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PADRONIZAÇÃO DA TÉCNICA DE DOSAGEM DE 8-OH-dGUANOSINA POR HPLC, EM CÉLULAS DE SERTOLI TRATADAS COM RETINOL

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SUMÁRIO

Lista de Abreviaturas

DNA: ácido desoxirribonucleico

EAO: espécies ativas de oxigênio

O_2: Oxigênio molecular

O_2^-: ânion superóxido

H_2O_2: peróxido de Hidrogênio

H_2O: água

•OH: radical hidroxil

Fe_2^+: ferro II

Fe_3^+: ferro III

SOD: superóxido dismutase

8-oxo-dGua ou 8-OH-dG: 8-oxo-7,8-2'-dsoxiguanosina

ODC: ornitina decarboxilase

TBARS: espécies reativas do ácido tiobarbitúrico

HMG: proteínas do grupo de alta mobilidade

Scavenger: qualquer substância capaz de "limpar" radicais livres de sistemas biológicos/químicos

HBSS: solução salina tamponada de Hank

PBS: solução salina tamponada

CRBP: proteína citoplasmática ligante de retinol

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RESUMO

Nos últimos anos tem sido crescente o interesse pelo mecanismo bioquímico através do qual as Espécies Ativas de Oxigênio modificam estruturas biológicas, em particular as alterações no DNA. Células de Sertoli cultivadas, submetidas ao tratamento com retinol, mostraram-se sensíveis a ação prejudicial das EAO, quanto a peroxidação lipídica, aumento de atividade de enzimas antioxidantes e sensibilidade da cromatina a ação de DNAses. A espécie ativa de oxigênio apontada como principal causadora dos danos a biomoléculas é o radical hidroxil. O ferro presente no núcleo das células, através da reação de Fenton, pode gerar este radical, que ataca o DNA *in situ*. O principal produto deste ataque às bases do DNA, é a 8-oxo-dGuanosina. Neste trabalho foram quantificados os níveis de 8-oxo-dGua, em DNA de células de Sertoli, tratadas ou não com retinol 7μM, através da técnica de HPLC por análise espectrofotométrica e eletroquímica em série. Os resultados mostraram um aumento significativo dos níveis de 8-oxo-dGua em DNA de células de Sertoli tratadas com retinol, indicando que o tratamento em doses acima de 7μM, pode causar danos no DNA. Esses danos podem levar à mutações e transformações celulares malignas, visto que o aumento dos níveis deste aduto no DNA está relacionado com mutações e câncer.

I. INTRODUÇÃO

I. 1. Retinol e Células de Sertoli

Entre os vários processos celulares dependentes de vitamina A, encontra-se a espermatogênese, que na ausência desta vitamina não ocorre. A administração de ácido retinóico a animais deficientes em vitaminas A restabelece as funções basais das células, mas somente o retinol e seus ésteres restabelecem a função testicular (KIM *et al.*, 1993).

O principal alvo do retinol no testículo é a célula de Sertoli, que são células somáticas encontradas nos túbulos seminíferos, essenciais para desenvolvimento da espermatogênese. Estas células fornecem suporte para as células germinativas e formam a barreira hemato-testicular, provendo-as de fatores essenciais para seu correto desenvolvimento. Como outras células de origem epitelial, as células de Sertoli possuem altos níveis de proteína celular ligante do retinol (CRBP), portanto sendo responsivas ao tratamento com retinol, podendo exercer controle sobre as concentrações de alguns dos derivados da vitamina A presentes no interstício, tornando-os disponíveis para as células germinativas.

Culturas de células de Sertoli, tratadas com diferentes concentrações de retinol (5, 7, 10 e 20 μM), mostraram que o tratamento com retinol acima de 5μM tornava-as mais sensíveis à ação da DNAse I. Este efeito foi revertido pelo tratamento simultâneo com 1,10-fenantrolina, quelante de Fe (II), indicando a possível participação de espécies ativas de oxigênio via reação de Fenton nestes processos (MOREIRA *et al.*, 1997). Quando pré-tratadas com retinol em doses acima de 5μM e submetidas a UVC apresentaram maior mortalidade 48 horas após a irradiação. Este fato sugere que a associação do

retinol com UVC potencializa os danos induzidos ao DNA, pois o efeito também foi parcialmente revertido pelo tratamento com fenantrolina. O tratamento com retinol também modificou os níveis de fosforilação das proteínas nucleares histonas e HMGs (MOREIRA *et al.*, 1994).

I.2. Espécies Ativas de Oxigênio e Estresse Oxidativo

Em condições fisiológicas, de todo o oxigênio molecular capturado nas mitocôndrias e processado na cadeia transportadora de elétrons, até 5% escapam e formam radicais livres. A via de escape se dá através de sucessivas reduções monovalentes (ou seja, oxigênio é reduzido em um elétron de cada vez) produzindo intermediários como o ânion superóxido (O^{-}_{2}), peróxido de hidrogênio (H_2O_2) e o radical hidroxil ($^{\bullet}OH$), (HALLIWELL e GUTTERIDGE, 1999).

As EAO são capazes de reagir indiscriminadamente com qualquer tipo de molécula orgânica, extraindo elétrons e gerando novos radicais em reações em cadeia e altamente citotóxicas (AMES *et al.*, 1993).

As células porém têm mecanismos de defesa capazes de inativar as EAO, como a ação de algumas enzimas específicas e scavengers de EAO. As principais enzimas antioxidantes de eucariotos superiores são: superóxido dismutase, que converte o ânion superóxido em peróxido de hidrogênio e oxigênio molecular; a catalase que converte peróxido de hidrogênio em água e oxigênio molecular e a glutationa peroxidase importante para a proteção contra peróxidos orgânicos (ROOH) e peróxido de hidrogênio. Já contra o radical hidroxil as células não apresentam defesa específica e por isto é

provavelmente a EAO mais nociva de todas, sendo capaz, de iniciar reações em cadeia formadoras de radicais, peroxidação lipídica e danos ao DNA.

O desequilíbrio entre a produção de EAO e a defesa antioxidante gera o que chamamos de estresse oxidativo.

I.3. Ferro e Espécies ativas de Oxigênio

O ferro e o cobre são metais presentes na estrutura de muitas enzimas e proteínas das células. Como elementos de transição, suas formas iônicas participam de reações de transferência de elétrons que podem gerar radicais livres. Entre estas reações, tem-se a reação de Fenton, na qual o radical hidroxil, altamente reativo, é gerado:

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\bullet}OH$$

A reação de Fenton também pode ocorrer com o cobre (Cu II), no entanto o ferro provavelmente é o principal metal envolvido na síntese de $^{\bullet}OH$ a partir do peróxido de hidrogênio, devido a maior disponibilidade intracelular (MELLO-FILHO e MENEGHINI, 1991).

I.4. O dano oxidativo ao DNA

Entre as diversas moléculas afetadas pelo estado pró-oxidante nas células, o DNA é de singular importância, pois é o centro da informação genética. Muitos tipos de modificações no DNA são produzidas por espécies

ativas de oxigênio (EAOs), como quebras de cadeia, danos nos açúcares (desoxirriboses) e modificações nas bases púricas e pirimídicas. O modelo geral destas modificações aponta o radical hidroxil como seu principal causador (HALLIWELL e ARUOMA, 1993).

Diversas modificações induzidas no DNA por EAOs são mutagênicas e um dos principais produtos formados pelo ataque de radicais hidroxil em bases do DNA é a 8-oxo-dGuanosina (HALLIWELL e GUTTERIGE, 1989; SHINEGAGA *et al.*, 1990).

O Ferro no estado II associado à estrutura da cromatina pode reagir com o peróxido de hidrogênio, originado da desmutação do ânion superóxido. O radical hidroxil formado nesta reação ataca o DNA *in situ*, (MURATA *et al.*, 2000). O ataque do radical hidroxil à base desoxiguanosina leva a formação do aduto 8-oxo-dGuanosina (KASAI e NISHIMURA, 1984; FLOYD *et al.*, 1988,), promovendo assim, quando em grande número, alterações que levam a mutações, a transformações malignas e eventualmente à morte celular.

Muitas técnicas são empregadas para quantificar lesões ao DNA, a técnica mais comumente utilizada para se quantificar modificações induzidas por estresse oxidativo no DNA é a determinação dos níveis de 8-oxo-dGua. Diversos estudos demonstraram que os níveis aumentados desse aduto de DNA estão relacionados ao desenvolvimento de processos carcinogênicos (FLOYD, 1990).

