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RESEARCH ARTICLE

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Effect of resveratrol on Sertoli cell proliferation

Agostina Gorga | Gustavo Marcelo Rindone | Mariana Regueira | Eliana Herminia Pellizzari | María del Carmen Camberos | Selva Beatriz Cigorraga | María Fernanda Riera | María Noel Galardo | Silvina Beatriz Meroni

Centro de Investigaciones Endocrinológicas, "Dr César Bergadá," CONICET-FEI, División de Endocrinología, Hospital de Niños Ricardo Gutiérrez, Buenos Aires, Argentina

Correspondence
Silvina Beatriz Meroni, Investigadora Independiente CONICET, Centro de Investigaciones Endocrinológicas, "Dr César Bergadá," CONICET-FEI, División de Endocrinología, Hospital de Niños Ricardo Gutiérrez, Gallo 1330 C1425EFD, Buenos Aires, Argentina.
Email: smeroni@cedie.org.ar

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Abstract

Resveratrol (RSV), a polyphenolic compound largely found in red grape skin, has been used as a nutritional supplement as it exhibits beneficial health effects, such as anticancer, cardioprotective, antiaging, and anti-inflammatory. Particularly, it has been shown that it participates in the mechanisms involved in cell proliferation. Sirtuin 1 (SIRT1) is considered a well-known RSV effector. Noteworthy, *Sirt1*-knockout animals are infertile. The aim of this study was, first, to determine whether RSV has any effect on Sertoli cell proliferation and, second, whether SIRT1, a putative target of RSV, is present in immature Sertoli cells. Sertoli cell cultures obtained from 8-day-old rats, which actively proliferate, were treated with RSV (10 and 50 μM) under basal and follicle-stimulating hormone (FSH)-stimulated conditions. Bromodeoxyuridine (BrdU) incorporation and the expression of *cyclins D1*, *D2*, *D3*, *E1*, and *E2* and the Cip/Kip cell cycle inhibitors $p21^{Cip}$ and $p27^{Kip}$ were analyzed. RSV decreased BrdU incorporation and *cyclins D1*, *D2*, *E1*, and *E2* expression and increased $p21^{Cip}$ and $p27^{Kip}$ messenger RNA (mRNA) levels. RSV also decreased FSH-stimulated BrdU incorporation and *cyclins D1* and *D2* mRNA levels. The effect of RSV on cMYC was also analyzed. RSV treatment did not modify basal and FSH-stimulated *cMyc* expression; however, it inhibited basal and FSH-stimulated cMYC transcriptional activity, suggesting a role of cMYC in RSV effects. Additionally, *Sirt1* was detected in immature Sertoli cells. Altogether, these results suggest that RSV possibly, by activating SIRT1 and regulating cMYC transcriptional activity, participates in the regulation of immature Sertoli cell proliferation.

KEYWORDS

proliferation, resveratrol, Sertoli, sirtuin 1

1 | INTRODUCTION

Resveratrol (3,5,4′-trihydroxystilbene; RSV) is a polyphenolic compound found in a variety of plants, such as grapes, peanuts, and berries. Previous studies have demonstrated that RSV has a number of biological activities, such as anticancer, cardioprotective, neuroprotective, antiaging, and anti-inflammatory. Importantly, particular attention to the antitumoral effect of RSV has been paid because of its ability to suppress cell proliferation.[1,2] In this respect, many reports have shown that RSV imparts inhibitory effects on several types of cancers, such as colon cancer, lymphoma, and breast cancer.[3-5] As for the mechanisms involved, it has been observed that RSV treatment affects the expression of

several cell cycle regulators such as cyclins and cell cycle inhibitors. In this context, it has been shown that RSV treatment induces cell cycle arrest at the G1 phase regulating *cyclin D1*, cyclin dependent kinase 4 (*CDK4*), and $p21^{Cip}$ expression in gastric cancer[6]; *cyclin D1*, *CDK2*, *CDK4*, and $p21^{Cip}$ levels in human colon cancer cell lines;[7] and *cyclins D1*, *D2*, *E,* and $p21^{Cip}$ levels in human epidermoid carcinoma A431.[8] It is worth mentioning that RSV affects proliferation of not only tumor cells but also normal cells such as keratinocytes, smooth muscle cells, and endothelial cells.[9-12]

Sertoli cells proliferate at defined periods of lifespan in rats, and the proliferation is associated with cell immaturity. Around 15 to 20 days of age, Sertoli cell proliferation ceases and the blood-testis barrier is formed.[13] Once the blood-testis barrier is established, Sertoli cells initiate a process of terminal differentiation that allows them to sustain spermatogenesis. It is worth remembering that the final number of Sertoli cells reached during the proliferative periods determines the sperm production capacity in adulthood. This relationship occurs because each Sertoli cell has a fixed capacity for the number of germ cells that it can support.[14] The gonadotrophic hormone follicle-stimulating hormone (FSH) has a major role in Sertoli cell proliferation at early postnatal life and in differentiation after the cessation of mitosis at puberty.[15] To this respect, it is worth mentioning that the mechanisms involved in the cessation of mitosis and the initiation of terminal maturation have not been fully clarified in Sertoli cells.

The sirtuin 1 (SIRT1) nicotinamide adenine dinucleotide (NAD^+)-dependent type III histone and protein deacetylase are among the molecular targets for RSV. Noticeably, *Sirt1*-knockout animals are infertile, and this fact is accompanied by Sertoli cell immaturity.[16] This scenario is consistent with a fail of Sertoli cells to cease proliferation and assume terminal differentiation. Even though SIRT1 is not apparently present in the adult Sertoli cells,[17] its presence in immature Sertoli cells has not been ruled out. The aim of this study was, first, to determine whether RSV has any effect on Sertoli cell proliferation and, second, whether SIRT1, a putative target of RSV, is present in immature Sertoli cells.

