T_3 preserves ovarian granulosa cells from chemotherapy-induced apoptosis

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Abstract

Infertility is a dramatic and frequent side effect in women who are undergoing chemotherapy. Actual strategies are mainly focused on oocyte cryopreservation, but this is not always a suitable option. Considering the key role that granulosa cells play in follicle life, we studied whether thyroid hormone 3,5,3′-triiodothyronine (T_3) protects rat ovarian granulosa cells from chemotherapy-induced apoptosis. To this aim, a cell line was established from fresh isolated rat granulosa cells and named rGROV. Cells were exposed to paclitaxel (PTX) and T_3, and apoptosis, cell viability, and cell cycle distribution were analyzed under different conditions. First, the integrity of the steroidogenic pathway was demonstrated, and the presence of thyroid receptors, transporters, and deiodinases was confirmed by quantitative PCR. Cells were then exposed to PTX alone or contemporary to T_3. MTT and TUNEL assays revealed that while there was a relevant percentage of dying cells when exposed to PTX (40–60%), the percentage was sensibly reduced (20–30%) in favor of living cells if T_3 was present. Cell cycle analysis showed that cells exposed to PTX alone were first collected in G2 and then died by apoptosis; on the other hand, the T_3 granted the cells to cycle regularly and survive PTX insult. In addition, western blot and FCM analyses confirmed that caspases activation, casp 3 and Bax, were downregulated by T_3 and that Bcl2 and cyclins A and B together with cdk1 were upregulated by T_3. In conclusion, we demonstrated that thyroid hormone T_3 can counteract the lethal effect of taxol on granulosa cells.

Journal of Endocrinology (2012) **215,** 281–289

Introduction

Exposure to chemotherapy causes various adverse effects on the ovaries, including premature ovarian failure and infertility. Over the last decades, great attempts have been made to minimize the toxic effects of chemotherapy on the ovaries (Meirow & Nugent 2001, Ben-Aharon *et al.* 2010, Tomao *et al.* 2010). Moreover, cancer-related infertility is still considered a marginal aspect of the quality of life in cancer patients. Adjuvant chemotherapy causes a progressive dose-related depletion of ovarian follicles and granulosa cells leading to oligoamenorrhea and subsequent chemotherapy-induced amenorrhea (CIA). This condition can be strongly invalidating when occurring in premenopausal women, being related to a rapid depletion of the oocyte reserve, mediated by the apoptotic cell death and ovarian atrophy with disappearance of primordial follicles. The incidence of CIA varies with the type, dosage, and schedule of chemotherapy and is directly related to age (Padmanabhan *et al.* 1986, Swain *et al.* 2010). Reversible amenorrhea will result when maturing follicles are destroyed, differently when all primordial follicles are destroyed, permanent amenorrhea, or premature ovarian failure will happen. Recent data suggest that the major concern of patients receiving chemotherapy is that of premature menopause and infertility (Maltaris *et al.* 2008, Kim *et al.* 2011). This is becoming a more pressing issue as, over the last 30 years, there has been a trend toward delaying child bearing. Although loss of fertility is an important issue for young cancer survivors, there is often little discussion about fertility preservation before initiation of adjuvant therapy. The impact of infertility on the quality of life of the patient who survives breast cancer explains the increase in the demand for fertility preservation techniques. Neither embryo nor oocyte cryopreservation is a practical option for women who cannot delay cancer treatment. These aspects are more relevant in breast cancer patients, mainly in the early phase of the disease, due to the high probability to retain reproductive possibilities also after aggressive and integrated therapies. In addition, breast cancer is the most common cancer in fertile premenopausal women and constitutes

DOI: 10.1530/JOE-12-0153
0022–0795/12/0215–281
Online version via http://www.endocrinology-journals.org

an emerging reproductive problem for three different reasons: the increasing rate of young patients in fertile age, the lack of specific guidelines for gonadic failure prevention after chemotherapy, and the conflicting and unresolved debates about the prognosis and risks of pregnancy occurring in women after breast cancer (De la Haba-Rodriguez & Calderay 2010).

Chemotherapeutic agents can be grouped depending on the severity of their impact on ovarian reserve. Taxanes are classified as high risk, provoking amenorrhea in 62–83% of cases (Najafi *et al*. 2011, Peréz-Fidalgo *et al*. 2010). Paclitaxel (PTX) and mitoxantrone, frequently used drugs for the treatment of advanced breast cancer, are microtubule-stabilizing agents, which kill malignant cells by inducing both cellular necrosis and apoptosis. They exert their cytotoxic effects via different mechanisms, including the inhibition of microtubule reorganization during interphase followed by cell cycle arrest, cell cycle nonspecific interaction with DNA and DNA cleavage, and the activation of several caspases (Blagosklonny & Fojo 1999). It has been demonstrated that the cytotoxic effects of PTX are due to apoptosis (Georgadis *et al*. 1997, Fan 1999, Stumm *et al*. 2004), being crucial its action on Bcl2 and consequently on Bax.