II. OBJETIVO

Determinar e quantificar possíveis lesões ao DNA de células de Sertoli tratadas com retinol, induzidas por espécies ativas de oxigênio, através de análise eletroquímica e espectrofotométrica por HPLC.

III. MATERIAIS E MÉTODOS

III. 1. Cultura de células:

As células de Sertoli foram isoladas de ratos Wistar machos de 15 dias de idade, do ratário do Depto de Bioquímica – ICBS-UFRGS. Após os animais terem sido mortos por asfixia com éter, os testículos foram removidos e lavados com tampão salino HBSS (pH 7,4). Os testículos foram descapsulados com tripsina (Difco, Detroit, MICH, USA). As células peritubulares foram separadas por digestão enzimática com colagenase tipo I e por sucessivas lavagens com HBSS e centrifugações, conforme descrito pelo método modificado de Dorington (MOREIRA *et al*, 1994). A viabilidade celular foi estabelecida por exclusão de tripan azul (Trypan blue) e o nível de contaminantes, (células peritubulares que permaneceram na cultura) foi determinado pelo teste de fosfatase alcalina, nunca sendo superior a 2%.

As células foram semeadas numa densidade de 6,7 X 10^6 células por placa. Após 24 horas de cultura com Meio 199 (Sigma, ST. Louis, MO, USA) pH 7,6 suplementado com 1% de soro bovino à 34°C, a camada de células aderidas às placas foram lavadas com HBSS (tampão salino) e cultivadas por

outras 24 horas em Meio 199 livre de soro. Após este período, foi realizado o tratamento com meio199 suplementado com retinol nas concentrações de 7 μM e 20μM por mais 24h. As células controle receberam meio 199 ou meio 199 com etanol (solvente do retinol) na mesma concentração das células tratadas, correspondendo a 0,1% de etanol.

III. 2. Extração e purificação do DNA genômico:

Após 24 horas de tratamento, foi realizada a extração do DNA genômico utilizando técnica adaptada de Teixeira e Meneghini (TEIXEIRA e MENEGHINI, 1996).

O meio de cultura foi coletado e as células raspadas das placas e centrifugadas juntas com o meio a 1000 rpm por 5 minutos. O pellet foi lavado com PBS e novamente centrifugado. O sobrenadante foi descartado e as células lisadas com uma solução de DES/SDS (5mM EDTA, 1M LiCl, 50mM TrisCl, 1M Uréia, 0,2% SDS, pH 8,0) e o lisado foi incubado com Proteinase K (50 μg /mL) por 12 horas (over-night). O DNA foi precipitado pela adição de 0,55 V de isopropanol, incubado por 15 minutos e a seguir centrifugado por 15 minutos a 8000 rpm. O precipitado, então, foi lavado com etanol 70%, seco ao ar e dissolvido em TE – Tris EDTA (pH 7,4) a temperatura ambiente por, pelo menos, 4 horas. Após a dissolução, seguiu-se uma incubação com RNAse A e RNAse T1 por 90 minutos à 37 °C. Após, o DNA foi purificado com fenol:clorofórmio (1:1) e depois com apenas clorofórmio. A fase aquosa foi então precipitada com 0,1 volume de acetato de sódio 3M (pH 5,2) e 3 volumes de etanol, e centrifugação de 8000 rpm por 15 minutos, o precipitado lavado com etanol 70%, seco ao ar e então, ressuspenso

em uma solução de acetato de sódio 20mM (pH 5,2) e quantificado por A_{260nm}

III. 3. Análise do DNA por HPLC:

Nesta análise utilizou-se o método descrito por Shinegaga (SHINEGAGA *et al.*, 1990).

Através de purificação por hidrólise enzimática o DNA foi digerido com nuclease P1 (50 μg /100 μg de DNA) por 3h, a 37 °C. Após este período, foram adicionados 0,1 volume de tampão Tris.Cl (1M, pH 8,0) e fosfatase alcalina de intestino de bezerro (1U/100 μg de DNA). A incubação prosseguiu por 90 minutos a 37 °C.

Para análise dos nucleosídeos, foi utilizado um HPLC Shimadzu modelo LC-10AD e uma coluna Techsphere modelo CDS 5U (150 x 4,6 mm). Como fase móvel utilizou-se tampão fosfato (50 mM KH_2PO_4, 10% Metanol, 2,5 mM EDTA, pH 5,5), filtrado e degaseificado. As separações foram efetuadas a um fluxo de 1mL/min.

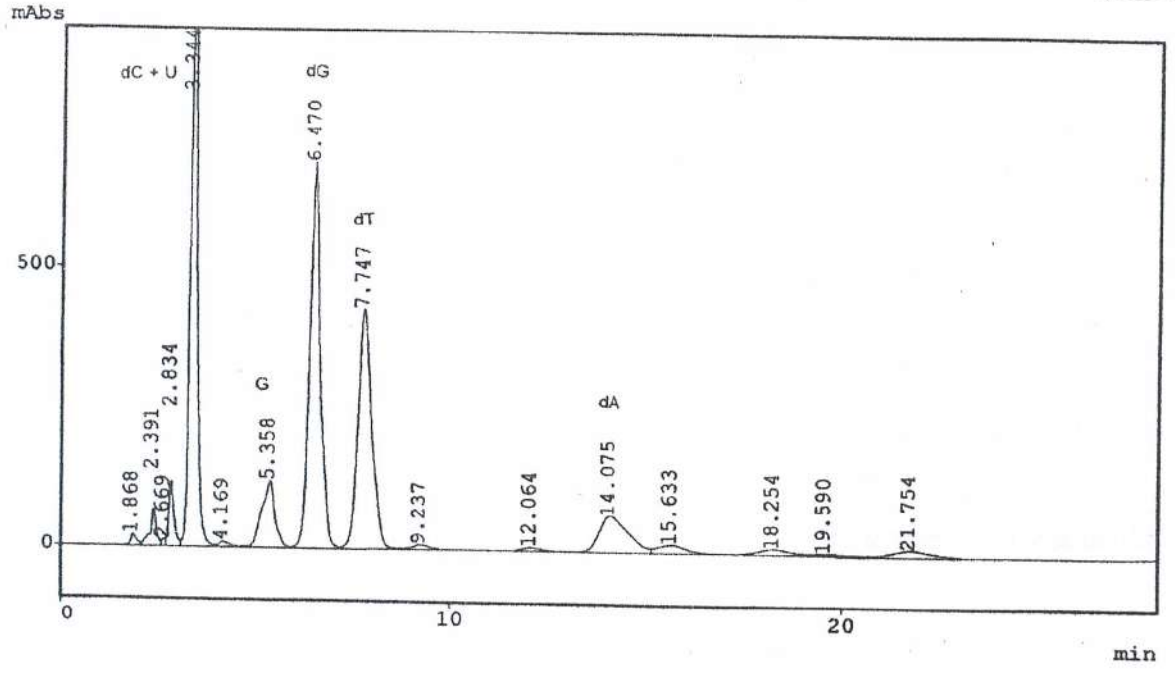
As amostras foram analisadas por um detector espectrofotométrico e um eletroquímico colocado em série. A detecção espectrofotométrica (285 nm) e a detecção amperométrica (+0,6 V) foram efetuadas por uma unidade Shimadzu modelo LC-10AD. Os cromatogramas foram analisados pelo software EZ Chrom (Scientific Software, Inc.).

A quantificação da 8-oxo-dGuanosina foi feita por comparação com curvas padrões da mesma e de 2'- desoxiguanosina. O resultado da análise foi expresso em fentomoles de 8-oxo-dG/ μg de DNA.