2 | MATERIALS AND METHODS

2.1 | Materials

RSV tissue culture media and all other drugs were purchased from Sigma-Aldrich (St Louis, MO). Ovine FSH (NIH-oFSH-S-16) was obtained from the National Hormone and Pituitary Program, NIDDK (Bethesda, MD).

2.2 | Sertoli cell isolation and culture

Sertoli cells from 8-day-old Sprague-Dawley rats were isolated as previously described.[18] Animals were housed and used according to the guidelines recommended by the National Institute of Health and approved by the Institutional Ethics Committee. Animals were killed by cervical dislocation. Briefly, testes were removed, decapsulated, and incubated in culture medium containing 0.03% collagenase and 0.003% soybean trypsin inhibitor for 5 minutes at room temperature. Culture medium consisted of a 1:1 mixture of Ham's F-12 and Dulbecco modified Eagle medium, supplemented with 0.1% bovine serum albumin, 100 IU/mL penicillin, 2.5 μg/mL amphotericin B, and 1.2 mg/mL sodium bicarbonate. After the initial dispersion, seminiferous tubules were sedimented, and the supernatant was discarded to remove interstitial cells. Seminiferous tubules were treated with 1M glycine-2 mM ethylenediaminetetraacetic acid (pH 7.4) to remove peritubular cells. Tubules washed with culture medium and recovered by sedimentation were incubated for 10 minutes at room temperature with a solution of 0.03% collagenase, 0.003% soybean trypsin inhibitor, and 0.03% DNase in the culture medium. The resultant Sertoli cell suspension, collected by centrifugation at 200*g* for 2 minutes, was resuspended in the culture medium described above without bovine serum albumin and with the following additions: 10 μg/mL transferrin, 5 μg/mL insulin, 5 μg/mL vitamin E, and 4 ng/mL hydrocortisone. Sertoli cells were cultured in 6-, 24-, or 96-well plates and in eight-chamber Permanox (Sigma-Aldrich, St. Louis, MO) slides (5 μg DNA/cm^2) at 34°C in a mixture of 5% CO_2:95% air.

2.3 | Culture conditions

Sertoli cells were allowed to attach for 48 hours in the culture medium mentioned above. The medium was replaced at this time (day 3) with a fresh medium without insulin. To evaluate bromodeoxyuridine (BrdU) incorporation, cells treated with different doses of RSV (10 or 50 μM) in the absence or presence of 100 ng/mL of FSH on day 3 and harvested on day 4 were used. To perform reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis, different treatments were carried out and cells were harvested on day 4. For dose-response studies, cells incubated for 2, 4, or 12 hours with 10 or 50 μM RSV were used. For time-course studies, cells incubated for 2, 4, 6, 12, and 24 hours with 50 μM RSV were utilized. Finally, to analyze the effects of RSV in FSH-stimulated cultures, cells incubated for 4 hours with 100 ng/mL FSH in the absence or presence of 50 μM RSV were used.

2.4 | Reverse-transcription polymerase chain reaction

Purified Sertoli cells obtained from 8-, 20-, and 30-day-old rats,[18-20] germ cells from 30-day-old rats,[21] and liver and adult testicular tissues were utilized to isolate total RNA using TRI reagent (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of RNA was estimated by spectrophotometry at 260 nm. RT-PCR was performed as previously described.[22] The complementary DNA (cDNA) encoding *Sirt1* was amplified using specific gene primers (FWD: 5′-ACAGTTCCAGCC ATCTCTGTG-3′; REV: 5′-GCAAAGGAACCATGACACT G-3′). The PCR products were resolved by 2% w/v agarose gel and stained with ethidium bromide.

2.5 | BrdU incorporation

Sertoli cell monolayers cultured in eight-chamber Permanox slides were incubated with BrdU (10 μM) for the last 24 hours of culture. Subsequently, cells were washed with phosphate-buffered saline (PBS) and fixed with methanol:acetic acid (3:1) for 30 minutes. DNA denaturation was carried out by incubating the slides in 70% ethanol/0.2M NaOH for 3 minutes followed by 1-minute incubation with 70% cold ethanol and 1-minute incubation with absolute ethanol allowing the plate to dry. To rehydrate the cells, three rinses with PBS were performed for 3 minutes. Endogenous peroxidase was blocked with H_2O_2 for 30 minutes, and nonspecific sites were blocked with horse serum (1:50) in PBS for another 30 minutes. Finally, cells were incubated with monoclonal BrdU antibody (1:200; Dako, Glostrup, Denmark) in a wet chamber at 4°C overnight. Immunoperoxidase staining was performed according to the manufacturer-recommended protocol for universal labeled streptavidin-biotin system horseradish peroxidase (VECTASTAIN Elite ABC HRP Kit; Vector Laboratories, Burlingame, CA). Nuclei were counterstained with hematoxylin, and over 2000 cells were counted. The percentage of proliferating (BrdU-positive) Sertoli cells was calculated as: (BrdU-positive cells/total Sertoli cells) × 100.

2.6 | Reverse-transcription quantitative polymerase chain reaction

Total RNA was isolated from Sertoli cells cultured in six-well plates with TRI reagent (Sigma-Aldrich) according to the manufacturer's recommendations. The amount of RNA was estimated by spectrophotometry at 260 nm. RT was performed as previously described.[22] Real-time PCR was performed using Step One Real Time PCR System (Applied Biosystems, Warrington, UK). Table 1 shows the specific primers used to analyze *cyclins D1* to *D3*, *cyclins E1* to *E2*, $p21^{Cip}$, $p27^{Kip}$, hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*), and β2 microglobulin (*B2m*) expressions. Amplification was carried out as recommended by the manufacturer: 25 μL reaction mixture containing 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), the appropriate primer concentration, and 1 μL of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalized to *Hprt1* and *B2m*. The amplification program included the initial denaturation step at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative $\Delta\Delta C_t$ method was used to calculate relative gene expression.