Apoptosis has been identified as a key event in the depletion of oocytes and follicles in both aged and chemotherapy-treated women (Fujino *et al*. 1996, Perez *et al*. 2007*a*,*b*). The main compromised compartment is the granulosa cell layer. It is well known, in fact, that ovarian granulosa cells play a key physiological role in supporting the development and selection of the ovarian follicle by controlling oocyte maturation and by producing the steroid hormones that are critical for maintenance of the ovarian cycle (Richards & Hedin 1988). Indeed, follicular atresia is initiated by apoptosis of the granulosa cells (Amsterdam & Selvaraj 1997).

Thyroid hormones play important roles in proliferation and apoptosis, as reviewed by Oetting & Yen (2007). However, little information is available concerning their role in proliferation and apoptosis of granulosa cells, although a link between thyroid and ovaries has widely been observed (Stradtman 1993, Wakim *et al*. 1995, Doufas & Mastorakos 2000). Our previous work has demonstrated how the granulosa cell population can be considered a thyroid hormone target, being as how their survival is induced by 3,5,3′-triiodothyronine (T_3) under specific circumstances via cell cycle and metabolism regulation (Verga Falzacappa *et al*. 2009), strengthening our consideration of this hormone as a survival factor. In addition, a recent work has evidenced an antiapoptotic action on rat granulosa cells exerted by T_3 together with FSH (Zhang *et al*. 2011). The purpose of this study was to elucidate the effect of T_3 on granulosa cell proliferation and apoptosis after treatments with chemotherapeutic agents, demonstrating that T_3 is able to protect rat ovarian granulosa cells from chemotherapy-induced apoptosis.

Materials and Methods

Chemicals

T_3 was obtained from Sigma–Aldrich. PTX was purchased from Teva Pharmaceutical Industries – Teva Italia (Milan, Italy).

Animals

Female Wistar rats (about 18 days old) were used as cell donors. The animals had free access to tap water and pelleted food throughout the course of the study. The Local Animal Ethics Committee approved all experiments.

Isolation and culture of rat granulosa cells

Ovaries were isolated from female Wistar rats by standard surgical procurement followed by needle puncture and follicular fluid collection. In brief, animals anesthetized with ketamine 70 mg/kg + domitor 0·5 mg/kg injected i.p. were killed by CO_2 inhalation. For the exposure of the whole ovaries, the abdominal wall was opened via a midline incision. The whole ovaries were excised and transferred to a Petri capsule containing DMEM:F12 (Lonza, Basel, Switzerland); the visible follicles were punctured with a 25 gauge needle and the follicular fluid was collected. The fluid was centrifuged at 250 ***g*** for 5 min, and the pellet was resuspended in DMEM:F12. rGROV cells were then cultured in DMEM:F12 supplemented with 10% fetal bovine serum (FBS), L-glutamine 2 mmol/l, and penicillin 100 μg/ml–streptomycin 50 μg/ml in 24 multiwells (BD Biosciences, San Jose, CA, USA), expanded, and freezed in FBS containing DMSO 10%. Then cells were thawed and cultured for various cycles at 37 °C in a humidified atmosphere of 5% CO_2.

PTX or vehicle alone was added to culture medium 24 h after seeding. The hormonal treatment was performed by adding T_3 or vehicle alone to culture medium cells contemporary to PTX. Every 24 h, fresh aliquots of T_3 (10^{-3} M) were added to culture medium in all the experiments.

RNA isolation and quantitative RT-PCR analysis

Total cellular RNA was isolated from rGROV cells using SV Total RNA Isolation Kit (Promega), according to the manufacturer's instructions. RNA (1 μg) was subjected to RT using a cDNA synthesis kit OmniScript (Qiagen). cDNA was amplified to determine expression by real-time PCR as described previously (Canettieri *et al*. 2008) using the primer pairs listed in Table 1. All primers were synthesized by MWG Oligo Synthesis Report (Eurofins MWG Operon, Edersberg, Germany).

Cell growth analysis

Cell growth was analyzed by determining Trypan Blue-negative cell number in a Thomas's hematocytometer. PTX 7 μM was added together with the hormone. At 24, 48,

