ORIGINAL ARTICLE: REPRODUCTIVE BIOLOGY

Estrogenic regulation of testicular expression of stem cell factor and c-kit: implications in germ cell survival and male fertility

Sara Correia, M.S., Mário R. Alves, M.S., José E. Cavaco, Ph.D., Pedro F. Oliveira, Ph.D., and Sílvia Socorro, Ph.D.

CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal

Objective: To study the effect of estrogens regulating the testicular expression of stem cell factor (SCF) and c-kit.
Design: Experimental study.
Setting: University research center.
Animal(s): Male Wistar rats.
Intervention(s): Rat seminiferous tubules (SeT) cultured in the presence or absence of 17β-estradiol (E_2).
Main Outcome Measure(s): Expression of SCF and c-kit as well as apoptotic factors, FasL, FasR, Bcl-2, and Bax analyzed via quantitative reverse transcription-polymerase and Western blot; enzymatic activity of apoptosis effector caspase-3 assessed by colorimetric assay; proliferation index in SeT epithelium determined via fluorescent immunohistochemistry of nuclear proliferation marker Ki67.
Result(s): E_2 (100 nM) induced a decrease in c-kit expression while increasing expression of SCF. Altered expression of the SCF/c-kit system relied on apoptosis of germ cells, as evidenced by the up-regulated expression of FasL/FasR, the increased ratio of proapoptotic/antiapoptotic proteins (Bax/Bcl-2), and the augmented activity of caspase-3. Decreased proliferation was also found in SeT in response to E_2.
Conclusion(s): A 100 nM dose of E_2 unbalance the SCF/c-kit system, with a crucial impact on germ cell survival and thus male fertility. These findings contribute to our knowledge of the mechanisms underlying male idiopathic infertility associated with hyperestrogenism and open new perspectives on treatment targeting estrogen-signaling mechanisms. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, c-kit, estrogens, male infertility, SCF

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/correias-estrogenic-regulation-testicular-expression-scf-ckit/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

Spermatogenesis is a complex cellular process involving mitosis, differentiation, and meiosis, which gives rise to the male haploid germ cells (1). Successful spermatogenesis also depends on the strict control of the balance between germ cell proliferation and apoptosis (2), which is of the uttermost relevance for male fertility. In fact, increased rates of apoptosis (3–5) together with altered expression of apoptosis-related genes (6–9) have been described in the testis of infertile men.

Experimental evidence has associated estrogens with testicular apoptosis and diminution of germ cell numbers (6, 10–13). Moreover, it has been documented that prenatal exposure to estrogens or estrogen-like substances has deleterious effects on spermatogenic output (14). Clinical studies also have suggested that augmented intratesticular production of estrogens is linked to spermatogenic failure and thus to male infertility because

Received February 3, 2014; revised March 29, 2014; accepted April 6, 2014.
S.C. has nothing to disclose. M.R.A. has nothing to disclose. J.E.C. has nothing to disclose. P.F.O. has nothing to disclose. S.S. has nothing to disclose.
Supported by the Portuguese Foundation for Science and Technology (FCT) under Program COMPETE (PEst-C/SAU/UI0709/2011), under the program Ciência 2008 (to P.F.O.), and fellowship SFRH/BD/60945/2009 (to S.C.).
Reprint requests: Sílvia Socorro, Ph.D., CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Avenue Infante D. Henrique, 6200–506 Covilhã, Portugal (E-mail: ssocorro@fcsaude.ubi.pt).

Fertility and Sterility® Vol. ■, No. ■, ■ 2014 0015-0282/$36.00
Copyright ©2014 American Society for Reproductive Medicine, Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.fertnstert.2014.04.009

increased levels of 17β-estradiol (E_2) have been detected in the testis of idiopathic infertile patients (15, 16). However, the molecular targets underpinning the estrogenic effects in mammalian testis are not fully elucidated.

Stem cell factor (SCF), a membrane-bound cytokine in Sertoli cells (17), and its tyrosine kinase receptor c-kit, present on the surface of adjacent germ cells (18, 19), have been indicated as a powerful mechanism for the control of germ cell proliferation and apoptosis. Multiple experimental approaches have shown that blocking SCF/c-kit interaction results in increased apoptosis and reduced proliferation of germ cells, demonstrating the importance of this system for successful spermatogenesis (20–22).

Although it has been demonstrated that E_2 regulates the expression of SCF and c-kit in distinct cell types (23–26), the effect of estrogens in controlling the testicular expression of these factors has been unknown. We analyzed the effect of E_2 stimulation on SCF and c-kit expression in rat seminiferous tubules (SeT) cultured ex vivo. We used a 100 nM dose of E_2 to mimic the elevated concentration of estrogens found in the testis of infertile patients and determined the expression and/or activity of proliferation and apoptosis markers.

MATERIALS AND METHODS

Chemicals

All chemicals, culture media, and antibodies unless otherwise stated were purchased from Sigma-Aldrich.

Animals

We housed 90-day-old Wistar (*Rattus norvegicus*) male rats under a 12-hour light/dark cycle, with food and water available ad libitum, and all animals were handled in compliance with the guidelines established by the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the European Union rules for the care and handling of laboratory animals (Directive no. 86\609\EEC). In addition, the animal protocol was approved by the local institutional animal care and use committee. All rats were euthanized under anesthesia (Clorketam 1000; Vetoquinol).