2.7 | Transfection and luciferase reporter assay

Sertoli cells cultured on 24-well plates were allowed to attach for 48 hours in the presence of insulin, and the medium was replaced at this time with fresh medium without insulin. Afterward, transfection was performed with the plasmids pGL3-E-box[23] or pGL3-Basic (Promega Corporation, Madison, WI) using Lipofectamine 2000

TABLE 1 Rat-specific primers sets for analysis by RT-qPCR

Gene	Primer sequence
Ccnd1	FWD: 5′-CTACCGCACAACGCACTTTC-3′ REV: 5′-AAGGGCTTCAATCTGTTCCTG-3′
Ccnd2	FWD: 5′-CTGACCAAGATCACCCACAC-3′ REV: 5′-CTCTTGACGGAACTGCTGAAG-3′
Ccnd3	FWD: 5′-GAAACCACACCCCTGACTATTG-3′ REV: 5′-AGGTCCCACTTGAGCTTCC-3′
Ccne1	FWD: 5′-ACAGCTTATTGGGATTTCAGC-3′ REV: 5′-GGAGCAAGCACCATCAGTAAC-3′
Ccne2	FWD: 5′-AGCCAGACTCTCCACAAGAAG-3′ REV: 5′-ATTCCTCCAGACAGTACAGGTG-3′
Cdkn1a	FWD: 5′-GTCTTGCACTCTGGTGTCTCA-3′ REV: 5′-GCACTTCAGGGCTTTCTCTT-3′
Cdkn1b	FWD: 5′-TTCGACGCCAGACGTAAAC-3′ REV: 5′-TTCAATGGAGTCAGCGATATG-3′
Hprt1	FWD: 5′-AGTTCTTTGCTGACCTGCTG-3′ REV: 5′-TTTATGTCCCCCGTTGACTG-3′
B2m	FWD: 5′-CGTGATCTTTCTGGTGCTTG-3′ REV: 5′-ATTTGAGGTGGGTGGAACTG-3′

Abbreviation: Ccnd1, cyclin D1; Ccnd2, cyclin D2; Ccnd3, cyclin D3; Ccne1, cyclin E1; Ccne2, cyclin E2; Cdkn1a, p21Cip; Cdkn1b, p27Kip; B2m, β2 microglobulin; FWD, forward; Hprt1, hypoxanthine-guanine phosphoribosyltransferase; REV, reverse.

(Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The plasmid pMIR-REPORT (Promega Corporation) expressing the β-galactosidase gene was used for normalization. The medium was replaced 24 hours after transfection and the cells were treated with 10 or 50 μM RSV in the absence or presence of FSH 100 ng/mL for 24 hours. Then, cells were washed with PBS and total lysates prepared using passive lysis buffer. Samples were processed with the Dual-Luciferase Reporter System (Promega Corporation), and luciferase activity was measured using Junior LB9509 Luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase activity was normalized to β-galactosidase activity and expressed as arbitrary units.

2.8 | Cell viability assay

The monolayers were washed twice with PBS, and then the cells were recovered by trypsinization. The cell suspension was then centrifuged for 5 minutes at 400*g*, and the cell pellet was recovered. Cells were resuspended in a fresh medium and 0.4% trypan blue was added. Cells that excluded the colorant (viable) and those that were stained (nonviable) were evaluated in a Neubauer chamber (Sigma-Aldrich, St Louis, MO).

2.9 | Western blot analysis

Sertoli cells cultured in six-well plates were washed once with PBS at room temperature. Then, 200 μL PBS containing 2 μL protease inhibitor cocktail, 1 mM NaF, 1 mM egtazic acid, 1 mM ethylenediaminetetraacetic acid, 50 nM okadaic acid, and 2 mM phenylmethylsulfonyl fluoride were added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. For Western blot analysis, 2× Laemmli buffer (4% [w/v] sodium dodecyl sulfate, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, 0.004% [w/v] bromophenol blue, and 0.125M Tris-HCl, pH 6.8) was added and thoroughly mixed.[24]

Samples were immersed in a boiling water bath for 5 minutes and then immediately settled on ice. Proteins were resolved in 5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (5% acrylamide/bisacrylamide for the resolving gel and 4.3% acrylamide/bisacrylamide for the stacking gel) in a Mini Protean 3 Cell (Bio-Rad, Hercules, CA). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis, gels were equilibrated in transfer buffer for 10 minutes and electrotransferred at 100 V for 60 minutes onto polyvinylidene fluoride (Hybond-P; Amersham Pharmacia Biotech, Little Chalfont, Bucks. H, UK) using Mini Trans-Blot Cell (Bio-Rad). Membranes were probed with commercial antibodies for phospho-ACC (P-ACC) (Ser79) and AKT (New England Biolabs Inc, Beverley, MA) that allow specific recognition of both P-ACC and total-AKT protein. The intensities of autoradiographic bands were estimated by densitometric scanning using NIH Image software (Scion Corporation, Frederick, MD).

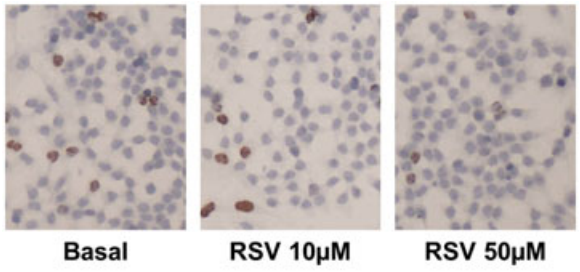
2.10 | Statistical analysis

All experiments were run in triplicates and repeated three to four times. One-way analysis of variance and post hoc analysis using Tukey-Krämer's multiple comparisons test were performed using GraphPad InStat version 6.00 (GraphPad Software, San Diego, CA). *P* values of less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Effect of RSV on Sertoli cell proliferation and on cyclins and cell cycle inhibitors expression