IV. RESULTADOS

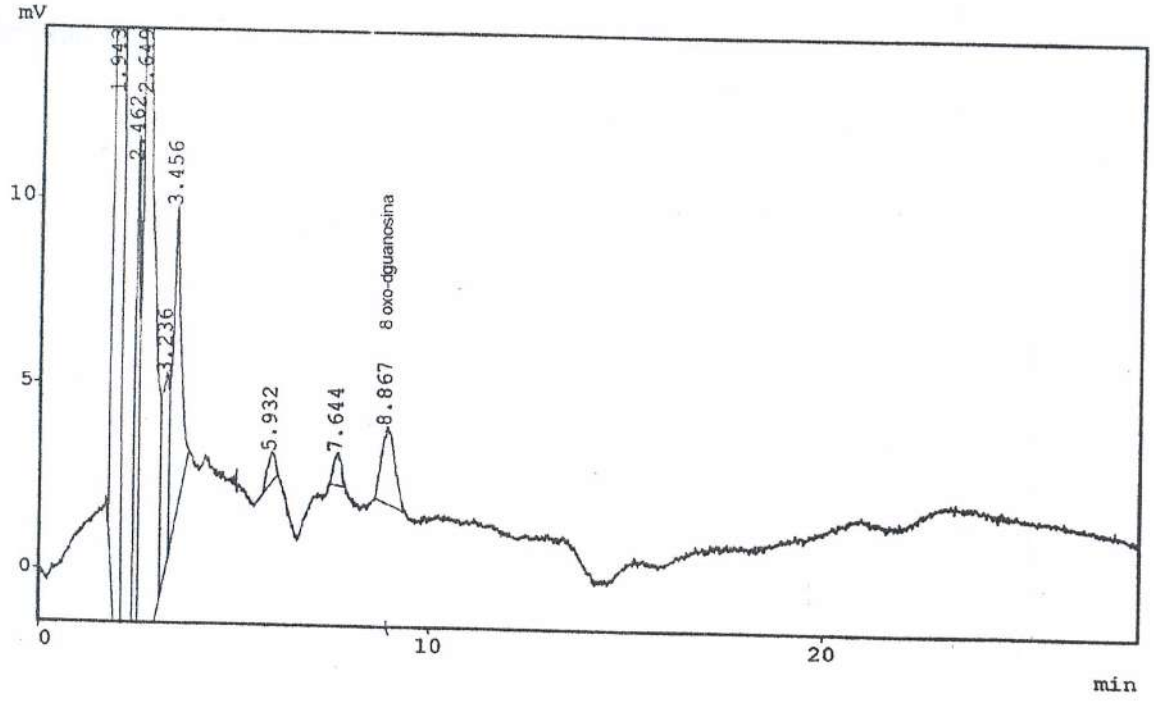
Com o objetivo de testar o equipamento de HPLC e verificar e o funcionamento da técnica foi realizado um primeiro experimento utilizando DNA comercial de esperma de salmão.

Figura 1. Análise de nucleosídeos de DNA comercial de esperma de salmão. Detector U.V.



A análise dos nucleosídeos foi feita pelo detector U.V (285nm), observando-se na figura, os tempos de retenção na coluna, o pico da guanosina por volta dos 5,4 minutos e o pico da desoxiguanosina em 6,5 minutos.

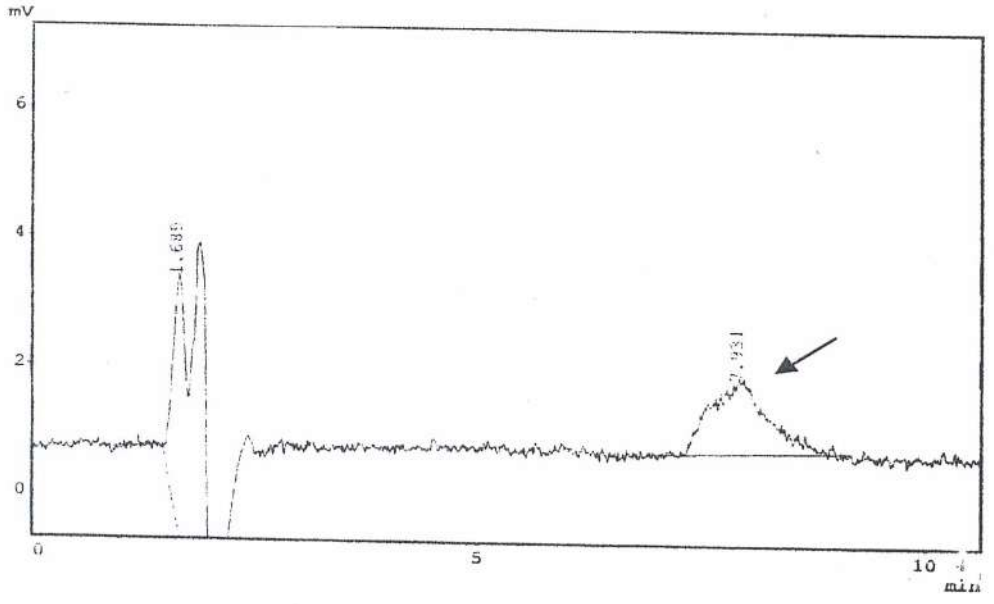
Figura 2. Análise de 8-oxo-dGua em DNA de esperma de salmão. Detector eletroquímico.



Neste cromatograma foi observado o pico da 8-oxo-dGua, indicando que o aparelho e a técnica estavam em perfeito funcionamento.

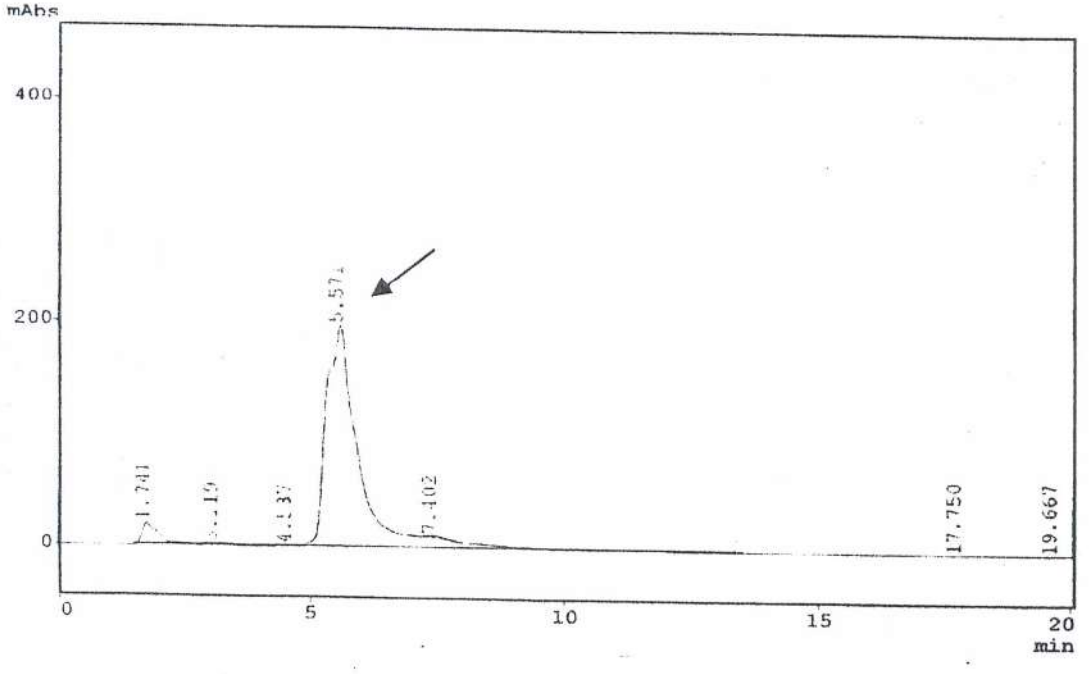
Após este primeiro experimento, deu-se seqüência a análise dos padrões e amostras de DNA das células tratadas com retinol.

Figura 3. Cromatograma do padrão de 8-oxo-dGuanosina (2ng/ml). Detector eletroquímico.