Ex Vivo Culture of SeT

Rat SeT were cultured as previously described elsewhere (6, 27). Briefly, testes (n = 5) were removed, trimmed free of fat, washed in cold phosphate-buffered saline (PBS) and placed in Dulbecco's modified Eagle's medium/Ham's F-12 culture medium supplemented with 20 mg/L of gentamicin sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 μg/L of bovine serum albumin (BSA) at 33°C. Tunicae were cut and peeled back to expose tubules. Ten fragments of SeT (~1 cm each) were cultured with or without 100 nM of E_2 for 24 hours and 48 hours at 33°C in an atmosphere of 5% CO_2. The 100 nM dose of E_2 was intended to mimic the elevated concentrations of estrogens found in the intratesticular milieu of infertile patients, as normal physiologic concentrations range from 0.5 to 57 nM (28–30). At the end of the experiment, SeT were recovered from the culture medium, snap-frozen in liquid nitrogen, and stored at −80°C until RNA or protein isolation. The SeT were also collected at 0, 24, and 48 hours and fixed in 4% paraformaldehyde for paraffin embedding and histologic processing.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from rat SeT using TRI reagent according to the manufacturer's instructions. To assess the quantity and integrity of total RNA, its optical density was determined (NanoPhotometer; Implen), and an agarose gel electrophoresis analysis was performed. We synthesized cDNA in a final volume of 20 μL using 1 μg of each RNA sample, 160 IU Moloney Murine Leukemia Virus reverse transcriptase (Promega), 0.5 μg random primers (Invitrogen), and 10 mM each deoxynucleotide triphosphates (dNTP) (GE Healthcare) according to the protocol supplied by the manufacturer. Synthesized cDNA was stored at −20°C until further use.

Real-time Quantitative Polymerase Chain Reaction

Expression analysis of *SCF* and *c-kit* RNA in rat SeT was performed by quantitative polymerase chain reaction (qPCR). The *SCF* and *c-kit* specific primers sets were, respectively, [1] sense: ATGGCTTGGGAAATGTCTG; antisense: GCTGATGCTACGGAGTTAC; [2] sense: CCGTCTCCACCATC CATCC; antisense: TTCGCTCTGCTTATTCTCAATCC. As an internal reference for normalization of expression of interest target genes, we used *β-actin* (sense: ATGGTGGGTATGGGT CAG; antisense: CAATGCCGTGTTCAATGG) and *GAPDH* (sense: GTTCAACGGCACAGTCAAG; antisense: CTCAGCAC CAGCATCACC). Reactions were performed in an iQ5 system (Bio-Rad Laboratories), and the efficiency of the amplifications was determined for all primer sets using serial dilutions of cDNA (1, 1:5, and 1:25). The primer concentration and annealing temperature were optimized before the assay, and the specificity of the amplicons was determined by melting curve analysis. The annealing temperature was 60°C for all primer sets except *SCF* (58°C). Each reaction consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Bio-Rad Laboratories), sense and anti-sense primers (200 nM for all primer pairs), and 1 μL of cDNA in a final volume of 20 μL. Also, a no-template control was included for each reaction, and all reactions were performed in triplicate. Normalized expression values were calculated according to a published mathematical model proposed by Hellemans et al. (31).

Western Blot

Total protein was isolated from rat SeT using radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, and 1 mM EDTA) supplemented with protease inhibitors cocktail. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories), and proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels and electrotransferred to a polyvinylidene difluoride membrane (GE Healthcare).

Membranes were incubated overnight at 4°C with rabbit anti-SCF (1:500, H-189: sc-9132; Santa Cruz Biotechnology), rabbit anti-c-kit (1:500, C-19: sc-168; Santa Cruz Biotechnology), rabbit anti-FasL (1:500, C-178: sc-6237; Santa Cruz Biotechnology), rabbit anti-FasR (1:500, A-20: sc-1023; Santa Cruz Biotechnology), rabbit anti-Bcl-2 (1:5,000, no. 2876; Cell Signaling Technology), or rabbit anti-Bax (1:1,000, no. 2772; Cell Signaling Technology). A mouse anti-α-tubulin monoclonal antibody (1:5,000, T9026) was used for protein loading control in all Western blot analyses. Goat anti-rabbit IgG-AP (1:5,000, NIF1317; GE Healthcare) or goat anti-mouse IgG+IgM-AP (1:5,000, NIF1316, GE Healthcare) were used as secondary antibodies. Membranes were developed with ECF substrate (GE Healthcare) for 5 minutes and scanned with Molecular Imager FX Pro plus MultiImager (Bio-Rad Laboratories). Band densities were obtained according to standard methods using the Quantity One Software (Bio-Rad Laboratories) and normalized by division with the respective α-tubulin band density.

Ki67 and c-Kit Fluorescent Immunohistochemistry

Formalin-fixed paraffin sections (5 μm) of SeT were deparaffinized in xylene and rehydrated in graded alcohols. After heat-induced antigen retrieval (citrate buffer bath, 10 mM, pH 6.0), the sections were permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Unspecific staining was blocked by incubation with PBS containing 1% (w/v) BSA (PBA) and 0.3 M glycine (Fisher Scientific) for 30 minutes at room temperature. Sections were incubated overnight at 4°C with rabbit anti-Ki67 (ab16667; Abcam) or rabbit anti-c-kit (C-19: sc-168; Santa Cruz Biotechnology) primary antibodies diluted 1:50 in PBA. Sections were then incubated with Alexa Fluor 546 goat anti-rabbit IgG or Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies (Invitrogen) diluted 1:500 in PBA for 1 hour at room temperature. Cell nuclei were stained by incubation with Hoechst 33342 (10 μg/mL, Invitrogen) for 5 minutes. Sections were then washed with PBS for 10 minutes and mounted in Dako fluorescent mounting medium. Specificity of the staining was assessed by the omission of primary antibody. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss). The proliferation index was determined by the percentage of Ki67-positive cells out of the total number of Hoechst stained nuclei in 20 randomly selected ×40 magnification fields in each section.