Table 1 qPCR primers

rFSHR	F: TGGATTTGGAGACCTGGAGAA
	R: AATTTCATGCAACTTGGGTAGGTT
rTHRA1	F: AGACGAGCAGTGTGTCGTGTGT
	R: CTGGATTGTACGGCGAAAGAA
rStaR	F: GCAAAAGGCCTTGGGCATA
	R: CACACCTGGCACCACCTTACT
rCyp17a1	F: CAGTGATCATCGGCCACTATCA
	R: GACAAGAGGCTTTGAGTCACCAT
rHsd3b	F: ATCCACACCGCTGCTGTCA
	R: TACCGGCCTCCAATAGGTTCT
rp450scc	F: CCTATTCCGCTTTGCCTTTG
	R: GATGAACCGCTGGGACTCA
rMCt8	F: GCCAAGTGGAGTTCCAAGCA
	R: CAGCCCAAACGGTCAGTGA
rDIO1	F: GCCTCCACAGCTGACTTCCT
	R: CTGCCTGATGTCCACGTTGT
rDIO2	F: CTCCTAGACGCCTACAAACAGGTT
	R: AGGCACAATTGTTACCTGCTTCA
rDIO3	F: CCCGTTGGTGCTCAATTTTG
	R: GTCAACGTCGCGCTGGTACT
rCyp19a	F: GGTCCGCCCTTTCTTCATG
	R: ACCCAGCCTGTCCAAATGC
rTHRB	F: AGGACCCAGCATGACTACTAACCT
	R: CCTGGCCTCGGTCTGGAT

and 72 h of continuous exposure, viable cells were harvested and counted. Cell number was determined, and data presented as mean ± S.D.

MTT assay

Cell viability in the apoptosis studies was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Promega). Cells were plated in 96 multiwells and treated as described previously. A solution of a tetrazolium salt was added to the culture medium and, after 3 h, the metabolic formazan product was solubilized in an organic solution. After 1 h of solubilization, the absorbances at 570 and 630 nm were recorded using a 96-well plate reader.

Cell cycle and apoptosis analyses

The cell cycle and apoptosis were evaluated using PI staining and flow cytometry (FCM) analysis. Adherent cells alone for cell cycle or floating and adherent cells for apoptosis were harvested, fixed in 70% ethanol, and, after removing alcoholic fixative, stained with a solution containing 50 μg/ml PI (MP Biomedicals, Santa Ana, CA, USA) and 75 KU/ml RNase (Sigma–Aldrich) in PBS 1× for 30 min at room temperature in the dark.

Samples were then measured using a FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA). Twenty thousand events per sample were acquired. The percentages of the cell cycle distribution were estimated on linear PI histograms using the MODFIT Software (Verity Software House, Topsham, ME, USA). Scattering light was used to denote the cell size.

TUNEL assay

The induction of apoptosis was evaluated by TUNEL assay (Roche Diagnostics) using FCM. Briefly, trypsinized adherent cells and floating cells were pooled, washed once with PBS (Lonza), and fixed in 4% paraformaldehyde (Sigma–Aldrich) for 30 min. Samples were then permeabilized in 0·1% Triton X-100 (Sigma–Aldrich) in 0·1% sodium citrate (Sigma–Aldrich) and washed with PBS (Lonza). Each sample was incubated in 50 ml reaction mixture (terminal deoxynucleotidyl transferase, TdT, and fluorescein-UTP) for 1 h at 37 °C, washed in PBS (Lonza), and then measured by FCM at the indicated hours.

Active caspase assay

The activation of caspases has been evaluated by flow cytometry, using the CaspGlow Red Active Caspase Staining Kit (MBL, 4-5-3 Sakae, Naka-ku, Nagoya, Japan). Briefly, cells were cultured and exposed to PTX and T_3 alone or combined as described previously. After 48 h of culture, supernatant and cells were collected, and the centrifuged pellets were stained with Red-VAD-FMK as indicated by the manufacturer, 1 h, 37 °C, 5% CO_2. Then cells were resuspended in the provided buffer and samples were analyzed by FCM using the FL-2 channel.

Western blot

Cells were lysed and the total cellular protein content was measured using the Bradford method (Bio-Rad). Total extracts (40 μg) per sample were loaded onto an 8–10–12% SDS–polyacrylamide gel, electrophoresed, and then blotted onto nitrocellulose membranes (Bio-Rad). Membranes were incubated 16 h at 4 °C with TRA/B (1:500; Santa Cruz Biotechnology, Inc.), FSHR (1:400; Santa Cruz Biotechnology, Inc.), cyclin A (1:500; Santa Cruz Biotechnology, Inc.), cyclin B (1:500; Santa Cruz Biotechnology, Inc.), cdk1 (1:500; Santa Cruz Biotechnology, Inc.), Bax (1:500; BD Pharmingen), caspase 3 (1:500; Santa Cruz Biotechnology, Inc.), Bcl2 (1:500; BD Pharmingen), and 1 h at RT with β-actin (1:1000; Sigma). After three washes, the membranes were incubated with the secondary HRP antibodies (anti-mouse and anti-rabbit; Sigma) 1:4000 for 45′ at RT. Immunoreactivity was visualized by the ECL immunodetection system (Amersham Corp.) following the manufacturer's instruction. The relative band intensity was evaluated by densitometric analysis (TotalLab, Nonlinear Dynamics, Newcastle, UK) and normalized to β-actin.

Hormonal assays

Culture media were collected. The secreted 17β-estradiol (E_2) content was measured using a competitive chemiluminescent immunoassay (Roche) and normalized for the milligram of total protein obtained from cells as described

previously. To test the ability of the cells to secrete E_2 in response to testosterone, the cells were shifted in a serum-free medium and exposed to testosterone 10 uM for the last 6 h of a 48 h cell culture. Then E_2 was measured.