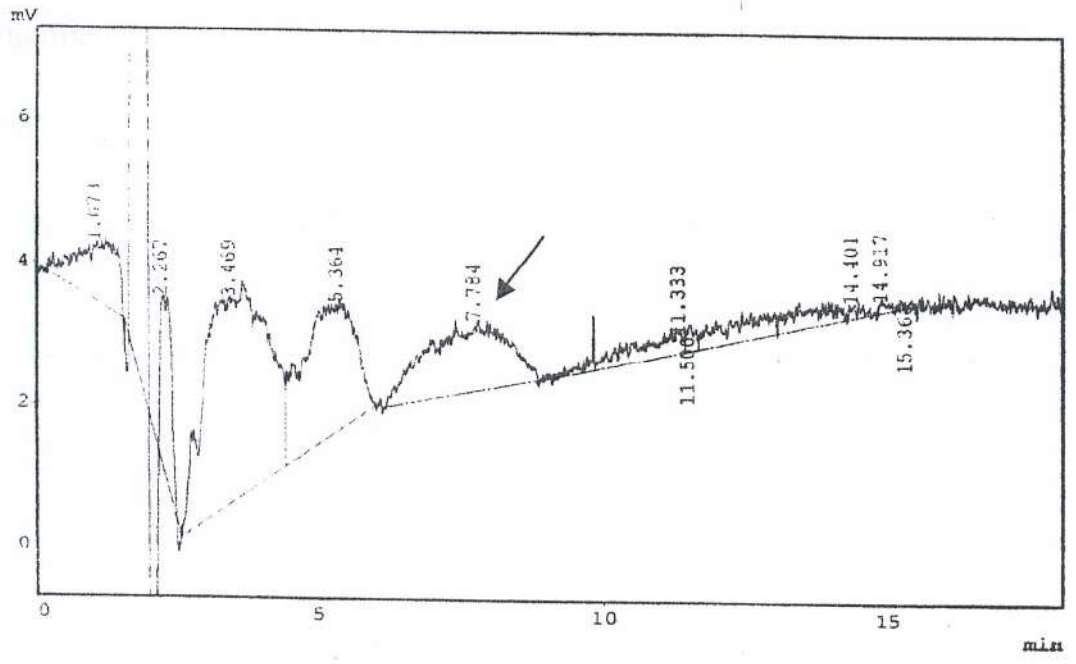
A seta indica o pico do padrão de 8-oxo-dGua, retido na coluna por volta dos 7,9 minutos.

Figura 4. Cromatograma do padrão de 2´-desoxiguanosina (6ng/ml). Detector U.V.

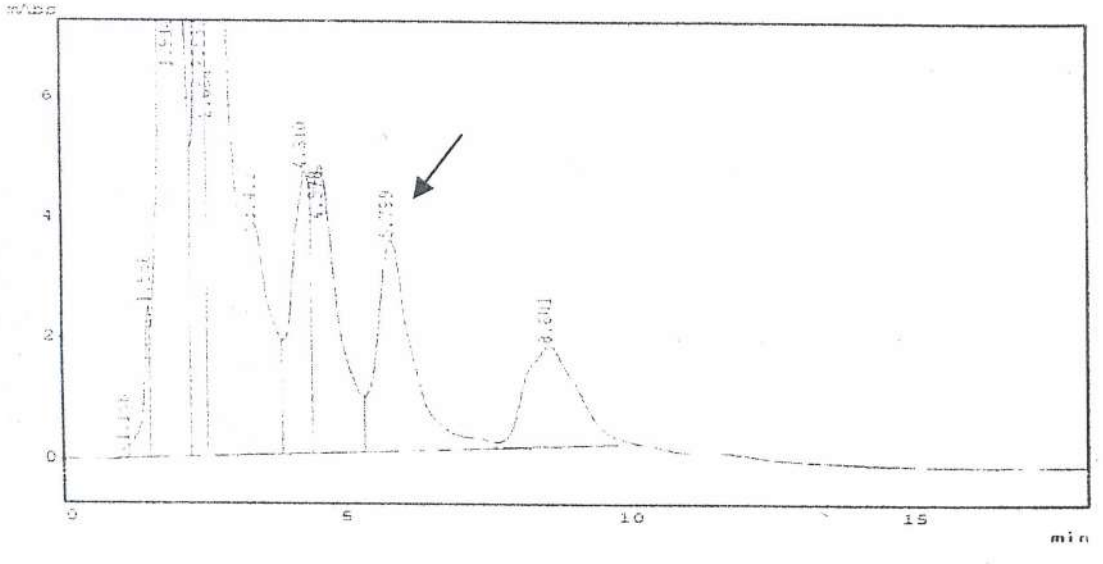


A seta indica o pico do padrão de 2´-desoxiguanosina.

Figuras 5 e 6. Análise dos nucleosídeos de amostra de DNA, de células de Sertoli tratadas com retinol 7μM. Detector eletroquímico e detector U.V.



A seta indica o pico de 8-oxo-dGua.



A seta indica o pico da desoxiguanosina.

Tabela 1. Formação de 8-oxo-dG no DNA de células de Sertoli tratadas com retinol

Tratamento	fmol 8-oxo-dG/ μg de DNA (± SD)
Controle-meio	1740,7 ± 98,9
Controle- etanol 0,1%	2697,6 ± 307,2 *
Retinol 7 μM	12.981 ± 3168 *#
Retinol 20 μM	9140,5 ± 39,7 *#

As células foram cultivadas em meio 199, suplementado ou não com retinol por 24h, mantidas a 37°C, 5% de CO_2, em atmosfera umidificada. O DNA foi extraído, digerido e o conteúdo de 8-oxo-dGuanosina foi determinado por HPLC. As médias e desvios referem-se a dois experimentos em triplicata.

* $p < 0,05$ em relação ao meio e # $p < 0,05$ em relação ao 0,1% etanol; Teste ANOVA

V. DISCUSSÃO

O dano ao DNA genômico ocorre espontaneamente e pode promover o desenvolvimento de mutações. Estas, por sua vez, podem induzir uma variedade de lesões ao DNA, incluindo modificações em bases, fragmentação e quebras de fita simples e duplas. Quando não reparados, estes danos interferem na transcrição, replicação do DNA e na segregação cromossômica, resultando na perda da viabilidade celular (HALLIWELL e GUTTERIDGE, 1999). Para garantir sua sobrevivência, as células são equipadas com mecanismos de reparo dessas lesões ao DNA (necessários para o atraso na progressão do ciclo celular), a fim de evitar a replicação ou a segregação do DNA danificado. As células de organismos multicelulares podem, ainda, optar pela ativação do processo de morte celular programada (apoptose), em resposta ao dano no DNA.

Todo o antioxidante é de fato um agente redox, protegendo da ação de radicais livres em algumas circunstâncias e em outras, promovendo a geração destes (HERBERT, 1996). A vitamina A é um ótimo aceptor e doador de elétrons em reações químicas, propriedades estas que parecem proteger as proteínas ligantes de retinol (OLSON, 1996). Em humanos os valores normais de retinol no soro são por volta de 360-1200μg/L. Estima-se que a concentração fisiológica de retinol seja aproximadamente 5μM (LIVREA e PACKER, 1993). Em condições normais, as células não estão expostas a altas concentrações de retinol livre (DAVES e ONG, 1995). O consumo excessivo de suplementos de vitamina A e β-caroteno satura as proteínas ligantes de compostos livres, o que pode levar à citotoxicidade (MURATA *et al*, 2000).

Resultados anteriores do grupo de pesquisa do Centro de Estudos em Estresse Oxidativo, mostraram aumento de peroxidação lipídica (TBARs e

dienos conjugados) e apoptose em células de Sertoli tratadas com retinol. Tais resultados corroboram com a hipótese do retinol como um agente pró-oxidante que leva a danos oxidativos no DNA e membranas celulares, além de alterações na atividade de enzimas antioxidantes (DAL PIZZOL *et al*, 2001), como por exemplo, de ornitina decarboxilase (ODC).

A ODC é uma enzima-chave no metabolismo de biossíntese das poliaminas, relacionada com a proliferação e marcadora da fase final de G1 do ciclo celular. Os níveis de poliaminas são elevados durante o processo de crescimento. A atividade da ODC declina em sistemas de crescimento lento e aumenta durante processos de proliferação rápida, incluindo células transformadas malignas. Trabalhos anteriores demonstraram que o tratamento com retinol induziu um aumento na atividade da ODC dependente do estado redox celular em células de Sertoli cultivadas (KLAMT *et al*, 2000).

Segundo Moreira e colaboradores (1996), o tratamento com retinol além de estimular a síntese de DNA altera os mecanismos de reparo, pois em tratamento simultâneo com hidroxi-uréia, um forte inibidor de síntese de DNA, ainda é observado a incorporação de timidina marcada, sendo que estes processos dependem de um *pool* diferente de nucleotídeos do que os utilizados para síntese. Em outro trabalho foi demonstrado que o tratamento com retinol levou a uma maior fragilidade do DNA à ação das DNAses (MOREIRA *et al.*, 1997), ou seja o DNA tornava-se mais sensível à degradação, por estas enzimas. Modificações no padrão de fosforilação de HMGs de células de Sertoli tratadas com retinol foram demonstradas por Moreira e colaboradores em 2000. Sabe-se que mudanças químicas (fosforilação e acetilação) em proteínas associadas ao DNA, como histonas, HMGs estão relacionadas a processos de regulação do crescimento e diferenciação da célula, e transformação celular (DAVIE, 1996).

