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# Mitogenic Signaling Mediated by Oxidants in Retinol Treated Sertoli Cells

FELIPE DAL-PIZZOL[a,b], FÁBIO KLAMT[a], RODRIGO J.S. DALMOLIN[a], ELENA A. BERNARD[a] and JOSÉ CLÁUDIO F. MOREIRA[a,*]

[a]*Laboratório de Estresse Oxidativo, Departamento de Bioquímica, ICBS-Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2600, Porto Alegre, RS 90035-003, Brazil;* [b]*Departamento de Medicina, Universidade do Extremo Sul Catarinense, Criciúma, Brazil*

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Recent intervention studies revealed that supplementation with retinoids resulted in a higher incidence of lung cancer. Recently the causal mechanism has begun to be clarified. We report here that retinol-induced oxidative stress is accompanied by cellular proliferation. Retinol (7 μM) significantly induced thiobarbituric acid reactive species (TBARS) formation, which was inhibited by trolox, superoxide dismutase, *N*-acetylcysteine and ethanol. This was accompanied by an increase in DNA synthesis and focus formation in cultured rat Sertoli cells. Antioxidants and ethanol inhibited retinol-induced DNA synthesis. Our findings suggest that retinol-induced oxidative stress was associated with cellular proliferation complementing our understanding of the significance of retinol supplementation in neoplastic transformation.

*Keywords*: Retinol; Vitamin A; Oxidative stress; TBARS; Proliferation; Focus

## INTRODUCTION

It is well known that retinol regulates cellular division and differentiation.[1] In spite of the important physiological functions of retinol, the effects of supplementation with supra-physiological doses of retinol are not well-defined. Many authors propose a protective role of retinoids in the development of cancer.[2–6] On the other hand, several reports suggested a positive association of retinoids intake and increased incidence of cancer.[7–12]

The production of reactive oxygen species (ROS) can lead to progressive accumulation of biomolecular damage and, consequently, are involved in many physiological (i.e. aging) and

*Corresponding author. Tel.: +55-51-316-5549. Fax: +55-51-316-5535. E-mail: pizzol@ez-poa.com.br



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pathological (i.e. cancer) processes.[13] Recently, Irani *et al.*[14] proposed that constitutive production of ROS in Ras-transformed cells activates intracellular pathways that contribute to phenotype transformation.

Recently, we[15,16] and others[17] demonstrated that retinol supplementation could induce oxidative stress damage in different cell models, and this effect probably involves iron[15] and superoxide production.[16,17]

Since ROS production could induce cell proliferation and phenotype transformation we report here that retinol-induced oxidative stress is accompanied by cellular proliferation, which is reverted by antioxidants and ethanol. We also report that retinol treatment lead to focus formation in cultured Sertoli cells.

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## MATERIALS AND METHODS

### Materials

Type I collagenase, medium 199, HBSS, all-*trans* retinol, thiobarbituric acid, superoxide dismutase, and NADPH were purchased from Sigma, St. Louis, MO, USA. Trypsin was purchased from Difco, Detroit, MI, USA. Trolox was purchased from Aldrich Chemical, Milwaukee, USA. [$^3$H] thymidine was purchased from Amersham International, Amersham, UK.

### Cell Culture

Sertoli cells from 15-day-old Wistar rats were prepared and cultured essentially as previously described.[18] In brief, the animals were killed by ether asphyxiation; testes were removed and washed in saline pH 7.4. Sertoli cells were isolated by enzymatic digestion of decapsulated testes with trypsin and type I collagenase. A small percentage (3–4%) of contamination by peritubular cells, determined by histochemical demonstration of alkaline phosphatase activity, was present in these Sertoli cell preparations.

After isolation, Sertoli cells were counted in a Neubauer chamber and cultivated at a plating density of $3.2 \times 10^5$ cells/cm$^2$ in Petri dishes containing Medium 199 pH 7.4 supplemented with 1% fetal bovine serum (v/v). Cells were maintained at 34°C in a humidified atmosphere of 5% $CO_2$ in air. The medium was replaced after 24 h by serum free medium to remove unattached Sertoli and germinal cells. Experiments were performed on cells treated with retinol 7μM with or without 0.3–1% ethanol, superoxide dismutase (EC 1.15.1.1) (SOD) (200 U/ml), 0.1 mM trolox, 1 mM *N*-acetylcysteine or inactivated SOD (200 U/ml, boiled for 5 min to destroy enzyme activity). Control cultures received only the retinol solvent (0.1% ethanol, v/v). To control the effect of ethanol in all experimental procedures, a group without the addition of ethanol was analyzed, and no significant differences between this and control group were encountered on all the parameters measured. The formation of oxidized retinol metabolites was monitored by spectroscopic scan of all retinol solutions before use. Cell viability and morphology did not differ significantly between all tested groups (data not shown), as assessed by trypan blue exclusion.

