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# Differential effects of retinol and retinoic acid on cell proliferation: A role for reactive species and redox-dependent mechanisms in retinol supplementation

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**Abstract**
While some authors suggest that retinoids are potential anti-proliferative and antioxidant agents, evidence has suggested those present pro-oxidant properties, which might lead to malignant proliferation. These discordances stimulated one to investigate the proliferative/anti-proliferative properties of two major retinoids, retinol (ROH) and retinoic acid (RA). In Sertoli cells, ROH increased proliferation while RA was anti-proliferative. ROH increased DNA synthesis, decreased p21 levels and induced cell cycle progression. ROH increased reactive species (RS) production and stimulated p38, JNK1/2 and ERK1/2 MAPKs activation. Antioxidant treatment with Trolox blocked ROH-induced RS production, MAPKs activation and proliferation; MAPKs inhibition blocked proliferation. The potential sites of RS indicate that ROH-induced RS is promoted via mitochondria and xanthine oxidase. In contrast, RA induced neither RS production nor MAPKs activation. RA decreased DNA synthesis and increased p21 leading to cell arrest. Overall, data show that ROH, but not RA, is able to induce proliferation through non-classical and redox-dependent mechanisms.

**Keywords:** *Retinol, retinoic acid, proliferation, MAPKs, reactive species*

## Introduction

Vitamin A (Retinol, ROH) and its derivatives, retinoids, are important regulators of the cell cycle, playing a role on proliferation, apoptosis and differentiation of diverse cell types [1]. In recent years, the influence of retinoids on cell growth and differentiation has been investigated. There is a growing body of *in vitro* data demonstrating that active retinoids as retinoic acid (RA) antagonize cell growth in a variety of normal and tumour cells, characterizing them as potential chemotherapeutic agents [2–4]. In addition, some authors suggest that retinoids act as antioxidants and could be potential agents in antioxidant supplementation protocols for treatment and prevention of malignant and neurodegenerative diseases [3,4].

On the other hand, a growing body of evidence has suggested that retinoids present pro-oxidant properties in biological systems, which might induce cell damage, proto-oncogene activation, proliferation and neoplasic transformation [5–21]. Clinical trials have been carried out based on the potential antioxidant role of vitamin A, but some of them had to be discontinued due to increased mortality related to lung cancer incidence [5,6]. It was suggested that many of the deleterious effects observed in these clinical trials could be related to free radicals genera-

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tion in vitamin A supplementation [5]. These discordances between vitamin A pro-oxidant/antioxidant and proliferative/anti-proliferative properties are especially so when it is considered that excessive intake of vitamin A supplements has been used as 'antioxidant therapies' for prevention and treatment of diseases such as cancer, Parkinson's and Alzheimer's [5,6,22–24].

Studies have suggested that ROH and its principal metabolite RA present different redox-active properties in biological systems [9,10]. Previous works reported that ROH and retinaldehyde, but not RA, induce redox-dependent cell death in human fibroblasts [10]. In PC12 cells was observed an increased RS formation following ROH treatment, which was not detected in RA treated-cells [9]. In Wistar rats, retinol palmitate supplementation increased lipoperoxidation, protein damage and mitochondrial dysfunction in the brain, which were accompanied by depression and locomotory deficiency [18–20]. However, the pro-oxidant retinoid molecules, the mechanisms underlying its pro-oxidant effects, and its consequences on cell proliferation/death remain to be better determined. This is important when it is considering that RS are important signalling molecules by regulating map kinases (MAPKs) signalling cascades and several other redox-sensitive pathways involved in cell proliferation, death and transformation [25].

Our group has long been studying ROH actions in Sertoli cells [11–17]. They are one of the principal physiological targets and stockers of vitamin A in mammalian, playing important functions in male reproductive system [26–28]. In Sertoli cells, ROH induces antioxidant enzymes activation, lipoperoxidation and DNA damage [12,15–17]. In addition, a pre-transformed and proliferative phenotype was reported in Sertoli cells following ROH treatment [11,12]. However, it was not previously established whether ROH effects are attributed to ROH *per se* or to its principal intracellular metabolite RA, its mechanisms and the contribution of RS in ROH and/or RA effects.

In particular, the apparently conflicting data regarding the pro-oxidant/antioxidant and proliferative/anti-proliferative potential of different retinoid molecules and our aforementioned data stimulated us to compare the effect of two major intracellular retinoids, ROH and RA, on cell proliferation and its mechanisms in a model of primary, undifferentiated and low-proliferative Sertoli cells [12,29]. Here, we report that ROH increased cell proliferation while RA presented an anti-proliferative effect. These differential effects were dependent on ROH, but not RA, ability to increase RS production in mitochondria leading to a redox-sensitive activation of MAPK pathways.

## Materials and methods

### *Materials*

All-*trans* retinol alcohol (ROH), all-*trans* retinoic acid (RA), 2′,7′-dichlorohydrofluorescein diacetate (DCFH-DA), phospho-JNK1/2 (Thr 183/Tyr 185), phospho-p38 (Thr 180/Tyr 182) and phospho-ERK1/2 (Thr 185/Tyr 187) specific antibodies were from Sigma Chemical Co. (St Louis, MO). SP600125 was from Promega Corporation (Madison, USA). SB203580 were from Merck Biosciences (Darmstadt, Germany) and UO126 were from Biomol Research Laboratories (Plymouth Meeting, PA). ROH, RA and inhibitors were dissolved in dimethylsulphoxide (DMSO). Solvent controls were performed for each condition.

