vitamin E along with tocopherol; chemically composed of chromanol ring with an attached side-chain. Both of them exists in four isomers (alpha, beta, gamma, and delta) named according to their methyl groups [18,19]. T3 is capable of scavenging and quenching free radicals, by its antioxidative activity, however, resides mainly with their chain breaking property, which neutralizes peroxyl and alkoxyl radical generated during lipid peroxidation according to their methyl groups [18-39]. In addition, the antioxidant impact of T3 was

found to use as neuroprotective (63). A study indicated that T3-rich palm Vitamin E improves the percentage of pregnancies and the number of fertilized embryos retrieved following superovulation in nicotine-treated rats [20,21]. The beneficial effect of γ-T3 supplementation was clearly seen when the zona pellucida thickness is increased and remained intact despite nicotine exposure and reduce the oocytes apoptosis [22,23]. The protein damage caused by nicotine was neutralized by the addition of the gamma-T3 [21-24]. Whereas several studies have indicated the anticancer effect of tocotrienol, relatively little is known about the effect of T3 in preserving fertility in patients undergoing chemotherapy.

METHODS

Animal husbandry

Fifty ICR (Imprinting Control Region) female mice aged between 10 and 12 weeks of average weight of 30 g were used in this study. They were housed in plastic cages in air-conditioned house. Mice were fed with normal pellet and tap water as given to the mice *ad libitum*. Mice were equally divided into five groups and given different treatment for 4 weeks as follows: Group 1 was administrated i.p injection with 0.15 ml normal saline as a control group. Group 2 was given i. p injection with 10 mg/kg B. w of CPA, this dose was chosen based on using as chronic treatment with ovary according to Letterie [25]. Group 3 was coadministrated i. p injection 10 mg/kg B. w CPA and 60 mg/kg B. w T3 orally this dose was chosen based on using with oocyte in previous literature according to Rajikin *et al*. [21]. Group 4 was only given 60 mg/kg B. w T3 orally second control group. Group 5 was only given 0.15 ml of corn oil orally third control group. All experimental animals were subjected to superovulation induction to synchronize ovulation as formerly described by Luo *et al*. [26].

Real-time polymerase chain reaction (PCR)

Both ovaries were eviscerated and grossed to prepare for RT-PCR technique and histological processing steps. The ovaries were frozen in liquid nitrogen immediately after dissection and total RNA extraction from ovary homogenates that was carried out according to manufactures instructions in the kit (imnu PREP RNA mini kit, Repfon, USA), mRNA extraction followed by synthesis of the cDNA. The primers for quantitative RT-PCR in this study comprised of four primers pairs. Two of the primer pairs were references genes α-*actin* and glyceraldehyde-3-phosphate dehydrogenase and the other two primer pairs were target genes; *ER* and *PR*. The melting temperature (Tm) for the primer was set to around 60°C–40°C to ensure proper hybridization to the target sequence. This study applied the SYBER Green I Dye system.

Immunohistochemistry

The assay immunohistochemistry (IHC) procedure was done according to the previous described method with slight modification [27]. All IHC reactions kit was obtained from DAKO company animal research kit, two types of antibodies were used, ER and PR, for the detection of ER and PR localization. The optimization procedure was performed to ensure that the staining is reproducible and specific for the protein tested. The slides were subjected to antigen retrieval pretreatment by heat-induced method, 0.3% hydrogen peroxide and incubated for 5 min. Biotinylated primary antibody modified biotinylated anti-mouse immunoglobulin in Tris-HCl buffer was prepared by diluting 1/200 stock ER and PR. Streptavidin conjugated to horseradish peroxidase secondary antibody was applied and incubated. After the above step, substrate buffered solution, pH7.5, containing hydrogen peroxide and DAB chromogen solution was applied. Slides were immersed in a bath of hematoxylin. The presence of ER was assessed by brown cytoplasmic staining intensity as well as analyzed using image analysis software (Olympic, Japan), brown stain was measured and expressed as a percentage of the entire ovarian area.

Statistical analysis

All quantitative evaluation of ER and PR-A immunoreactivity expression by percentage. For statistical comparison between different treatment groups, analysis of variance one-way is used. All data were used Statistical Package for the Social Sciences version 20. Significant differences are indicated by asterisks in the figures (*p<0.05; **p<0.001).

