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Retinol and retinoic acid increase MMP-2 activity by different pathways in cultured Sertoli cells

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Abstract
Diseases such as atherosclerosis, arthritis and cancer have been related with imbalance in ROS production and failures in regulation of the MMPs. Authors suggested a relationship between MPP activity and ROS. Our research group has demonstrated that retinol 7μM induced changes in Sertoli cell metabolism linking retinol treatment and oxidative stress. We verified MMP activity in Sertoli cells treated with vitamin A using gelatin zymography. We found that retinol (7μM) and retinoic acid (1nM) induced MMP-2 activity in Sertoli cells. Antioxidants reversed retinol-induced but not retinoic acid-induced MMP-2 activity. Moreover, retinol but not retinoic acid increased ROS production quantified by DCFH-DA oxidation. We found that retinol and retinoic acid induced ERK1/2 phosphorylation, but only retinol-increased MMP-2 activity was inhibited by UO126, an ERK1/2 phosphorylation inhibitor. Our findings suggested that retinol-induced MMP-2 activity, but not retinoic acid-induced MMP-2 activity, was related to ERK1/2 phosphorylation and ROS production.

Keywords: *MMP, retinol, retinoic acid, ROS, Sertoli cells*

Introduction

Reactive oxygen species (ROS) are constantly generated in aerobic metabolism and play an important role in cellular processes, such as signaling pathways, when in a basal rate [1]. The term "oxidative regulation" has been proposed to indicate the functionality of redox modifications of proteins in regulation of their biological activities. Oxide-reductive reactions of biomolecules were known as "oxidative stress". Now were considered as "signals" and might contain biological information that is necessary to maintain cellular homeostasis [2]. However, an imbalance between anti-oxidant defenses and ROS production can lead to oxidative damages in biomolecules playing a causative role in numerous diseases such as atherosclerosis, arthritis, and cancer [3]. The modulation of an important family of proteases, matrix metalloproteinases (MMP), seems to be intimately related to these diseases [4].

MMPs are traditionally denominated as calcium-dependent zinc-containing endopeptidases, structurally and functionally related [5]. The main function of the MMPs is to promote the extracellular matrix (ECM) degradation. In the past years, their specific proteolytic targets have expanded to several other extracellular proteins, including an assortment of other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor binding proteins, cell surface receptors, and adhesion molecules [6]. Thus, MMP can influence directly or indirectly several cellular properties such as growth, death and migration [7].

MMPs activity is precisely regulated at different levels: transcription, activation of the precursor

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zymogens, interaction with specific ECM components, and inhibition by endogenous inhibitors. The loss of control of the MMP activity is involved in a large range of diseases [8]. Numerous signaling pathways involved in the control of MMP transcription are redox-responsive and authors have proposed a relationship between MPP activity and ROS [9]. An important member of MMP family is MMP-2, also known as gelatinase-A [10]. MMP-2 is expressed at high levels in several human tumour samples [11] and authors have described their activity in Sertoli cells [12–14].

Sertoli cells are epithelial cells that provide structural and nutritional support to developing germinal cells and participate in the architecture of seminiferous tubules**,** establishing interactions with peritubular cells and ECM in a MMP-dependent process [13,14]. Our group has used Sertoli cells as a model to study oxidative stress and redox signaling induced by vitamin A. Researchers have described a protective role of vitamin A in several diseases, which was related to its ability to scavenge toxic forms of oxygen and other free radicals in living systems [15]. In addition, the physiological function of retinoids in several cellular processes, such as division and differentiation, is well-known. On the other hand, our previous results have demonstrated that retinol supplementation induces oxidative damage in biomelecules, [16–18] upregulation of antioxidant enzymes, [19,20] preneoplasic transformation, [21] and activation of phosphorylation signaling pathways [22]. In this work, we investigated the MMP-2 activation by treatment with retinol and retinoic acid in cultured Sertoli cells. We evidenced that both retinol and retinoic acid increased MMP-2 activity. We also investigated the role of ROS in this activation. As described before, retinol treatment is able to cause ERK1/2 MAPK phosphorylation by radical overproduction [22]. We confirmed these results and verified a retinoic acid-dependent ERK1/2 MAPK phosphorylation, as well as the relationship between ERK1/2 MAPK phosphorylation and MMP-2 upregulation. Eventually, we analyzed the morphological aspect of Sertoli cell cultures for five days after the onset of retinol or retinoic acid treatment.

