Free Rad. Res., Vol. 34, pp. 395-404
Reprints available directly from the publisher
Photocopying permitted by license only

© 2001 OPA (Overseas Publishers Association) N.V.
Published by license under
the Harwood Academic Publishers imprint,
part of The Gordon and Breach Publishing Group.
Printed in Malaysia

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

Retinol Supplementation Induces Oxidative Stress and Modulates Antioxidant Enzyme Activities in Rat Sertoli Cells

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

[a]*Laboratório de Estresse Oxidativo, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil and* [b]*Departamento de Biofísica, Universidade Federal do Rio Grande do Sul Porto Alegre, Brazil*

Accepted for publication by Prof. B. Halliwell

(Received 20 Juin 2000; In revised form 4 August 2000)

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 caused cellular oxidative stress and modulated superoxide dismutase, catalase and glutathione peroxidase activities. Retinol (7 μM) significantly increased TBARS, conjugated dienes, and hydroperoxide-initiated chemiluminescence in cultured Sertoli cells. In response to retinol treatment superoxide dismutase, catalase and glutathione peroxidase activities increased. TBARS content and catalase activities were decreased by a free radical scavenger. These findings suggest that retinol may induce oxidative stress and modulate antioxidant enzyme activities in Sertoli cells.

Keywords: retinol, oxidative stress, reactive oxygen species, lipid peroxidation, catalase, glutathione peroxidase, superoxide dismutase

INTRODUCTION

Retinol occurs naturally in certain foods of animal and vegetal origin and can be obtained direct from the diet or by the intake of provitamin A (compounds that can be converted to vitamin A in the body such as beta-carotene). It is well known that retinol regulates cellular division and differentiation[1]. Apart from these important physiological functions, the effects and safety of the supplementation with retinol are not well defined.

In the last decade many epidemiological studies have shown an association between low dietary intake of retinol and the development of cancer, specifically, lung[2], liver[3], oral and pharyngeal[4], oesophagus[5] and gastric[6]. On the other hand, in an animal model of lung cancer retinol increased the malignant transformation induced by gamma irradiation[7] and Badr *et al.*[8] demonstrated that retinol increased the induction of chromosomal aberrations in human lymphocyte cultures. In addition, two reports suggested a positive association of vitamin A

* To whom correspondence and requests for reprints should be addressed: Departamento de Bioquímica, ICBS – Universidade Federal do Rio Grande do Sul Ramiro Barcelos, 2600, Porto Alegre, RS, Brasil. 90035–003 Fax: 55 51 316 5535 e-mail: pizzol@ez-poa.com.br

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

intake and increased incidence of prostate cancer[9,10]. More recently one randomized, controlled clinical trial demonstrated that supplementation with a combination of beta carotene and retinol increases the incidence and mortality from lung cancer in 18,314 smokers, former smokers or workers exposed to asbestos[11]. In another randomized controlled study with 29,133 smokers in Finland[12] a higher incidence of lung cancer in patients exposed to the pro-vitamin A, beta-carotene was demonstrated. However, attempts to use retinoids and carotenoids for cancer chemoprevention and therapy are ongoing[13–15]. Therefore, the casual mechanisms should be elucidated to establish safe approaches in cancer chemoprevention.

The production of reactive oxygen species (ROS) is recognized as a cause of immediate cellular injury leading to cell death or apoptosis. ROS can also lead to progressive accumulation of DNA damage and, consequently, are involved in many physiological (i.e. aging) and pathological (i.e. cancer) processes[16]. The hydroxyl ($^{\bullet}OH$) radical is probably the most potent of the ROS, and the probable initiator of the chain reactions which form lipid peroxides and organic radicals.

Our previous studies demonstrated an increased in chromatin sensitivity to DNAse I [17], an increase in [methyl-^{3}H] thymidine incorporation into DNA[18], and significant changes in nuclear protein phosphorylation[19] in Sertoli cells treated with retinol (7 μM). Many of these effects were inhibited by the addition of 1,10-phenanthroline (iron chelator), suggesting the participation of a Fenton reaction in these retinol-induced effects. Recently, we[20] and others[21] demonstrated that retinol supplementation could induce oxidative DNA damage, and this effect probably involves metal ions.

We report here that retinol supplementation caused oxidative damage to other cellular constituents, besides DNA, and this was accompanied by an activation of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

MATERIAIS AND METHODS

Materials

Type I collagenase, medium 199, HBSS, all-*trans* retinol, thiobarbituric acid, mannitol, *tert*-butyl hydroperoxide, hydrogen peroxide, reduced glutathione, glutathione reductase, and NADPH were purchased from Sigma, St. Louis, MO, USA. Trypsin was purchased from Difco, Detroit, MI, USA.