Conforme observado na tabela 1, o tratamento com retinol aumentou significativamente os níveis de 8-oxo-dGua nas células tratadas em relação ao controle. Entretanto não houve diferença significativa dos níveis de 8-oxo-dG entre os tratamentos 7 μM e 20 μM de retinol. Este mesmo efeito foi observado nas análises de peroxidação lipídica e apoptose nestas células, o que pode ser explicado pela alta mortalidade das células expostas a 20 μM de retinol (60%), e sendo que as análises são feitas com as células que sobrevivem.

O aumento dos níveis de 8-oxo-dGua mostra um dano direto no DNA das células de Sertoli causado pelo radical hidroxil, indicando o potencial mutagênico do retinol em concentrações acima de 7 μM (DAL PIZZOL *et al*, 2000), uma vez que há clara associação entre o aumento dos níveis deste aduto no DNA e o surgimento de mutações e câncer (SHINEGAGA *et al.*, 1990; HALLIWELL e GUTTERIGE, 1999).

Apesar dos danos oxidativos induzidos pelo retinol, trabalho, foi demonstrado, recentemente, que células de Sertoli apresentam um maior índice de síntese de DNA e formação de focos proliferativos (DAL PIZZOL *et al*, 2001), indicando que as células resistentes são selecionadas e dão início a um processo proliferativo, que pode ou não estar associado à transformação maligna, em virtude dos inúmeros danos ocorridos ao DNA.

O desenvolvimento do câncer em animais é um processo lento que envolve três estágios: iniciação, promoção e progressão tumoral. A iniciação é causada por uma alteração irreversível no DNA. Esta alteração não garante por si a iniciação do tumor. É necessário algum grau de replicação do DNA e proliferação celular para permitir que a mutação se perpetue no DNA antes que mecanismos de reparo possam removê-la. A proliferação celular pode

ocorrer normalmente em tecidos ativos, ou pode ser estimulada pelo próprio carcinógeno. A iniciação é seguida pela promoção. Os promotores tumorais causam a expressão do fenótipo latente das células iniciadas através da seleção e expansão clonal. Muitos carcinógenos podem agir tanto como iniciadores quanto como promotores. Durante a promoção tumoral, o material genético da célula iniciada se torna expresso através de mudanças na expressão de genes que regulam a diferenciação e o crescimento celular. O estágio final da carcinogênese, é a evolução de uma lesão pré-maligna em lesões malignas, envolvendo novos danos ao DNA (HALLIWELL e GUTTERIDGE, 1999).

Em virtude da importância do dano ao DNA no desenvolvimento da carcinogênese, têm-se também como agentes carcinógenos as espécies ativas de oxigênio por serem capazes de causar modificações em biomoléculas.

Muitos estudos ainda são necessários para a elucidação dos inúmeros mecanismos envolvidos em processos carcinogênicos, e necessários para a avaliação dos efeitos da suplementação com retinol em modelos *in vivo* de transformação celular.

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Retinol Supplementation Induces DNA Damage and Modulates Iron Turnover in Rat Sertoli Cells

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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 caused cellular DNA damage probably involving cellular iron accumulation. Retinol (7μM) significantly induced DNA single strands breaks, DNA fragmentation and production of 8-oxo-7, 8-dihydro-2'-deoxyguanosine in cultured Sertoli cells. In contrast, lower doses seemed not to induce single-strands break in this experimental model. The breaks in DNA were inhibited by an iron scavenger; and 7μM retinol treatment modulated iron turnover leading to iron accumulation, suggesting that iron ions were required for the retinol cellular effects. These findings suggest that retinol-induced DNA damage was associated with the modulation of iron turnover, and these characteristics could be responsible for the increased incidence of lung cancer associated with retinoids supplementation.

Keywords: retinol; vitamin A; DNA damage; oxidative stress; iron; iron metabolism

INTRODUCTION

At least two major human problems, aging and cancer, involve damage to DNA[1]. In recent years tremendous advances have been made in our understanding of the mechanism of gene expression and the role of reactive oxygen species (ROS) in producing DNA damage.

ROS produce a number of lesions in DNA, probably by direct chemical attack[2], instead of the activation of calcium dependent endonucleases[3], and many such lesions are known to be mutagenic[4,5]. Neither superoxide nor hydrogen peroxide causes any strand breakage or chemical modification of the purines or pyrimidines in the absence of transition metal ions[6–8]. Their toxicity *in vivo* is thought to result from their metal ion dependent conversion to hydroxyl radicals, which are very reactive towards organic compounds. Mello-Filho and Meneghini demonstrated that in mammalian cells it appears that iron-mediated, as compared to the copper-mediated, intranuclear Fenton reaction is responsible for DNA damage [9].

Instead of the important physiological functions of retinol [10], the effects of supplementation with supra-physiological doses of retinol are not well defined. Many authors propose a pro-

* 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. Tel.: 55 51 316 5549, Fax: 55 51 316 5535, e-mail: pizzol@ez-poa.com.br

tective role of retinoids in the development of cancer [11–15]. On the other hand, in animal models of lung cancer retinol increases the malignant transformation induced by gamma irradiation [16] and Badr et al [17] demonstrated that retinol increases the induction of chromosomal aberrations in human lymphocyte cultures. In addition, two reports suggest a positive association of vitamin A intake and increased incidence in prostate cancer [18,19]. 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 [20]. In another randomized controlled study with 29,133 smokers in Finland[21] 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 [22–24]. Therefore, the casual mechanisms should be elucidated to establish safe approaches in cancer chemoprevention.

Our previous studies demonstrated an increase in chromatin sensitivity to DNAse I [25], an increase in methyl[3H]- thymidine incorporation into DNA[26], changes in nuclear protein phosphorylation[27], an increase in ornithine decarboxylase (ODC) and catalase activity[28], and an increase in lipid peroxidation[28] in Sertoli cells treated with retinol (7μM). These effects were not observed with lower concentrations of retinol [28], and were abolished with the addition of hydroxyl (·OH) scavengers and metal ions chelators[25,28]. These data suggested that the effects of retinol supplementation could be induced by Fenton-mediated ·OH production. Recently, Murata et al[29] demonstrated retinol and retinal-induced oxidative DNA damage probably by the dismutation of superoxide to hydrogen peroxide in the presence of endogenous metals.

We report here that retinol supplementation caused cellular DNA cleavage, and induction of 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodG). To clarify the role of metal ions in retinol induced DNA damage, we performed experiments using an iron chelator and we also investigated iron turnover in retinol treated Sertoli cells.

MATERIALS AND METHODS

Materials

Type I collagenase, medium 199, HBSS, all-*trans* retinol, 1,10 phenanthroline, proteinase K, RNAse A, RNAse T1, nuclease P_1, calf intestinal alkaline phosphatase and human apotransferrin, were purchased from Sigma, St. Louis, MO, USA. Trypsin was purchased from Difco, Detroit, MI, USA. [^{3}H] thymidine (3.15 TBq/mmol) was purchased from Amersham Place, Little Chalfont, England. [^{55}Fe] ($^{55}FeCl_3$ – 111 GBq/g) was purchased from NEN Life Science Products, Bedford, MA, USA.

Cell Culture

Sertoli cells from 15-day-old Wistar rats were prepared and cultured essentially as previous described [26]. 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 with or without 100μM 1,10 phenanthroline simultaneously. 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 (data not shown).

The formation of oxidized retinol metabolites was monitored by spectroscopic scan of all retinol solution before its use. Cell viability was assessed by trypan blue exclusion.

Determination of DNA Single Strand Breaks (SSB)

The determination of DNA SSB was made basically as described by Olive [30]. Briefly, cells were labeled for 24 h with 3 μCi/ml of [^{3}H] thymidine before retinol treatment. After retinol treatment, cells were exposed to a solution containing 10mM Tris, 10mM EDTA, 2% SDS, 50mM NaOH, pH 12.4. After this cells were treated with 120mM KCl, incubated for 10 min at 65°C and centrifuged at 2,400g for 10 min at 4°C. The supernatant and the remaining pellet were counted. The SSB was calculated by dividing the counts in the supernatant by the total counts.