Materials and methods

Animals and chemicals

Type I collagenase, hyalorunidase, medium 199, HBSS, all-*trans* retinol and all-*trans* retinoic acid were purchased from Sigma, St. Louis, MO, US. Trypsin was purchased from Difco, Detroit, MI, US. Anti-phospho-ERK1/2 (Thr202/Tyr204) was obtained from Cell Signaling Technology (Beverley, MA, US). Horseradish peroxidase-coupled anti-IgG antibody was from Amersham Pharmacia Biotech (Piscataway, NJ, US). The West Pico chemiluminescent kit was obtained from Pierce (Rockford, IL, US). Pregnant Wistar rats were housed individually in Plexiglas cages. The animals were maintained in a 12h light/dark cycle at a constant temperature of 23°C, with free access to commercial food and water.

Sertoli cell culture and treatment

Sertoli cells from 15-days-old Wistar rats were isolated and cultured as described before [17,20]. In short, animals were killed by ether asphyxiation, testes were removed, decapsulated, washed in saline pH 7.4, digested enzymatically with trypsin (30min at 34°C), and centrifuged (750 × g for 5min). After, the trypsin was inhibited, the sample was centrifuged (750 × g for 5 min), the supernatant was discarded, the pellet incubated with collagenase and hyaluronidase (30min at 34°C), and once more centrifuged (10 min at 40 × g). Isolated cells were counted by Tripan blue exclusion probe in Neubauer chamber and maintained at 34°C in a humidified atmosphere of 5% CO_2 in air, growing in a plating density of 3.2×10^5 cells/cm^2 in medium 199 (pH 7.4) supplemented with 1% fetal bovine serum (v/v) in the first 24h. After that, the medium was replaced by serum free medium and cells were maintained for more 24h. Experiments were performed on cells treated with retinol (7μM), retinoic acid (1nM), and retinol vehicle (ethanol 0.1% v/v), or co-treated with Trolox® (a hydrophilic analogue of vitamin E, 100μM), the ·OH radical scavenger mannitol (1mM), the thiol antioxidants dithiothreitol (DTT; 1mM) and N-acetyl-cysteine (NAC, 1mM), and UO126 (inhibitor of ERK1/2 phosphorylation) for 24h, except in the ERK1/2 phosphorylation and DCFH-DA experiments. All treatments were performed in a light-protected environment.

Gelatin zymography

Zymography was performed as previously described [12]. In brief, culture media were removed, centrifuged (1000 × g for 5min) to eliminate cell debris, and concentrated ten times using Centricon (cut-off at 30kDa; Millipore, Bedford, MA, US). Equal amounts of cell proteins (approximately 40 μg/lane) were electrophoresed (120V) at 4°C on 8% polyacrylamide gels containing 0,2% gelatin (Sigma Chemical Co.) in the absence of reducing agent. Following electrophoresis, gel was washed in Triton X-100 2.5% pH 7.5 (three times of 30min at room temperature) to remove SDS and washed again in distilled water (three times of 30min at room temperature). The gel was subsequently incubated at 37°C for 16h in reaction buffer (50mM Tris–base, NaCl 150mM, $ZnCl_2$ 1μM, sodium azide 0.02%, CaCl 10mM, pH 8). In these conditions, gelatinases present in the samples is renatured and autoactivated.

White zones of lysis indicating gelatin-degrading activity were revealed by staining with Coomassie Brillant blue R-250 0.1%. Densitometric analyses of the gels were performed with the ImageJ® software. Data were normalized by mean of control and expressed in arbitrary units.

Determination of intracellular ROS production

Intracellular ROS production was detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). This reagent is known to enter the cells and react predominantly with highly oxidizing species of ROS, producing the fluorophore dichlorofluorescein (DCF) [23]. Briefly, cells were seeded in 96-well plates and cultured for 48h. 100μM DCFH-DA dissolved in medium containing 1% FBS was added to the cell culture 30min before retinol or retinoic acid incubation to allow cellular incorporation. Then, the medium was discarded and cells treated for 40min. The DCFH-DA oxidation was monitored with 10min interval at 37°C from the fluorescence emission intensity in a 96-well plate fluorescence reader (model F2000, Hitachi Ltd., Tokyo, Japan) with an emission wavelength set at 535nm and an excitation wavelength set at 485nm. Data were expressed in arbitrary fluorescence units.

Western blotting

SDS-PAGE and Western blotting were carried out as before reported [22]. Sertoli cells were previously cultured by 48h and retinol or retinoid acid treatments were added in culture media. After different times of treatment (15, 30, 60 and 120 minutes for retinoic acid and 1, 5, 10, 15 and 30 minutes for retinol), culture media was removed and cells were lysed in Laemmli-sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol) and equal amounts of cell proteins (approximately 35 μg/lane) were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and electroblotted onto polyvinyledilene difluoride (PVDF) membranes. Protein loading and electrobloting efficiency were verified by Ponceau S staining, and the membrane was then blocked in Tween–Tris buffered saline (TTBS; 100mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin and incubated overnight with the primary antibody to be tested. The membrane was washed in TTBS and incubated with horseradish peroxidasecoupled anti-IgG antibody, washed again and the immunoreactivity was detected by enhanced chemiluminescence.