Sertoli cell cultures were incubated for 24 hours with 10 or 50 μM RSV, and a cell viability test performed at the end of the incubation period showed that the drug does not modify cell viability (data not shown). As a measure of the proliferation of Sertoli cells, BrdU incorporation in cell cultures was analyzed. Figure 1 shows that both doses of RSV decreased BrdU incorporation. Furthermore, the expression of cyclins and cell cycle inhibitors was evaluated. The left panels in Figure 2 show time-course studies—2-, 4-, 6-, 12-, and 24-hour incubations with 50 μM RSV—while the right panels show dose-response studies—10 and 50 μM RSV in 12-hour (A) and 4-hour (B-E) incubations—for the expression of cyclins. The left panel on Figure 2A shows that incubations with 50 μM RSV for 4, 6, 12, and 24 hours decreased *cyclin D1* messenger RNA (mRNA) levels, while a 2-hour treatment did not modify them. The right panel on the same figure shows that 50 μM RSV promoted a decrease of *cyclin D1* expression in a 12-hour incubation. The left panel on Figure 2B shows that *cyclin D2* mRNA levels diminished in 4-, 6-, and 12-hour incubations with 50 μM RSV. The right panel of Figure 2B shows that only the 50 μM RSV decreased *cyclin D2* expression in a 4-hour incubation. Right and left panels in Figure 2C show that RSV treatments did not modify *cyclin D3* expression. The left panel in Figure 2D shows that *cyclin E1* mRNA levels decreased in 2-, 4-, 6-, and 12-hour incubations with 50 μM RSV, and the right panel in the same figure shows that this decrease was observed in 4-hour incubation with both doses studied. The left panel in Figure 2E shows that *cyclin E2* mRNA levels decreased in 6- and 12-hour incubations with 50 μM RSV, and the right panel in the



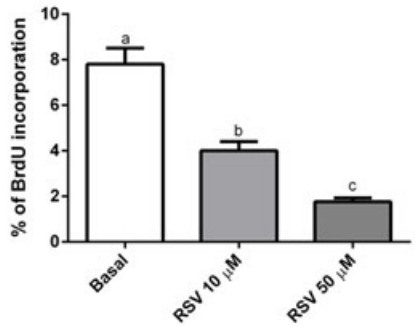


FIGURE 1 Effect of resveratrol on Sertoli cell proliferation. Sertoli cell monolayers obtained from 8-day-old rats were maintained under basal conditions or incubated with RSV (10 or 50 μM) for 24 hours. At the end of the culture period, BrdU incorporation was evaluated. Representative images of Sertoli cells showing immunoreactivity for BrdU (brown) are shown. Each bar represents percentage of BrdU-positive cells (2000 cells per group). Results are presented as mean ± SD of four independent experiments. Different letters indicate statistical significant differences ($P < 0.05$). BrdU, bromodeoxyuridine; RSV, resveratrol

same figure shows that this decrease was observed only in 4-hour incubations with 50 μM RSV.

As for the expression of the Cip/Kip family cell cycle inhibitors, the left panel in Figure 3A shows that 50 μM RSV increased $p21^{Cip}$ mRNA levels in 4-, 6-, 12-, and 24-hour incubations, and the right panel in the same figure shows that both doses of RSV utilized in this study promoted an increase in $p21^{Cip}$ mRNA levels in a 4-hour incubation. Moreover, the left panel in Figure 3B shows that 50 μM RSV promoted an increase in $p27^{Kip}$ mRNA levels in 4-hour incubations, while the right panel shows that only the 50 μM RSV dose increased $p27^{Kip}$ mRNA levels in a 4-hour incubation.

3.2 | Effect of RSV on FSH regulation of Sertoli cell proliferation

Considering that FSH is the major Sertoli cell mitogen, a possible role of RSV on FSH-regulated Sertoli cell proliferation was assessed. First, Sertoli cell cultures were incubated with 10 or 50 μM RSV in the presence of 100 ng/mL FSH for 24 hours and BrdU incorporation was analyzed. Figure 4A shows that 10 and 50 μM RSV inhibited FSH stimulation of BrdU incorporation. Second, to analyze a possible role of RSV on FSH regulation of cyclins expression, Sertoli cell cultures were treated with FSH in the absence or presence of RSV 50 μM. It is worth mentioning that FSH increased *cyclins D1* and *D2* expression but did not modify *D3*, *E1*, and *E2* expression (data not shown). Figure 4B shows that RSV inhibited FSH-stimulated *cyclins D1* and *D2* expression.

3.3 | Possible participation of cMYC in the action of RSV

cMYC regulates the expression of cyclins and cell cycle inhibitors.[25] In this context, we hypothesized that cMYC regulation might be a mechanism by which the effects of RSV on cell proliferation are produced. To evaluate this hypothesis, *cMyc* mRNA levels and cMYC transcriptional activity were determined in immature Sertoli cell cultures treated with RSV in the absence or presence of FSH. The left panel in Figure 5 shows that 50 μM RSV did not modify basal and FSH-stimulated *cMyc* mRNA levels. As for cMYC transcriptional activity, Figure 5 right panel shows that RSV decreased basal and FSH-stimulated E-box-mediated reporter-gene transcription, and this result may be interpreted as a decrease in transcriptional activity of cMYC.

3.4 | *Sirt1* expression in immature Sertoli cells

As mentioned in the introduction, RSV can activate SIRT1, and, in fact, many investigators consider RSV as a tool to analyze the effects resulting from the activation of this deacetylase. Moreover, the molecular basis involved in the stimulation by RSV of SIRT1 activity has been defined.[26] In addition to SIRT1, AMP-activated protein kinase (AMPK) activation as a result of increasing doses of RSV has been demonstrated.[27-29] These latter studies opaque the hypothesis establishing that RSV effects are related exclusively to SIRT1 activation. In the present experimental conditions, no stimulation of AMPK (analyzed as P-ACC levels) was observed when Sertoli cell cultures from 8-day-old rats were treated with 10 μM RSV (data not shown). This fact allows us to speculate that RSV effects, at least with the 10 μM dose, might be accounted for by the activity of SIRT1.