RESULTS

Gene expression study

The current study found highly significant expression of ERα gene in CPA-exposed ovary compared to the control group, which was by 5.9-fold (p<0.01). Meanwhile, significant downregulation of ERα gene was evident in coadministration of CPA with T3 compared to CPA group (p<0.01), with a value of 2.17-fold compared to 5.97-fold, respectively. In addition, there were no significant differences between ERα transcript level for CPA + T3 group compared to all control groups as shown in (Fig. 1). In term of, CPA treated ovary showed significant increase in the *PR-A* transcript level of approximately 3.48 fold as compared to control group (p<0.01). On another hand, there was a 1.69 fold significant reduction in *PR-A* transcript level in CPA and T3 group compared to CPA group (p<0.01) as shown in Fig. 2.

Protein expression study

Estrogen receptor (ER)-α immunoreactivity

An assessment of ER-α distribution was most within ovarian components with high expression and largely observed in the cytoplasm of the CPA- treated ovary. In the control group, ER-α localization in ovarian tissue ranged in low expression in ovarian components. Our results showed that coadministration of CPA and T3 led to the restoration of localization of ER-α to near normal. ER-α immunoreactivity in CPA and T3 group exhibited moderate to low expression in follicles with clear oocyte membrane as shown in Fig. 3. In addition, quantitative analysis showed a significant reduction in the percentage of immunoreactivity staining, i.e. 26.56 versus 30.57% (CPA and T3 vs. CPA, respectively); p<0.05 whereas there were no significant increases in staining percentage expression in CPA and T3 versus control group: 26.56 versus 24.16%; p=0.095 (Fig. 4).

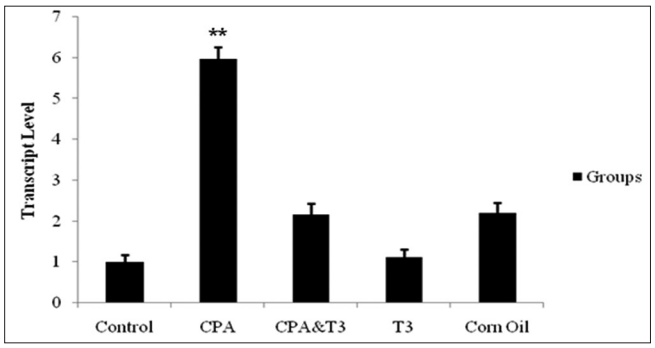

Fig. 1: ERα transcript level of all groups: There was significant downregulation of *ER* in CPA and T3 group compared to CPA (p<0.01). ER: Estrogen receptor, PR: Progesterone receptor

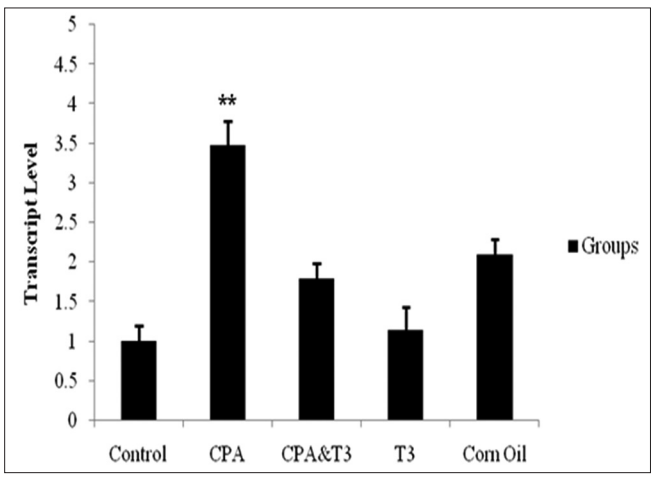

Fig. 2: *PR-A* transcript level of all groups: There was significant downregulation of *PR-A* in CPA and T3 group compared to CPA (p<0.01). PR: Progesterone receptor

