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Ações Extranucleares da Vitamina A mediadas pela Produção de Espécies Reativas do Oxigênio

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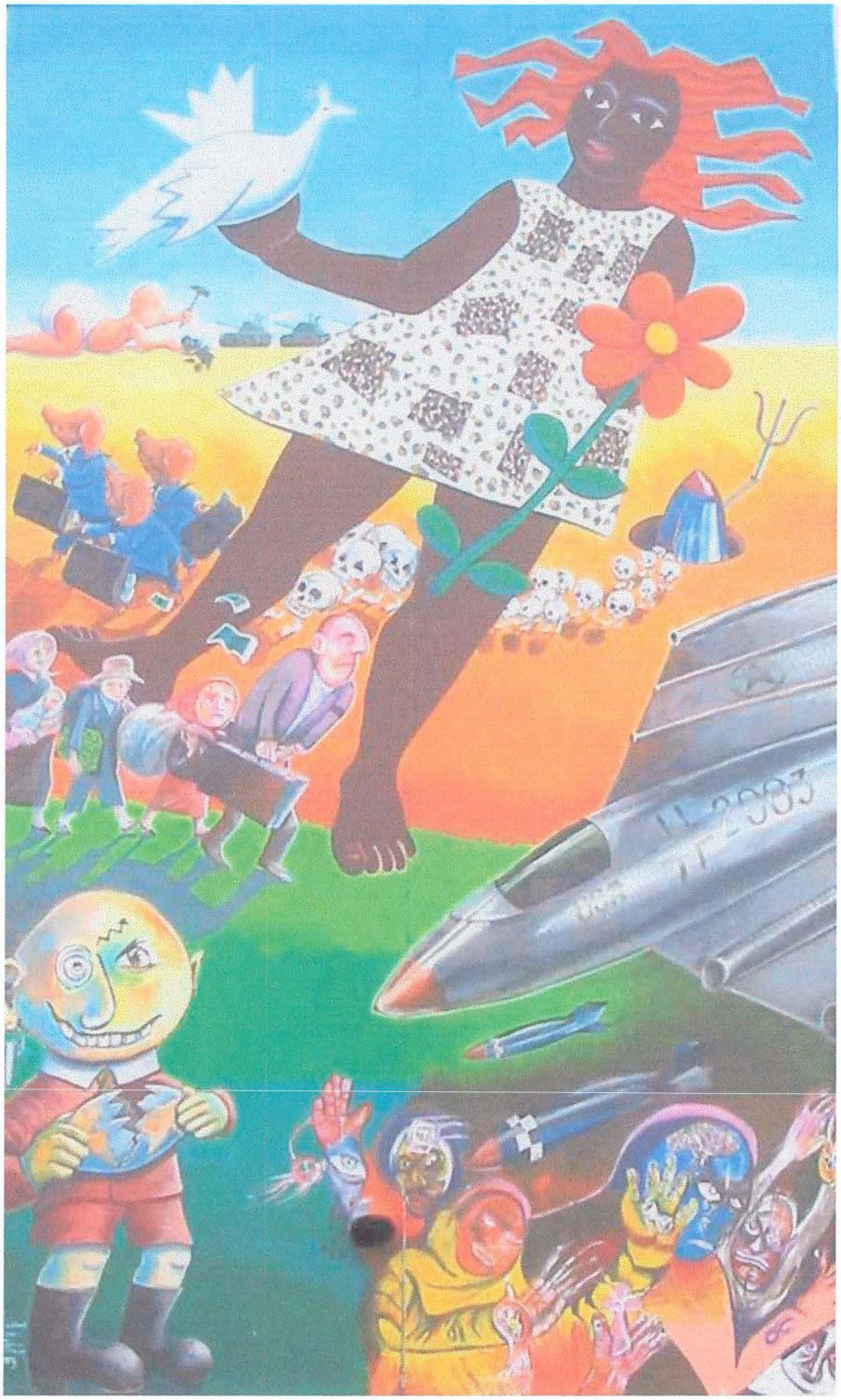

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Só me dói viver se não for por amor.

Gabriel García Márques

Aos meus pais e minha irmã, com amor.