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 in a plating density of 3.2×10^5 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 (0.1 to 7 μM) with or without 1 mM mannitol simultaneously. Control cultures received only the retinol solvent (0.1% ethanol, v/v). Doses above 10 μM lead to extensive cell death (data not shown) and this make unsuitable the analyses of oxidative stress parameters in these concentrations. To control for the effect of ethanol, in all experimental procedures a group without the addition of ethanol was analyzed, and no significant differ-

RIGHTSLINK

ences between this and control group were encountered on all the parameters measured (data not shown). 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.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

Determination of Conjugated Dienes

The level of lipid peroxidation was estimated by assay of conjugated diene double bonds[22]. Briefly, Sertoli cells were homogenized in 2 mL of 0.25 M sucrose/5mM EDTA and centrifuged at 14500 × g for 15 min at 4°C. From the postmitochondrial supernatant the microsomes were spun down at 105000 × g for 60 min at 4°C. The extraction of lipid was made as described by Folch *et al.*[23]. The lipids extracted were dried under vacuum at room temperature and then dissolved in 1 mL of cyclohexane. The lipid/cyclohexane solution was immediately scanned from 300 to 220 nm and second-derivative spectrum was recorded, which confers greater sensitivity for the diene conjugation method[24]. An absorbance spectrum plots absorbance (A) against wavelength (λ). A first-derivative spectrum plots rate of change of absorbance with wavelength ($dA/d\lambda$) against wavelength. The second-derivative spectrum plots the rate of change of this rate of change ($d^2A/\ d^2\lambda$). In the second-derivative spectrum the conjugated dienes signal was characterized by the height of the minimum peak at 233 nm, and is expressed in arbitrary units[24].

Thiobarbituric Acid Reactive Species (TBARS)

As an index of ROS production we used the formation of TBARS during an acid-heating reaction, which is widely adopted as a sensitive method for measurement of lipid peroxidation, as previously described[25]. Briefly, the samples were mixed with 1 ml of trichloroacetic acid 10% (TCA) and 1ml 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) thiobarbituric acid reactive species (TBARS) were determined by the absorbance at 535 nm.

Hydroperoxide-Initiated Chemiluminescence

To evaluate the oxidant status in these cells we measured the hydroperoxide-initiated chemiluminescence. This method measures the balance between pro-oxidants and antioxidants present in these cells[26]. Briefly, the cell nomogenates were diluted to a final concentration of 1 mg of protein/mL and 3mM of *tert*-butyl hydroperoxide was added and chemiluminescence was measured in a scintillator counter as previously described[26].

Antioxidant Enzyme Activities

Enzyme assays were performed in cells extracts obtained as follows. Cells were harvested and washed three times with saline. To determine CAT and GPx activities cells were sonicated in 50mM phosphate buffer (pH 7.0) and the resulting suspension was centrifuged at 3000 g for 10 min. The supernatant was used for enzyme assays. CAT activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm[27]. For GPx activity NADPH oxidation was followed at 340 nm[28] in the presence of reduced glutathione, *tert*-butyl hydroperoxide, and glutathione reductase. SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described[29].

Protein Quantification

All the results were normalized by the protein content[30].