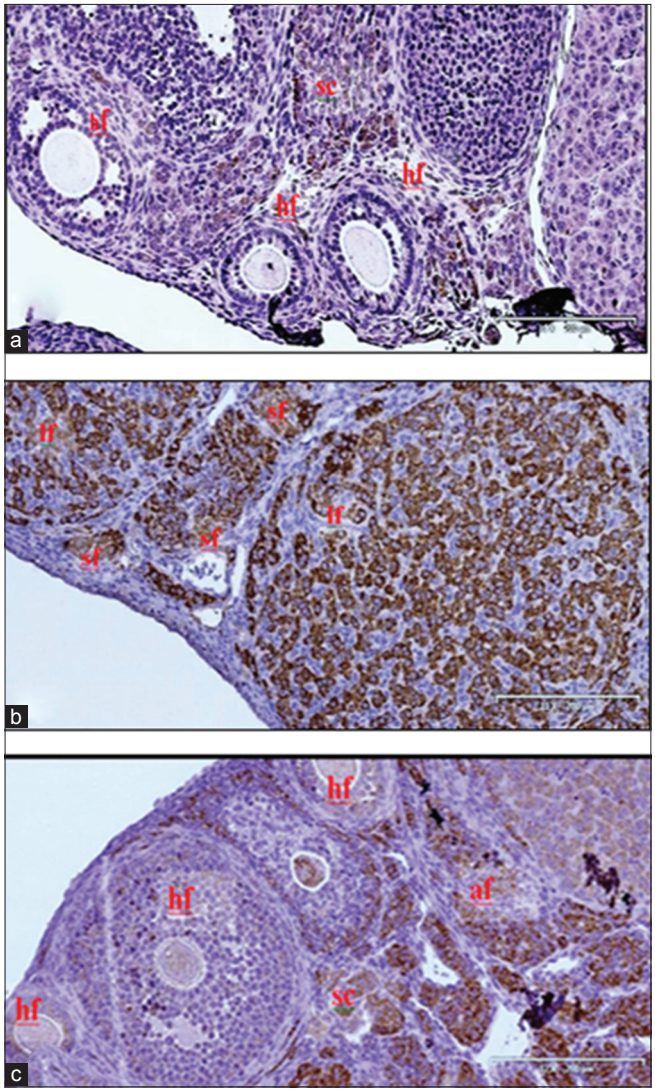

Fig. 3: ER-α expression in the normal saline-treated ovary (a). ER-α expression in CPA-treated ovary showing a high number of ER-α positive cells in small and large atretic follicles as indicated (b). Expression in CPA snd T3 showing a moderate number of ER-positive cells group (c). ER: Estrogen receptor, PR: Progesterone receptor

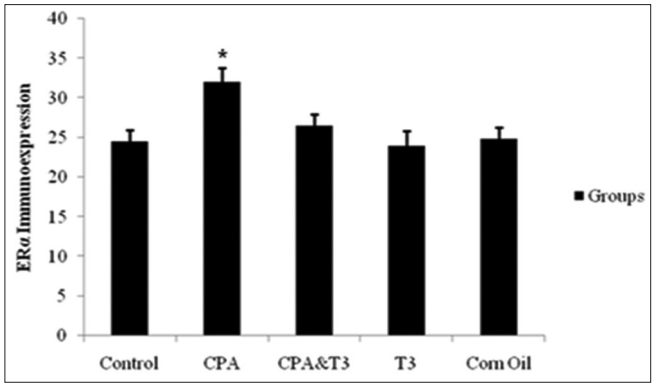

Fig. 4: Qualitative evaluation of ER-α immunoexpression: There was significantly increased expression in the CPA-treated ovaries compared to CPA and T3 group (p<0.05). ER: Estrogen receptor