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RESUMO

Apesar de ter sido umas das primeiras vitaminas descobertas, o conhecimento do amplo espectro de atividade biológica da vitamina A (retinol) permanece desconhecido. É bem descrita a ação biológica dos retinóides sobre a modulacão da expressão gênica celular através de receptores nucleares. Entretanto, uma grande quantidade de evidências tem demonstrado um aparente aumento na carcinogênese induzida por retinol, mas os retinóides são, ainda hoje, comumente utilizados na prevenção e tratamento de tumores. Estudos clínicos recentes demonstraram que a suplementação com retinóides resulta em um aumento na incidência de tumores e, além disso, tem-se descrito um efeito pró-oxidante do retinol em sistemas biológicos. Processos biológicos diversos são modulados por radicais livres. Como, classicamente, os retinóides regulam proliferação, diferenciação e morte celular, este trabalho teve como objetivo a caracterização do possível papel do estresse oxidativo gerado pela suplementação com retinol nestes fenômenos biológicos. A suplementação com retinol (7 μM) causou um aumento no dano oxidativo celular mediado pela irradiação ultravioleta, na atividade da enzima ornitina decarboxilase (ODC), na genotoxicidade e na transformação pré-neoplásica. Todos esses efeitos foram revertidos ou atenuados com a co-administração de antioxidantes. Também demonstramos que o retinol ativou a maquinaria apoptótica mitocondrial em dois modelos experimentais, de forma dependente da produção de radicais livres. Estes resultados, somados, sugerem que fenômenos biológicos modulados pelo retinol são relacionados, ao menos parcialmente, à produção de espécies reativas de oxigênio.

ABSTRACT

In spite of being one of the first vitamins to be discovered, the full range of biological activities of vitamin A (retinol) remains unknown. Biological actions of retinoids on modulation of cellular gene expression by nuclear receptors have been widely known. However, a growing body of evidence has demonstrated an apparent enhancement of carcinogenesis induced by retinol, but attempts to use retinoids for cancer prevention and therapy are, nevertheless, ongoing. Recent intervention studies revealed that supplementation with retinoids resulted in a higher incidence of cancer and besides that, a prooxidant effect of retinol in biological system has been described. Diverse biological processes are modulated by free radicals. Since classically retinoids modulates cellular proliferation, differentiation and cell death we decide to characterizes the possible role of oxidative stress generated by retinol supplementation on these biological processes. Retinol (7 μM) supplementation caused an enhancement in the UV-mediated cellular oxidative damaged, in ornithine decarboxylase (ODC) activity, genotoxicity and preneoplasic transformation. All these effect have been attenuated or reverted by antioxidant co-administration. We also demonstrated that retinol activates a prooxidant-dependent fashion the mitochondrial apoptotic machinery in two experimental models. Taken together, these findings suggest that the biological actions modulated by retinol may be partially related to free radical generation.

ÍNDICE

ÍNDICE DE FIGURAS

LISTA DE ABREVIATURAS

$\Delta\Psi_m$: potencial de membrana mitocondrial

$^{\bullet}OH$: radical hidroxil

ADH: álcool dehidrogenases

AIF: fator de indução à apoptose

ALDH: aldeído dehidrogenases

AP-1: proteína ativadora-1

AR: ácido retinóico

Bcl: família de proteínas anti e pró-apoptóticas descritas de linfomas de células B

CARET: estudo da eficácia do beta-caroteno e retinol

CAT: catalase

cit. c: citocromo c

cRaf: Ser/thr proteína quinase ativada por RAS-GTP

CRBP: proteína citoplasmática ligante a retinol

CuZnSOD: superóxido dismutase dependente de cobre e zinco

DNA: ácido desoxirribonucéico

GPx: glutatião peroxidase

GSH: glutatião reduzido

GSSG: glutatião oxidado

H_2O_2: peróxido de hidrogênio

JNK: c-Jun amino-terminal quinase

MAPK: proteína quinase ativada por mitógeno

MnSOD: superóxido dismutase dependente de manganês

mPT: permeabilidade transitória mitocondrial

NAD^+: adenina nicotinamida dinucleotídeo

NADH: adenina nicotinamida dinucleotídeo reduzida

NFkB: fator nuclear kappa B

NO: óxido nítrico

$O_2^{\bullet-}$: radical superóxido

ODC: ornitina decarboxilase

PKC: proteína quinase C

PTP: poro de permeabilidade transitória

RAR: receptor de ácido retinóico

Ras: proteína G monomérica

ROS: espécies reativas de oxigênio

RXR: receptor de retinóides X

SOD: superóxido dismutase

Capítulo I

Introdução

1. INTRODUÇÃO

1.1 *A vitamina A (Retinol)*

A vitamina A (all-*trans* retinol) e seus derivados naturais, coletivamente referidos como retinóides, são nutrientes essenciais provenientes da dieta, responsáveis pela regulação de uma gama diversa de processos fisiopatológicos que envolvem desde fenômenos de embriogênese, reprodução e visão, até diferenciação e manutenção da homeostase tecidual, proliferação celular e morte de células normais e neoplásicas. A despeito de ter sido um dos primeiros compostos antioxidantes a ser descoberto, em 1913, a ampla ação biológica dos retinóides ainda nos dias de hoje permanece desconhecida [1].