Caspase-3 Activity Assay

Caspase-3 activity assay was performed as previously described elsewhere (32). Briefly, 25 μg of total protein extracted from SeT were incubated with reaction buffer (25 mM HEPES, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 10% sucrose, and 10 mM dithiothreitol, pH 7.5) and 200 μM of caspase-3 substrate (Ac-DEVD-pNA) for 2 hours at 37°C. Upon caspase cleavage, p-nitro-aniline (pNA) is released producing a yellow color, which is measured spectrophotometrically at 405 nm. The amount of generated product was calculated by extrapolation of the standard curve of free pNA.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling

We performed the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis using the In Situ Cell Death Detection Kit, TMR red (Roche) following the manufacturer's instructions. Briefly, SeT sections (5 μm) were deparaffinized in xylene, rehydrated in graded alcohols, rinsed in 0.15 M PBS (pH 7.4), and permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 30 minutes at room temperature. Thereafter, sections were incubated in a humidified atmosphere for 1 hour at 37°C in the dark with TUNEL reaction mixture (enzyme solution equilibrated in label solution). After an additional rinse in PBS, the cell nuclei were stained by incubation with Hoechst 33342 (10 μg/mL; Invitrogen) for 5 minutes. Sections were then washed with PBS for 10 minutes and mounted in Dako fluorescent mounting medium. Images were acquired using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss).

Statistical Analysis

Statistical significance of differences between controls and hormonal treatment were evaluated by unpaired t-test with Welch's correction, using GraphPad Prism v5.00 (GraphPad Software). $P<.05$ was considered statistically significant.

RESULTS

E_2 Treatment Decreases c-Kit Expression while Increasing Expression of Its Ligand SCF

Because SCF and c-kit have been identified as estrogen-target genes in several tissues, we investigated the effect of E_2 on their expression in testicular cells. When SeT was cultured ex vivo for 24 hours in the presence of 100 nM E_2, it showed decreased expression of c-kit receptor. An approximately 0.4-fold-reduction ($P<.01$) was observed both at the mRNA (Fig. 1A) and protein (see Fig. 1B) level.

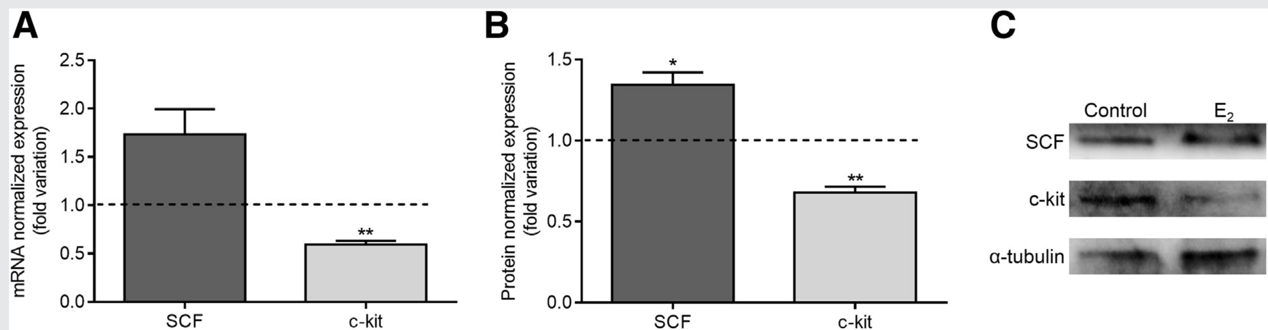
Regarding SCF, both RNA (see Fig. 1A) and protein (see Fig. 1B) expressions were increased in response to E_2-treatment. However, only the protein levels displayed statistically significant differences (1.34-fold variation relative to control; $P<.05$).

Apoptosis is Favored and Proliferation Index is Decreased in Response to E_2 Stimulation

Germ cell death in human, rat, and mouse testis has been associated with the activation of the extrinsic pathway of apoptosis involving the death factors FasL (ligand) and FasR (receptor) (8, 33). Western blot analysis showed that protein levels of both FasL and FasR (Fig. 2A) were statistically significantly increased in response to 100 nM of E_2 with a fold induction of approximately 1.5 relative to the control group in both cases ($P<.01$).

The Bax and Bcl-2 proteins are, respectively, proapoptotic and antiapoptotic members of the Bcl-2 family of apoptosis

FIGURE 1



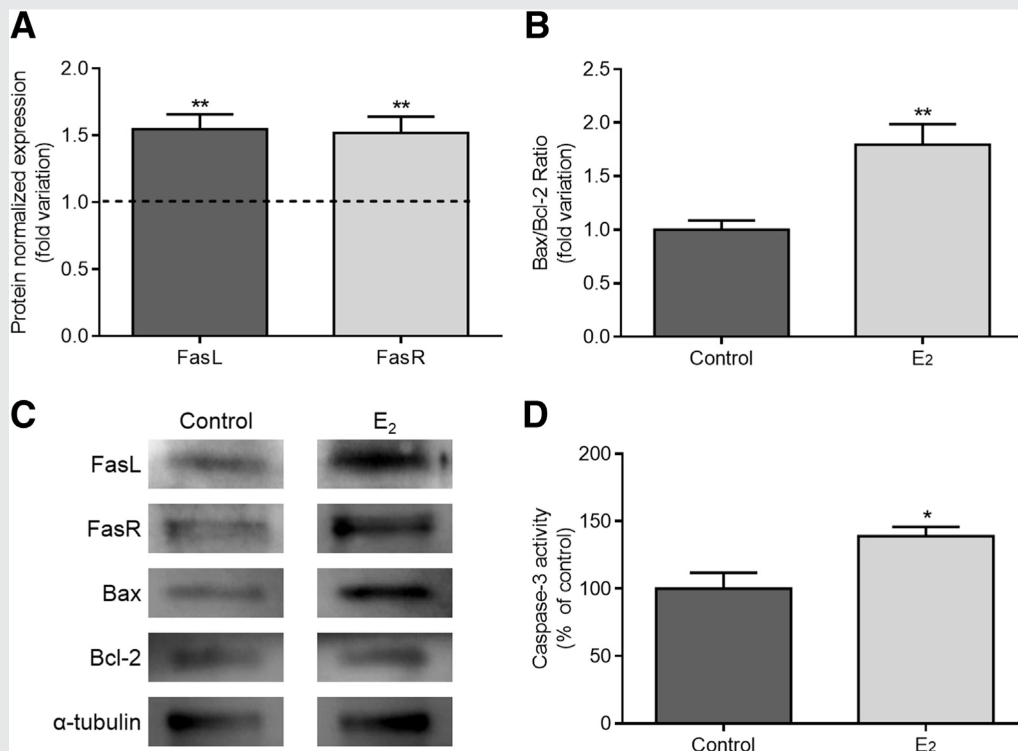
Effect of 100 nM E_2 on mRNA (A) and protein (B) expression of SCF and c-kit in rat SeT cultured ex vivo for 24 hours. The mRNA expression was determined by qPCR after normalization with β-actin and GAPDH housekeeping genes. Protein expression was determined by Western blot analysis after normalization with α-tubulin. Results are expressed as the fold variation relative to 0 nM E_2 (*dashed line*). *$P<.05$; **$P<.01$. Error bars indicate mean ± standard error of the mean (n = 5). (C) Representative immunoblots.