As mentioned in the introduction, the presence of SIRT1 in immature Sertoli cells had not been previously analyzed. The aim of this last set of experiments was to examine a possible expression of *Sirt1* in immature Sertoli cells. The presence of *Sirt1* in cells obtained from

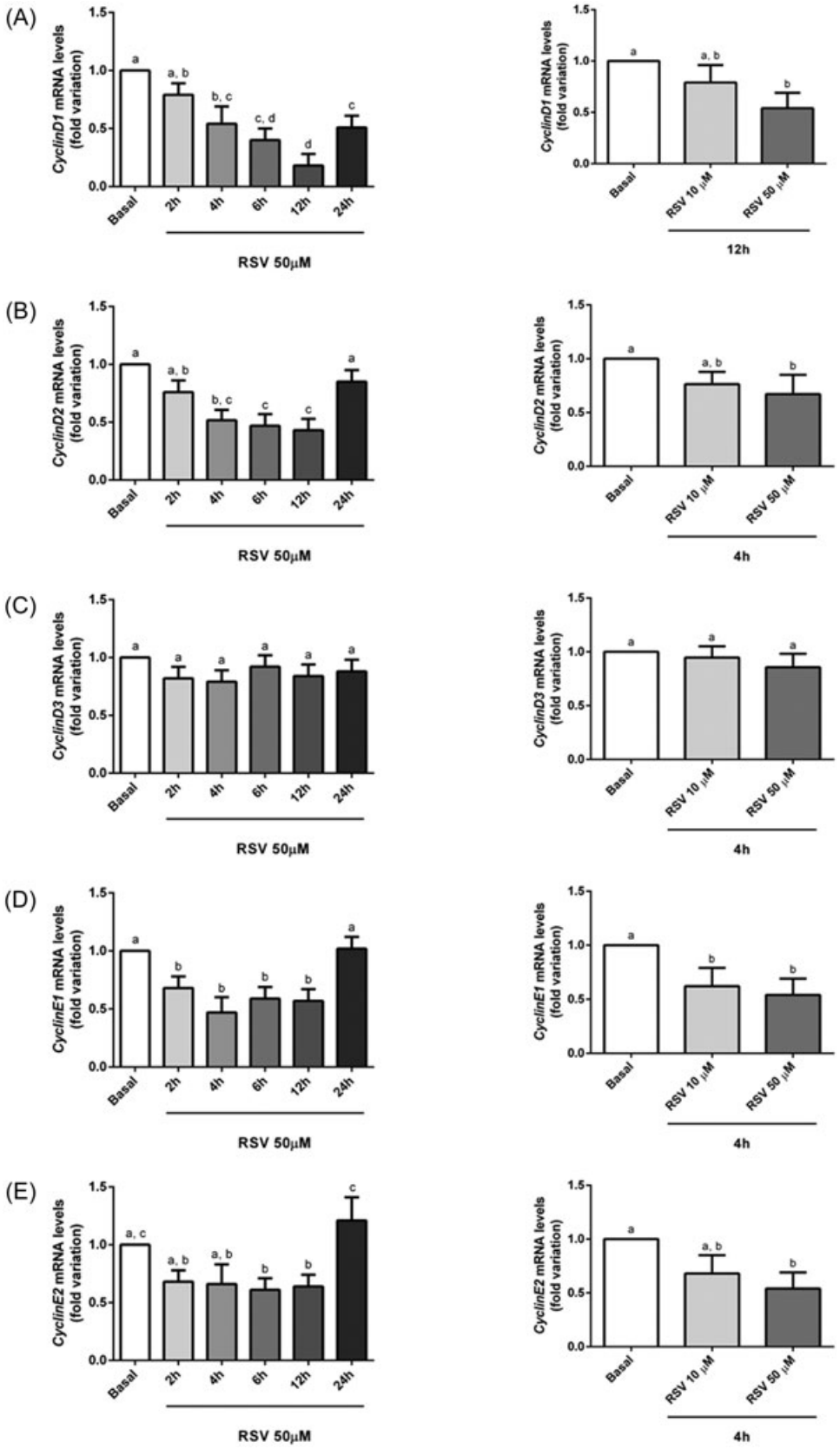


FIGURE 2 Continued.