### Thiobarbituric Acid Reactive Species (TBARS)

As an index of lipid peroxidation we used the formation of TBARS during an acid-heating reaction as previously described.[19] Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% (TCA) and 1 ml of thiobarbituric acid 0.67% (TBA), then heated in a boiling water bath for 15 min. Butanol (2:1 v/v) was added and after a centrifugation (800*g*/5 min) TBARS were determined by the absorbance at 535 nm.

### Cell Proliferation Assay

As an index of cell proliferation we used the incorporation of [$^3$H] thymidine as previously

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described.[20] Briefly, after indicated treatments cells were washed with HBSS and [$^3$H] thymidine (3 μCi/ml) was added. After incubation for 4 h, cells were washed, harvested and [$^3$H] thymidine incorporation was determined by precipitation in 10% trichloroacetic acid, solubilization in 0.2 M NaOH, and liquid scintillation counting.

### Focus Formation Assay

Sertoli cells were cultured as described under "Cell culture". After retinol (7 μM) treatment cells were maintained in Medium 199 pH 7.4 supplemented with 10% fetal bovine serum and fresh medium was added every 3 days. Cell foci were scored 15 days after retinol treatment after fixing with methanol/acetone (1:1) and staining with trypan blue. Morphology was examined under a light microscope.

### Protein Quantification

All the results were normalized by the protein content.[22]

### Statistical Analysis

Results are expressed as means; $p$ values were considered significant when $p < 0.05$. Differences in experimental groups were determined by ANOVA two-ways. Comparison between means was carried out using Newman–Keuls test.

## RESULTS

As we described previously,[16] 7 μM retinol increased TBARS production (Table I). Lipid peroxidation was inhibited by SOD, trolox and *N*-acetylcysteine (Table I). In the same way, 0.3 and 1% ethanol inhibited retinol-induced lipid peroxidation (Table I). In contrast, treatment with inactivated SOD did not result in an inhibition in retinol-induced TBARS production (Table I).

It is well established that ROS have numerous effects on cell function including induction of growth, regulation of kinase activity and cellular transformation.[14] Retinol (7 μM) treated cells displayed a greater rate of DNA synthesis than did control cells (Table I). In addition, retinol

TABLE I Determination of TBARS and [$^3$H] thymidine incorporation in cells treated with retinol. Cultured Sertoli cells were treated with the indicated concentrations of retinol dissolved with ethanol (0.1%) for 24 h. Depending on the experimental procedure cells were treated with ethanol 0.3–1%, trolox (0.1 mM), *N*-acetylcysteine (1 mM), superoxide dismutase (200 U/ml) or denatured superoxide dismutase for 24 h. For the determination of TBARS samples were mixed with trichloroacetic acid 10% (TCA) and thiobarbituric acid 0.67% (TBA) and the TBARS were determined as described under "Materials and Methods" ($n = 3$). For [$^3$H] thymidine incorporation cells were incubated with [$^3$H] thymidine (3 μCi/ml) for 4 h and radioactivity in trichroloacetic acid-precipitable material was measured as described under "Materials and Methods" ($n = 3$). Results are expressed as means±S.D.

| Treatment | TBARS content (nm/mg protein×10) | | [$^3$H] thymidine incorporation (cpm/mg protein) | |
|---|---|---|---|---|
| | Without retinol | With retinol | Without retinol | With retinol |
| Control | 1.25±0.05 | 1.53±0.09* | 1111.43±165.8 | 2140±48.2* |
| Ethanol 0.3% | 1.38±0.24 | 1.07±0.01† | 1025.95±88.6 | 1312.42±72.5† |
| Ethanol 1% | 1.33±0.05 | 0.97±0.14† | 1114.4±110.1 | 1065.4±203.7† |
| Trolox (0.1 mM) | 1.12±0.01 | 0.89±0.08† | 1493.6±71.86 | 1236±155.8† |
| Superoxide dismutase (200 U/ml) | 1.04±0.02 | 1.12±0.04† | 1070.1±95.8 | 1744±254.8*,† |
| Denatured superoxide dismutase | 0.98±0.08 | 1.45±0.13 | 1013.6±45.6 | 2304.2±78.7* |
| *N*-acetylcysteine (1 mM) | 0.99±0.1 | 0.87±0.08† | 1129.9±67 | 1038.9±31† |

* Different from control; $p < 0.05$.
† Different from retinol 7 μM; $p < 0.05$.