Correia. Estrogens and male fertility. Fertil Steril 2014.

regulators (34), and the Bax/Bcl-2 protein ratio has been considered a powerful indicator of cell commitment to undergo apoptosis (35, 36). In the stimulation experiments of SeT with 100 nM of E_2 (48 hours), the calculation of the Bax/Bcl-2 protein ratio demonstrated a 1.8-fold variation in the E_2-treated animals compared with the control group ($P<.01$) (see Fig. 2B).

Activation of cell death systems invariably leads to stimulation of caspase activity, culminating in the activation of caspase-3 (34). Thus, we determined caspase-3 activity as a measurement of apoptosis. An increase of almost 40% in caspase-3 activity was observed in cultured SeT in response to 100 nM E_2 stimulation for 48 hours ($P<.05$) (see Fig. 2D).

FIGURE 2



Apoptosis in rat SeT cultured ex vivo for 48 hours in presence (E_2) or absence (control) of 100 nM of E_2. (A) Expression of death factors FasL and FasR (24 hours). (B) Ratio of proapoptotic (Bax) and antiapoptotic (Bcl-2) proteins. Protein expression was determined by Western blot analysis after normalization with α-tubulin. (C) Representative immunoblots. (D) Caspase-3 activity. Results are represented as mean ± standard error of the mean (n = 5). *$P<.05$; **$P<.01$.

Correia. Estrogens and male fertility. Fertil Steril 2014.

The presence of apoptotic cells in the SeT with and without E_2 treatment (100 nM) was analyzed by detection of DNA fragmentation using the TUNEL assay. Representative photomicrographs showing TUNEL-positive cells in the control and E_2-treated groups during the time-course of the experiment are provided in Figure 3A.

The c-kit has been widely recognized as a germ cell marker highly expressed in spermatogonia and early spermatocytes of adult testis (19, 37–39). Therefore, c-kit immunofluorescent labeling was used as an indicator of the germ cell population in control and E_2-treated groups (see Fig. 3B).

We also sought to determine the effect of E_2 on proliferation of testicular cells, which was assessed by means of Ki67 immunofluorescence analysis. Ki67 is detected in the nucleus of proliferating cells in all active phases of the cell division cycle but is absent in nonproliferating cells (40). The cell proliferation index, determined by the number of Ki67-positive cells relative to the total cell number, was reduced by approximately half in SeT treated with 100 nM of E_2 for 48 hours in comparison with the control group ($P<.001$) (Fig. 4).

DISCUSSION

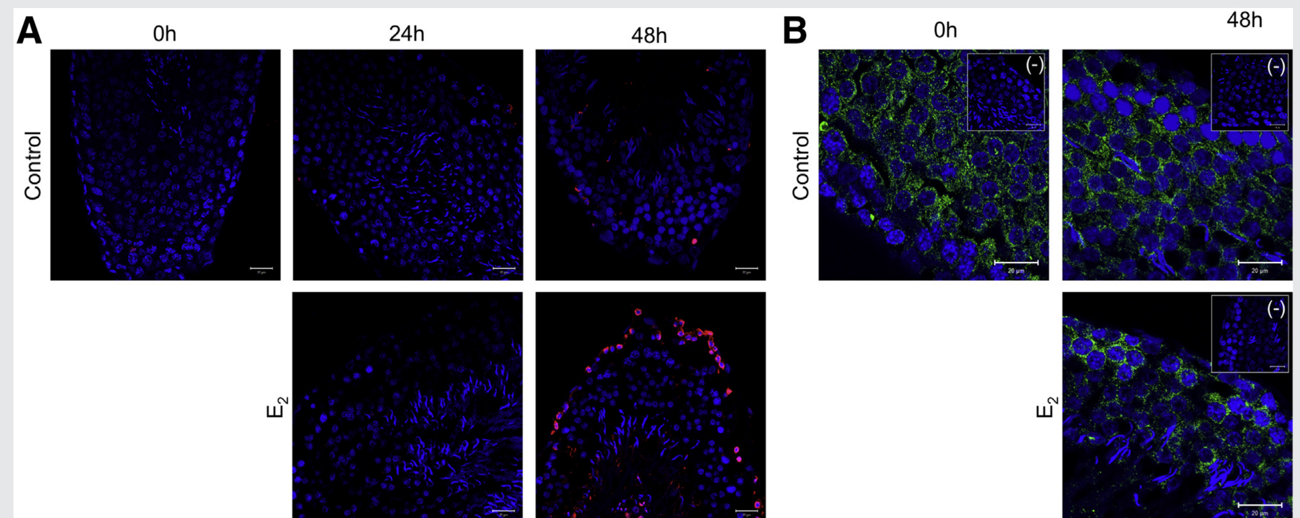
Achievement of successful spermatogenesis relies on an accurate regulation of germ cell proliferation and apoptosis, which has been associated with the activity of SCF/c-kit system. The SCF, present on the membrane of Sertoli cells (41), has been regarded as an important germ cell survival regulator (42), acting through the c-kit receptor, which is present on the surface of adjacent germ cells (18). Evidence also exists to demonstrate that loss of c-kit signaling causes increased apoptosis of germ cells (5, 20). Thus, enhanced or diminished expression of c-kit should disturb germ cell and Sertoli cell communication, with a profound impact on germ cell survival.