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

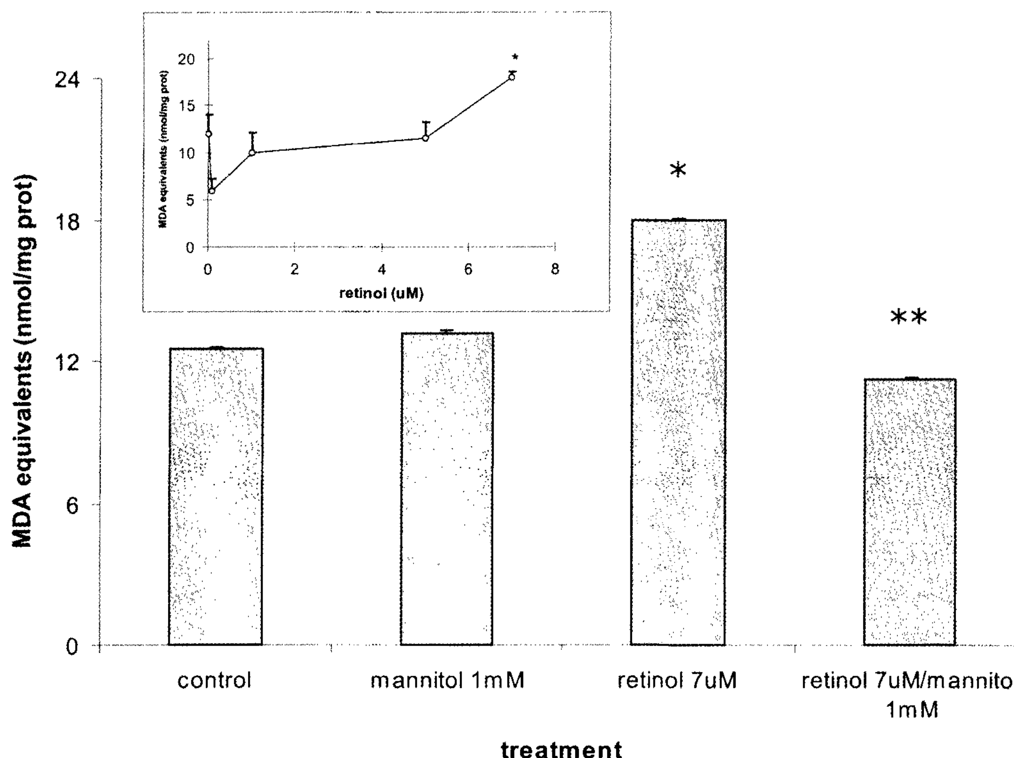


FIGURE 1 Determination of TBARS in cells treated with retinol. Cutured Sertoli cells were treated with the indicated concentrations of retinol dissolved with ethanol (0.1%) for 24 h; controls also contained 0.1% ethanol (insert box). Depending on the experimental procedure cells were treated with mannitol (1 mM) for 24 h. For the determination of TBARS samples were mixed with 1 ml of trichloroacetic acid 10% (TCA) and 1ml of thiobarbituric acid 0.67% (TBA) and the TBARS were determined as described under "Materials and Methods" (n=3). *different from control; $p < 0.01$ **different from retinol 7 μM; $p < 0.01$

Statistical Analyses

Results are expressed as means; *p* values were considered significant when $p<0.01$. Differences in experimental groups were determined by ANOVA. Comparison between means was carried out using a Newman-Keuls test.

RESULTS

Sertoli cells were exposed to retinol and the lipid peroxidation was estimated by conjugated dienes and TBARS concentration as described in Material and Methods.

Retinol treatment induced production of TBARS only at higher doses (Fig. 1 – insert box). Retinol at 0.1 to 5 μM seems not to induce TBARS production in cultured Sertoli cells. This pattern was also described previously with retinol-induced DNA oxidative damage[20].

Fig. 1 shows the effect of mannitol on TBARS production. Lipid peroxidation was inhibited by the free radical scavenger, mannitol, suggesting that retinol induced lipid peroxidation is mediated by free radicals.

Since only 7 μM retinol increased TBARS content we investigated if this dose could increase other markers of oxidative stress. As shown in Table I, the concentration of conjugated dienes in