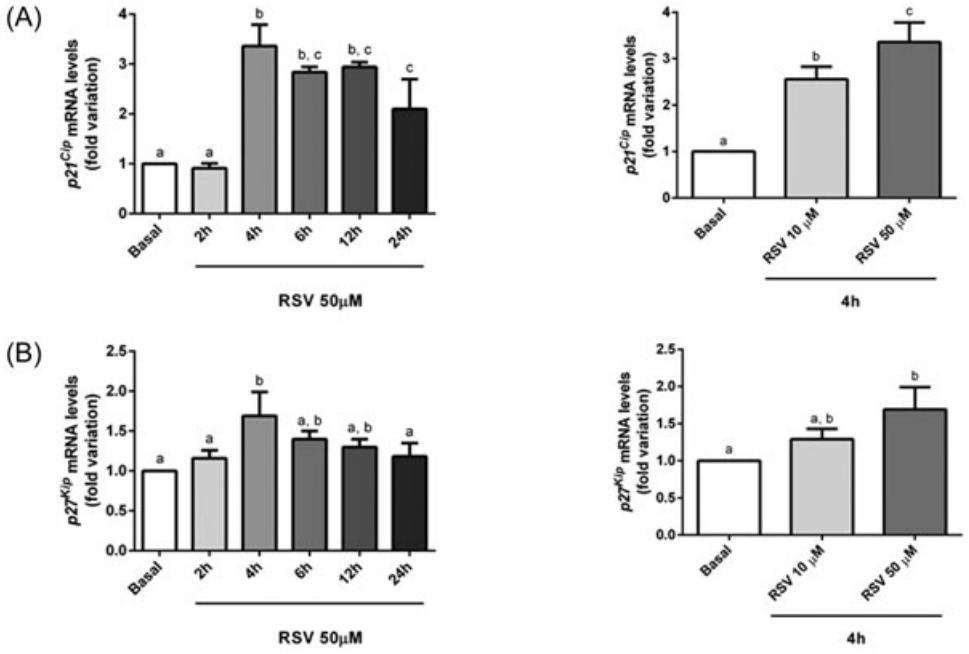


FIGURE 3 Effect of RSV on $p21^{Cip}$ (A) and $p27^{Kip}$ (B) expression. Sertoli cells obtained from 8-day-old rats were maintained under basal conditions or incubated with RSV 50 μM for 2, 4, 6, 12, and 24 hours (left panel) or with 10 or 50 μM RSV for 4 hours (right panel). Total RNA was extracted and RT-qPCR was performed. The comparative $\Delta\Delta C_t$ method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed, indicating a fold variation in mRNA levels relative to basal. Results are expressed as mean ± SD. Different letters indicate statistical significant differences ($P < 0.05$). mRNA, messenger RNA; RSV, resveratrol; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

8-, 20-, and 30-day-old rats was determined by RT-PCR analysis. Figure 6A shows that *Sirt1* was detected in immature Sertoli cells. *Sirt1* was also detected in isolated germ cells, testis, and liver. RT-qPCR studies were performed on RNAs isolated from 8-, 20-, and 30-day-old rat Sertoli cells. Figure 6B shows that significantly higher levels of *Sirt1* expression in 8-day-old Sertoli cells than in 30-day-old Sertoli cells were observed.

4 | DISCUSSION

An adequate Sertoli cell shift from an immature proliferating phenotype to a functionally differentiated adult phenotype is essential for spermatogenesis and male fertility. A few hormones related to this transition and some underlying mechanisms that may govern the cessation of Sertoli cell proliferation have been proposed.[30]

As stated before, RSV antiproliferative actions have been related to the regulation of the expression of several cell cycle regulators inducing cell cycle arrest at G1/S transition. The present investigation shows that RSV reduces Sertoli cell proliferation. Sertoli cell proliferation under diverse stimuli has been correlated with the levels of *cyclins D1*, *D2*, and *E1* in different experimental models.[31-36] Moreover, $p21^{Cip}$ and $p27^{Kip}$ expressions have been associated with the detention of proliferation and with the increase in the levels of differentiation markers of Sertoli cells.[18,30,37] Results obtained herein show that the decreased proliferation caused by RSV treatment in 8-day-old Sertoli cells is accompanied by decreased expression of *cyclins D1*, *D2*, *E1,* and *E2* and increased expression of $p21^{Cip}$ and $p27^{Kip}$, suggesting that the observed effect of RSV on proliferation under basal conditions may be explained, at least in part, by the variation of these cell cycle regulators.

The role of FSH in Sertoli cell proliferation has been largely investigated.[18,31,38-42] Considering that FSH regulates Sertoli cell proliferation during fetal and postnatal life, it is important to analyze factors that might counteract FSH

FIGURE 2 Effect of RSV on *cyclins D* (A-C) and *E* (D,E) expression. Sertoli cells obtained from 8-day-old rats were maintained under basal conditions or incubated with 50 μM RSV for 2, 4, 6, 12, and 24 hours (left panel) or with 10 or 50 μM RSV (right panel) for 12 hours (A) or 4 hours (B-E). Total RNA was extracted and RT-qPCR was performed. The comparative $\Delta\Delta C_t$ method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed, indicating a fold variation in mRNA levels relative to basal. Results are expressed as mean ± SD. Different letters indicate statistical significant differences ($P < 0.05$). mRNA, messenger RNA; RSV, resveratrol; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