Several studies have indicated that estrogens are apoptosis inducers in the testis (10, 11, 13, 43), and reports exist demonstrating its capacity in regulating the expression of SCF and c-kit in several cell types (23–26). This led to our hypothesis that E_2 action toward an increase in germ cell apoptosis depends on the control of the testicular expression of SCF and c-kit.

In our present study, we investigated the effect of 100 nM E_2 on the expression of SCF and c-kit in ex vivo cultures of rat SeT. A statistically significant decrease of c-kit expression (see Fig. 1A and B) was observed in SeT in response to E_2 treatment for 24 hours. However, 100 nM of E_2 increased SCF expression in SeT (see Fig. 1B). To the best of our knowledge, our is the first study to report on the regulation of SCF/c-kit in response to estrogenic stimuli in testicular cells, which could have a relevant impact on germ cell numbers. As a growth factor receptor that transduces growth regulatory signals (44) to the germ cells, c-kit ensures the maintenance of the self-renewal and differentiation ratio of spermatogonia (45). It has been shown that mice with a mutant form of c-kit (46) or allele inactivation (20) are sterile due to reduced proliferation and increased apoptosis of spermatogonia. Therefore, despite the up-regulation of SCF levels in response to E_2, a decreased expression of c-kit suggests lower proliferation and increased apoptosis of germ cells.

Proliferation analysis by means of fluorescent immunohistochemistry using the proliferation marker Ki67 confirmed the decreased proliferation index in SeT treated with 100 nM E_2 (see Fig. 4), as suggested by the decreased expression of c-kit (see Fig. 1). Although they did not study the engagement of c-kit signaling, Chimento et al. (43) also found that 100 nM E_2

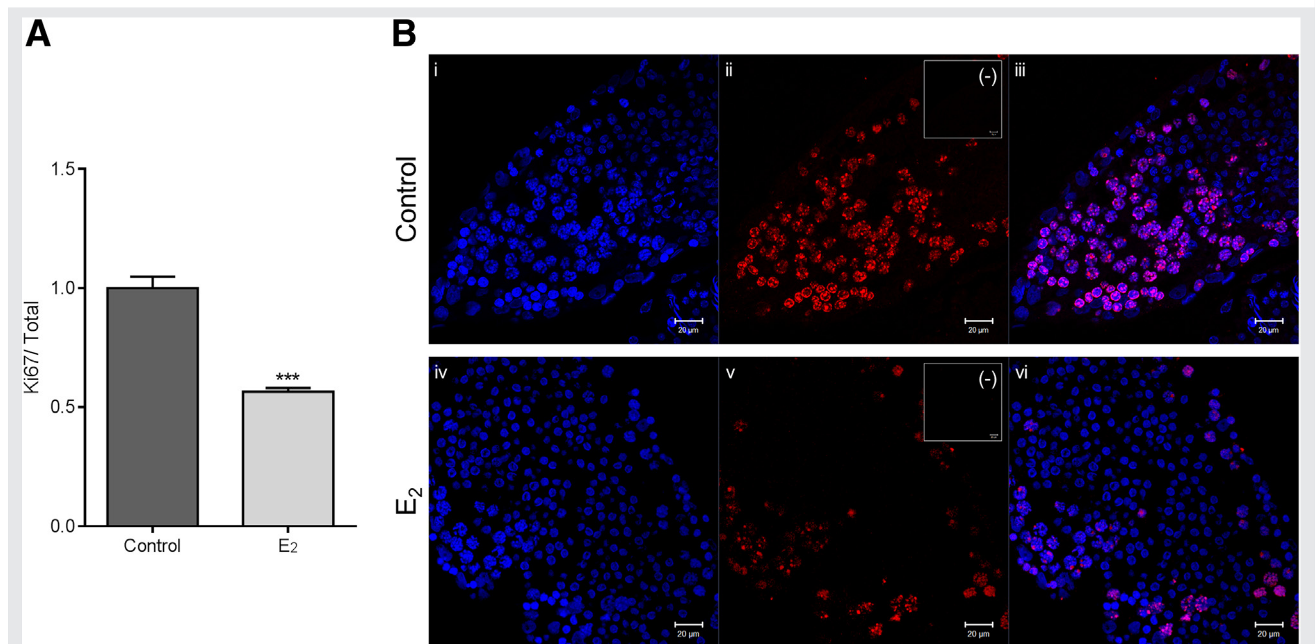
FIGURE 3



Representative confocal microscopy images showing TUNEL (A) and c-kit (B) positive cells in rat SeT cultured ex vivo in presence (E_2) or absence (control) of 100 nM of E_2 at different experimental time-points (0, 24, and 48 hours). Nuclei are stained with Hoechst 33342 (*blue*), and fluorescence for TUNEL- and c-kit-positive cells is red and green, respectively. Negative controls for c-kit obtained by omission of the primary antibody are provided as insert panels (–).