Statistical analysis

The data were presented as mean ± S.D. A comparison of the individual treatment was conducted by Student's *t*-test or, if there were more than two groups, by one-way ANOVA, followed by Dunnett or Tukey's *post hoc* analyses.

Results

Cell line establishment and characterization

Rat granulosa cells were obtained from follicle injection as described in the Materials and Methods section. Cells were then cultured and passed through various freezing and thawing passages, maintaining their typical morphology and aspect, and the cell line rGROV has been established (Fig. 1A). To ascertain that the main features of granulosa cells were not altered, steroidogenesis key genes have been analyzed by qRT-PCR and compared with rat follicles. As shown in Fig. 1B, the amplicons for cyp19, cyp17a, p450 scc, StAR, 3BHSD, and FSHR were present. When compared with rat follicles, the resultant genes were lower (about 1/5th ÷ 1/10th); this in accordance with the E_2 production. To consider the gene expression and hormone production levels as significant, they were also compared with non-steroidogenic tissues (thyroid) and resulted much higher (about 20 times). The receptor for FSH, which is usually lost during cell culture, was found to be relievable even at the protein level. To confirm that the steroidogenic pathway was also functioning, the ability of the cells to synthesize and secrete E_2 was analyzed at different time points. The most representative 48-h point is shown in Fig. 1C; as evident, the hormone was present in the cell-collected media, in a quantity lower when compared with follicles, but significantly higher when compared with nonsteroidogenic tissues and media alone. In addition, the ability of the cells to secrete E_2 in response to testosterone alone has been successfully tested (Fig. 1D), and the resultant rGROV cells were suitable for our following experiments.

Thyroid hormone receptors, transporters, and deiodinases are expressed in rGROV cells

To assess that our cell model was appropriate for thyroid hormone action studies, the key molecules in the 'thyroid hormone machinery' were searched for both mRNAs and

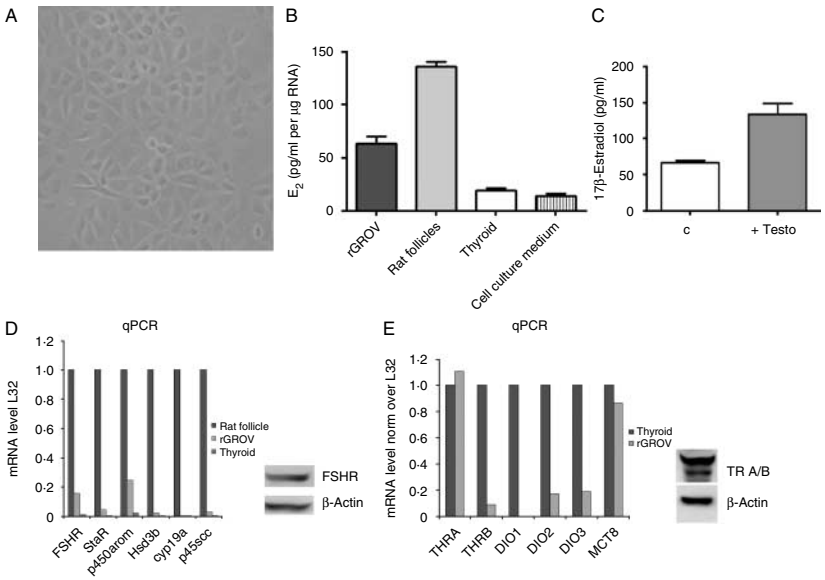


Figure 1 Rat granulosa cells rGROV. (A) Rat granulosa cells were isolated as described. Optical microscopy (40×) photos were taken to show their morphology. (B) 17BE was measured in media collected at 24, 48, and 72 h from three different cultures of rGROV, rat follicles, and thyroid cells and measured by chemoluminescence, as described in the Materials and Methods section. Complete cell culture media were also measured. The concentrations for the 48 h time point are shown in the histogram. (C) 17BE was measured in media collected at 48 h of complete medium cell culture (c) and serum-free testosterone 10 µM medium 6-h cell culture (+Testo). Concentrations are shown in the histogram. (D and E) qRT-PCR: total RNA was extracted from cells in three different culture sets and qRT-PCR was performed as described in the Materials and Methods section. Results represent the mean ± S.E.M. of three separate experiments.

protein levels. As shown in Fig. 1D, qRT-PCR showed that the cells express levels of the thyroid receptors α and β, the T_3 transporter MCT8, and the selenoenzymes desiodases DIO2 and DIO3, at lower levels than a control thyroid cell line, but easily detectable and sometimes even comparable. In addition, TRα/β presence was confirmed by western blot analysis.