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

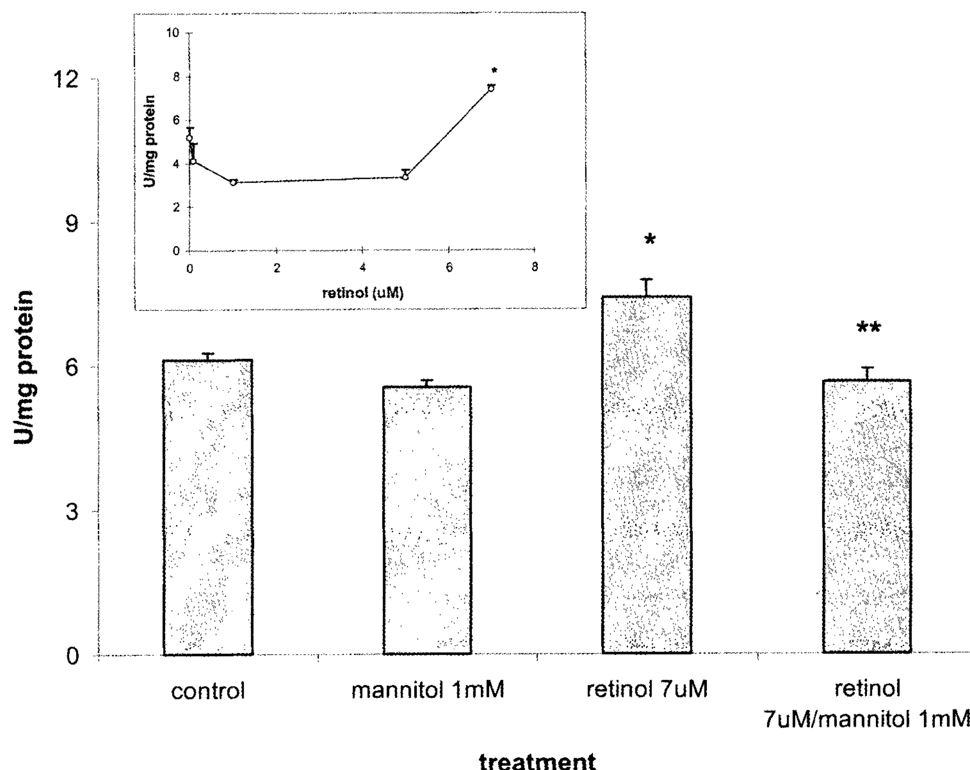


FIGURE 2 **Catalase activity in cells treated with retinol**. Cells were treated as described in the legend to Fig. 1. For the determination of catalase activity the rate of decrease in H_2O_2 absorbance at 240 nm was measured as described under "Materials and Methods" (n=3). *different from control; $p < 0.01$ **different from retinol 7 μM; $p < 0.01$

Sertoli cells treated with 7 μM retinol was significantly higher than that of the control. In the same way, the chemiluminescence initiated by hydroperoxide was significantly increased in 7 μM retinol treated cells (Tab. 1). These findings suggest that 7 μM retinol doses could induce lipid peroxidation and alter the balance between pro-oxidants and antioxidants present in these cells

In normal conditions, there is a steady state balance between the production of ROS and their destruction by the cellular antioxidant system. However, this balance can be broken either by increasing the ROS production or by decreasing the defense system. When cells are exposed to an oxidative stress various defense mechanisms are induced, including SOD, CAT and GPx[31].

In accordance with data presented in fig. 1, in fig. 2 (insert box) retinol treatment induced catalase activity only at higher doses, suggesting that this dose could induce oxidative stress in Sertoli cells. In the same way, the GPx activity was significantly increased in 7 μM retinol treated cells (Figure 3). CAT and GPx activities were inhibited by mannitol (Fig. 2 and Fig. 3), suggesting a role of oxidative stress in the antioxidant defense activation, and not a direct retinol effect. In contrast, SOD activity increased in a dose-dependent manner, from 1 to 7 μM retinol (Figure 4).

RIGHTSLINK

TABLE I Effect of retinol treatment (7 μM) for 24h on conjugated dienes and hydroperoxide-initiated chemiluminescence. nata represent mean ± SEM of at least thrcc replicates per independent experiment and three separate experiments

Assay	*Control*	*Retinol 7 μM*
Conjugated dienes (arbitrary units)	0.05 ± 0.05	0.57 ± 0.083[a]
Hydroperoxide-initiated chemiluminescence (cpm/mg protein)	414 ± 40	717 ± 52[a]