### *Sertoli cells cultures*

Sertoli cells were isolated as previously described [12]. Briefly, testes of 15-day-old rats were removed, decapsulated, digested enzymatically with trypsin and centrifuged at $750 \times g$ for 5 min. The pellet was mixed with soybean trypsin inhibitor, then centrifuged and incubated with collagenase, hyaluronidase and deoxyribonuclease for 30 min at 37°C. After centrifugation (10 min at $40 \times g$), the pellet was taken to isolate Sertoli cells. Cells were plated in multi-well plates ($2.1 \times 10^5$ cells/cm$^2$, 80% confluence) in Medium 199, pH 7.4, 1% FBS and maintained at 37°C for 24 h to attach. The medium was then changed to serum-free medium and cells were taken for assay after 48 h of culture.

### *Cell proliferation assays*

[Methyl-$^3$H] thymidine incorporation was assessed as indicative of the DNA synthesis and proliferation rate in Sertoli cells [30]. At 24 h prior treatments, cells were pre-warmed with 0.5 μCi/mL [Methyl-$^3$H] thymidine (248 GBq mmol$^{-1}$; Amersham, UK) in order to stabilize the intracellular nucleotide pool. Protein kinase inhibitors and antioxidants were added 30 min prior to ROH/RA incubation. At the end of 30 min, the medium was changed and a new medium containing ROH or RA was added for an additional 24 h. After treatments, 1 μCi/ml of [methyl-$^3$H] thymidine diluted in medium was added for an additional 12 h. To confirm that thymidine incorporation is due to DNA synthesis and not to DNA repair mechanisms, cells were treated with hydroxyurea, a potent inhibitor of DNA synthesis [31]. At the end of treatments, 3 mm hydroxyurea was incubated for an additional 2 h. After, the 1 μCi/ml radiolabelled thymidine solution was added. Cells were washed with PBS and 500 μl TCA 10% was added. TCA insoluble pellet was collected by centrifugation ($1000 \times g$, 10 min) and resuspended in 0.1% SDS containing 1 mg/mL

bovine albumin. After, 1 mL liquid scintillant was added and incorporated radionucleotide was measured using a Packard Tri-Carb Model 3320 scintillation counter. To assess the effect of ROH/RA on cell number/growth, cells were plated in 96-well plates ($1.1 \times 10^5$ cells/well). At 24 and 48 h after ROH/RA incubation, cells were trypsinized and counted in a hemocytometer.

*Cell cycle analysis*

After treatments, cells were harvested with trypsin-EDTA, washed twice with PBS containing 5 mM EDTA and fixed in 70% ethanol at $-20^{\circ}$C. After pre-treatment with 50 μg/ml RNAse A, cells were stained with 50 μg/ml propidium iodide (PI; Sigma) solution for at least 30 min on ice. Stained and single cells were analysed by flow cytometry (FACScalibur system, Becton and Dickinson, San Jose, CA). Ten thousand cells were analysed per sample.

*SDS-PAGE and immunoblot*

Proteins (20 μg) were separated by SDS-PAGE on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide gels and electrotransferred onto nitrocellulose membranes. Membranes were then incubated in TBS-T (20 mM Tris–HCl, pH 7.5, 137 mm NaCl, 0.05% (v/v) Tween 20) containing 1% (w/v) non-fat milk powder for 1 h at room temperature. Subsequently, the membranes were incubated for 12 h with the appropriate primary antibody (dilution 1:200–1:1000). After washing in TBS-T, blots were incubated with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG antibodies (1:10 000), 1.5 h at room temperature. Bands were detected using chemiluminescence and densitometric analysis was performed by Image-J® software.

*Determination of intracellular RS production*

Intracellular RS production was detected using 2′, 7′ dichlorodihydrofluorescein diacetate, DCFH-DA [32]. This reagent enter the cells and react predominantly with highly oxidizing species of RS such as hydroxyl radicals (OH), hydroperoxides and peroxynitrite, thus producing the fluorophore dichlorofluorescein (DCF). Briefly, cells were seeded in 96-well plates, and 50 μM DCFH-DA dissolved in medium containing 1% FBS was added 30 min before ROH/RA incubation to allow cellular incorporation. Then, the medium was discarded and cells were treated. The DCFH oxidation was monitored at 37°C in a 96-well plate fluorescence reader with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm.

*Superoxide production in submitochondrial particles*

Submitochondrial particles (SMP) were obtained as previously described by Boveris [33]. Superoxide production in SMP was determined using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37°C (E480 nm 4.0/mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl (pH 7.4), SMP (0.3–1.0 mg protein/mL), 0.1 mM catalase and 1 mM epinephrine. Succinate (7 mM) was used as substrate. Superoxide dismutase was used at 0.1–0.3 μM final concentration to give assay specificity.

*Xanthine oxidase activity*

The xanthine oxidase (XOD) activity was measured spectrophotometrically at 295 nm and 37°C using xanthine as the substrate [14]. The formation of uric acid from xanthine increases the absorbance. One unit of activity was defined as 1 μmol uric acid formed per minute at 37°C and pH 7.4 and was expressed in units/mg protein.

*Protein quantification*

Protein contents were measured by the Lowry et al. [34] method.

*Statistical analysis*

Data are expressed as means ± SD and were analysed by one-way ANOVA followed by Duncan's post-hoc test. Differences were considered significant at $p < 0.05$.