T_3 grants cell cycle progression against PTX

It is well known that PTX can alter cell cycle in almost every cell (Blagosklonny & Fojo 1999). In particular, its ability to induce at first a G2 arrest that can be resolved in apoptotic death has been diffusively proved. Given these considerations, our first goal was to assess whether T_3 could contrast PTX action on cell cycle and see where this eventual action could lead. To this aim, first cell cycle analysis was performed through flow cytometry on adherent cells at 6, 12, 24, and 48 h. As shown in Fig. 2A, cell cycle analysis showed that PTX produced the characteristic G2 block already at 12 h (20%), which increased at 24 h up to 35%. On the other hand, when T_3 was administered contemporary to PTX, the cells were well distributed through the various cell cycle phases. In particular, the G2 block was not relevant even at 24 h (15%) and a transient S-phase accumulation was evident (30%), thus indicating that cells were able to overcome the PTX-induced DNA damage and cycle regularly. Otherwise, at 48 h, the difference in G2 population was totally overcome.

T_3 can protect rGROV cells from PTX apoptosis thus preserving cell viability

PI staining and TUNEL assay were then performed to investigate apoptosis. To this aim, adherent and floating cells were pooled and either PI staining or TUNEL assay was then performed as described in the Materials and Methods section. As shown in panel B, even though a significant amount of apoptosis was revealed in the PTX cells (20%, 24 h and 60%, 48 h), the PTX+T_3 sample did not have such a massive amount of apoptotic cells (13%, 24 h and 20%, 48 h). The hormone-treated cells in fact, despite the PTX presence, were mainly vital and regularly cycling. The presence of T_3 alone was also tested and, as shown, the hormone-treated cells cycle regularly without significant alteration in phase distribution, except of the 48-h point in which it is evident that cells have been speeded up by T_3. Taking together the cell cycle and the apoptosis panels, data showed how T_3 can counteract the ability of PTX to induce G2 accumulation at first, consequently counteracting the strong apoptosis induced by PTX at 48 h. TUNEL assay was performed also by immunofluorescence on rGROV cells exposed to PTX alone or contemporary to T_3, for 24 and 48 h (data not shown), and the results were consistent with the FCM analysis. Coherently, cell metabolism analyzed by MTT (Fig. 3B) confirmed that the presence of T_3 could decrease the percentage of nonviable cells by about 10% at 24 h and 30% at 48 h, ensuring cell viability to be similar to control cells.

A

		G1	S	G2
6	Control	75±3	13±2	12±2
	T_3	73±2	14±2	13±1
	PTX	74±2	18±2	8±1
	PTX+T_3	75±3	11±3	14±2
12	Control	77±2	18±3	5±2
	T_3	70±3	25±3	8±2
	PTX	64±3	16±3	20±2
	PTX+T_3	64±3	26±2	10±2
24	Control	75±3	13±4	12±1
	T_3	69±2	26±3	5±1
	PTX	54±3	11±2	35±3
	PTX+T_3	55±3	30±2	15±3
48	Control	71±2	25±3	4±2
	T_3	85±1	6±3	9±1
	PTX	80±2	10±2	10±3
	PTX+T_3	85±2	13±3	2±2

Levels of significance

12 h *P*<0·0001 PTX vs control (S and G2)
24 h *P*>0·0001 T_3 vs control, PTX+T3 vs Ptx (S and G2)
48 h *P*<0·0001 T_3, PTX, PTX +T_3 vs control (S)
P<0·005 Ptx+T_3 vs PTX ; PTX vs control (G2)

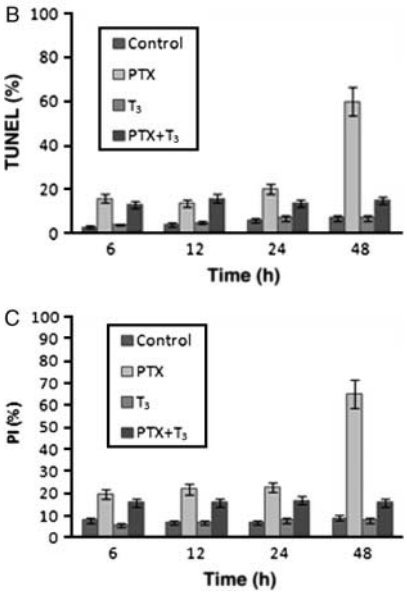
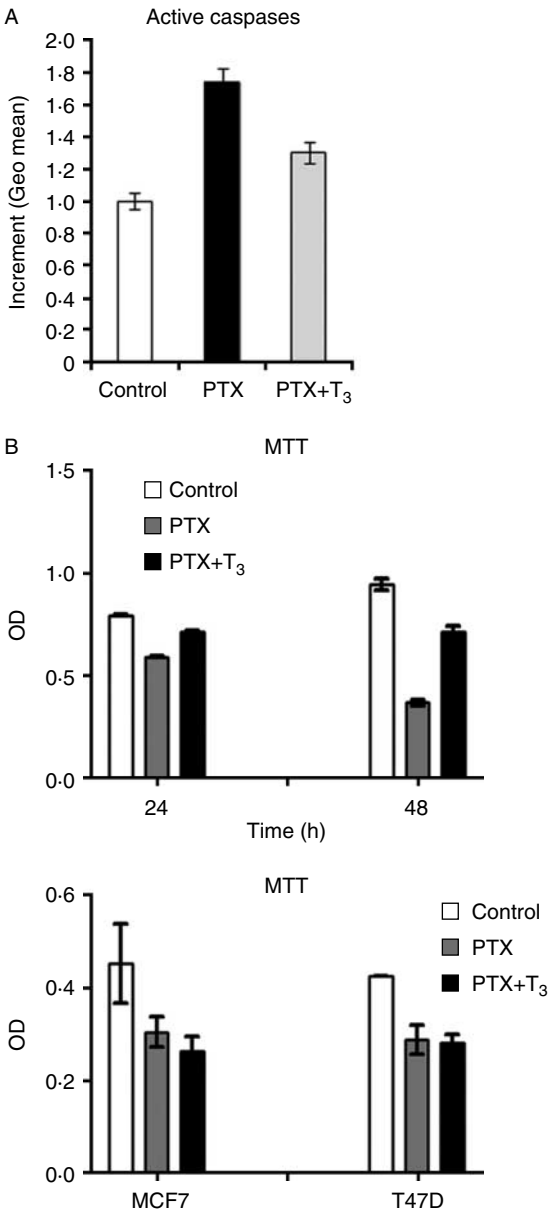