a. different from control; $p < 0.01$

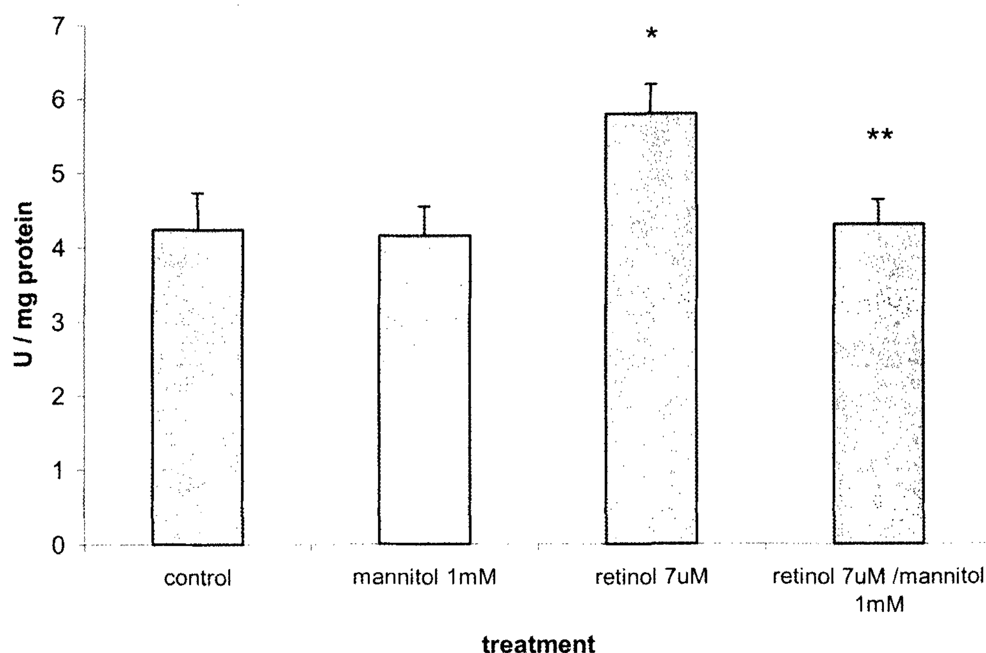


FIGURE 3 **Glutathione peroxidase activity in cells treated with retinol**. Cells were treated as described in the legend to Fig. 1. For the determination of glutathione peroxidase activity the rate of NADPH oxidation was followed at 340 nm as described under "Materials and Methods" (n=3). *different from control; $p < 0.01$ **different from retinol 7 μM $p < 0.01$

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

DISCUSSION

Any substance that can enhance ROS production is potentially carcinogenic once it can damage DNA, inducing mutations on specific genes responsible for cell proliferation control[16]. Oxidative stress can cause damage to all types of biomolecules, including DNA, proteins and lipids. In many situations it is unclear which is the most important target, since injury mechanisms overlap widely. The primary cellular target of oxidative stress can vary depending on the cell, the type of stress imposed and how severe the stress is.

We report here that supplementation with retinol caused lipid peroxidation. This damage seems to be induced only by 7 μM retinol, since 5 μM or lower doses did not induce TBARS, as we demonstrated previously for DNA damage[20]. In response, Sertoli cells increase antioxidant

RIGHTSLINK

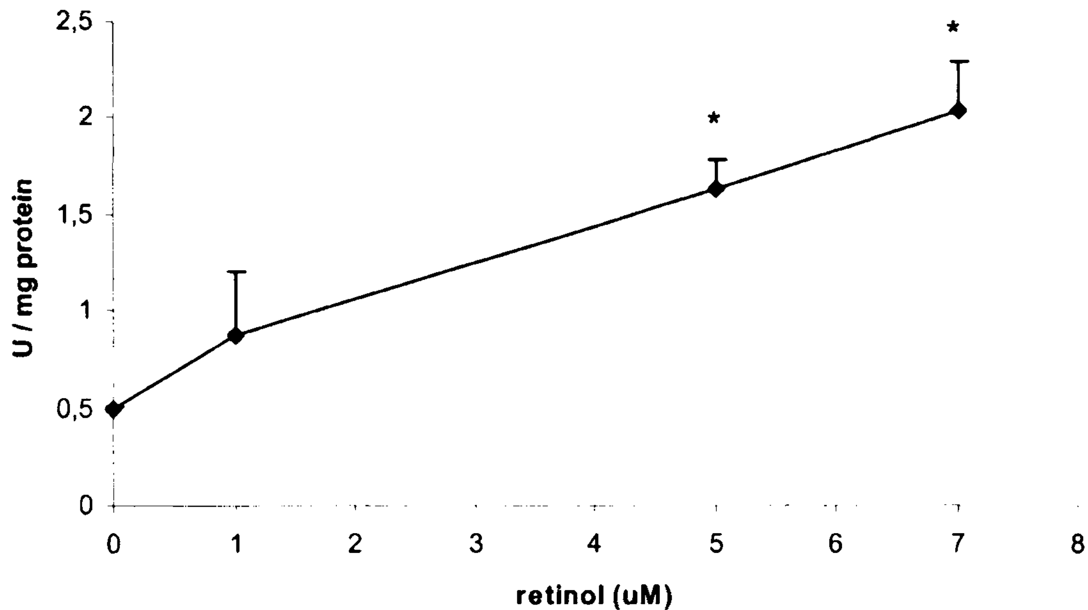


FIGURE 4 **Superoxide dismutase activity in cells treated with retinol**. Cells were treated as described in the legend to Fig. 1. For the determination of superoxide dismutase the inhibition of adrenaline auto-oxidation was measured as described under "Materials and Methods" (n=3). *different from control; $p < 0.01$

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

defense mechanisms to protect themselves against the injury caused by ROS. Lipid peroxidation and activation of antioxidant enzymes were inhibited by mannitol, suggesting a role for free radicals in the oxidative retinol effects.

In contrast, 5 μM or lower doses seemed to decrease TBARS and catalase activity, even though this difference did not reach statistical significance. This indicates that retinol in lower doses could act as an antioxidant, but this effect needs to be confirmed in different experimental protocols. Every antioxidant is in fact a redox agent, protecting against free radicals in some circumstances and promoting free radical generation in others[32]. Although vitamin A is a good acceptor and donor of electrons in chemical reactions, its properties appear to be very carefully protected by retinol-binding proteins[33]. Normal values of human retinol serum are around 360–1200 μg/L (1.25–4.1 μM), and it is estimated that the physiological retinol concentration in Sertoli cells is around 5 μM[1]. In normal conditions cells are not exposed to high concentration of free retinol[34]. However, pharmacological amounts of supplements may perturb key physiological processes. If excessive intake of supplements of vitamin A and β-carotene saturate binding proteins, free compounds may have cytotoxicity [21].