A principal fonte de retinóides naturais são, em animais, o tecido adiposo e o fígado – local de grande reserva na forma de ésteres de retinol – e os vegetais amarelos e verdes, que possuem grandes quantidades de carotenóides. Os ésteres de retinol ingeridos são hidrolizados a retinol por uma enzima hidrolase presente na luz do intestino. Tanto o retinol quanto os carotenóides são prontamente absorvidos pela mucosa intestinal. Uma vez absorvidos, os retinóides são incorporados aos quilomicrons para serem transportados, via circulação linfática, para o fígado, além de, em menor grau, testículos, pulmão, rins, tecido adiposo e músculo esquelético [2]. Uma vez dentro das células alvo, a vitamina A é convertida ao ácido retinóico – a forma descrita mais ativa biologicamente – pela ação de duas famílias de álcool dehidrogenases. O primeiro passo é a conversão oxidativa reversível de retinol em retinal, mediado pela família de enzimas microssomais álcool dehidrogenases (ADH), enzimas estas que utilizam NAD^+ oxidado como cofatores, gerando

NADH. O próximo passo, a conversão oxidativa irreversível de retinal em ácido retinóico, que é catalisada pelas enzimas aldeído dehidrogenases (ALDH) e por isoenzimas da família do citocromo P450 (CYP) (Figura 1). Uma vez no citoplasma, o ácido retinóico e algumas classes de retinóides ligam-se a duas classes de receptores nucleares: receptores de ácido retinóico (RAR) e receptores de retinóides X (RXR); receptores estes que possuem diferentes isoformas (-α, -β, e RAR-γ; -α, -β, e RXR-γ) [3]. Estes receptores nucleares formam homo- e hetero-dímeros, ligando-se a regiões promotoras de genes responsivos a retinóides, através dos elementos responsivos ao receptor de ácido retinóico (RARE) e elementos responsivos ao receptor de retinóides X (RXRE), modulando a expressão de diversos genes (Figura 1) [4].

Classicamente, diversos autores relacionam os efeitos biológicos dos retinóides à sua conversão a ácido retinóico e este como regulador metabólico da célula alvo, através da modulação da expressão de genes. A capacidade dos retinóides de induzir maturação celular pela promoção de diferenciação celular tem sido amplamente utilizada em intervenções terapêuticas, principalmente no tratamento de tumores epiteliais e leucemias do tipo promielocíticas agudas mediadas pela translocação cromossomal t(15;17), que causa uma deficiente expressão de RAR-α [5]. Em tumores epiteliais, o tratamento com ácido retinóico é efetivo em diversos estágios durante o processo de carcinogenesis, como um inibidor da metaplasia escamosa e como um agente preventivo na formação de novos tumores.

Porém, recentemente, diversos trabalhos têm demonstrado que os retinóides possuem ações biológicas que não envolvem sua interação com receptores nucleares [6]. Dentre estes efeitos, destacam-se a modulação direta da atividade de proteínas relacionadas