Figure 2 Flow cytometry. (A) Cells were exposed to T_3 or PTX alone or concurrently to PTX and T_3 for 6, 12, 24, and 48 h and then processed as described in the Materials and Methods section for PI staining. The distribution of the cells in the various cell cycle phases is indicated in Panel A. All the data are presented as mean±S.D. and are the results of at least three individual experiments. (B) Percentage of apoptotic cells in control, PTX-treated, T_3-treated PTX+T_3 cells by using TUNEL (left panel) or PI (right panel) at the indicated hours (*x* axis). All the data are presented as mean±S.D., and are the results of at least three independent experiments. Data were analyzed by one-way ANOVA and Tukey's *post hoc* test. The pairing was significantly effective ($P<0·0001$) and the means were significantly different.

As our preface is to determine whether T_3 can preserve ovarian cells from a chemotherapy-induced apoptosis in a mammalian cancer treatment regimen, it is an essential condition that T_3 could not protect the mammalian cancer cells as well. To exclude this chance, two different human mammalian cancer cells, namely T47D and MCF7, were exposed to the same experimental condition as rGROV, and cell viability was tested, too. As shown, in both cases, the ability of PTX to affect cell viability was not overcome by T_3, thus granting the chemotherapy efficacy on the cancer cells.



T_3 prevents caspases activation by PTX

It is well known that caspase activation is one of the key events in apoptosis. To better analyze the T_3 antiapoptotic effect on rGROV cells, caspase activation was analyzed by FCM as described in the Materials and Methods section. As shown, when active caspases were measured by FCM, while in the PTX-treated sample there was a positivity of the FL-2 signal, the presence of T_3 maintained the caspase activation at a lower level, comparable to the control one, in accordance with the TUNEL data.

T_3 regulates cell cycle molecule expression

To investigate deeper into the mechanisms underlying the T_3 ability to counteract PTX action on the cell cycle, the G2 cyclins A and B1, and their partner CDK1 levels were analyzed by western blot (Fig. 4A). In accordance with the cell cycle data, the cell cycle molecules were strongly downregulated by PTX, mainly CYCA1 and CDK1, but the presence of the hormone was able to ensure that the said molecule levels were as high as control, thus allowing cell cycling. To investigate the mechanism of this regulation, cell cycle molecule expression was also analyzed by RT-PCR at earlier time points (data not shown), and a trend coherent with the proteic one was observed, thus suggesting that T_3 may act at different regulation levels.

T_3 regulates apoptotic factors

To moreover confirm the ability of T_3 to regulate the apoptotic process, the proapoptotic factors Bax and Casp 3 and the antiapoptotic factor Bcl2 levels were analyzed by western blot (Fig. 4B). As shown, PTX could sensibly upregulate the expression of both the proapoptotic factors and downregulate the expression of the antiapoptotic Bcl2. However, the presence of T_3 maintained the levels of both Bax and active Casp 3 as low as the control ones, and the Bcl2 levels as high as the control, which is consistent with T_3 antiapoptotic action.