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treatment induced focus formation in cultured Sertoli cells, in contrast to control cells (Fig. 1).

To test whether retinol-induced oxidative stress mediated this mitogenic response, we treated cells with SOD, trolox, *N*-acetylcysteine or inactivated SOD. Trolox and *N*-acetylcysteine effectively inhibited DNA synthesis in retinol treated cells to control levels (Table I). SOD treatment did not result in a reduction of DNA synthesis to an amount comparable to trolox and *N*-acetylcysteine treatment (Table I). As we demonstrated to TBARS production, treatment with inactivated SOD did not result in an inhibition in retinol-induced DNA synthesis (Table I). Ethanol treatment also inhibited DNA synthesis in retinol treated cells to control levels (Table I).

## DISCUSSION

We[15,16] and others[17] demonstrated that retinol treatment could induce oxidative stress in different cell models. The contribution of retinol-induced oxidative stress to cell growth and phenotype is not completely clarified. We report here that supplementation with retinol caused oxidative stress and that this is important to cell proliferation. We also demonstrated that retinol induces focus formation in cultured Sertoli cells. Besides antioxidants, ethanol also diminished retinol effects on oxidative stress and cell proliferation parameters. It is important to note that Sertoli cells obtained from a 15-day-old rat will have essentially ceased mitotic activity, and exhibit contact growth inhibition. In this situation, an increase in DNA synthesis and focus formation induced by retinol is suggestive of loss of normal growth control and phenotype transformation. This is not necessarily related to malignant transformation, since our model is not one of neoplastic transformation.

We previously demonstrated that retinol treatment induced ornithine decarboxylase (ODC) activity in a redox dependent way.[23] ODC is the rate-limiting enzyme in the biosynthesis of the polyamines, which plays key role in DNA synthesis.[24] The intracellular levels of polyamines are elevated during growth processes. ODC activity declines in slow-growing systems and rises during rapid proliferation, including malignant transformed cells.[25,26] The importance of chronic mitogenesis for mutagenesis and carcinogenesis is well-known.[27] Cell division increases mutagenesis for several reasons, and apparently ROS could induce mitogenesis in several cell models.[14,28–30] Irani *et al.*[14] demonstrated that the constitutive production of ROS in Ras-transformed A6 cells activates intracellular pathways that correlate with the ability of these cells to progress through the cell cycle in the absence of growth factors. This phenomenon was also observed for Rac1 and PKC pathways,[28,31] and suggests that modulation of the redox state of the cell plays an important role in the control of cell growth and may provide one mechanism to explain the observation that some antioxidants appear to exert protective effects against human cancers.

Murata *et al.*[17] demonstrated a dose-dependent manner of superoxide generation induced by retinol oxidation. This is supported by our results of a dose-dependent increased activity of SOD induced by retinol treatment.[16] We supposed that retinol oxidation induces ROS production that could activate redox dependent intracellular pathways that lead to cell proliferation. Instead of the possible redox mediated effects induced by retinol on cell proliferation, recent findings demonstrated that retinoids could directly modulate activation of the cRaf and PKC families of kinases.[32–34]

Trolox, *N*-acetylcysteine and SOD blocked the stimulation of DNA synthesis and TBARS production induced by retinol. This suggested that retinol-induced oxidative stress is required for its growth promoting effects. The effects of ethanol could be related to its interference in retinol oxidation by alcohol dehydrogenase,[35] with diminution of retinol oxidation.