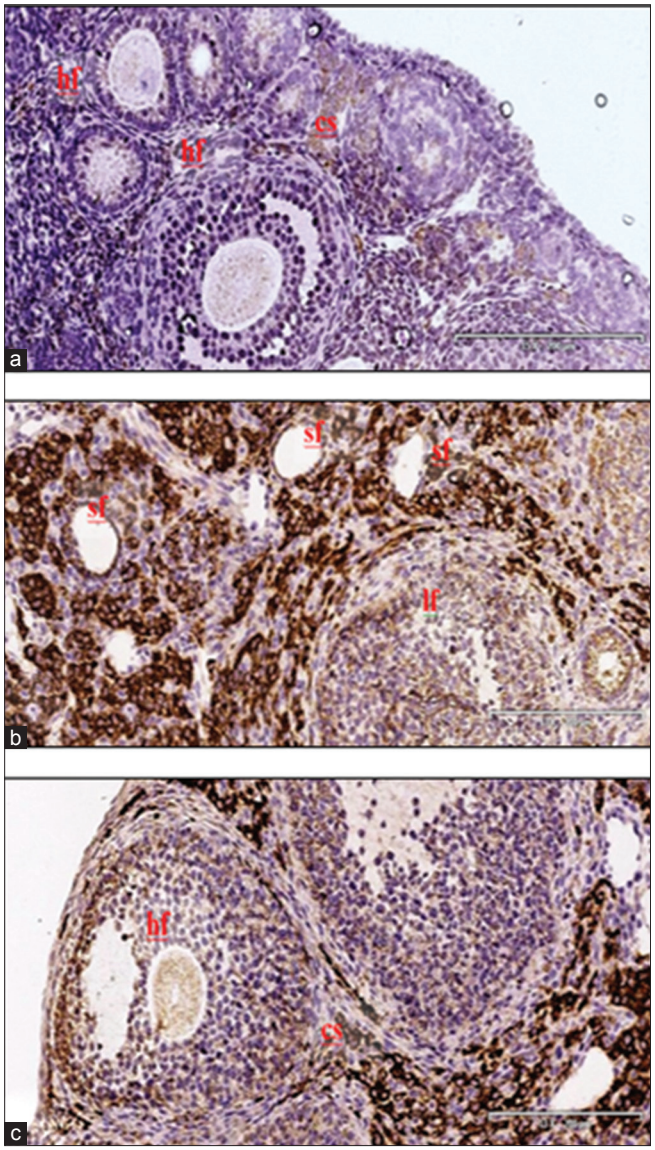

Fig. 5: PR-A expression in the normal saline-treated ovary, there was low PR-A immunostaining in the stromal cells and the healthy follicles (a). PR-A expression in CPA group showing a high number of positive stain in follicles (b). PR-A expression in CPA and T3 groups showing a moderate number of positive stain in the healthy follicles (c). ER: Estrogen receptor, PR: Progesterone receptor

Progesterone receptor (PR)-A immunoreactivity

In CPA treatment, the immunoexpression of PR-A ranged from moderate to high expression. High immunoreactivity of PR-A was observed in certain ovarian components. In the control group, immunoreactivity of PR-A is weak in most of the ovarian structures. Throughout the CPA and T3 treatment, it was found that the PR-A immunoreactivity was less ranging from low to moderate PR-A expression, including a number of oocytes in tertiary and large follicles. These observations were near to that seen in normal saline control, as shown in Fig. 5. In addition, quantitative analysis showed a significant reduction in the percentage of immunoreactivity staining (Fig. 6).

DISCUSSION

Estrogen receptor (ER)-α and progesterone receptor (PR)-A 0ver-expression with CPA therapy

CPA caused high expression of ER-α gene as indicated by the present study. This is a novel finding in the elucidation of the mechanism of infertility in CPA treatment. It is thus proposed that ER-α overexpression may be used as a new biomarker to monitor CPA chemotherapy side effects on ovary impairment. Investigations focusing on the alterations of the ERs

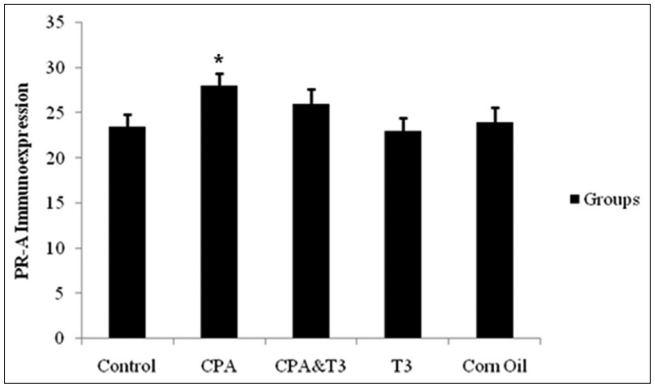

Fig. 6: Qualitative evaluation of PR immunoexpression, CPA and T3 group showed no significant difference in PR expression compared to control. PR: Progesterone receptor