## Results

*Proliferation, cell cycle analysis and p21 levels in ROH- and RA-treated cells*

Radiolabelled thymidine incorporation into DNA was assessed to measure DNA synthesis and the proliferative activity of Sertoli cells [30]. ROH and RA concentrations were chosen based on the physiologic range for these compounds which is in micromolar to ROH and in nanomolar to RA [35,36]. Results from thymidine incorporation assay showed that, at the end of 24 h treatment, 7 and 10 μM ROH significantly increased the rate of proliferation while 1–100 ηM RA presented an opposite effect by decreasing the rate of thymidine incorporation into DNA (Figure 1A). Treatment with 3 mM hydroxyurea for 2 h before thymidine incubation drastically reduced thymidine incorporation into DNA in both untreated and 7 μM ROH groups, suggesting that, in our experiments, thymidine incorporation involved DNA synthesis (Figure 1A). At 48 h after treatments,

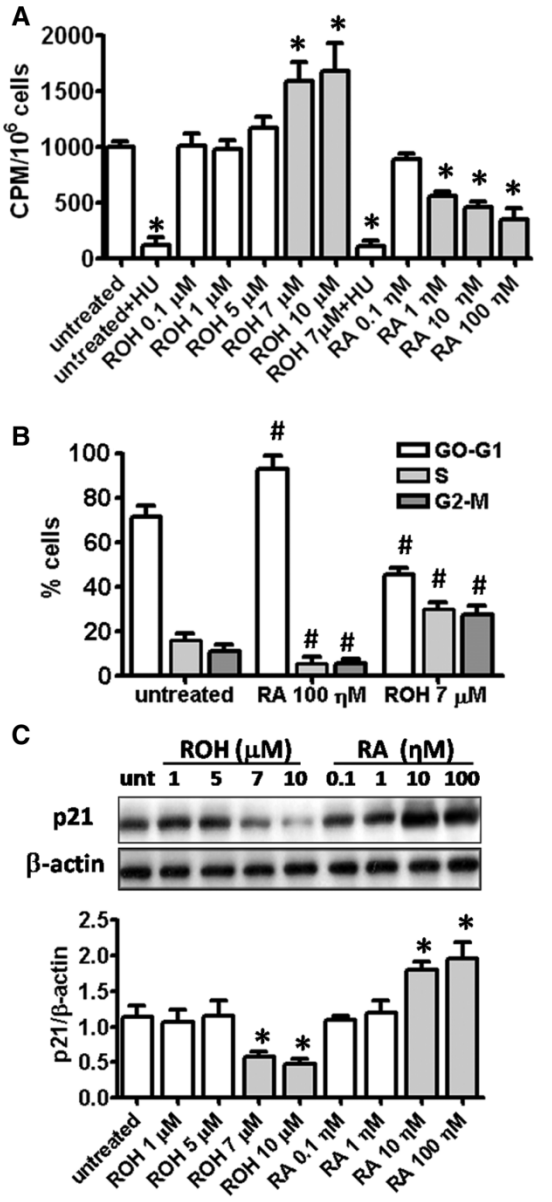


Figure 1. Cell proliferation in ROH and RA-treated Sertoli cells. Cells were treated for 24 h with different ROH and RA concentrations. (A) Radiolabelled thymidine incorporation into DNA. (B) Cells were treated with 7 μM ROH or 100 ηM RA during 24 h. After, cell cycle analysis was determined by flow cytometry. (C) Representative immunobloting with densitometry analysis of the CDK inhibitor p21 content after 24 h ROH/RA treatment. ROH, retinol; RA, retinoic acid; HU, hydroxyurea, 3 mM. Representative from three independent experiments ($n=3$). *Different from untreated cells; #different from untreated cells considering the same cell cycle phase.

cell number increased $33 \pm 5\%$ ($1.41 \pm 0.07 \times 10^5$ cells/well) in 7 μM ROH treatment compared to untreated cells ($1.05 \pm 0.06 \times 10^5$ cells/well), confirming that ROH stimulates cell growth. RA (100 ηM) did not alter cell number ($1.01 \pm 0.08 \times 10^5$ cells/well). Corroborating results from cell cycle analysis showed that 7 μM ROH induced a significant increase in S phase and G2-M phase cells suggesting cell cycle progression (Figure 1B). On the other hand, 100 ηM RA increased cell number in G1–GO phase with subsequent decrease in S and G2-M phase compared to untreated cells, suggesting suppression of proliferation (Figure 1B). No sub-G1 cells were detected in 100 ηM RA. In agreement with cell proliferation assay and cell cycle analysis, immunodetection of the cyclin-dependent kinase (CDK) inhibitor p21 showed that 7 and 10 μM ROH decreased p21 levels, while RA increased p21 after 24 h treatment (Figure 1C). These data suggest that ROH stimulated cell proliferation while RA presented an anti-proliferative effect.

### *ROH-induced cell proliferation is dependent on MAPKs; RA anti-proliferative effect is refractory to MAPK inhibitors*

To determine whether MAPK signalling pathways are involved on ROH/RA proliferative/anti-proliferative effect, we pre-treated Sertoli cells with selective MAPK inhibitors, such as SP600125 (JNK1/2 inhibitor); SB203580 (p38 inhibitor); and UO126 (MEK1/2-ERK1/2 inhibitor) at 10 μM for 30 min prior to 7 μM ROH or 100 ηM RA incubation and the rate of thymidine incorporation was assessed. After 24 h treatment, pre-treatment with JNK1/2, p38 and ERK1/2 inhibitors attenuated ROH-induced proliferation (Figure 2A). On the other hand, MAPK inhibitors did not alter RA anti-proliferative effect (Figure 2A). Besides inhibiting ROH-induced proliferation, the JNK1/2 inhibitor SP600125 also inhibited a decrease in p21 in 7 μM ROH-treated cells (Figure 2B). SB203580 (Figure 2B) and UO126 had no effect (not shown). Such as in the proliferation assay, RA effect on p21 was refractory to MAPK inhibitors.