Figure 3 Caspases and vitality analyses. Cells were exposed to PTX alone or contemporary to PTX and T_3 for 24 and 48 h. (A) Active caspases assay: floating and adherent pooled cells were stained for active caspases and the fluorescence positivity was measured by FCM. Geo mean obtained in the FL2 channel was compared with control, the increment is shown in the histogram, as mean ± S.D., and is the result of at least five independent experiments. A comparison of the individual treatment was conducted using one-way ANOVA and Tukey's *post hoc* test. The pairing was significantly effective ($P<0{\cdot}0001$) and the means were significantly different ($P<0{\cdot}0001$ PTX vs control and $P<0{\cdot}005$ PTX + T_3 vs PTX). (B) MTT assays were performed as described in the Materials and Methods section. Data are presented as percentage of the OD (570 nm) control value (taken as 100%) on the y axis, as mean ± S.D., and are the results of at least five independent experiments. A comparison of the individual treatment was conducted by using one-way ANOVA and Tukey's *post hoc* test. The pairing was significantly effective ($P<0{\cdot}0001$) and the means were significantly different ($P<0{\cdot}0001$ PTX vs control 24 h; $P<0{\cdot}0001$ PTX vs control 48 h).

Discussion

Although CIA represents a severe side effect of cancer treatment, the concept of intervention on the ovary to protect ovarian follicular and steroidogenic function from chemotoxicity remains unpopular. Very few models of protection of the ovary from chemotherapy toxicity have been reported in the literature (Morita *et al.* 2000, Perez *et al.* 2007*a*,*b*, Yeh *et al.* 2008, Gonfloni *et al.* 2009, Salih 2011). In this context, one of the major compartments deserving such protection is represented by granulosa cells, which are mainly responsible for hormone production and thus follicular progression. Those cells did indeed initiate the death process that is responsible for atresia. Protection of granulosa cells by direct intervention on the survival pathways might constitute another means for chemoprotection of the ovary.

Our previous study on granulosa cancer cells COV 434 (Verga falzacappa *et al.* 2009) together with the data from Zhang *et al.* (2011) gave strong indications of the ability of T_3 to counteract apoptosis in those cells. Nonetheless, growing evidence indicates that the thyroid hormones might induce cell survival against various insults, both physiological and pharmacological, in different cellular systems (Laoag-Fernandez *et al.* 2004, Sukocheva & Carpenter 2006, Verga Falzacappa *et al.* 2006).

We investigated the effects of T_3 treatment on granulosa cell survival after chemotherapy treatment, focusing on the molecular aspects of these effects. To this aim, we established a new nontumoral cell line named rGROV cells. Granulosa cells were isolated from follicles obtained by a 18-day-old rat females and settled for cell culture. Their ability to secrete E_2 and the good expression of the main steroidogenic genes were tested. This study demonstrates for the first time that thyroid hormone T_3 is able to block the cytotoxic effect of taxanes on rGROV granulosa cells, granting the cells to cycle regularly, escaping apoptosis.

As taxanes represent a highly affective agent concerning ovarian function and are often used in breast cancer treatment, we utilized PTX for our study. The ppc of PTX for breast cancer treatment is 175 mg/m^2, corresponding to 4·7 μM (as reported in the Pharmacopeia); so we decided to use this dose for our experiments. PTX is well known to induce cell cycle arrest at the metaphase–anaphase transition of mitosis (Blagosklonny & Fojo 1999). Inhibition of mitotic progression and arrest correlates with PTX cytotoxicity and the mitotic cells are the most sensitive to PTX arrest (Jordan *et al.* 1996). In accordance, we observed a strong G2 block in the cycling cells exposed to PTX. T_3 is considered a primary liver mitogen (Francavilla *et al.* 1994, Malik *et al.* 2003) and its action on various cell cycle molecules has been demonstrated (Barrera-Hernandez *et al.* 1999, Pibiri *et al.* 2001, Verga Falzacappa *et al.* 2006). In our cell system, T_3 was able to contrast the G2 arrest induced by PTX. As this action was accompanied by a strong regulation of the G2 regulator molecules, we can speculate that even a T_3 direct action on the cell cycle could be implied in its survival effect against PTX.

When PTX is administered to cycling cells, apoptosis does not usually occur immediately but starts after PTX exposure of at least 16 h or longer, when slippage from mitosis may