in human disease helped to explain the functions of the ERs in normal and diseased states. This is important for the development of relevant therapeutic strategies [28,29]. In addition, the findings in this study may result in the impairment of ovarian function also raise the possibility of considering CPA as an estrogenic agent. Moreover, a previous study showed that overexpression of ERα is associated with poor disease-free survival [10,30-32]. It has been suggested that estrogenic chemicals may bind to ER-α and block endogenous estrogen from binding to the receptors [33], thus hindering the normal functioning of the receptor as well as cause aberrant gene transcription causing induction of ER-α gene expression in the ovary [5,34]. Thus, disturbances in the delicate balance of ER-α expression in the ovaries may lead to a considerable change in the way that ovaries respond to estrogenic chemicals [35,36]. This elevation in gene expression could be attributed to the increased sensitivity of ER-α ovarian cells to toxicity induced by CPA.

On another hand, previous studies using transgenic mice; ER-α was inducibly overexpressed in reproductive tissues (ER-α over-expressers) and suggested to link with inhibition of female fertility [2,7]. Overexpression of ER-α tends to make the ovary more susceptible to compromise fertility due to an increased number of binding sites for the estrogenic compound. It has also been reported that ER-α overexpression *in vivo* caused an increase in the percentage of atretic antral follicles and decreased fertility [9,36,37]. The focus of the present study was to determine whether overexpression of ERα results in increased susceptibility of ovary function impairment to infertility induced by CPA. It is well-known that CPA caused temporal infertility throughout the depletion of follicle numbers in different stages of folliculogenesis [38-40].

In this study, it was focused on action CPA mediated by overexpression of ER-α in the ovary resulted in ovary function impairment to induce infertility. As previous reports have pointed out, administration of MXC in ER-α overexpressed situation *in vivo* caused decreased fertility compared to control animals [9,37]. The above reports are in agreement with the findings in the current study, which showed increased expression of ER-α in ovarian cells exposed to CPA. Consequently, it could be suggested that the deleterious effects of CPA were mediated by ER-α gene upregulation in an estrogen-independent pathway. This is possibly a new mechanism in CPA-induced ovarian damage. This hypothesis is based on the previous reports by other researchers that ER overexpression had been found to account for reduced fertility females [2,7]. Thus, this study showed aberrations in the transcript level of ER-α occur right across the spectrum of ovarian tissue, which played a key role in fertility. This might be the reason attributable to the adverse effect exerted by CPA administration.

In term of PR-A over-expression, this current study had shed sight on the disturbance of *PR-A* transcript level in CPA chemotherapy. Accordingly, it was found CPA treatment resulted in upregulation of PR-A gene in the ovary. This probably linked with the same as found in a study done by Fleisch *et al*. and Maedeh *et al*. using transgenic PR-A mice model where aberration in the relative expression of the two isoforms of PR lead to multiple and complex abnormalities in endometrial physiology as based on PR-A over-expression in the mammary glands of transgenic mice strongly suggest a high predisposition to the development of tumors [14,43,44]. Therefore, this study suggested that long-term treatment with chemotherapy affects PR-A expression balance in normal cells and it involves different pathways. However, an earlier report indicated that there was notable evidence in cisplatin-treated ovarian cancer cell line upregulating PR-A through the PI3K inhibitor to cisplatin-based chemotherapy [45,46].

The current finding suggested that the ability of CPA to induce upregulation of PR-A is possibly indicating a novel mechanism that shows it occurs either through PR-A dependent or independent hormone transcriptional action. It could be proposed that PR-A overexpression could reflect the reduction of ovulation and abnormal morphometric features of the ovary. Thus, the critical role of PR-A in hindering folliculogenesis must be assessed *in vivo*. The current study is the first to demonstrate in an *in vivo* model that PR-A plays an important role in compromised fertility with chemotherapy.