### *ROH, but not RA, induces activation of MAPKs*

Activation/inhibition of MAPKs is directly linked to the regulation of the cell proliferation/death [25,37,38]. Data presented in Figure 2 suggest that JNK1/2, p38 and ERK1/2 MAPKs are involved in ROH-induced proliferation, but not in RA effects. MAPK activation was assessed by Western blot with antibodies capable of recognizing phophorylated (i.e. activated) forms of these proteins. As shown in Figure 3A, 7 μM ROH induced a rapid activation of JNK1/2, p38 and ERK1/2. The phosphorylated forms of p38 and ERK1/2 peaked between 15–30 min and decreased after 60 min incubation; JNK1/2 activation started at 15 min and continued for at least 60 min after ROH treatment (Figure 3A). In contrast, 100 ηM RA did not present any effect upon JNK1/2, p38 or ERK1/2 phosphorylation (Figure 3A). Thus, a 15-min incubation period was used for subsequent experiments. We also tested the effect of different ROH concentrations on MAPKs phosphorylation (Figure 3B). JNK1/2 and ERK1/2 phosphorylation were

stimulated from 5 μM ROH in a dose-dependent manner up to 10 μM. Phosphorylation of p38 was detected from 7 μM ROH. The incubation with an antibody that recognizes total JNK1/2, p38 or ERK1/2 proteins (i.e. phosphorylated plus non-phophorylated forms) confirmed that ROH treatment specifically increased MAPK phosphorylation but did not alter its total immunocontent at 15 min incubation (not shown).

The rapid (few minutes) activation of MAPKs by ROH suggest a mechanism different from RAR/RXR nuclear receptor-mediated gene transactivation and subsequent new protein synthesis, which in general takes hours or even days [39]. In fact, pre-incubation with a general inhibitor of the protein synthesis cycloheximide (CHX 5 μg/mL) for 30 min prior to 7 μM ROH incubation did not alter the pattern of the ROH-induced JNK1/2, ERK1/2 and p38 phosphorylation, suggesting that *de novo* protein synthesis is not required for activation of these kinases (Figure 3C).

### *ROH, but not RA, increases RS production*

Previous works from our group reported that ROH induces oxidative damage to proteins, lipids and

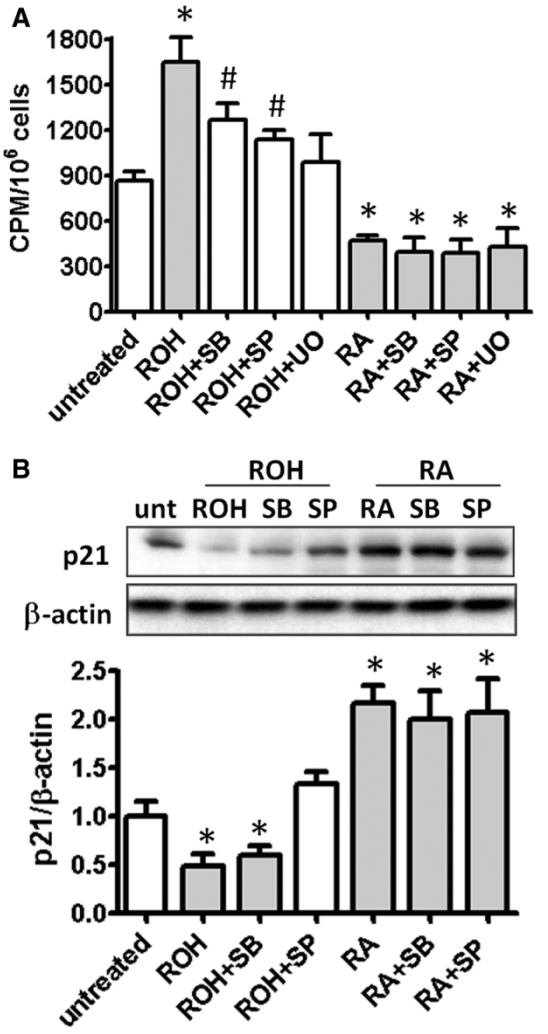


Figure 2. MAPKs mediate ROH-induced cell proliferation. (A) Radiolabelled thymidine incorporation in 7 μM ROH and 100 ηM RA-treated Sertoli cells in the presence or absence of the JNK1/2, p38 or ERK1/2 inhibitors. (B) Representative immunobloting showing p21 protein after 24 h treatment with 7 μM ROH or 100 ηM RA in the presence of MAPKs inhibitors. Inhibitors were added 30 min prior ROH or RA. SP, SP600125, JNK1/2 inhibitor; SB, SB203580, p38 inhibitor; UO, UO126, MEK1/2-ERK1/2 inhibitor. Data from three independent experiments ($n=3$). *Different from untreated cells; #different from untreated and from ROH-treated cells.