Morphological aspect of Sertoli cell cultures

Sertoli cells were cultured as described. After 24h, the treatments were removed and cultures received media 199 pH 7.4 supplemented with 10% fetal bovine serum. Supplemented medium was replaced every two days. Before medium replacement, the cultures were observed and photographed under a phase-contrast microscopy.

Protein quantification

All the results were normalized by protein content, measured as described by Lowry [24].

Statistic analysis

Data are expressed as means ± S.E.M. Differences were considered to be significant at the $p < 0.05$ and were analyzed by one-way ANOVA followed by Duncan's post hoc test.

Results

Retinoic acid and retinol increase MMP-2 activity

Sertoli cells were treated with retinoic acid or retinol in different concentrations and gelatin zymography assay was carried out as described in *Materials and Methods*. MMP-2 migrates as a triplet around 72, 66 and 62 kDa (Figure 1.B) [12]. However, bands can appear fused and/or incompletely identifiable (Figure 1.A) as a result of gelatinolytic activity of the enzyme. We observed an increased enzyme activity in retinoic acid 1nM treatment as well as retinol 7μM treatment (Figure 1). This increase is not detected in higher concentrations indicating a no dose-dependent relationship leading us to choose these concentrations for further experiments.

Antioxidant treatment reverses MMP-2 activation induced by retinol but not by retinoic acid

Sertoli cells received Trolox® 100μM, mannitol 1mM, DTT 1mM or NAC 1mM in co-treatment with retinoic acid 1nM or retinol 7μM (Figure 2). The elevation on MMP-2 activity induced by retinoic acid was reversed only by DTT (Figure 2.A). DTT was also capable to reverse MMP-2 activation induced by retinol treatment. In addition, both

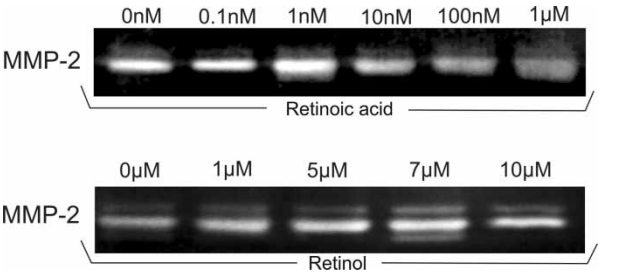

Figure 1. Retinoic acid and retinol induced MMP-2 activity. Concentrated media of cultured Sertoli cells treated for 24 hours with retinoic acid or retinol were analyzed by gelatin zymography. White lytic bands correspond to MMP-2 activity. Experiments were repeated three times and representative experiments are shown.