Correia. Estrogens and male fertility. Fertil Steril 2014.

FIGURE 4



Proliferation index in rat SeT cultured ex vivo for 48 hours in presence (E_2) or absence (control) of 100 nM of E_2. Proliferation was determined by Ki67 immunofluorescence analysis. (A) Percentage of Ki67-positive cells relative to the total cell number. Results are expressed as the fold variation compared with control. Error bars indicate mean $\pm$ standard error of the mean (n = 5 in each group). ***$P<.001$. (B) Representative images of Hoechst stained nuclei (i, iv), Ki67 immunofluorescence (ii, v), and corresponding merged images (iii, vi) in control and E_2-treated groups. Negative controls for Ki67 obtained by omission of the primary antibody are provided as insert panels (−).

Correia. Estrogens and male fertility. Fertil Steril 2014.

has a detrimental effect on germ cell proliferation, drastically down-regulating the expression of cell cycle regulators cyclins A1 and B1. Curiously, promotion of spermatogonia proliferation through SCF/c-kit action seems to involve the phosphatidylinositol 3-kinase (PI3K) survival pathway, including up-regulation and nuclear accumulation of cyclin D3 (47). Further studies are needed to determine whether cyclins A1 and B1 might be the targets of c-kit signaling within the testis.

As previously noted, besides governing germ cell proliferation, the SCF/c-kit system also influences germ cell apoptosis. Protease inactivation of c-kit (48) or blocking of SCF/c-kit interaction (49) has been shown to induce apoptosis of spermatogonia. Therefore, attenuated c-kit signaling due to diminished expression levels of c-kit protein in consequence of E_2 stimulation could be responsible for increased apoptosis of germ cells.

The interplay between apoptotic death factors FasL and FasR has been identified as a crucial mechanism for determining germ cell death through the activation of the extrinsic pathway of apoptosis (33). Our results have demonstrated that the expression of both FasL and FasR is increased in cultured SeT in presence of 100 nM E_2 (see Fig. 2A).

The protein ratio of mitochondria-related apoptosis regulators, namely, Bax (proapoptotic) and Bcl-2 (antiapoptotic), is a recognized indicator of the activation of apoptotic pathways and was included as a measurement of apoptosis. An increased Bax/Bcl-2 protein ratio was found in the E_2-treated group (see Fig. 2B). The imbalance toward proapoptotic proteins is due to increased expression of Bax, but the Bcl-2 levels remained virtually unchanged. This finding is concordant with the up-regulated expression of Bax observed when the SCF/c-kit interaction is blocked (49). The use of an anti-c-kit antibody diminishing c-kit activity, as could happen when c-kit levels are reduced as we reported herein, was associated with increased protein ratio of proapoptotic/antiapoptotic proteins due to elevated expression of Bax (49).

The process of apoptosis independent of the triggered pathway converges on the activation of caspase-3, an effector caspase, which is considered a remarkable end point of apoptosis (50). Determination of caspase-3 enzymatic activity in SeT cultured in the presence or absence of 100 nM E_2 demonstrated an increase of almost 40% in caspase-3 activity (see Fig. 2D) in response to E_2. The up-regulated expression of FasL and FasR, the increased ratio of proapoptotic/antiapoptotic proteins, and the augmented activity of caspase-3 suggest augmented apoptosis in response to E_2, which was confirmed by the TUNEL-labeling assay (see Fig. 3A).

A considerable amount of data have established that germ cells essentially express the death receptor FasR whereas Sertoli cells mainly present the ligand FasL (51, 52). Therefore, the increased expression of FasL (see Fig. 2A) concomitant with an increased response of germ cells to death signals, because FasR expression is augmented (see Fig. 2A), indicates that an augmentation of apoptosis is occurring in the germ cell population. Because c-kit is a known germ

cell marker (19, 37–39), the reduced labeling of c-kit in SeT treated with E_2 for 48 hours (see Fig. 3B) demonstrated the loss of germ cells. This is also strongly supported by our recent report on isolated Sertoli cells that showed that 100 nM of E_2 diminishes Sertoli cells apoptosis by reduction of Bax/Bcl-2 ratio and caspase-3 activity (12). In addition, the increased expression of Bax, as reported herein, has been considered a feature of germ cells undergoing apoptosis in response to E_2 (10, 43).

The question of E_2 induction of apoptosis in testicular cells has remained a matter of controversy for in vivo and in vitro studies, with the use of natural or synthetic estrogens and/or different doses and distinct routes of administration producing not always concordant results. In fact, there are reports pointing to E_2 as a germ cell survival factor that inhibits apoptosis and restores spermatogenesis upon a harmful stimulus (28, 53). However, these studies have used low doses of E_2 within the range of physiologic concentrations (0.5–57 nM) (28–30).

In our study, using ex vivo cultures of SeT, we demonstrated that elevated concentrations of E_2 (100 nM), mimicking those found in the intratesticular milieu of infertile patients, unbalanced the expression of the SCF/c-kit system, disrupting the survival and death communication between germ cells and Sertoli cells toward germ cell apoptosis. In sum, our results showed that E_2 induced a decrease in c-kit expression, which was coupled with augmented apoptosis as evidenced by the increased expression of FasL, FasR, and Bax/Bcl-2 ratio as well as caspase-3 activity. Moreover, a decreased proliferation was found in response to E_2 downregulation of c-kit expression, indicating diminished survival and increased apoptosis of germ cells in the epithelium of SeT. These data are of paramount importance, considering hyperestrogenism-related cases of idiopathic infertility (15, 16) and the observed diminished expression of c-kit in subfertile testicular tissues (5).