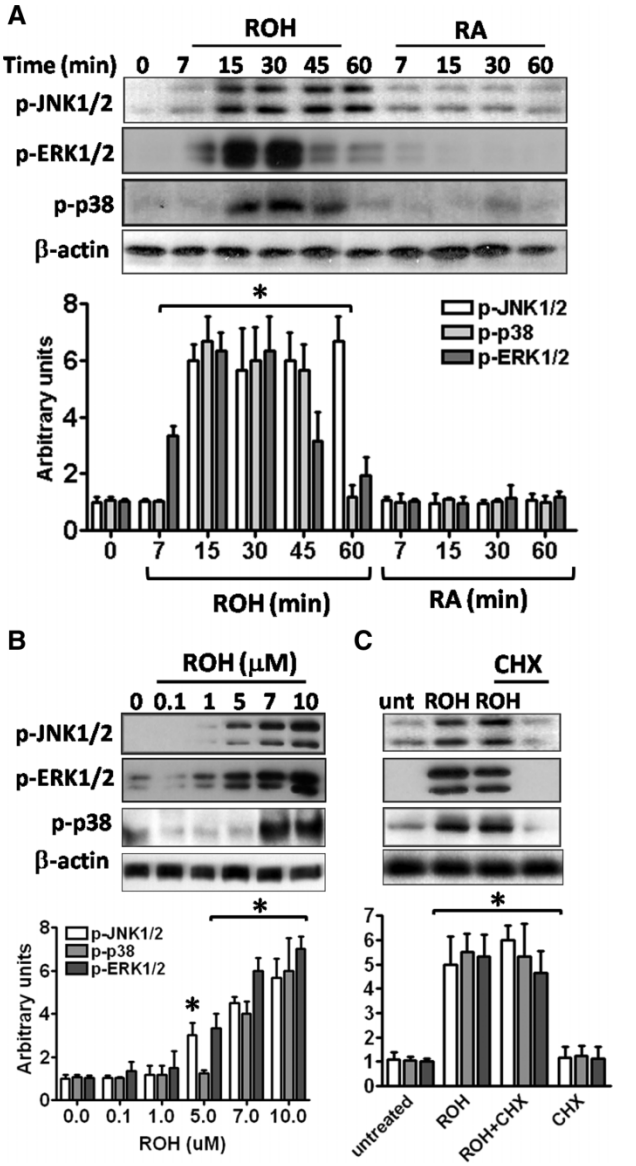


Figure 3. Phosphorylation of JNK1/2, p38 and ERK1/2 in ROH and RA-treated cells. (A) Cells were incubated for different times (min) with 7 μM ROH or 100 ηM RA and phosphorylated forms (i.e. activated forms) of JNK1/2, ERK1/2 and p38 were detected by Western blotting with specific antibodies as described in Materials and methods. (B) Effect of different ROH concentrations (μM) on MAPK phosphorylation at 15 min incubation. (C) Effect of the protein synthesis inhibitor cycloheximide (CHX, 5 μg/mL) on 7 μM ROH-elicited MAPK activation. unt, untreated cells. Representative immunoblots from three independent experiments ($n=3$). *Different from untreated cells.

DNA [12,15–17]. Since MAPK pathways are known to be activated by pro-oxidant agents [25], we decided to evaluate the possible involvement of RS on ROH-induced JNK1/2, p38 and ERK1/2 phosphorylation. Consistent with the time-course effect of ROH upon MAPKs, the DCF assay showed that ROH induces a rapid and dose-dependent increase in RS during the first 6 h incubation (Figure 4A). The increase in RS was observed between 7–20 μM ROH (Figure 4A). In contrast, RA treatment did not increase RS production at any tested concentration (Figure 4B). Pre-treatment with a RS scavenger, the alpha-tocopherol analogue Trolox (50 μM), blocked 7 μM ROH-induced RS production (Figure 5A).

*RS scavenger pre-treatment blocks ROH-induced MAPK activation, cell proliferation and p21 suppression*

To assess the involvement of RS on ROH-induced MAPK activation, cells were pre-treated with 50 μM Trolox for 30 min prior to 7 μM ROH incubation and MAPKs phosphorylation was determined (Figure 5B). Besides inhibiting RS production (Figure 5A), Trolox also blocked ROH-induced JNK1/2, p38 and ERK1/2 phosphorylation (Figure 5B). In addition, Trolox inhibited ROH-induced cell proliferation as assessed by thymidine incorporation assay. However, the anti-proliferative effect of RA was refractory to Trolox pre-treatment (Figure 5C). Trolox also inhibited the decrease in p21 in ROH-treated cells, but did not alter RA-induced p21protein (Figure 5D). These results altogether suggest that ROH induces redox-dependent activation of MAPKs and p21 suppression inducing cell proliferation, which were not observed in RA-treated cells.

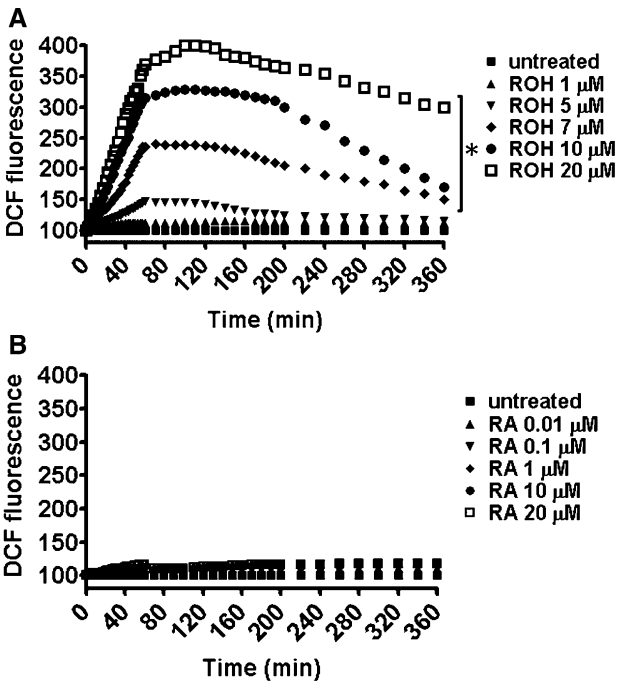


Figure 4. ROH, but not RA, induces RS production in Sertoli cells. Representative time-course effect of different concentrations of ROH (A) and RA (B) on RS production. Cells were pre-incubated for 30 min with 50 μM DCFH-DA to allow cell incorporation; after ROH or RA was added, and DCF fluorescence was monitored for 6 h in a microplate fluorescence reader. ROH lanes, different concentration of retinol, μM; RA lanes, different concentrations of retinoic acid, μM. *Different from untreated cells.