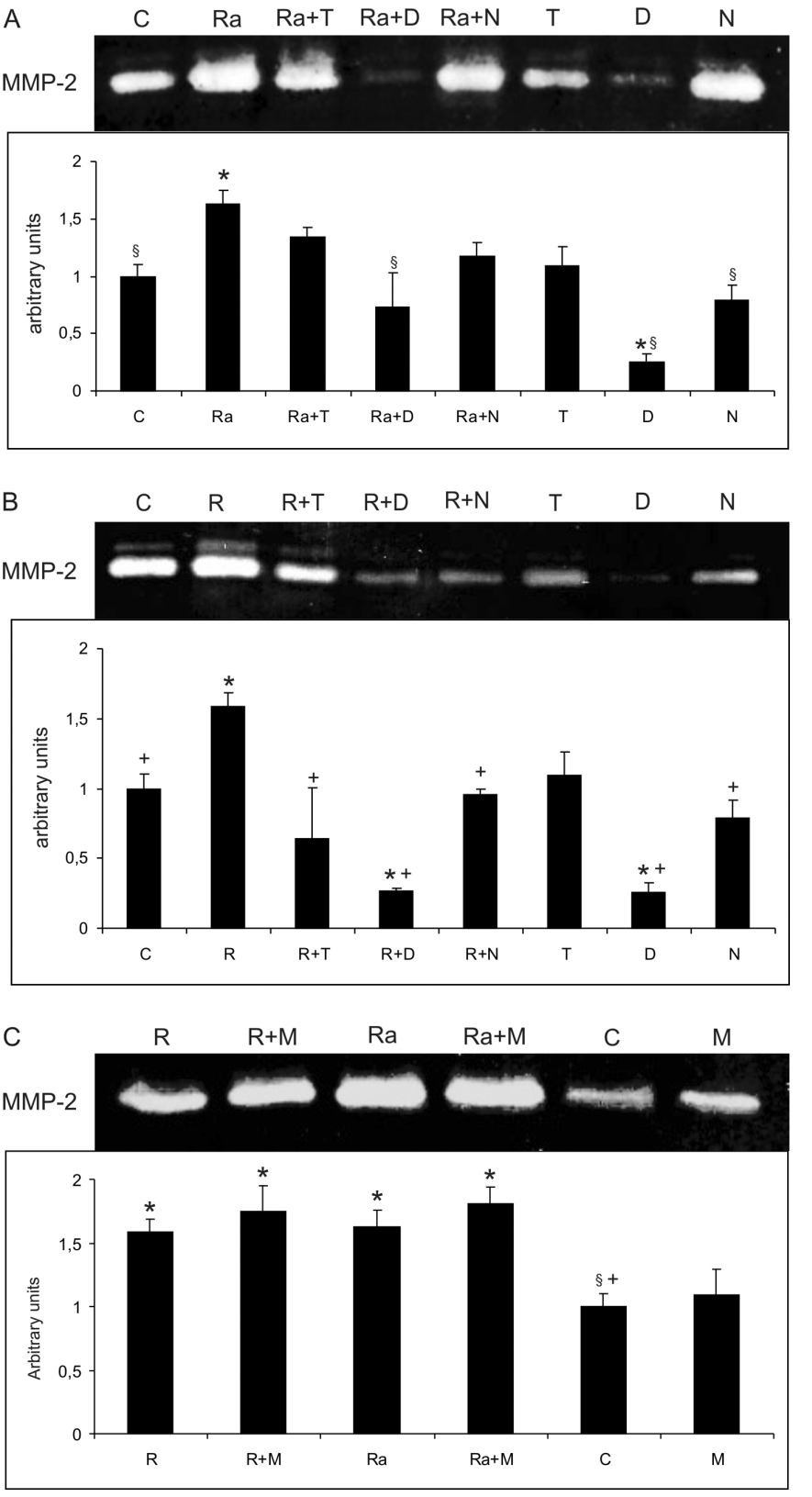

Figure 2. MMP-2 activity in cultured Sertoli cells treated with retinoic acid or retinol and co-treated with antioxidants. Gelatin zymography of concentrated culture media of Sertoli cells treated for 24 hours with**:** retinoic acid 1nM and co-treated with dithiothreitol 1mM, N-acetyl-cysteine 1mM, or Trolox® 100μM (A); retinol 7μM and co-treated with dithiothreitol 1mM, N-acetyl-cysteine 1mM, or Trolox® 100μM (B); retinol 7μM or retinoic acid 1nM and co-treated with mannitol 1mM (C). Experiments were repeated four times and representative experiments are shown. (⋆) different of control, (§) different of retinoic acid and (+) different of retinol. $p < 0.05$. C = control, Ra = retinoic acid 1nM, R = retinol 7μM, T = Trolox® 100μM, D = dithiothreitol 1mM, N = N-acetyl-cysteine 1mM and M = mannitol 1mM.

Trolox® and NAC were able to attenuate retinol-induced MMP-2 activation (Figure 2.B). Mannitol was incapable of reversing the MPP-2 activation induced by both retinoids (Figure 2.C) suggesting that ·OH radical does not seem to be involved in this MPP-2 activation.

Retinol but not retinoic acid treatment induces ROS production

Figure 3 shows that retinol 7μM enhanced intracellular ROS production during 40min period evaluated and Trolox® 100μM co-treatment blocked this effect. In contrast, retinoic acid 1nM did not increase ROS production. In DCF assay, we used hydrogen peroxide 100μM as positive control for ROS production (not shown).

Retinoic acid and retinol increase ERK1/2 phosphorylation

We perform a Western blotting with a specific antibody able to detect ERK1/2 only when dually phosphorylated (i.e. when activated; pERK1/2 [25]). Both retinoic acid and retinol induced an increase in ERK1/2 phosphorylation (Figure 4.A). A peak was identify around 15 minutes of treatment and decreasing at 30 minutes in both treatments, suggesting a rapid and transient activation of these members of MAPK family.

ERK1/2 inhibitor reverses MMP-2 activity induced by retinol but not by retinoic acid

Figure 4.B shows that the co-treatment with UO126, a classical inhibitor of ERK1/2 which impedes MEK1/2 to act in ERK1/2 phosphorylation, [26,27] reversed MMP-2 activity induced by retinol. UO126 did not reverse MMP-2 activity induced by retinoic acid (Figure 4B).