Estrogen receptor (ER)-α and progesterone receptor (PR)-A Immunoreactivity in CPA-exposed ovary

The study found increased immunoreactivity of ER-α and PR-A protein in ovarian tissue in CPA-treated mice compared to control. This may be critical to explain the further mechanism of ovary function impairment with CPA treatment. The location of ER-α and PR-A proteins was observed in the cytoplasm of the cells [47-49]. Hence, these observations in the present study, that the immuno-expression of ERα and PR-A in mice ovarian cells was localized in the cytoplasm of follicular and luteal cells, were consistent with previous reports. Ruijin *et al*. demonstrated that the expression of both PR isoforms in mouse granulosa cells was rapidly upregulated by hCG treatment and dramatically downregulated when the granulosa cells were undergoing luteinization, also with cystic ovarian disease exhibited upregulation of ER protein expression with no significant change in PR expression protein compared to normal ovaries, it has been reported that ER-α was highly expressed in cystic follicles compared to other follicular categories [16]. This work investigated the immunoreactivity of PR-A in mice ovarian cells to determine the quantitative evaluation of aberrant signaling of PR-A pathway following treatment by CPA.

It is intriguing to speculate that such components of ER-α and PR-A pathway might determine the differential regulatory patterns of ER-α and PR-A signaling and thus, defining new emerging properties that could account for the different estrogen and progesterone-dependent or independent outcomes. That was clarifying the roles of post-translational modifications of emerging up regulation proteins, which could be expected to be clear evidence in contribution to the disorder of ERα and PR-A responsiveness. Eventually, this study explored the possible role of T3 in modulating PR-A expression when administered along with the CPA.

As this research confers a profound understanding of the immunoreactivity aberrations of the ERα and PR-A might emerge target components for therapeutic strategies for the treatment of chemotherapy-compromised fertility. As corresponding to the previous study which showed that ERα overexpression was observed on SH-SY5Y cells under chronic minimal peroxide (CMP) treatment; CMP-induced ROS generation Chaddwick *et al*., which caused stress. It was indicated that pre-treatment of cells with the neuronal degenerative disorder before CMP-induced stress prevented CMP-induced upregulation of ERα [52]. Ikeda *et al*. identified the estrogen-responsive genes based on the DNA binding properties of ERs and proposed that the diversity of ER regulatory agents reflected the biologic importance of precisely coordinating the extent of ER expression and suggested that

investigators working in a number of fields can contribute relevant molecular knowledge that may result in the clinical use of novel molecular determinants of ER expression.

In this study, the findings showed high immune-staining of ER and PR proteins in the stroma, and atretic follicles in different developmental stages, especially small follicles in the CPA treated group compared to control. CPA exerts sub-cytotoxic chronic stress, which is possible to result in overexpression of ER and PR involved in a large area in cytoplasmic ovarian cells. Thus, in depressed ovarian cells, upregulation cytoplasmic estrogen and PRs could be mediated the deleterious effects of CPA in the mice ovary.

Estrogen receptor (ER)-α and progesterone receptor (PR)-A assessment transcriptional level and localization in CPA + T3-treated ovary

In this study, we examine in detail the molecular targets of tocotrienols and their roles in CPA-comprised fertility at both (experimental) preclinical and clinical levels. The findings in this study have provided insights concerning consistent changes in genes and protein expression of ER and PR that are linked with fertility dysfunction in CPA-exposed ovary. ER-α and PR-A over-expression expands our knowledge of the events that occur in the ovary during ovulation and luteinization; thus, possibly identifying novel gene targets of T3. T3 modulation of ER over-expression in ovary exposed to CPA might be attributed by T3 antioxidant properties which were also seen in a study done by Barbati *et al*. [52]. They suggested a potential implication of ROS generation in the regulation of mERα by pre-treating SH-SY5Y cells with N-acetyl-L-cysteine, a known chemical that exert antioxidant activity by acting as a thiol group supplier [54]. Thereby, it is notable that the mechanism of ERα which is responsible for regulating the expression of many genes to control physiological processes as that cellular responsiveness or susceptibility to estrogen [3-55]. Selective ER modulators (SERMs) known as nonsteroidal compounds, which modulated ERα activities throughout binding to ER and induced change in the biological activity of the receptor depending on the tissue type [56]. Several studies highlight the emerging role of the many common SERMs which are composed of synthetic non-steroidal agents such as tamoxifen and raloxifene [57-59]. Remarkably, coadministration of T3 with CPA resulted in modulation of ERα transcript level in the ovary; thus, T3 may be considered as an alternative substance in situations requiring such intervention. SERMs have been shown to have a distinct therapeutic application in medicine; particularly, in the treatment of breast cancer. The actions of T3 as a nonsteroidal anti-estrogens were clearly evident in this study.