*Mitochondria and xanthine oxidase are involved on ROH-induced RS production*

Next, in order to clarify the precise source of RS in ROH treated cells, the effect of some RS-generating enzyme inhibitors was investigated. Allopurinol (the inhibitor of xanthine oxidase, 100 μM) and rotenone (the inhibitor of mitochondrial electron transport chain complex I, 10 μM) significantly decreased ROH-induced RS generation. ROH-induced RS was unaffected by DPI (the inhibitor of the NADPH oxidase, 100 μM) (Figure 6A). Rotenone (Rot) inhibitory effects on RS occurred at early times of incubation (few minutes) compared to allopurinol (Allop). Inhibitory effects of allopurinol on RS production were detected only after 3 h incubation with ROH (Figure 6A). These results suggest that xanthine oxidase and mitochondrial electron transport chain may act as the primary sites for the generation of RS by ROH in Sertoli cells. In addition, submitochondrial particles (SMP) isolated after 1–6 h of 7 μM ROH treatment presented a significant increase in the rate of superoxide production compared to untreated cells, suggesting that ROH lead to impairment on electron transfer system (Figure 6B). Consistent with the time course effect of allopurinol on RS production, xanthine oxidase (XOD) activities are increased only after 3 and 6 h of 7 μM ROH incubation (Figure 6C). In addition, it was tested whether these inhibitors could prevent the ROH-induced MAPKs activation. Only the pre-treatment with 10 μM rotenone inhibited ROH-induced p38, JNK1/2 and ERK1/2 activation allopurinol presented any inhibitory effect (Figure 6D).

## Discussion

Although vitamin A (ROH) is accepted as essential for various vital cellular processes, its ability to act as a pro-oxidant factor has been neglected. We and others have postulated that retinoids might induce oxidative stress in biological systems [8–21]. For example, incubation of Sertoli cells with ROH induces antioxidant enzymes activation, increases lipoperoxidation and DNA damage; besides, it induces chromatin sensitivity to DNAse I, ornithine decarboxylase activation, HMG dephosphorylation and phosphorylation of the H3 histone, which are important markers of entry into the S phase of cell cycle [12,15–17,40]. In the herein presented work,

we demonstrate that ROH, through redox signalling, caused the switch of cell cycle from a low-proliferative to a proliferative condition. The proliferative effect of ROH was dependent on redox-sensitive activation of JNK1/2, p38 and ERK1/2 which was not observed in RA-treated cells. ROH mitogenic signalling was accompanied by a RS and JNK1/2-dependent decrease in the CDK inhibitor p21, which is considered a classical inhibitor of cell cycle entry into S phase by maintaining cells in G1–G0 [41,42]. ROH not only decreased p21, but also increased the cell number in S and G2-M phase and the rate of DNA synthesis in a mechanism mediated by RS. Interestingly, ROH-induced proliferation was observed with the administration of 7 and 10 μM ROH, which were able to increase RS. ROH concentrations between 1–5 μM—which are considered to be within the physiological range for Sertoli cells [36]—increased neither RS production nor proliferation. Murata and Kawanishi [43] demonstrated that even lower ROH concentrations (2–5 μM) could induce DNA oxidative damage in HL-60 cells. Allen and Haskell [44] suggested that a serum ROH concentration of 3.5 μM is associated with vitamin A toxicity in infants. These data suggest the importance of keeping ROH status within the physiological range, since slight variations in the ROH levels may trigger important changes in the cellular redox state.

On the other hand, the ROH structurally related compound RA did not present any pro-oxidant effect in the physiological (0.1–10 ηM) or supra-physiological concentrations tested (100 ηM to 20 μM) [45]. Previous studies showed that only RA precursors as ROH and retinaldehyde presented pro-oxidant effects in fibroblasts and PC-12 cells [9,10]. Our data show cell differentiation events in RA-treated cells [29,41]. Particularly in Sertoli cells physiology, the decrease in cell proliferation coupled with an