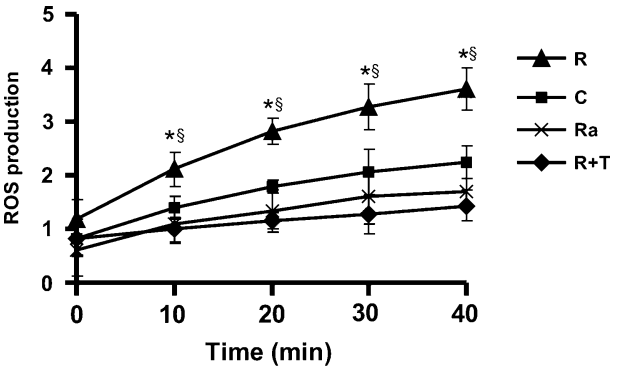

Figure 3. Intracellular ROS production determined by DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) oxidation in cultured Sertoli cells treated with retinoic acid 1nM and retinol 7μM for different times. The DCFH-DA oxidation was monitored with 10min interval at 37°C from the fluorescence emission intensity. Data were expressed in arbitrary fluorescence units. (⋆) different of control and (§) different of retinoic acid. $p < 0.05$. R = retinol 7μM, C = control, Ra = retinoic acid 1nM and T = Trolox® 100μM.

Modifications on morphological aspect of Sertoli cell cultures treated with vitamin A

Figure 5 demonstrated that cell cultures treated with retinol or retinoic acid for 24 hours presented lower density compared to control cells. After three days, cells established a monolayer and there was a similarity in the morphological aspect among the three groups (retinoic acid, retinol and control). High cell density was observed after five days and, at this time, we could observe cell agglomeration over the monolayer.

Discussion

The activity of MMPs is regulated at several levels and the loss of control of their activity leads to a breakdown in tissue homeostasis. Unregulated control of MMP activities changes tissue functionality and seems to be deleterious in biological systems [4]. Nelson and Melendez (2004) proposed an oxidative activation of pro-MMP-1 [9]. MMPs are secreted as inactive zymogens and its catalytic domain contains a highly conserved zinc-binding site bound by a cysteine residue within the propeptide region. This folded conformation is required to keep the MMP in its inactive proform. Oxidants may react and oxidatively modify the thiol residue releasing the zinc-binding domain and turning an unstable MMP-1 intermediate susceptible to cleavage by autocatalytic processing, therefore activating MMP-1. Proteolytic cleavage of MMPs is an important mechanism of zymogen activation. Given the conserved cysteine-switch domain and the general activation mechanism of most MMP family members, the activation by oxidation may regulate several MMPs [9,28].

We and others have demonstrated that retinol 7μM might induce oxidative stress in different cell systems [21,29]. Figure 3 confirms that retinol 7μM treatment generated ROS in Sertoli cells and Trolox® co-treatment decreased ROS production. Figure 2.B shows that both Trolox® and NAC were able to reverse retinol-induced MMP-2 activity at control levels and DTT decreased enzyme activity to lower levels than baseline. Altogether, these results suggested that ROS were involved in MMP-2 activity induced by retinol. In contrast, Figure 2.C shows that mannitol co-treatment was unable to reverse retinol-induced MMP-2 activity. This result contrasted to previous reports that showed mannitol preventing some of retinol biological effects [22,30].

Under normal metabolic conditions, 1–2% of the oxygen consumed by the cell is converted to $^{-}O_2$ by electron leakage at mitochondrial complex I and complex III [31] and other sites of ROS production