Although the causes are not totally known, it has been reported that an increase in the intratesticular concentration of E_2 in infertile men is accompanied by an augmented ratio of estradiol/testosterone (15, 16). It seems likely that this hormone deregulation may occur as a result of the increased expression and/or activity of the aromatase enzyme. Lardone et al. (16) found increased levels of aromatase mRNA in the testis of infertile men with mixed atrophy and Sertoli cell-only syndrome. More recently, it was shown that the use of aromatase inhibitors for treatment of infertile men with high estradiol/testosterone ratios improved both hormone and semen parameters (54), which supports that the cause of the disorder is on the abnormal activity of aromatase. Because elevated levels of E_2 (15, 16) and increased rates of apoptosis have been found in the testis of infertile men (5–9), we may extrapolate that the increased estrogenic response underlies the impairment of spermatogenesis and infertility by causing depletion of germ cells due to augmented apoptosis and reduced proliferation in consequence of diminished expression of c-kit.

Acknowledgments: The authors acknowledge Catarina Ferreira for technical assistance in tissue processing and fluorescent immunohistochemistry.

REFERENCES

1. Oakberg EF. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 1956;99:391–413.
2. Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 2000;22:423–30.
3. Lin WW, Lamb DJ, Wheeler TM, Abrams J, Lipshultz LI, Kim ED. Apoptotic frequency is increased in spermatogenic maturation arrest and hypospermatogenic states. J Urol 1997;158:1791–3.
4. Takagi S, Itoh N, Kimura M, Sasao T, Tsukamoto T. Spermatogonial proliferation and apoptosis in hypospermatogenesis associated with nonobstructive azoospermia. Fertil Steril 2001;76:901–7.
5. Feng HL, Sandlow JI, Sparks AE, Sandra A, Zheng LJ. Decreased expression of the c-kit receptor is associated with increased apoptosis in subfertile human testes. Fertil Steril 1999;71:85–9.
6. Laurentino S, Gonçalves J, Cavaco JE, Oliveira PF, Alves MG, de Sousa M, et al. Apoptosis-inhibitor Aven is downregulated in defective spermatogenesis and a novel estrogen target gene in mammalian testis. Fertil Steril 2011;96:745–50.
7. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, de Sousa M, Barros A, et al. Regucalcin, a calcium-binding protein with a role in male reproduction? Mol Hum Reprod 2012;18:161–70.
8. Kim S-K, Yoon Y-D, Park Y-S, Seo JT, Kim J-H. Involvement of the Fas–Fas ligand system and active caspase-3 in abnormal apoptosis in human testes with maturation arrest and Sertoli cell–only syndrome. Fertil Steril 2007;87:547–53.
9. Weikert S, Schrader M, Müller M, Krause H, Miller K. Expression of the apoptosis inhibitor survivin in testicular tissue of infertile patients. Int J Androl 2004;27:161–5.
10. Chaki S, Misro M, Gautam DK, Kaushik M, Ghosh D, Chainy G. Estradiol treatment induces testicular oxidative stress and germ cell apoptosis in rats. Apoptosis 2006;11:1427–37.
11. Mishra DP, Shaha C. Estrogen-induced Spermatogenic Cell Apoptosis Occurs via the Mitochondrial Pathway role of superoxide and nitric oxide. J Biol Chem 2005;280:6181–96.
12. Simões V, Alves M, Martins A, Dias T, Rato L, Socorro S, et al. Regulation of apoptotic signaling pathways by 5α-dihydrotestosterone and 17β-estradiol in immature rat Sertoli cells. J Steroid Biochem Mol Biol 2012;135C:15–23.
13. D'Souza R, Gill-Sharma MK, Pathak S, Kedia N, Kumar R, Balasinor N. Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. Mol Cell Endocrinol 2005;241:41–8.
14. Toppari J. Environmental endocrine disrupters and disorders of sexual differentiation. Semin Reprod Med 2002;20:305–12.
15. Levalle OA, Zylbersztein C, Aszpis S, Mariani V, Ponzio R, Aranda C, et al. Serum luteinizing hormone pulsatility and intratesticular testosterone and oestradiol concentrations in idiopathic infertile men with high and normal follicle stimulating hormone serum concentrations. Hum Reprod 1994;9:781–7.
16. Lardone M, Castillo P, Valdevenito R, Ebensperger M, Ronco A, Pommer R, et al. P450-aromatase activity and expression in human testicular tissues with severe spermatogenic failure. Int J Androl 2010;33:650–60.
17. Rossi P, Albanesi C, Grimaldi P, Geremia R. Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. Biochem Biophys Res Commun 1991;176:910–4.
18. Sorrentino V, Giorgi M, Geremia R, Besmer P, Rossi P. Expression of the c-kit proto-oncogene in the murine male germ cells. Oncogene 1991;6:149–51.
19. Yoshinaga K, Nishikawa S, Ogawa M, Hayashi SI, Kunisada T, Fujimoto T, et al. Role of c-kit in mouse spermatogenesis—identification of spermatogonia as a specific site of c-kit expression and function. Development 1991;113:689–99.
20. Guerif F, Cadoret V, Rahal-Perola V, Lansac J, Bernex F, Panthier JJ, et al. Apoptosis, onset and maintenance of spermatogenesis: evidence for the involvement of Kit in Kit-haplodeficient mice. Biol Reprod 2002;67:70–9.
21. Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development 2000;127:2125–31.