DNA Fragmentation Determination

The determination of DNA fragmentation was made basically as described by Venable et al [31]. Briefly, cells were labeled for 24 h with 3 μCi/ml of [^{3}H] thymidine before retinol treatment. After retinol treatment, the medium was aspirated and counted. The cells were lysed with PBS containing 1% Triton X-100 and 2μM EDTA. The cells were centrifuged for 15 min at 14,000 rpm in a microcentrifuge. The supernatant was counted, and the remaining pellet containing larger DNA fragments was counted. The DNA fragmentation was calculated by adding the counts in the medium and the supernatant and dividing by the total counts.

Analysis of 8-oxodG Formation in Sertoli Cells Treated With Retinol

This was done essentially as previous described [32,33]. In brief, DNA was extracted from cell cultures and digested to nucleosides by nuclease P_1 and alkaline phosphatase. The 8-oxodG and 2'-deoxyguanosine (dG) contents were determined by HPLC using a electrochemical detector and UV detection, respectively.

Iron Uptake and Secretion in Sertoli Cells Treated With Retinol

Human apotransferrin was labeled with ^{55}Fe $^{55}(FeCl_3)$ using nitrilotriacetate as the iron carrier[34]. After retinol treatment, Sertoli cells were incubated in the presence of 12 μg/ml [^{59}Fe]-transferrin at 4°C for 10 min as previously described (as determined previously this is the saturating concentration) [34]. Then, they were washed in ice-cold HBSS and transferred to medium 199 at 34°C. After various times of incubation the medium was removed and counted to determine the amount of ^{55}Fe secretion. The amount of cellular ^{55}Fe uptake was determined by washing and lysing the cells and determining the radioactivity in a liquid scintillation counter [35]. Iron concentration was calculated by comparing cpm of samples with cpm of known amounts of ^{55}Fe $^{55}(FeCl_3)$.

Iron Distribution in Sertoli Cells Treated With Retinol

Cultured cells were exposed, during retinol treatment, to physiological [^{55}Fe]-transferrin concentrations (200 μg/dL) for 24h. After this period · nuclear-enriched, mitochon-

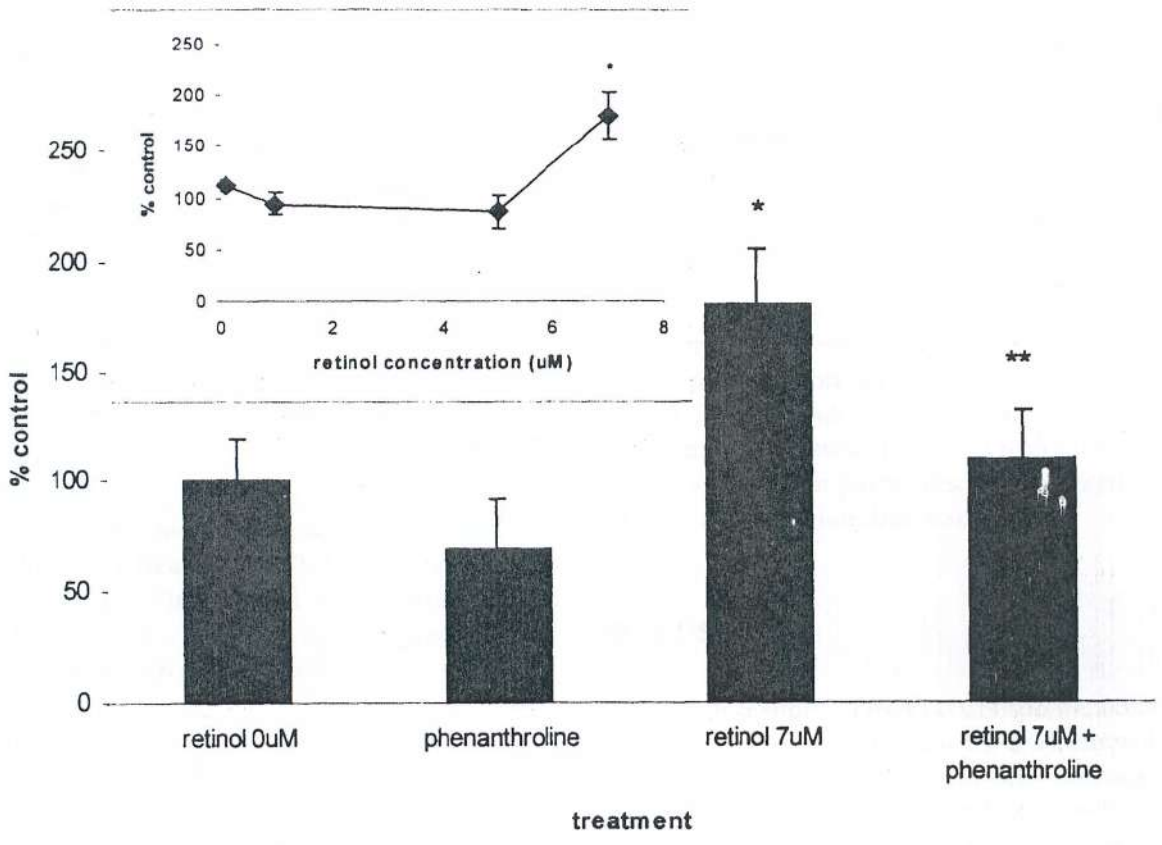

FIGURE 1 DNA single strand breaks (SSB) determination in cells treated with retinol. Cutured Sertoli cells were treated with the indicated concentrations of retinol dissolved with ethanol (0.1%) for 24 h; controls also contained 0.1% ethanol (insert box). Depending of the experimental procedure cells were treated with 1,10 phenanthroline (100 μM) for 24 h. For the determination of DNA single strand breaks cells were labeled for 24 h with 3 μCi/ml of [^{3}H] thymidine, DNA was extracted as described under "Materials and Methods" and the radioactivity of the fractions collected from the tube was measured in a liquid scintillation counter. Values were expressed as percent from control (n=4). * different from control, $p<0.01$ ** different from 7μM retinol group, $p<0.01$

drial-enriched, and cytoplasmatic-enriched compartments were isolated[36] and the radioactivity was counted in a liquid scintillation counter.

Statistical Analysis

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

RESULTS

Cellular DNA Damage Induced by Retinol

DNA SSB in cultured Sertoli cells treated with retinol were detected by thymidine labeled DNA as described in Materials and Methods. Retinol treatment induced SSB only at doses above 5 μm (Fig. 1 – insert box). Retinol at 0.1 to 5 μM seems not to induce SSB in cultured Sertoli cells. This pattern was also described previously with retinol-induced lipid peroxidation and catalase activation [28].

TABLE I Effect of retinol treatment (7μM) by 24h on DNA fragmentation and 8-oxodG content. Data represent mean ± SEM of at least three replicates per independent experiment and three distinct experiment

Assay	*Control*	*Retinol 7μM*
DNA fragmentation (% control)	100 ± 9	135 ± 7[a]
8-oxodG (8-oxodG/dGx10^5)	0.8 ± 0.09	3.86 ± 0.94[a]

a. Different from control; $p < 0.01$.

Since only 7μM retinol increased SSB we investigated if this dose could increase other markers of oxidative DNA damage. As shown in Table I, DNA fragmentation in Sertoli cells treated with 7μM retinol was significantly higher than that of the control. In the same way, 8-oxodG content was significantly increased in 7μM retinol treated cells (Tab. I). These findings suggest that 7μM retinol doses could induce DNA damage, probably by the generation of reactive oxygen species (ROS).

Effect of an Iron Chelator on DNA Damage Induced by Retinol

Fig. 1 shows the effect of 1,10 phenanthroline on retinol-induced SSB. The DNA damage was inhibited by the iron scavenger, suggesting that iron ions were required for the retinol cellular effects. We had previously shown that 1,10 phenanthroline inhibited ODC activation and conformational chromatin changes induced by retinol [25,28] in agreement with data presented here.