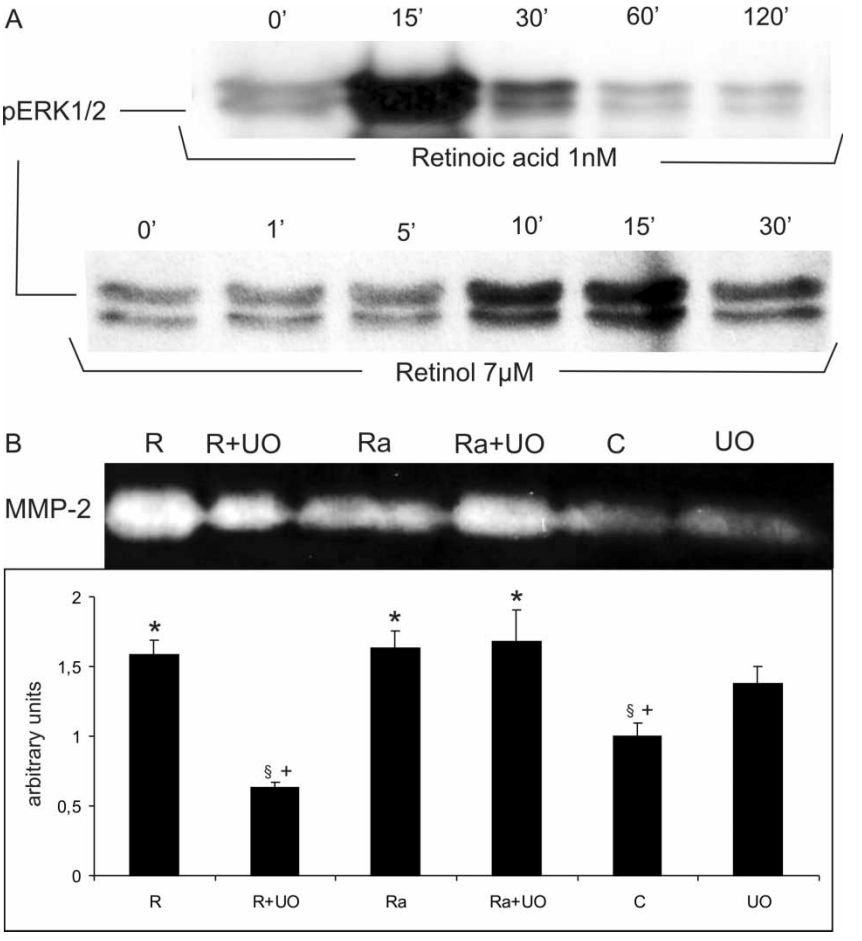

Figure 4. (A) ERK1/2 phosphorylation determined by Western blotting in cultured Sertoli cells treated with retinoic acid 1nM and retinol 7μM for different times. Sertoli cell treated with retinol or retinoid acid for different times (15, 30, 60 and 120 minutes for retinoic acid and 1, 5, 10, 15 and 30 minutes for retinol), culture media was removed, cells were lysed and immunoblot was performed. (B) Gelatin zymography of concentrated culture media of Sertoli cells treated for 24 hours with retinoic acid 1nM or retinoic acid 7μM and co-treated with UO126. Experiments were repeated three times and representative experiments are shown. (⋆) different of control, (§) different of retinoic acid and (+) different of retinol. $p < 0.05$. R = retinol 7μM, Ra = retinoic acid 1nM, C = control and UO = UO126.

such as hypoxanthine/xanthine oxidase and NADPH oxidase are known. Majority of ROS generated in these cellular systems are only moderately reactive (e.g. $^{-}O_2$ and H_2O_2) with other biological molecules [32]. At normal levels, reactive species can execute an important function in the regulation of the signaling pathways [1] acting in proteins, lipids, and nucleic acids by modifications in ROS-sensitive domains [33]. The most important redox-sensitive domain in proteins are cysteine residues, which can be modulated by alterations in their redox state, acting as redox sensor [34]. In recent years it has become clear that ROS regulate several genes, modulating process such as cell proliferation and differentiation as well as cell death and life machinery, acting as second messengers in signaling cascades [32].

One of the most important ROS that act in redox signaling is H_2O_2, that can be spontaneously or enzymaticaly generated from $^{-}O_2$ and is highly diffusible into cells [35]. However, $^{-}O_2$ and H_2O_2 can be converted into ·OH by Fenton or Haber-Weiss reactions [36]. ·OH seems inappropriate to act as a second messenger in signaling pathways as it is a high-reactive short-life radical that quickly induce irreversible damages to biomolecules [36]. The fact of the well-established hydroxyl radical scavenger mannitol [37] has not reversed MMP-2 activity induced by retinol suggest that other reactive species than ·OH mediated MMP-2 upregulation. Retinol treatment seems to generate $^{-}O_2$ and H_2O_2 as previous works showed increased activity of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase [19]. In addition, previous works have shown that an iron chelator (1,10-phenantroline) reversed oxidative damage induced by retinol [16], suggesting that Fenton reaction was involved in retinol-induced oxidative damage. However, retinol-induced MMP-2 activity seems to be independent of hydroxyl radical, in spite of it probably has been generated.

The extracellular signal-regulated kinases 1 and 2 (ERK1/2) are members of the superfamily of mitogen-activated protein kinases (MAPK). These kinases are important regulatory proteins through which