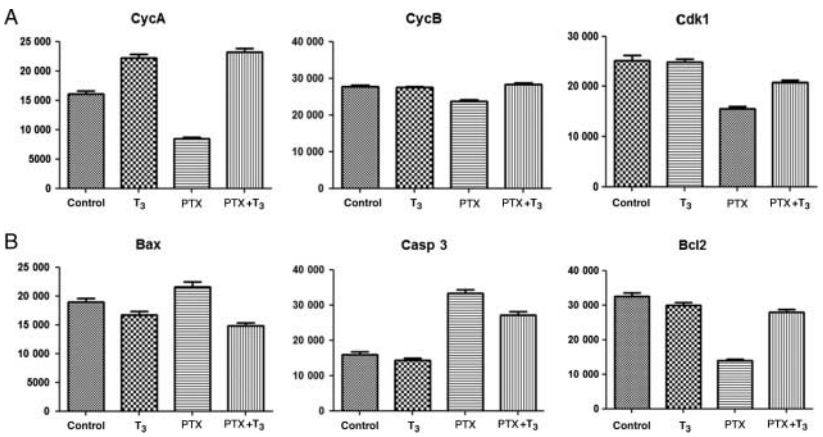


Figure 4 Cell cycle (A) and apoptosis (B) molecules. Cells were exposed to PTX alone or concurrently to PTX and T_3 for 48 h. Western blot analyses were performed as described in the Materials and Methods section and a specific band corresponding to the single molecules was detected. The expression of β-actin was analyzed as a control for gel loading. At least three different experiments were performed, and the means are shown here. Densitometric absorbance values from three separate experiments were averaged (±S.D.), after they had been normalized to β-actin for equal loading. Data are presented in the histogram as relative densitometric units (*y* axis). The different experimental groups are indicated on the *x* axis. A comparison of the individual treatment was conducted using one-way ANOVA followed by Tukey's *post hoc* test. The pairing was significantly effective ($P<0·0001$) and the means were significantly different (cycA, $P<0·0001$; cycB1, $P<0·005$; cdk1, $P<0·0001$; Bax, $P<0·005$; Casp 3, $P<0·0001$; Bcl2, $P<0·005$).

occur. Our results obtained by cytofluorimetry indicate that apoptosis occurs already after the first 24 h of PTX treatment but is evident only at 48 h. Coherently with the FCM indication, TUNEL assay revealed a strong antiapoptotic action of T_3 on PTX-treated cells. Also, the metabolic assay revealed how, acting on both cell cycle and apoptosis, T_3 was able to grant a vital cell metabolism.

The ability of T_3 to act as an antiapoptotic factor has been demonstrated recently in diverse cell systems (Laoag-Fernandez *et al.* 2004, Sukocheva & Carpenter 2006, Verga Falzacappa *et al.* 2006) and different pro- and antiapoptotic factors have been evidenced as targets of T_3 action. Caspase 3 is the most characterized effector caspase, required for granulosa cell apoptosis, as follicles from caspase 3 null ovaries do not show granulosa cell apoptosis in response to serum starvation (Matikainen *et al.* 2001). In this work, we demonstrated how the caspase 3 active form is strongly downregulated by T_3, even when there is a tight activation signal triggered by PTX.

PTX cytotoxicity involves signaling distinct from DNA-damaging drugs, but common to all microtubule-active drugs with the specific hallmark of Bcl2 hyperphosphorylation, resulting in increased levels of free Bax, favoring apoptosis. Moreover, Bax is one of the proapoptotic Bcl2 family members that has been demonstrated to be deeply implied in the ovarian apoptotic process. Its prominent role in oocyte death sustains DNA damage induced by both chemotherapeutic agents and aging (Greenfield *et al.* 2007). Inactivation of the *Bax* gene noticeably delayed ovarian senescence in female mice and reduced the number of oocytes undergoing spontaneous and chemotherapy-induced apoptosis. In addition, accumulation of both mRNA and proteins has been documented in oocytes of aged mice (Kujjo *et al.* 2010). Our previous data have already demonstrated how Bax could be targeted by T_3 while exerting its antiapoptotic action (Verga Falzacappa *et al.* 2006); in this work, we demonstrate how the strong increment of free Bax provoked by the presence of the chemotherapeutic agent is sensibly diminished by the presence of T_3, in accordance with the TUNEL and MTT result. In addition, the Bcl2 protein was also regulated. The caspases activation was also tested and resulted to be regulated by T_3; thus, we hypothesize that T_3 act also on the apoptotic pathway.

Compelling evidence indicates that PTX kills cells through the induction of apoptosis. The action of PTX on the cell cycle and apoptosis has been deeply discussed in the literature and it is somehow still not completely clear, as reviewed in Wang *et al.* (2000). However, it is noteworthy that these two processes are connected and involve various pathways. In particular, it has been shown that high concentrations of PTX cause massive microtubule damage, regulate gene expression, and activate kinases. Among genes that have been shown to be upregulated by PTX, Bax promotes apoptosis, whereas cyclin B1, together with its counterpart cdc2/cdk1, activates p34cdc2, which in turn facilitates the apoptotic process. All these events cooperate to finally activate caspases and lead to apoptosis. Considering this picture, we can speculate that all the changes we observed at the molecular levels are deeply interconnected and act together to lead to the final event of apoptotic cell death.

Taken together, our results suggest that the thyroid hormone T_3 can exert a strong pro-survival action on granulosa cells and overcome the PTX insult, enabling the cells to remain vital and cycling.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Author contribution statement

C V F and S M conceived and designed the experiments. C V F, E T, D A, D D A and P P performed the experiments. C V F, S M, and B B analyzed the data. S M, M C, and PP contributed reagents/materials/analysis tools. C V F and S M wrote the paper. M C and M G S did critical reading.

Acknowledgements

The authors would like to thank Drs Silvia Salatino and Luca Mauri for their technical assistance in hormonal assay and Drs Chiara Napoletano and Ilaria Zizzari for their technical assistance.

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Received in final form 17 August 2012
Accepted 21 August 2012
Made available online as an Accepted Preprint
21 August 2012