Differently from CAT and GPx, SOD activity increased in a dose-dependent manner in retinol treated Sertoli cells. These data are in accordance to the dose-dependent manner of superoxide generation induced by retinol treatment described by Murata *et al*[21]. Little controversy remains concerning the toxicity of superoxide and the physiological function of SOD as ascavenger of the this radical. The other side of the coin, that too much SOD may be toxic is now the much more interesting observation. Cells that

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

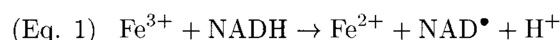
overexpress SOD[35] or mice transgenic for human SOD[35] have some abnormalities related to oxidative stress. It seems that any concentration of SOD other than the optimal leads to increased lipid peroxidation and therefore to increased oxidative stress[35]. Ours results, taken together with Murata et al results[21], suggest that, in Sertoli cells, the superoxide generation induced by retinol 7 μM treatment lead to an increased in SOD activity to a level that alters the oxidant/antioxidant cellular balance, inducing oxidative stress.

Recently, Chen *et al.*[36] provided the first demonstration of cellular ROS production induced by a natural retinoid. Anhydroretinol induced oxidative stress and cell death, measured by flow cytometry, in 5/2 and Jurkat cells. Murata *et al*[21] demonstrated that retinol and retinal cause oxidative damage to cellular and isolated DNA, probably involving the generation of superoxide anion and H_2O_2. Differently from our results[20], Murata *et al.*[21] demonstrated a dose-dependent effect of DNA damage induced by retinol (from 0.5 to 5 μM). This difference could be related to a different antioxidant environment or different metabolism of retinol in these two different cell lines.

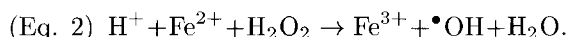
The limitations of our study should be considered. The methods used to determine oxidative stress have been criticized. There are basically two ways to detect free radical production. The only technique that can detect free radicals directly is electron spin resonance spectroscopy (ESR). An alternative to this is fingerprinting which measure products of ROS damage, i.e. TBARS, conjugated dienes and chemiluminescence. These are all older and criticized methods but they still are widely used[24] and when taken together strongly suggest the presence of oxidative stress in a biological system. The activation of enzymatic defenses against reactive oxygen species reinforces the idea of oxidative stress in a biological system. However, we do not know if the increases in antioxidant enzyme activity are due to induction of mRNA or protein activation.

The exact mechanism by which retinol increases ROS production is still not understood. We postulated that it involves a Fenton reaction since phenanthroline (iron chelator) may decreased retinol (7 μM) cellular effects[17,20]. We had previously demonstrated that retinol treatment modulates iron turnover in Sertoli cells[20], reinforcing the participation of a Fenton reaction on retinol-induced oxidative stress. Murata *et al.*[21] suggested that the oxidative damage to cellular and isolated DNA, probably involving the generation of superoxide and H_2O_2. Thus, these results taken together suggest the probable involvement of the Fenton reaction in retinol-induced oxidative stress.

Several studies have shown that the toxicity of hydrogen peroxide or organic peroxides to animal cells in culture can be increased by raising their iron content, and decreased by the presence of chelating agents (i.e. phenanthroline). Benfato et al[37] demonstrated that the transfection with transferrin receptor could induce cellular transformation to a neoplastic phenotype associated with iron accumulation. We also demonstrated that retinol increases the reduced nucleotide content in Sertoli cells (submitted data). This may also be responsible for the increased ROS production since it can propagate a free-radical chain as shown in the following reaction[38]:



$$\text{(Eq. 1)}\quad Fe^{3+} + NADH \rightarrow Fe^{2+} + NAD^{\bullet} + H^{+}$$



$$\text{(Eq. 2)}\quad H^{+} + Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O.$$

The $^{\bullet}OH$ radical produced in a Fenton reaction reacts with extremely high rate constants with almost every type of molecule found in living cells: sugars, amino acids, phospholipids and DNA bases. The oxidative agent generated by Fenton reactions can cause DNA strand breakage and lipid peroxidation in cells undergoing oxidative stress[39]. Hydroxyl radical attack upon DNA generates a whole series of modified purine and pyrimidine bases, many of which are known to be mutagenic[40,41].