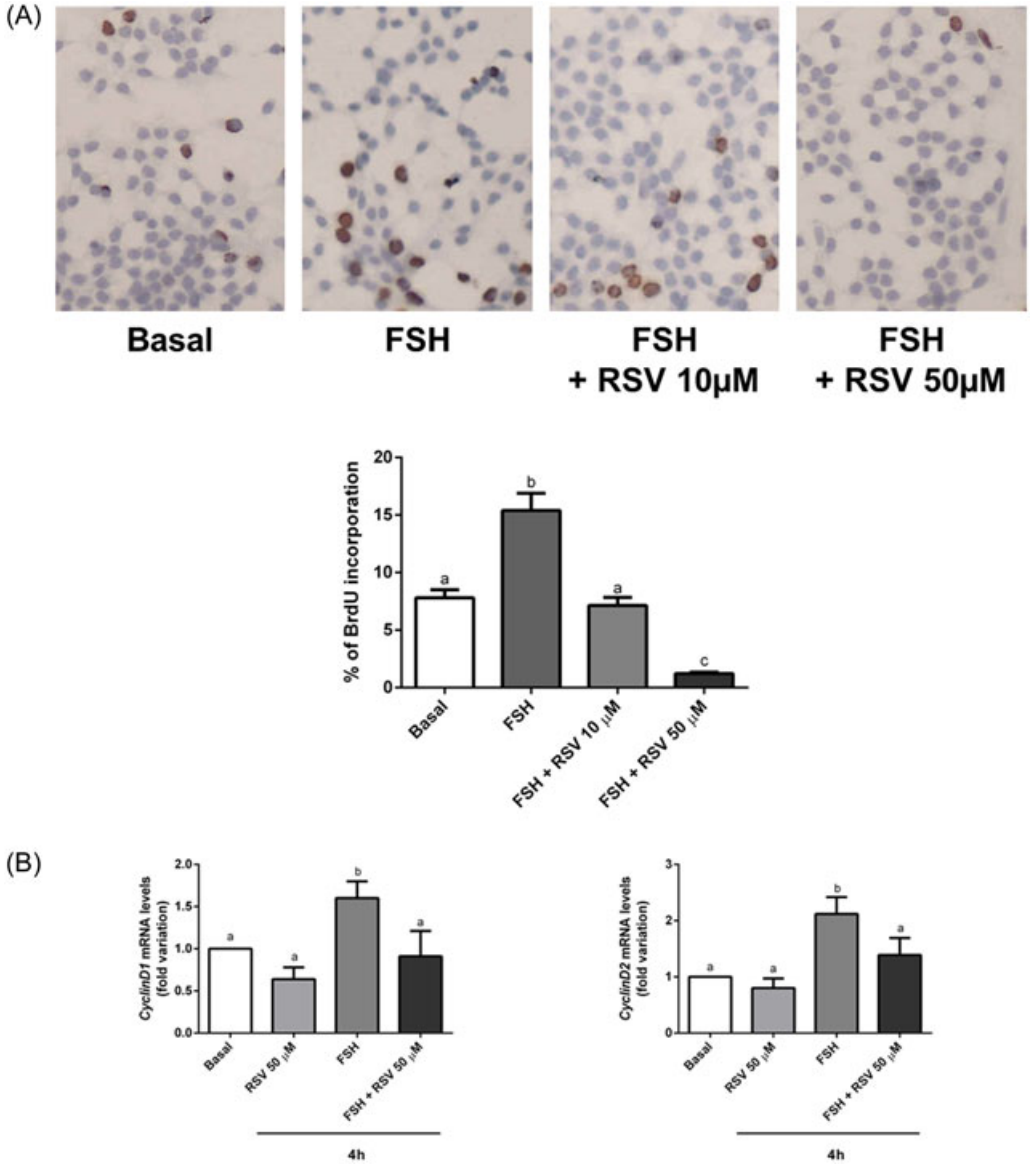


FIGURE 4 Effect of RSV on FSH stimulation of Sertoli cell proliferation. Sertoli cell monolayers obtained from 8-day-old rats were maintained under basal conditions or incubated with RSV (50 μM) in the absence or presence of FSH (100 ng/mL) for 24 hours. A, At the end of the culture period, BrdU incorporation was evaluated. Representative images of Sertoli cells showing immunoreactivity for BrdU (brown) are shown. Each bar represents percentage of BrdU-positive cells (2000 cells per group). Results are presented as mean ± SD of four independent experiments. Different letters indicate statistical significant differences ($P < 0.05$). B, Sertoli cells obtained from 8-day-old rats were maintained under basal conditions or incubated with RSV (50 μM) in the absence or presence of FSH (100 ng/mL) for 4 hours. Total RNA was extracted and RT-qPCR was performed. The comparative $\Delta\Delta C_t$ method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed, indicating a fold variation in mRNA levels relative to basal. Results are expressed as mean ± SD. Different letters indicate statistical significant differences ($P < 0.05$). BrdU, bromodeoxyuridine; FSH, follicle-stimulating hormone; mRNA, messenger RNA; RSV, resveratrol; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

action on Sertoli cell mitosis. In the present investigation, we show that RSV suppresses FSH-stimulated Sertoli cell proliferation. As for the regulation by FSH of the regulators of G1/S cell cycle transition, it has been shown that FSH can regulate, either directly or indirectly, Sertoli cell *cyclins D1* and *D2* levels in different experimental models.[31,43-45] To our knowledge, the regulation of the expression of other cyclins by FSH had not been analyzed so far. In the current study, it is shown that *cyclins D1* and *D2* expression are regulated by FSH, whereas *cyclins D3*, *E1*, and *E2* mRNA levels are not modified by the hormone. Concerning RSV action in FSH-stimulated cyclins mRNA levels, our results show that RSV treatment decreased FSH-stimulated *cyclins D1* and *D2* expression.

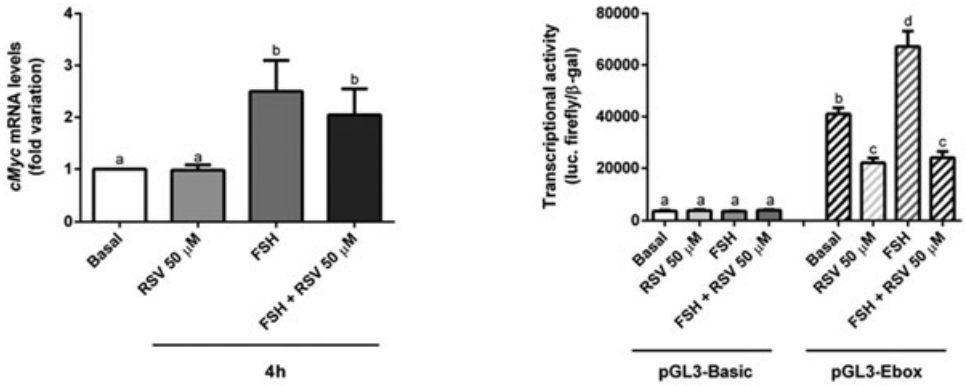


FIGURE 5 Effect of RSV on FSH stimulation of *cMyc* expression and transcriptional activity. Left panel shows Sertoli cells obtained from 8-day-old rats were maintained under basal conditions or incubated with RSV (50 μM) in the absence or presence of FSH (100 ng/mL) for 4 hours. Total RNA was extracted and RT-qPCR was performed. The comparative $\Delta\Delta C_t$ method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed, indicating fold variation in mRNA levels relative to basal. Results are expressed as mean ± SD. Different letters indicate statistical significant differences ($P < 0.05$). Right panel shows Sertoli cells obtained from 8-day-old rats transiently transfected with pGL3-E-box or pGL3-basic were treated with RSV (50 μM) in the absence or presence of FSH (100 ng/mL) for 24 hours. Results are expressed as Relative Light Units for firefly luciferase activity normalized to β-galactosidase activity. Data represent the mean ± SD of triplicate incubations in one representative experiment out of three. Different letters indicate statistical significant differences ($P < 0.05$). FSH, follicle-stimulating hormone; mRNA, messenger RNA; RSV, resveratrol; RT-qPCR, reverse-transcription quantitative polymerase chain reaction