Effect of Retinol Treatment on Iron Uptake and Secretion in Cultured Rat Sertoli Cells

The iron uptake was significantly faster only in the case of treatment with 7μM retinol as compared to control cells (Fig. 2). This effect is probably mediated by an increase in the number of transferrin receptors (TfR), but we can not exclude that retinol interfered with TfR kinetics. Although 7μM retinol increased iron uptake there was no significant difference in the secretion of iron in all but one time tested (120 min) (Fig. 3), indicating that there was an accumulation of iron in 7μM retinol-treated cells.

Effect of Retinol Treatment on Iron Distribution in Cultured Rat Sertoli Cells

Since 7μM retinol stimulated iron uptake in rat Sertoli cells, we decided to investigate iron distribution in different cellular compartments. Fig. 4 shows iron distribution in enriched nuclear, mitochondrial and cytoplasmatic compartments; 7μM retinol increased iron concentration in all three compartments, most markedly in nuclear and mitochondrial fractions.

DISCUSSION

Damage to genomic DNA occurs spontaneously and can be further enhanced by environmental mutagens. Chemical or physical mutagens induce a variety of lesions in DNA, including base modifications, cross-linking and strand breaks. If left unrepaired these damages interfere with transcription, DNA replication and chromosome segregation, resulting in the loss of cellular viability [37]. To ensure survival, cells must be equipped with mechanisms to repair these DNA lesions and need to delay cell-cycle progression to avoid the replication or the segregation of damaged DNA. As a safeguard, cells of multicellular organisms have the option of activating programmed cell death in response to DNA damage.

We report here that supplementation with retinol caused oxidative damage to cellular DNA as assessed by SSB, DNA fragmentation and 8-oxodG production. This damage seems to be induced only by 7μM retinol, since 5μM or lower doses did not induce SSB, as we demonstrated previously in the case of lipid peroxidation and catalase activity[28].

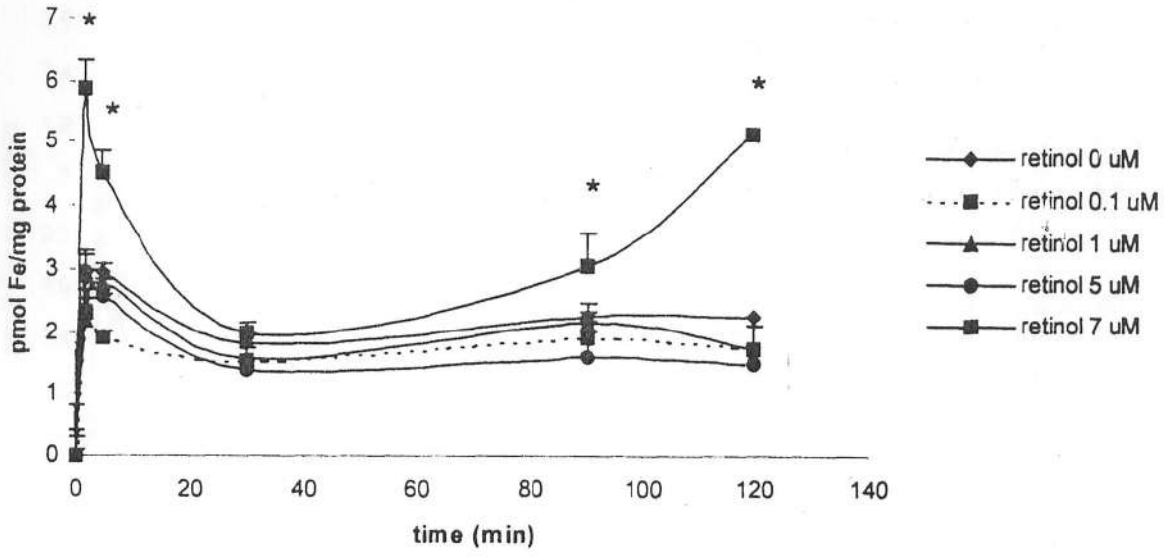

FIGURE 2 Iron uptake in Sertoli cells treated with retinol. Cells were treated as described in the legend to Fig. 1. After treatment, Sertoli cells were incubated in the presence of 12 μg/ml [^{55}Fe]-transferrin at 4°C for 10 min. After various times of incubation, cells were lysed and the radioactivity was counted in a liquid scintillation counter (n=4). * different from control, $p<0.01$

Every antioxidant is in fact a redox agent, protecting against free radicals in some circumstances and promoting free radical generation in others [38]. 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 [39]. Normal values of human retinol serum are around 360–1200 μg/L (1.25–4.1 μM), and is estimated that the physiological retinol concentration in Sertoli cells is around 5μM [10]. In normal conditions cells were not exposed to high concentration of free retinol[40]. However, pharmacological amounts of the supplements above physiological amounts may perturb key physiological processes. If excessive intake of supplements of vitamin A and β-carotene saturate binding protein, free compounds may have cytotoxicity[29].

Thus, ours results indicate that supplementation with retinol could induce oxidative DNA damage in this experimental model, and this effect could be, in part, responsible for the adverse effects of retinol supplementation. In contrast, Murata et al [29]recently demonstrated that even lower retinol doses (2 – 5 μM) could induce DNA damage in HL-60 cell line. This difference could be related to a different antioxidant environment or different retinol metabolism of these two different cell lines. Sertoli cells cultures seem to be a good model to study retinol effects on ROS production. Primary cells cultures are more representative of the cell type in the tissue from which they was derived, and do not have the disadvantages of continuous cell lines (i.e. greater chromosomal instability). Sertoli cells are epithelial in origin and, like other epithelial cells (i.e. skin and respiratory tract) are responsive to retinol treatment[25]. Sertoli cells are well characterized morphologically and biochemically and these could facilitate the identification of cellular effects mediated by retinol or ROS. In addition, their phagocyte function implies in the development of a complete oxidative defense system. Sertoli cells are also very

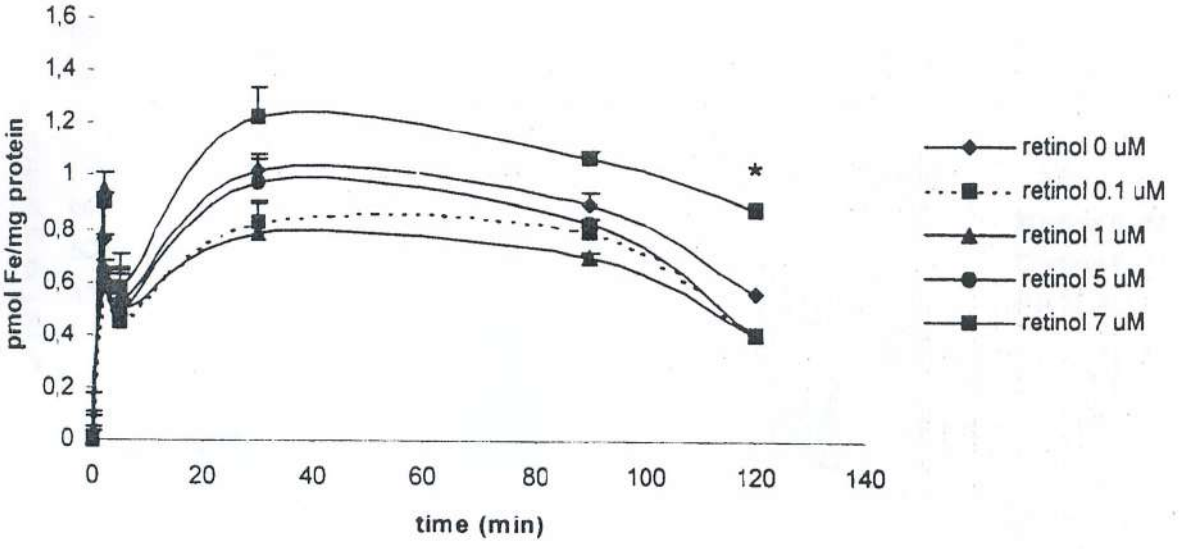

FIGURE 3 Iron secretion in Sertoli cells treated with retinol. Cells were treated as described in the legend to Fig. 1. After treatment, Sertoli cells were incubated in the presence of 12 μg/ml [^{55}Fe]-transferrin at 4°C for 10 min. After various times of incubation, the medium was removed and the radioactivity was counted in a liquid scintillation counter (n=4). * different from control, $p<0.01$

rich in iron and copper transport proteins, and could secrete transferrin to regulate the intracellular iron pool.