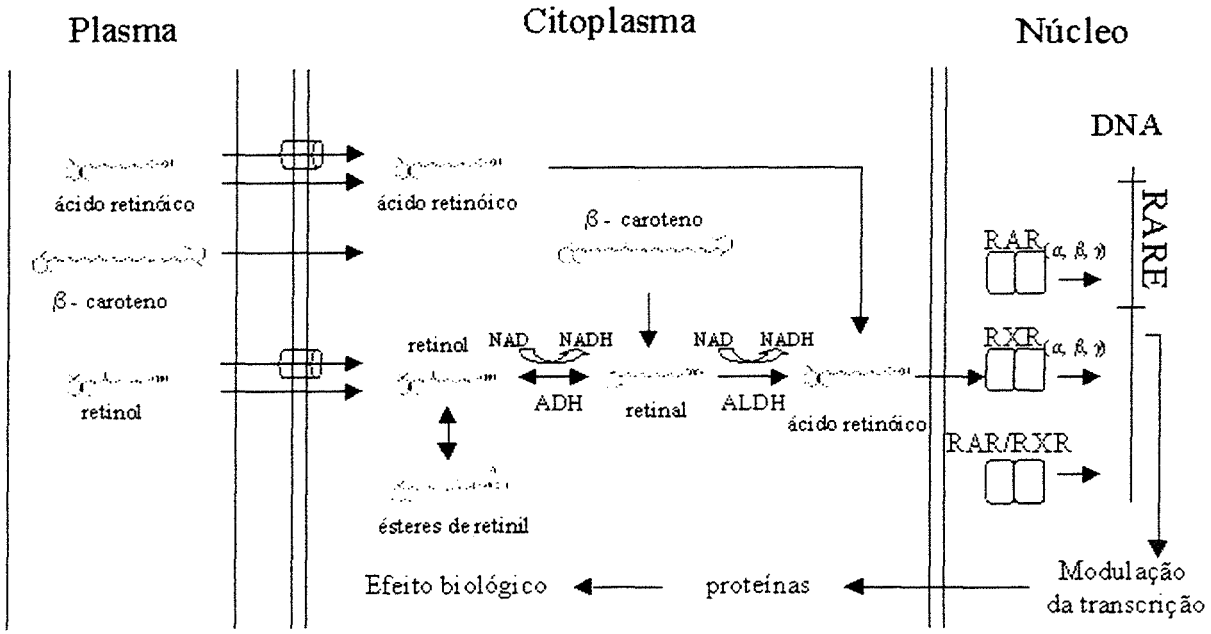

Figura 1: Mecanismo proposto da ação dos retinóides na expressão gênica. No plasma, os retinóides são transportados ligados a proteínas ligantes de retinóides (RBP), sendo absorvidas pelas células alvo por receptores específicos (RR) ou diretamente através da membrana plasmática. Uma vez no citoplasma, os retinóides são direcionados para o armazenamento (ésteres de retinil) ou metabolisados a ácido retinóico e direcionados ao núcleo, reprimindo ou ativando a expressão de genes alvos através da ligação dos receptores às seqüências responsivas (RARE). ADH, álcool dehidrogenases; ALDH, aldeído dehhidrogenases; RAR, receptores nucleares de ácido retinóico; RXR, receptores nucleares de retinóides X; RARE, elementos responsivos aos receptores nucleares de ácido retinóico. Adaptado de Roos *et al* [2].

com a sinalização celular, como a proteína quinase C (PKC) [7], a cRaf [8], e a tioredoxina redutase [9]. Como tem-se demonstrado que a atividade destas enzimas são dependentes do estado de oxidação de alguns resíduos críticos de cisteína, especula-se que este mecanismo de regulação dos retinóides seja por modificação do estado redox das proteínas alvo.

1.2 *Radicais Livres e Sistemas Biológicos*

Os radicais livres são conceitualmente denominados como *compostos, átomos ou moléculas capazes de difundir pelo sistema e que contém um ou mais elétrons desemparelhados em seu orbital mais externo*, sendo assim extremamente reativos [10]. O termo ROS – espécies reativas do oxigênio – abrange coletivamente os radicais de oxigênio ($O_2^{\bullet-}$, radical superóxido; $OH^{\bullet}$, radical hidroxil; $RO_2^{\bullet}$, radical peroxil; $RO^{\bullet}$, radical alkoxil) e os derivados não-radicais potencialmente oxidantes (H_2O_2, peróxido de hidrogênio; HOCl, radical hipoclorito; O_3, ozônio e singletos de O_2 – $^1O_2$) [11]. Existem as chamadas espécies reativas do nitrogênio (RNS), que corresponde ao radical NO (óxido nítrico), $ONOO^-$ (peróxido nitrito) entre outros [12]. Estes compostos, uma vez formados, são capazes de reagir com quase todas as biomoléculas celulares, através de cadeias de oxidorredução, causando inativação de enzimas, agregação ou fragmentação de proteínas, danos a molécula de DNA e lipídios de membrana.

Alberto Boveris e Britton Chance, juntos com outros grupos de pesquisa, demonstram que a principal fonte de produção de radicais livres em sistemas celulares é a cadeia transportadora de elétrons, presente nas mitocôndrias, durante o processo de

respiração celular, onde aproximadamente 4 % do oxigênio utilizado é parcialmente reduzido a $O_2^{\bullet-}$ e H_2O_2 [13].