Cell proliferation rates tightly correlate with *cMyc* expression levels in several cell types.[25] In rat Sertoli cells, *cMyc* expression varies throughout cell maturation. Particularly, *cMyc* mRNA is strongly detectable in proliferating 8-day-old Sertoli cells, while it is hardly detectable in cells obtained from 28-day-old rats with a nonproliferative mature phenotype.[46] Additionally, a clear role of cMYC in human Sertoli cell proliferation promoted by *Wnt/catenin b* has been demonstrated.[47] The results presented herein show that RSV does not modify *cMyc* mRNA levels but decreases cMYC transcriptional activity under basal conditions. It is worth mentioning that cMYC regulates a staggering number of genes[48,49] and many of these genes are related to cell cycle regulation.[50] Thus, it is not surprising that *cyclins D1 to D3* and *cyclins E1 to E2* are among those genes described as "Myc target genes."[25] Besides, it was observed that cMYC can inhibit $p21^{Cip}$ and $p27^{Kip}$ expression in human keratinocyte cells and immature B lymphoma cells, respectively.[51,52] Consequently, it is suggested that the observed decrease in cyclins and increase in cell cycle inhibitors expression in RSV-treated Sertoli cells under basal conditions can be mediated by the decreased cMYC transcriptional activity. As for FSH regulation of Sertoli cell *cMyc* mRNA levels, several reports show that the hormone induces *cMyc* expression in Sertoli cells from early pubertal and prepubertal rats.[18,53] Consequently, a possible regulation by RSV of *cMyc* mRNA levels and cMYC transcriptional activity regulated by FSH deserved evaluation. It was observed that RSV treatment does not modify FSH-stimulated *cMyc* mRNA levels, while it inhibits FSH-stimulated cMYC transcriptional activity. Altogether, our results point out a possible role of cMYC as a mediator of RSV effects on Sertoli cell proliferation in response to FSH.

As mentioned before, SIRT1 NAD^+-dependent type III histone and protein deacetylase and AMPK constitute major molecular targets for RSV. As for SIRT1, it appears to have physiological relevance in spermatogenesis.[17,54] To this respect and as mentioned in the introduction, *Sirt1*-knockout animals are infertile and Sertoli cells are immature.[16] In those studies, Sertoli cell immaturity was attributed to the hormone insufficiency that the animals present; however, a direct role of SIRT1 activation in Sertoli cells was not investigated so far. The current study shows that *Sirt1* is present in immature Sertoli cells at least up to 30-day-old rats. The presence of *Sirt1* in immature Sertoli cells points out a possible role of SIRT1 activation in Sertoli cell transition to a differentiated phenotype associated with cessation of mitosis. Considering that AMPK activation also has an antiproliferative effect[18] and that this kinase is also activated by RSV, it may be thought that the effects of RSV are mediated by this enzyme activity. However, by the fact that RSV does not modify P-ACC levels at doses that modify cell cycle behavior, it is tempting to speculate that the actions of this polyphenol are mediated by SIRT1 activation. Additionally, the results presented herein also show that *Sirt1* expression in Sertoli cells decreases with the age of the animal, thus suggesting a possible physiological role

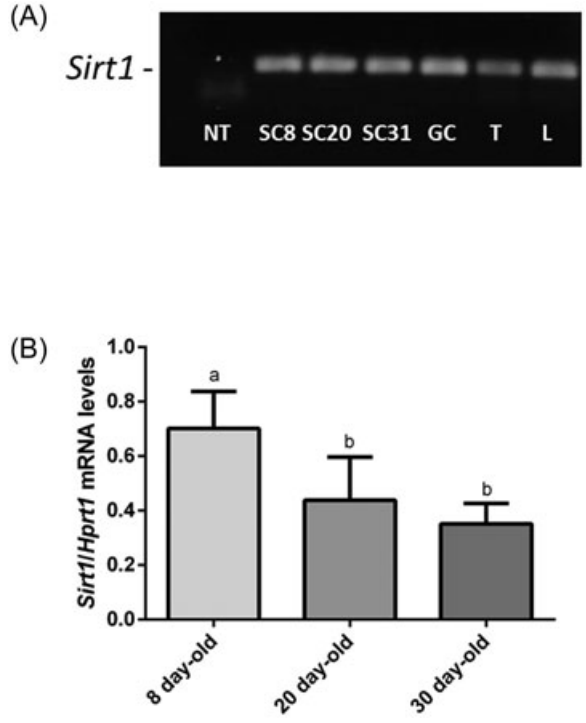


FIGURE 6 Characterization of *Sirt1* expression in Sertoli cells. A, Total RNA of Sertoli cells obtained from 8-, 20-, and 30-day-old rats (SC), germ cells (GC), adult rat testis (T), or liver (L) were extracted, analyzed by RT-PCR and visualized by ethidium bromide staining. NT indicates no template control. B, Total RNA of Sertoli cells obtained from 8-, 20-, and 30-day-old rats was extracted and RT-qPCR was performed. The data were normalized to *Hprt1*. Graphics show pooled data from four independent experiments. Results are expressed as mean ± SD. Different letters indicate statistical significant differences ($P < 0.05$). Hprt1, hypoxanthine-guanine phosphoribosyltransferase; mRNA, messenger RNA; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SIRT1, sirtuin 1

of this enzyme in immature Sertoli cells, which may be related to the cessation of mitosis and initiation of maturation.

In summary, the current study shows that RSV, possibly by activating SIRT1 and by regulating cMYC transcriptional activity, markedly decreases proliferation and antagonizes FSH action in immature Sertoli cells. Results obtained in the present investigation let us propose that SIRT1 activation might be considered as a molecular mechanism operating in immature Sertoli cells at the time of detention of proliferation and terminal maturation of this cell type aimed to sustain spermatogenesis.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Silvina Beatriz Meroni http://orcid.org/0000-0002-0313-7075

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