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

The results presented here reinforce the recently published effects of retinol on DNA damage[20,21], and indicate that retinol could not only damage DNA, but also other biomolecules.

Acknowledgements

This work was supported by grants from CAPES, FAPERGS, CNPq and FINEP (Brazilian agencies for research support). The authors wish to thank Dr. Richard Rodnight for critical English review.

References

[1] M.A. Livrea and L. Packer (1993) Retinoids – Progress in research and clinical applications. Marcel Dekker Inc, New York.

[2] G.A. Colditz, M.J. Stampfer and W.C. Willett (1987) Diet and lung cancer. A review of the epidemiologic evidence in humans. *Archives of Internal Medicine*, 147, 157–160.

[3] M.W. Yu, H.H. Hsieh, W.H. Pan, C.S. Yang and C.J. Chen (1995) Vegetable consumption, serum retinol level, and risk of hepatocellular carcinoma. *Cancer Research*, 55, 1301–1305.

[4] D.M. Winn, K.G. Ziegler, L.W. Piclke (1984) Diet on the etiology of oral and pharyngeal cancer among women from the Southern United States. *Cancer Research*, 44, 1216–1222.

[5] C. Mettlin, S. Graham, R. Prior, J. Marshall and M. Swanson (1981) Diet and cancer of the esophagus. *Nutrition and. Cancer*, 2, 143–147.

[6] W. Haenszel, M. Kurihara, M. Segi and R.K.C. Lee (1972) Stomach cancer among Japanese Hawaii. *Journal of the National Cancer Institute*, 9, 969–988.

[7] T.A. Mian, J.C. Theiss and T.F. Gesell (1984) Effect of vitamin A on lung tumorigenesis in irradiated and unirradiated strain A mice. *Cancer Letters*, 22, 103–112.

[8] F.M. Badr, O.H.M. El-Habit, M. Hamdy and G.A.R. Hassan (1998) The mutagenic versus protective role of vitamin A on the induction of chromosomal aberration in human lymphocyte cultures. *Mutation Research*, 414, 157–163.

[9] S. Graham, B. Haugey, J. Marshall, R. Priore, T. Byers, T. Rzepka, C. Mettlin and J.E. Pontes (1983) Diet in the epidemiology of carcinoma of the prostate gland. *Journal of the National Cancer Institute*, 70, 687–692.

[10] M.Y. Heshmat, L. Kaul, J. Kovi, M.A. Jackson, A.G. Jackson, G.W. Jones, M. Edson, J.P. Enterline, R.G. Worrell and S.L. Perry (1985) Nutrition and prostate cancer: a case-control study. *Prostate*, 6, 7–17.

[11] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, B. Valanis, J.H. Williams, S. Barnhart and S. Hammar (1996) Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *New England Journal of Medicine*, 334, 1150–1155.

[12] The Alpha-Thocopherol, Beta-Carotene Cancer Prevention Study Group (1994) The effects of vitamin E and beta-carotene on the incidence of lung cancer and other cancers in male smokers. *New England Journal of Medicine*, 330, 1029–1035.22.

[13] C. Rodrígues-Burford, R.A. Lubet, I. Eto, M.M. Juliana, G.J. Kelloff, C.J. Grubbs and V.E. Steele (1999) Effect of reduced body weight gain on the evaluation of chemopreventive agents in the methylnitrosourea-induced mammary cancer model. *Carcinogenesis*, 20, 71–76.

[14] A.R. Collins, B. Olmedilla, S. Southon, F. Granado and S.J. Duthie (1998) Serum carotenoids and oxidative DNA damage in human lymphocytes, *Carcinogenesis*, 19, 2159–2162.

[15] G. Pappalardo, G. Maiani, S. Mobarhan, A. Guadalaxara, E. Azzini, A. Raguzzini, M. Salucci, M. Serafini, M. Trifero, G. Illomei and A. Ferro-Luzzi, A. (1997) Plasma and tissue levels after supplementation with beta-carotene in subjects with precancerous and cancerous lesions of sigmoid colon. *European Journal of Clinical Nutrition*, 51, 661–666.

[16] B.N. Ames, M.K. Shigenaga and T.M. Hagen (1993) Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Science USA*, 90, 7915–7922.

[17] J.C.F. Moreira, F. Dal-Pizzol, D. Von Endt and E.A. Bernard (1997) Effect of retinol on chromatin structure in Sertoli cells: 1,10-phenanthroline inhibit the increased DNAse I sensitivity induced by retinol. *Medical Science Research*, 25, 635–638.