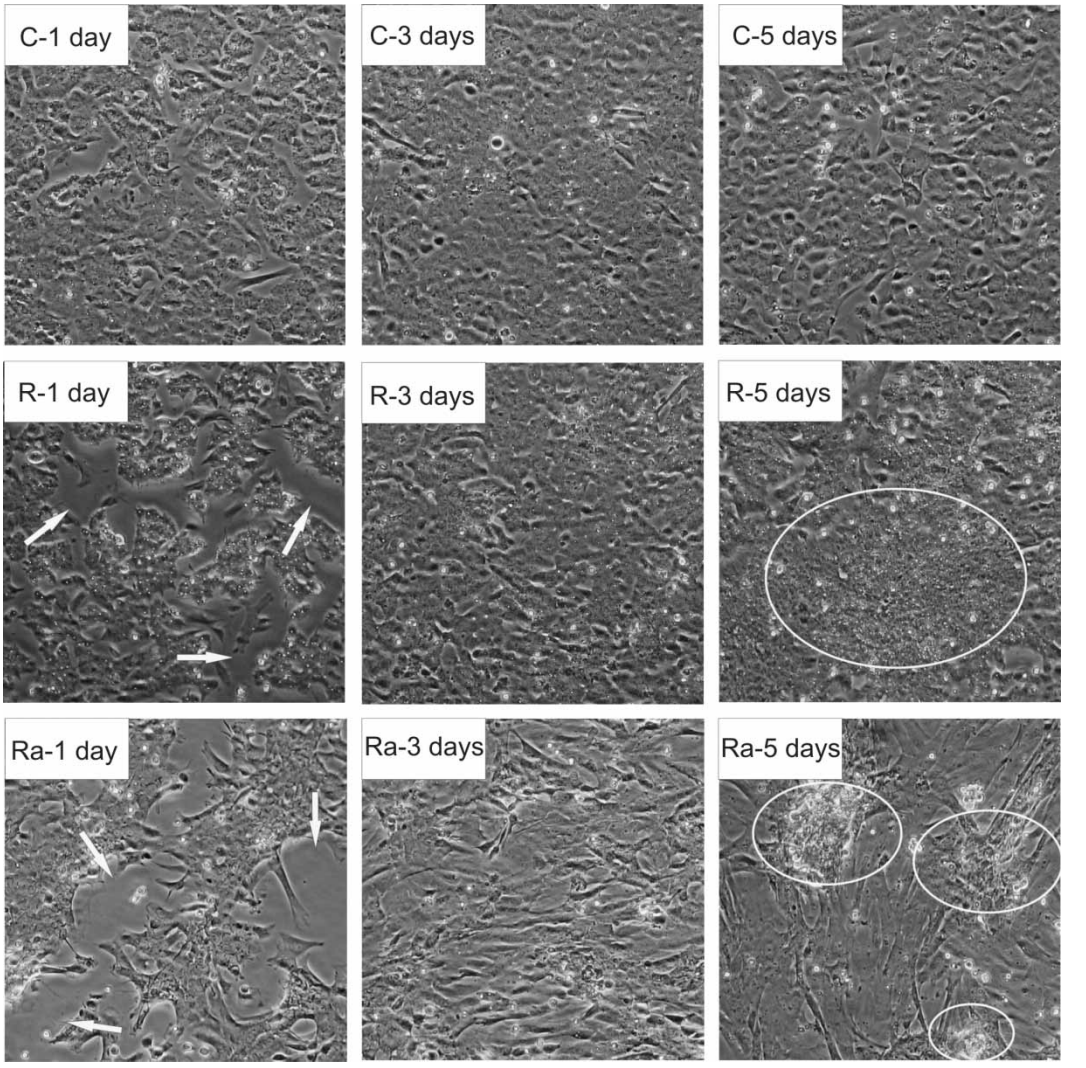

Figure 5. Morphological aspect of Sertoli cell cultures treated with retinol 7μM or retinoic acid 1nM. Sertoli cells were cultured and treated as described in *Material and Methods*. After 24 hours of treatment culture medium was replaced for 199 medium supplemented with 10% fetal bovine serum and fresh supplemented medium was added every two days. Before each medium replacement, the cultures were photographed under a phase-contrast microscope. Arrows indicate areas of low density. Ellipses indicate areas of over monolayer density. The experiments were repeated three times with nearly identical results. Representative photographs are shown. C-1 day = control after one day of culture, C-3 days = control after three days of culture, C-5 days = control after five days of culture, R-1 day = retinol 7μM after one day of culture, R-3 days = retinol 7μM after three days of culture, R-5 days = retinol 7μM after five days of culture, Ra-1 day = retinoic acid 1nM after one day of culture, Ra- 3 days = retinoic acid 1nM after three days of culture and Ra-5 days = retinoic acid 1nM after five days of culture.

extracellular signals are translated into intracellular events [38,39] and are related to MMP expression [40]. Gelain (2006) demonstrated that retinol treatment induced CREB phosphorylation mediated by ROS-dependent ERK1/2 activation [22]. CREB, once activated by phosphorylation at Ser-133, modulates cell cycle/apoptosis regulation genes, and also other transcription factors, such as different members of the AP-1 family, among others. One of the genes that CREB has been associated is MMP-2 [41]. In the Figure 4.A we show that retinol led to ERK1/2 activation. Generally, ERKs are mainly involved in anabolic processes, such as cell division and growth and can be activated by ROS such as oxygen peroxide [42,43]. Figure 4.B shows that UO126 co-treatment reverse totally retinol-induced MMP-2 activity, indicating that ERK1/2 pathway was involved in this increased enzyme activity.