The most likely mode of ·OH radical production *in vivo* is via the Fenton reaction, through reduction of H_2O_2 by ferrous ions. Cuprous ions are also an elemental producer of ·OH radicals in the Fenton reaction[41]. Copper is more active than iron as a Fenton reactant (42) but iron is much more abundant in biological systems [37]. The consequence is that iron is usually the redox cation that participates preponderantly in the cellular Fenton reaction involving DNA [9,43,44]. Murata et al [29], in isolated DNA, suggested that the existence of copper ions, but not iron, was required for retinol-induced DNA damage. We demonstrated that 1,10 phenanthroline inhibited retinol-induced SSB, indicating the participation of metal ions in retinol effects in cultured cells. Mello-Filho et al [9] demonstrated that in mammalian cell cultures 1,10 phenanthroline, but not neocrupoine prevented DNA strand-break production reinforcing the argument that an iron-mediated Fenton reaction may be the major contributor to DNA strand-breaks induced by ROS. Besides this, iron has been measured in the nucleus by analytical methods and Fe(II) and Fe(III)-DNA complexes has been demonstrated by XANES spectroscopy [45,46]. So, our results suggested that, in cell cultures, the existence of iron ions was required for the DNA damage induced by retinol, since 1,10 phenanthroline could inhibit retinol-induced SSB, and retinol treatment interferes with iron turnover in cultured Sertoli cells. This is in accordance with the predominant role of iron in the cellular Fenton reaction involving DNA.

Iron ions are potentially dangerous: their ability to undergo one-electron transfers enable them to be powerful catalysts of auto-oxidation reactions, conversion of hydrogen peroxide to ·OH and decomposition of lipid peroxides to reactive peroxyl and alkoxyl radicals. It is not only free metal ions that are catalytic: haem and

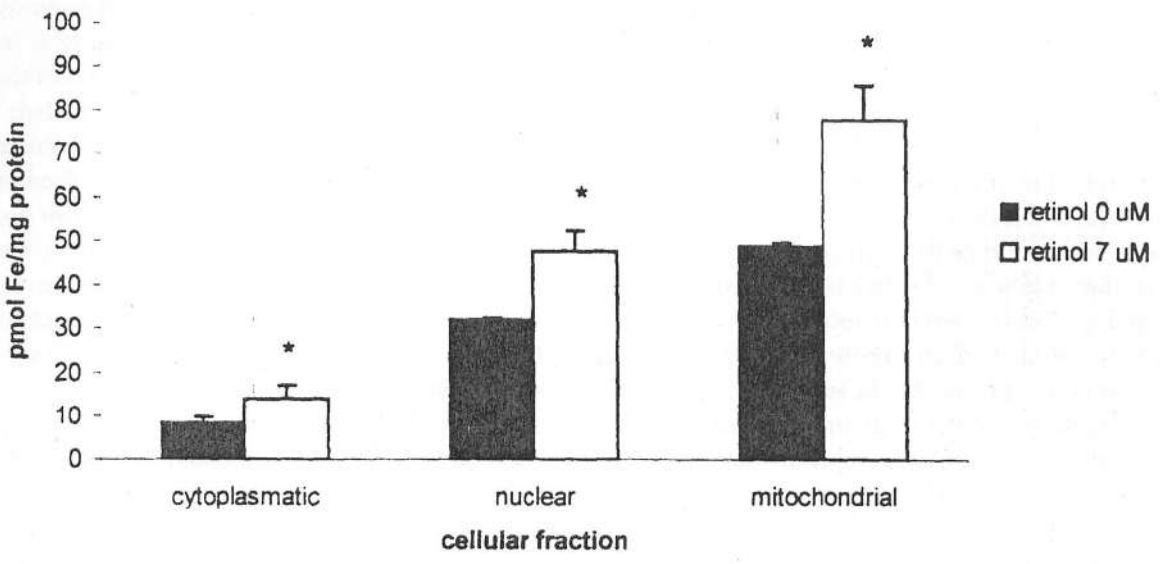

FIGURE 4 Iron distribution in Sertoli cells treated with retinol. Cultured cells were exposed to [^{55}Fe]-transferrin (200 µg/dL) for 24h. After this period nuclear-enriched, mitochondrial-enriched, and cytoplasmatic-enriched compartments were isolated and the radioactivity was counted in a liquid scintillation counter (n=4)

certain haem proteins can decompose lipid peroxides and interact with hydrogen peroxide to cause damage [47,48]. To overcome these problems higher organisms have developed iron-binding strategies and are also equipped with highly sophisticated mechanisms that prevent the expansion of a catalytically active intracellular iron pool while maintaining sufficient concentrations of the metal for metabolic needs [49].

It is a general assumption that the transport, uptake, use and storage of iron is undertaken in a very controlled way, given the potential of damage that production this ion can represent. There is a synchronized regulation of the synthesis of TfR (uptake) and ferritin subunits (storage) in mammalian cells, this involves cytoplasmatic iron regulatory proteins (IRP) [50]. Since the TfR plays a crucial role in iron uptake, the regulation of its expression is of great importance. In a broad sense, TfR expression is directly correlated with the concentration of intracellular iron in a poorly characterized metabolically active pool. The change in the number of TfRs in response to a change in iron levels is mainly due to mechanisms that affect the TfR mRNA pool by a post-transcriptional mechanism [51]. This process involves the binding of the IRP 1 and 2 to the iron responsive element (IRE) in the 3′ unstranslated region of the TfR mRNA. Apart from the post-transcriptional control of TfR expression, there are reports that the expression of the receptor may be controlled transcriptionally in some cells type [52,53]. The TfR gene promoter contains a TATA box and a sequence similar to the CAMP and phorbol ester-responsive elements [54–56]. Our results suggest that retinol supplementation could induce faster iron uptake, probably mediated by an increase in TfR number (Fig. 2), although, we can not exclude that retinol modifies TfR kinetics. We do not know if retinol regulates TfR via a transcriptional or post-transcriptional control and further studies will address this issue.

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 [57] Iron overload is well documented in patients suffering from idiopathic haemochromatosis [58]. The pathology resulting from iron overload in idiopathic haemochromatosis include elevated risk of hepatoma, esophageal cancer and skin melanoma [59]. If ·OH is attacking DNA, it must be produced very close to the DNA since this radical is so reactive that it cannot diffuse from its site of formation. Probably, iron ions are bound to DNA [45,46] and therefore that Fenton reaction generates .OH radical *in situ*[60]. Luo et al[61] and Henle et al[62] demonstrated that a higher amount of iron was associated with DNA when NADH was present, supporting the idea that a complex forms among DNA, iron and NADH. We had previously demonstrated that 7μM retinol induces an increase in NADH content in Sertoli cells (submitted data). Besides the increase in iron uptake demonstrated in Fig. 2, retinol supplementation also leads to iron accumulation in all three subcellular compartments studied, most markedly in mitochondrial-enriched and nuclear-enriched compartments (Fig. 4), providing an substrate to the intranuclear Fenton reaction induced by retinol treatment.

It is known that ROS are related to tumor-promoting potencies. On the basis of the finding that excessive retinol induced oxidative DNA damage, it is suggested that the oxidative DNA damage may be responsible for the initiation and/or tumor promotion/progression in multistage carcinogenesis. The results presented here reinforce the recently published effects of retinol and retinal on DNA damage in HL-60 cells[29]. Our results are the first evidence of retinol-induced modulation of iron metabolism and its relation to retinol-induced DNA damage in cultured cells.

In recent years advances have been made in our understanding of the molecular mechanism of retinol supplementation. We demonstrated that retinol induced conformational changes in chromatin [25], and altered phosphorylation pattern of nuclear proteins in Sertoli cells [27]. Retinol treatment induced lipid peroxidation [28]and DNA damage [29] in different cell lines, and induced the activation of antioxidant enzymes; ODC and catalase [28]. These effects could be attenuated by the addition of iron [25,28] or copper chelators[29] and·OH [28] or other radicals scavengers [29]. Taken together, these findings suggested that retinol-induced oxidative stress plays important roles in carcinogenesis in intervention studies using excess amounts of beta-carotene and retinol. Additional studies are required to understand the exact mechanism by which retinol supplementation leads to an increase in ROS production, the biochemical effects of long-term supplementation with retinol *in vivo* and its significance in neoplastic transformation of normal and previously injured cells.

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