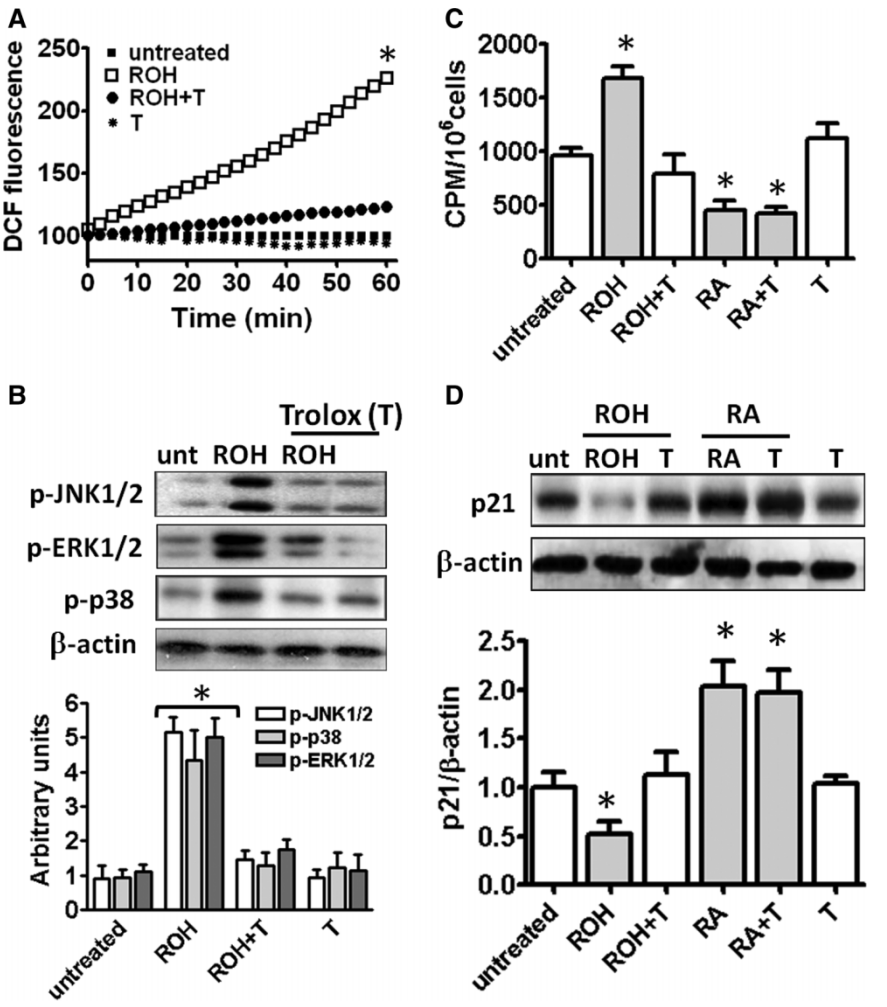


Figure 5. RS scavenger treatment inhibits ROH-induced RS production, MAPK activation, cell proliferation and $p21^{WAF1/CIP1}$ suppression. RA effects are refractory to RS scavenger. (A) Cells were pre-treated with 50 μM Trolox 30 min prior 7 μM ROH incubation and DCFH oxidation was monitored during 1 h. (B) Trolox pre-treatment inhibited ROH-elicited MAPKs phosphorylation. (C) ROH-induced proliferation and RA anti-proliferative effect in the presence of Trolox. Cells were treated with ROH or RA for 24 h and radiolabelled thymidine incorporation into DNA was assessed. (D) Representative immunoblotting showing the effect of Trolox pre-treatment on p21 levels in 7 μM ROH or 100 ηM RA-treated cells. ROH, 7 μM retinol; RA, 100 ηM retinoic acid; T, 50 μM Trolox. Representative from four independent experiments ($n = 4$). *Different from untreated cells.

increase in p21 levels—which occurred in RA-treated cells—are considered classical markers of the entry on differentiation phase [29,41]. RA positively induced p21, decreased DNA synthesis and increased cell number in G0–G1 in a way independent on RS or MAPKs. In this way, a RA genomic effect (e.g. RA binding on its nuclear receptors leading to gene transcription) leading to p21 gene induction has been accepted as a major mechanism of the RA-induced differentiation in diverse cell types [41,42,46].

Genomic actions of retinoids are mediated by two classes of nuclear receptors, RARs and RXRs [47,48]. Retinoid receptors are considered as ligand-regulated transcription factors, acting through binding to promoters of target genes and leading to changes in gene expression. These genomic actions of Vitamin A usually take hours or even days [39,48]. Recently, extra-nuclear mechanisms of signal transduction by retinoids have been reported [39,49]. These non-classical actions are rapid and do not rely on RAR/RXR-mediated gene transcription and new protein synthesis. Aggarwal et al. reported that retinoids induce CREB phosphorylation through a rapid stimulation of PKC and ERK1/2 in cells with silenced RAR/RXR [39]. In addition, ROH may act directly on the modulation of different PKC isoforms and modulate c-Raf activities [49]. Our data support that a rapid and extra-nuclear effect of ROH upon MAPK pathways plays a role in understanding ROH and RA differential effects on cell proliferation in Sertoli cells. ROH-induced MAPKs phosphorylation occurred in