Although the variety of steroidal PR modulators approved for the clinical use, the interactions of those modulators with other steroid receptors showing some unwanted side effects [60]. This study established that new development of PR modulators with an emphasis on natural compounds (T3) as certain PR modulators might be considered as compounds therapeutic options, it was reasonable to expect that the development of a chemoprotective agent such as T3 would lead to interest in whether naturally occurring compounds have similar effects to steroidal anti-estrogenic compounds. It is worthy-mention that T3 as natural-based PR antagonists and selective PR modulators are being evaluated clinically in improving fertility conditions.

The stain immunoreactivity analysis of ER and PR done in the present study indicated that T3 is capable to regulate ER and PR expression when under the influence of CPA. This observation may indicate that T3 inhibits translational activation to ER and PR proteins in stressed ovarian cells by certain chemotherapy. The delineation roles of genes and proteins were expressed in both assays RT-PCR and IHC could serve as potential targets for future fertility preservation and chemoprotection research exploitation. In gene study observation, T3 downregulate ER and PR genes overexpression by anti-estrogenic and anti-progesterone activity in CPA-stressed ovarian cells in mice. Subsequently, the current protein study reconfirms gene expressions throughout T3 regulate the intensity of abbreviation intensity of ER and PR proteins. Collectively, these data provide new insights into the intracellular mechanisms involved in mediating the regulation of ER and PR of combined CPA and T3 supplementation, through transcriptional and post-transcriptional signaling pathway, respectively. These findings also suggest that combined chronic duration CPA and T3 supplementation might provide significant fertility restoration during chemotherapy to women with cancer, while avoiding infertility, that is associated with long-term chemotherapy treatment strategies. In the quest to postulate the mechanism by which T3 inhibits the overexpression percentage of ER and PR proteins in the normal ovary during chemotherapy exposure, there is no study to date which is carried out to regulate ER and PR proteins in CPA-exposed ovary. We found that T3 is feasibly involved in proposed new molecular mechanisms as anti-estrogen and anti-progesterone effect in normal ovarian cells. Overall, T3 has previously been documented for their antioxidant and anticancer properties by different signaling pathways in various models whether cancer or normal cells *in vivo* and *in vitro*. However, the activity of T3 might not be confined to antitumor or antioxidant effects only. Furthermore, T3 has been reported to be involved in the modification of various cell-signaling pathways as mention by Aggarwal *et al*., with various effects reported is diabetes mellitus, cardiovascular, and neurological diseases. It is possible to suggest that T3 may exhibit anti-estrogenic and anti-progesterone effects by preventing the ER and PR proteins abbreviation to sustain fertility after chemotherapy treatment. The ability of T3 to prevent and treat cancers in animal studies is of potential importance for chemoprotective, but the impaction on maintenance fertility remains unresolved in CPA-compromised fertility whether the action directly on effected ovarian cells or indirectly through systemic interaction. This study found that chronic CPA administration caused an increase in ERα and PR-A expressions, with increase in the proteins of these two receptors in the cytoplasm of follicular and stromal cells. This is an original finding in chronic CPA exposure on the ovary, not previously reported in literature. T3 similarly regulates the expression of ER-α and PR-A at the genetic and cellular level. This study opens the gate to the idea that T3 is an essential candidate for the preservation of ovarian function in subjects undergoing chemotherapy.

AUTHORS' CONTRIBUTIONS

All authors who are contributed to this transcript, declare that there is no competing interest as well as, on behalf of all them, the corresponding author is responsible for this declaration.

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