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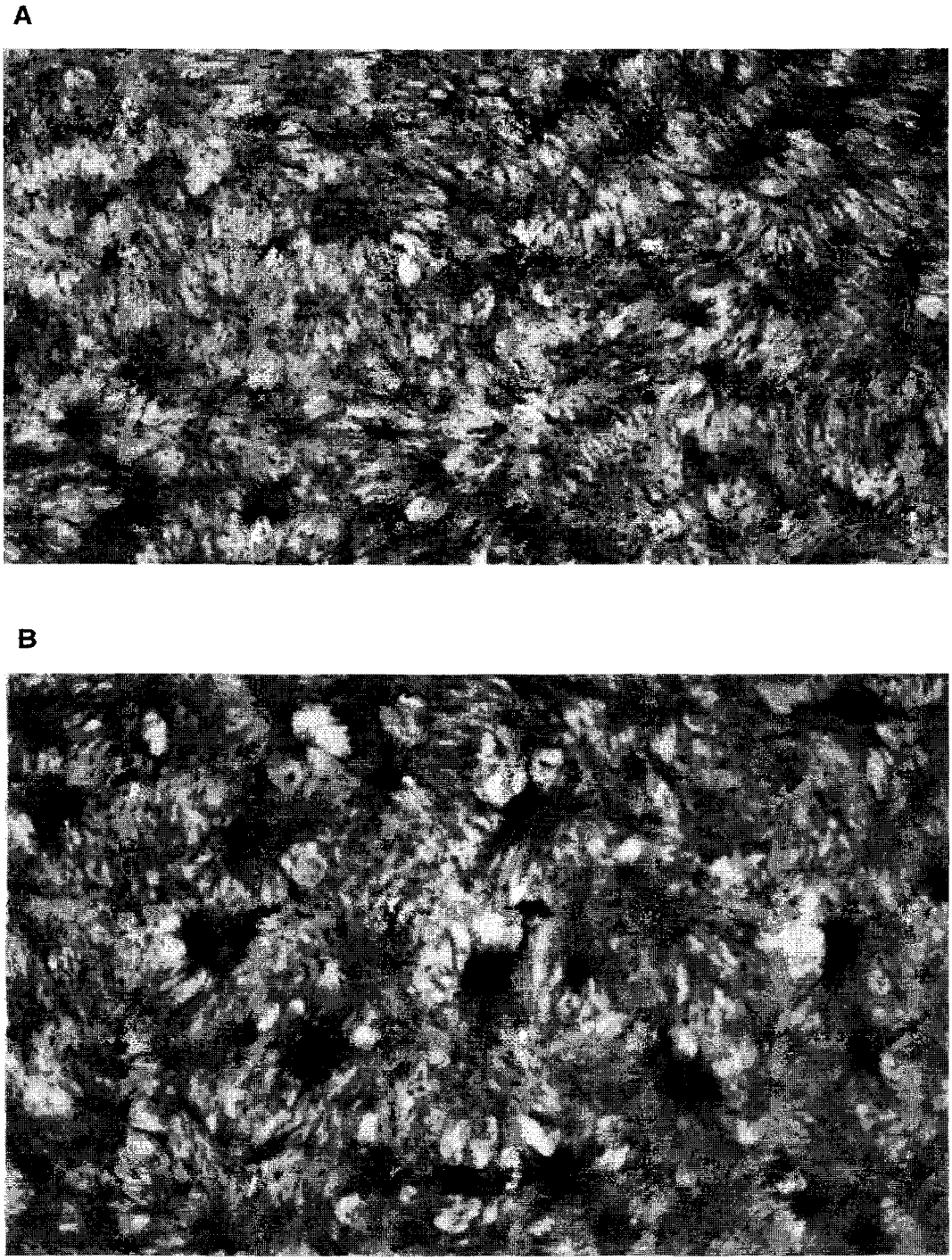


FIGURE 1 Focus formation in cells treated with retinol. Cells were treated as described in the legend to Table I and maintained in Medium 199 pH 7.4 supplemented with 10% fetal bovine serum for 15 days. Morphology was examined under a light microscope as described under "Materials and Methods". This experiment was repeated 3 times with nearly identical results. Representative photographs (×40) are shown. (A) Control cells; (B) retinol (7 μM) treated cells.

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SOD effects, probably are related to its enzymatic activity, instead of metal ion contamination of the commercial product, since denatured SOD did not have any significant effect on TBARS production and on thymidine uptake (Table I). This effect suggests that retinol mediated peroxidation and thymidine uptake is, in part, related to extracellular oxidative stress, since SOD does penetrate in intact cells. Recently, it was demonstrated that treatment with a wide range of ROS induces cell proliferation in different experimental models.[36,37] This upregulation of cell proliferation was suppressed by pre-treatment with hydroxyl radical scavengers, iron chelating agents and exogenous catalase and SOD mimic.[36,37] These results reinforce our proposed model of retinol induced cell proliferation.

In recent years advances have been made in our understanding of the molecular mechanism of retinol supplementation. It was demonstrated that retinol oxidation induced superoxide production.[17] This is accompanied by lipid peroxidation[16] and DNA damage,[15,17] and induced the activation of antioxidant enzymes[16] and ODC.[23] These effects could be attenuated by the addition of iron[15] or copper chelators[17] and OH[16,23] or other radical scavengers.[17] Ours results indicate that supplementation with retinol could induce oxidative stress and this is associated with cell proliferation and focus formation in cultured Sertoli cells. Additional studies are required to understand the significance of retinol supplementation in models of malignant transformation and the intracellular pathways responsible for these retinol effects.

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