[18] J.C.F. Moreira, F. Dal-Pizzol, F.C.R. Guma and E.A. Bernard (1996) Effects of pretreatment with hydroxiurea on the increase in [methyl-^{3}H] thymidine incorporation induced by retinol treatment in Sertoli cells. *Medical Science Research*, 24, 383–384.

[19] J.C.F. Moreira, F. Dal-Pizzol, A.B. Rocha, F. Klamt, N.C. Ribeiro, C.J.S. Ferreira and E.A. Bernard (2000) Retinol-induced changes in the phosphorylation of histones and high mobility group proteins from Sertoli cells. *Brazilian Journal of Medical and Biological Research*, 33, 287–293..

[20] F. Dal Pizzol, F. Klamt, M.L.C. Frota Jr, L.F. Moraes, J.C.F. Moreira and M.S. Benfato (2000) Retinol supplementation induces DNA damage and modulates iron turnover in rat Sertoli cells. *Free Radical Research, in press*.

[21] M. Murata and S. Kawanishi (2000) Oxidative DNA damage by vitamin A and its derivative via superoxide generation. *Journal of Biological Chemistry*, 275, 2003–2008.

[22] F.P. Corongiu, M. Lai and A. Milia (1983) Carbon tetrachloride, bromotrichloromethane and ethanol acute intoxication. *Biochemical Journal*, 212, 625–631.

[23] J. Folch, M. Lees and G.H. Sloane Stanley (1957) A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry*, 226, 497–509.

[24] B. Halliwell and J.M.C. Gutteridge (1999) Free Radicals in Biology and Medicine. Claredon Press, Oxford.

[25] H.H. Draper and M. Hadley (1990) Malondialdehyde Determination as Index of Lipid Peroxidation. *Methods in Enzymology*, 186, 421–431.

[26] B.G. Flecha, S. Llessuy and A. Boveris (1991) Hydroperoxide initiated chemiluminescence: an assay for oxida-

RIGHTS LINK

tive stress in biopsies of heart, liver, and muscle. *Free Radical Biology and Medcine*, 10, 93–100.

[27] H. Aebi (1984) Catalase in vitro. *Methods in Enzymology*, 105, 121–126.

[28] L. Flohé and W.A. Günzler (1984) Assays of glutathione peroxidase. *Methods in Enzymology*, 105, 114–121.

[29] J.V. Bannister and L. Calabrese (1987) Assays for SOD. *Methods Biochemistry Analytical*, 32, 279–312.

[30] O.H. Lowry, A.L. Rosebrough and R.J. Randall (1951) Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193, 265–275.

[31] C. Michiels, M. Raes, O. Toussaint and J. Remade (1994) Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radical Biology and Medicine*, 17, 235–248.

[32] V. Herbert (1996) Prooxidants effects of antioxidants vitamins. *Journal of Nutrition*, 126, 1197–1200.

[33] J.A. Olson (1996) Benefits and liabilities of vitamin A and carotenoids. *Journal of Nutrition*, 126, 1208–1212.

[34] J.T. Davis and D.E. Ong (1995) Retinol processing by the peritubular cell from the rat testis. *Biology of Reproduction*, 52, 356–364.

[35] J.M. McCord (1998) The importance of oxidant-antioxidant balance. In Oxidative stress in cancer, AIDS, and neurodegenerative diseases (ed. L. Montagnier, R. Olivier and C. Pasquier), Marcel Dekker Inc, New York, pp. 1–8.

[36] Y. Chen, J. Buck and F. Derguini (1999) Anhydroretinol induces oxidative stress and cell death. *Cancer Research*, 59, 3985–3990.

[37] M.S. Benfato, A.L.T.O. Nascimento and R. Meneghini (1997) Pro-Oxidant conditions induced by differential iron uptake in mouse L cells. *Cancer Journal*, 10, 279–84.

[38] J.A. Imlay and S. Linn (1988) DNA damage and oxygen radical toxicity. *Science*, 240, 1302–1309.

[39] C.R.A. Bertoncini and R. Meneghini (1995) DNA strand breaks produced by oxidative stress in mammalian cells exhibit 3'-phosphoglycolate termini. *Nucleic Acid Research*, 23, 2995–3002.

[40] R.A. Floyd (1990) The role of 8-hydroxyguanosine in carcinogenesis. *Carcinogenesis* 11, 1447–1450.

[41] M. Moriya (1993) Single-strand shuttle mutagenesis studies in mammalian cells: 8-Oxoguanosine in DNA induces targeted GXC – TXA transversions in simian kidney cells. Proceedings of the National Academy of Sciences of the USA 90, 1122–1126.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/22/11
For personal use only.

RIGHTSLINK