22. Yan W, Suominen J, Toppari J. Stem cell factor protects germ cells from apoptosis in vitro. J Cell Sci 2000;113:161–8.
23. Tanikawa M, Harada T, Mitsunari M, Onohara Y, Iwabe T, Terakawa N. Expression of c-kit messenger ribonucleic acid in human oocyte and presence of soluble c-kit in follicular fluid. J Clin Endocrinol Metab 1998;83:1239–42.
24. Huansheng D, Qingjie P, Hanqiong Z, Lianjun Z, Bo C, Wenbin Y. Estrogen inhibits the early development of mouse follicles through regulating the expression of Kit ligand. Biochem Biophys Res Commun 2011;410:659–64.
25. Song RX-D, Mor G, Naftolin F, McPherson RA, Song J, Zhang Z, et al. Effect of long-term estrogen deprivation on apoptotic responses of breast cancer cells to 17β-estradiol. J Natl Cancer Inst 2001;93:1714–23.
26. Jaita G, Candolfi M, Zaldivar V, Zárate S, Ferrari L, Pisera D, et al. Estrogens up-regulate the Fas/FasL apoptotic pathway in lactotropes. Endocrinology 2005;146:4737–44.
27. Laurentino SS, Correia S, Cavaco JE, Oliveira PF, Rato L, Sousa M, et al. Regucalcin is broadly expressed in male reproductive tissues and is a new androgen-target gene in mammalian testis. Reproduction 2011;142:447–56.
28. Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis in vitro. J Clin Endocrinol Metab 2000;85:2057–67.
29. Zhao M, Baker SD, Yan X, Zhao Y, Wright WW, Zirkin BR, et al. Simultaneous determination of steroid composition of human testicular fluid using liquid chromatography tandem mass spectrometry. Steroids 2004;69:721–6.
30. Roth M, Lin K, Amory J, Matsumoto A, Anawalt B, Snyder C, et al. Serum LH correlates highly with intratesticular steroid levels in normal men. J Androl 2010;31:138–45.
31. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007;8:R19.
32. Alves MG, Machado NG, Sardao VA, Carvalho RA, Oliveira PJ. Anti-apoptotic protection afforded by cardioplegic Celsior and histidine buffer solutions to hearts subjected to ischemia and ischemia/reperfusion. J Cell Biochem 2011;112:3872–81.
33. Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 1997;138:2081–8.
34. Lawen A. Apoptosis—an introduction. Bioessays 2003;25:888–96.
35. Harris M, Thompson C. The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ 2000;7:1182–91.
36. Mackey TJ, Borkowski A, Amin P, Jacobs SC, Kyprianou N. bcl-2/bax ratio as a predictive marker for therapeutic response to radiotherapy in patients with prostate cancer. Urology 1998;52:1085–90.
37. Strohmeyer T, Reese D, Press M, Ackermann R, Hartmann M, Slamon D. Expression of the c-kit proto-oncogene and its ligand stem cell factor (SCF) in normal and malignant human testicular tissue. J Urol 1995;153:511–5.
38. Bokemeyer C, Kuczyk MA, Dunn T, Serth J, Hartmann K, Jonasson J, et al. Expression of stem-cell factor and its receptor c-kit protein in normal testicular tissue and malignant germ-cell tumours. J Cancer Res Clin Oncol 1996;122:301–6.
39. Unni SK, Modi DN, Pathak SG, Dhabalia JV, Bhartiya D. Stage-specific localization and expression of c-kit in the adult human testis. J Histochem Cytochem 2009;57:861–9.
40. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133:1710–5.
41. Tajima Y, Onoue H, Kitamura Y, Nishimune Y. Biologically active kit ligand growth factor is produced by mouse Sertoli cells and is defective in Sld mutant mice. Development 1991;113:1031–5.
42. Manova K, Huang EJ, Angeles M, De Leon V, Sanchez S, Pronovost SM, et al. The expression pattern of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in oocyte growth and in proliferation of spermatogonia. Dev Biol 1993;157:85–99.
43. Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, et al. 17β-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ERα. Mol Cell Endocrinol 2010;320:136–44.
44. Blechman JM, Lev S, Barg J, Eisenstein M, Vaks B, Vogel Z, et al. The fourth immunoglobulin domain of the stem cell factor receptor couples ligand binding to signal transduction. Cell 1995;80:103–13.
45. Morimoto H, Kanatsu-Shinohara M, Takashima S, Chuma S, Nakatsuji N, Takehashi M, et al. Phenotypic plasticity of mouse spermatogonial stem cells. PLoS One 2009;4:e7909.
46. Blume-Jensen P, Jiang G, Hyman R, Lee K-F, O'Gorman S, Hunter T. Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3′-kinase is essential for male fertility. Nat Genet 2000;24:157–62.
47. Feng L-X, Ravindranath N, Dym M. Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. J Biol Chem 2000;275:25572–6.
48. Lizama C, Rojas-Benítez D, Antonelli M, Ludwig A, Bustamante-Marín X, Brouwer-Visser J, et al. TACE/ADAM17 is involved in germ cell apoptosis during rat spermatogenesis. Reproduction 2010;140:305–17.
49. Yan W, Samson M, Jégou B, Toppari J. Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. Mol Endocrinol 2000;14:682–99.
50. Dahmer MK. Caspases-2, -3, and -7 are involved in thapsigargin-induced apoptosis of SH-SY5Y neuroblastoma cells. J Neurosci Res 2005;80:576–83.
51. Sugihara A, Saiki S, Tsuji M, Tsujimura T, Nakata Y, Kubota A, et al. Expression of Fas and Fas ligand in the testes and testicular germ cell tumors: an immunohistochemical study. Anticancer Res 1997;17:3861–5.
52. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC. A role for CD95 ligand in preventing graft rejection. Nature 1995;377:630–2.
53. Porter KL, Shetty G, Shuttlesworth GA, Weng CC, Huhtaniemi I, Pakarinen P, et al. Estrogen enhances recovery from radiation-induced spermatogonial arrest in rat testes. J Androl 2009;30:440–51.
54. Gregoriou O, Bakas P, Grigoriadis C, Creatsa M, Hassiakos D, Creatsas G. Changes in hormonal profile and seminal parameters with use of aromatase inhibitors in management of infertile men with low testosterone to estradiol ratios. Fertil Steril 2012;98:48–51.