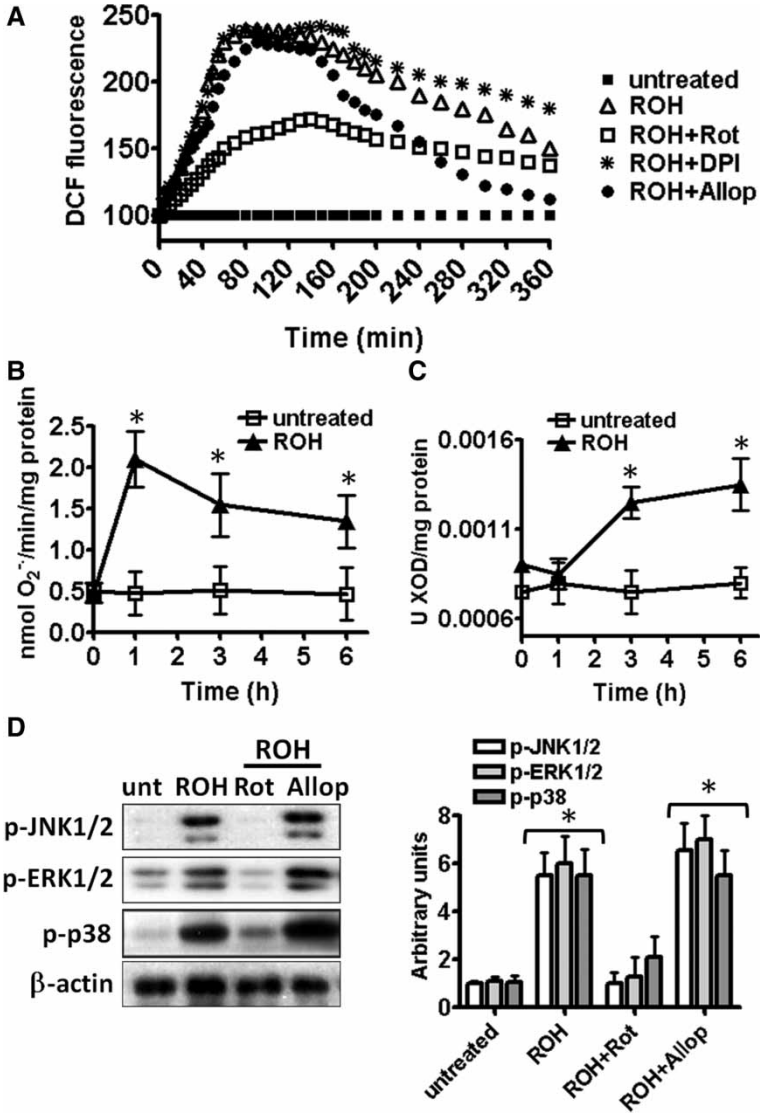


Figure 6. Mitochondria and xanthine oxidase mediate RS production in ROH-treated cells. (A) Representative DCF assay showing the effect of NADPH oxidase inhibitor (DPI), xanthine oxidase inhibitor (allopurinol), and electron chain transport inhibitor (rotenone) pre-treatment on 7 μM ROH-induced RS production. (B) Increased production of superoxide radical by submitochondrial particles isolated from cells treated with 7 μM ROH. (C) Xanthine oxidase (XOD) activity in 7 μM ROH-treated cells. (D) The effect of 10 μM rotenone or 100 μM allopurinol pre-treatment on ROH-induced MAPKs phosphorylation. DPI, diphenylene iodonium 100 μM; allop, allopurinol 100 μM; Rot, rotenone 10 μM. Data from three independent experiments ($n = 3$). *Different from untreated cells.

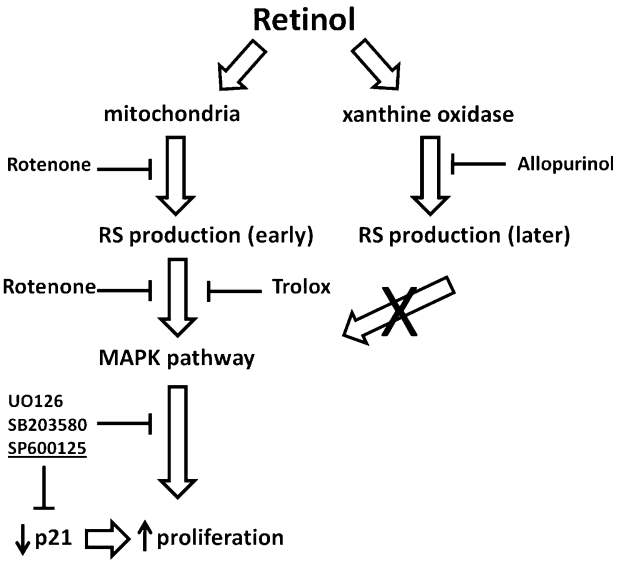


Figure 7. Proposed mechanism for the ROH-induced proliferation in Sertoli cells. ROH generates RS by modulating the mitochondrial electron transport system (early RS production, few minutes) and xanthine oxidase (later RS production, 3 h treatment). However, only the RS produced in mitochondria play a role in ROH-induced proliferation by stimulating MAPK pathways and by modulating cell cycle regulators as the CDK inhibitor $p21^{WAF1/CIP1}$.

as little as 15 min after ROH incubation, implying that the time frame is too short to trigger retinoid receptor-mediated genetic activation through transcription and translation. In addition, the data suggest that ROH effects upon MAPKs are dependent on its ability to increase RS, since the RS scavenger Trolox blocked both RS production and MAPKs activation. On the other hand, RA increased neither RS production nor MAPKs phosphorylation and its anti-proliferative effect was refractory to MAPK inhibitors.

Mitochondria-generated RS has been described as a major mechanism of the redox-stimulation of MAPKs [25,50]. In our model, ROH-induced RS formation and MAPKs activation were inhibited by rotenone, suggesting that a mitochondrial electron transfer system is involved in ROH redox signalling. The increased superoxide formation in isolated SMP suggests that ROH promotes dysfunctions in the electron transfer system, thus increasing the rate of superoxide formation. Corroborating, we previously reported superoxide over-production in liver isolated mitochondria incubated with ROH [21] and in SMP isolated from ROH supplemented rats [19]. Moreover, data presented here showed that xanthine oxidase plays a role in RS production, although only rotenone inhibited ROH-induced MAPKs activation. It possibly occurred since ROH-induced xanthine oxidase activation occurred at later time points (3 h) compared to the early mitochondria-mediated RS production and MAPKs activation (15 min).

In conclusion, ROH generates RS by modulating the mitochondrial electron transport system and xanthine oxidase activity. The RS produced in mitochondria play important roles in ROH-induced proliferation and cell cycle progression by activating MAPK pathways (Figure 7). Previous data [6–26] and data presented here are concerned that pharmacological amounts of ROH may perturb physiological processes leading to RS production and modulation of redox sensitive pathways. Although the consequences of this redox-signalling may vary in different cell types, these data reinforce the necessity of a better understanding of the redox and non-classical actions of vitamin A upon biological systems.

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