Altogether, these results suggest that retinol 7μM treatment increased ROS production, which activates ERK1/2 signaling pathway, increasing MMP-2 activity. It is rational to think that higher retinol concentrations would increase ROS production. However, elevated ROS generation can damage cellular systems to react with biomolecules in unspecific sites instead of acting in signaling pathways. This may explain why higher concentrations of retinol did not induce an increase in MMP-2 activity (Figure 1.B).

Despite retinol and its derivatives, the retinoids, are recognized as key regulators of cell growth and

differentiation, their exact mechanisms of action remain unknown [44]. Retinol represents the most abundant retinoid in blood and can be conversed to retinal and subsequently to retinoic acid by dehydrogenases. Retinoic acid is the most hormonally active retinoid and is not re-conversed to retinol in biological systems [45]. Figure 1 shows that retinoic acid 1nM increased MMP-2 activity. Only DTT co-treatment reversed retinoic acid-induced MMP-2 activity, as indicated in Figure 2.A. Treatment with potent thiol reducer DTT was able to decrease MMP-2 activity lower than control group changing enzyme baseline, as discussed above. NAC, Trolox® and mannitol (Figure 2.A and C) were unable to reverse retinoic acid-dependent MMP-2 activity suggesting that ROS were not involved in this activation. Indeed, DCF assay shows that retinoic acid did not produce ROS in Sertoli cells (Figure 3). These results contrasted to findings with retinol treatment suggesting that these two retinoids acted through different pathways in MMP-2 induction. In addition, a curious data was notorious: in spite of retinoic acid induced ERK1/2 phosphorylation – similarly to retinol-induced ERK1/2 phosphorylation – the UO126 co-treatment was unable to reverse MMP-2 activity induced by retinoic acid treatment (Figure 4). In fact, authors have related that retinoic acid induced ERK1/2 phosphorylation [46,47] and Cañón (2004) demonstrated that retinoic acid played rapid effects on CREB phosphorylation by ERK activation in neuronal cells [48]. On the other hand, the fact of the co-treatment with UO126 did not alter retinoic acid-induced MMP-2 activity suggest that ERK1/2 pathway seems not to be the major pathway involved in this activation. The most important targets of retinoic acid are the retinoic acid receptors (RARs and RXR) which are nuclear receptors that regulate several genes in response to retinoic acid [45]. Watson (2004) demonstrated that an increased amount of RAR-α was involved in MMP-1 over-expression in cutaneous ageing [49]. In this way, MMP-2 activity induced by retinoic acid in Sertoli cells may be related to direct activation of RARs. However, more studies are necessary to elucidate this fact, as well as to investigate the reason by which higher concentrations of retinoic acid did not increase MMP-2 activity (Figure 1.A).

Altogether, these data support that retinol and retinoic acid induced MMP-2 by different pathways in cultured Sertoli cells. Morphogenesis of seminiferous tubules depends upon interactions among Sertoli cells, peritubular cells and ECM (which is secreted by both cells types). In the adult life, Sertoli cells undergo cyclic structural modifications corresponding to a variation of their functional state during the cycle of the seminiferous epithelium according to maturation level of the germinal cells, leading to alterations on ECM mainly mediated by MMPs regulation [12–14,50]. Thus, alterations in MMP-2 activity can impair semniferous homeostasis. Longin (2002) suggested that MMP-2 was involved in FSH-induced changes in Sertoli cells [14] and El Ramy (2005) identify increased MMP-2 in Sertoli cells activity treated with FGF2, showing this treatment mediating interactions between peritubular and Sertoli cells [12]. Figure 5 shown that both retinoic acid and retinol changed morphological aspect of Sertoli cell cultures. Decreased density observed in both treated groups after 24h of treatment agrees with a recent work that demonstrated that retinol induced ROS-dependent apoptosis in Sertoli cells [51]. In addition, our previous papers showed that retinol treatment induced cellular proliferation, [52] cell cycle progression [21] as well as ERK1/2-dependen focus formation in Sertoli cells, [22] all in a ROS-dependent manner. These can explain the over-monolayer growth observed after five days in cells treated with retinol. However, retinoic acid did not generate ROS and also was able to induce morphologic changes in Sertoli cell cultures. Thus, in some extent, MMP-2 activity can be involved in morphologic alterations induced by retinoic acid and retinol.

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