A geração de espécies reativas de oxigênio é uma resultante do metabolismo celular basal aeróbico e este fenômeno possui implicações fisiológicas em sistemas biológicos. Existe a necessidade de produção de uma taxa basal de radicais livres, sendo estes necessários como segundo mensageiros (como é o caso do NO e do H_2O_2) e indutores de expressão gênica. Diversos processos celulares, como proliferação, sinalização, diferenciação, morte celular programada – apoptose – são dependentes da formação de radicais livres [14]. Mas doenças como câncer, síndrome da imunodeficiência adquirida, ateroesclerose, o processo natural de envelhecimento e diversas neuropatologias, como Parkinson e Alzheimer, estão correlacionadas com uma grande produção de espécies reativas do oxigênio [15].

Um complexo sistema de defesa antioxidante celular evoluiu junto com o metabolismo aeróbico, comprovando a sua citotoxicidade. Este sistema está baseado no balanço da atividade de diversas enzimas, como a *Cu/Zn*(citoplasmática)- e *Mn*(mitocondrial)-superóxido dismutase (SOD), responsáveis pela dismutação do radical superóxido formando o peróxido de hidrogênio, que é transformado em água pela atividade das enzimas catalase (CAT) e glutatião peroxidase (GPx); ambas possuem como substrato o peróxido de hidrogênio, no entanto, a GPx é mais específica para lipoperóxidos.

Além das enzimas antioxidantes, existe um conjunto de moléculas de baixa massa molecular que funcionam como tampões redox do ambiente celular. Destacam-se dentro desta classe o peptídeo glutatião (GSH) que, por apresentar cisteínas em sua composição, é um indicativo do estado redox das células e o ácido ascórbico (vitamina C). Em sinergia

com os mecanismos de defesa hidrossolúveis já descritos, existem os antioxidantes lipossolúveis que agem nas membranas celulares impedindo reações de cascata de lipoperoxidação. Entre estes estão as vitaminas como o α-tocoferol (vitamina E) e os derivados de β-carotenos (retinol ou vitamina A).

O α-tocoferol é o principal antioxidante celular lipossolúvel, sendo responsável pela captação dos radicais livres formados nas membranas celulares e, ao ser oxidado pelas ROS, gera um radical fenólico estável que é novamente reduzido e reciclado pelo ácido ascórbico através da atividade de enzimas redutases NADH/NADPH-dependentes [16]. Existem também alguns sistemas de defesa indiretos, como o de reparo de DNA, os quelantes endógenos de metais e a compartimentalização dos locais de produção de radiais livres [10]. Alguns trabalhos recentes demonstram que, em sistema nervoso central e na epiderme, o produto da atividade da enzima ornitina decarboxilase – as poliaminas – possuem uma função de apoio ao sistema antioxidante por funcionar como quelantes de metais [17, 18].

O processo patofisiológico chamado de estresse oxidativo se dá pelo desbalanço entre a produção de radicais livres e as defesas antioxidantes, isto é, um aumento da quantidade de ROS associado ou não com uma diminuição da capacidade celular de detoxificar estes compostos.

1.3 *Fisiologia dos radicais livres*

Como já dito anteriormente, a produção basal de radicais livres parece ter funções importantes em sistemas biológicos e não só vinculados a estados patológicos. Paradoxalmente, a formação de radicais livres está envolvida tanto na indução como no impedimento de fenômenos como morte celular programada e proliferação celular. Quantidades submicromolares de espécies reativas do oxigênio como o peróxido de hidrogênio estão relacionadas com processos fisiológicos como proliferação e diferenciação celular enquanto que doses maiores – na grandeza de milimolar – causam danos oxidativos em sistemas biológicos, que podem levar à formação de fenômenos fisiopatológicos como processos de inflamação, carcinogênese, necrose, entre outros [19].

A indução à apoptose é um processo fisiológico central para as células e a homeostase dos tecidos. Toda a resposta imune e ativação de células T é dependente de radicais livres. A morte de timócitos em amadurecimento é desencadeada por um súbito aumento (pulso) intracelular de oxidantes (H_2O_2, $O_2^{\bullet-}$), que sinaliza para estas células entrarem em apoptose [20]. Já em linhagens celulares U937, o peróxido de hidrogênio produzido por uma enzima de superfície celular, a γ-glutamil transpeptidase, difunde pela membrana plasmática e inibe a maquinaria de indução à apoptose e serve como um estimulo proliferativo [21]. Existem, basicamente, duas rotas propostas para a ativação da maquinaria apoptótica: uma dependente de receptores de membrana, levando à ativação de caspase-8; e a outra mediada pela liberação de fatores retidos na mitocôndria, principalmente o citocromo c e o fator de indução à apoptose (AIF) (Figura 2). Ambas vias culminam com a ativação de caspase-3, uma cisteína protease dita efetora, que leva ao

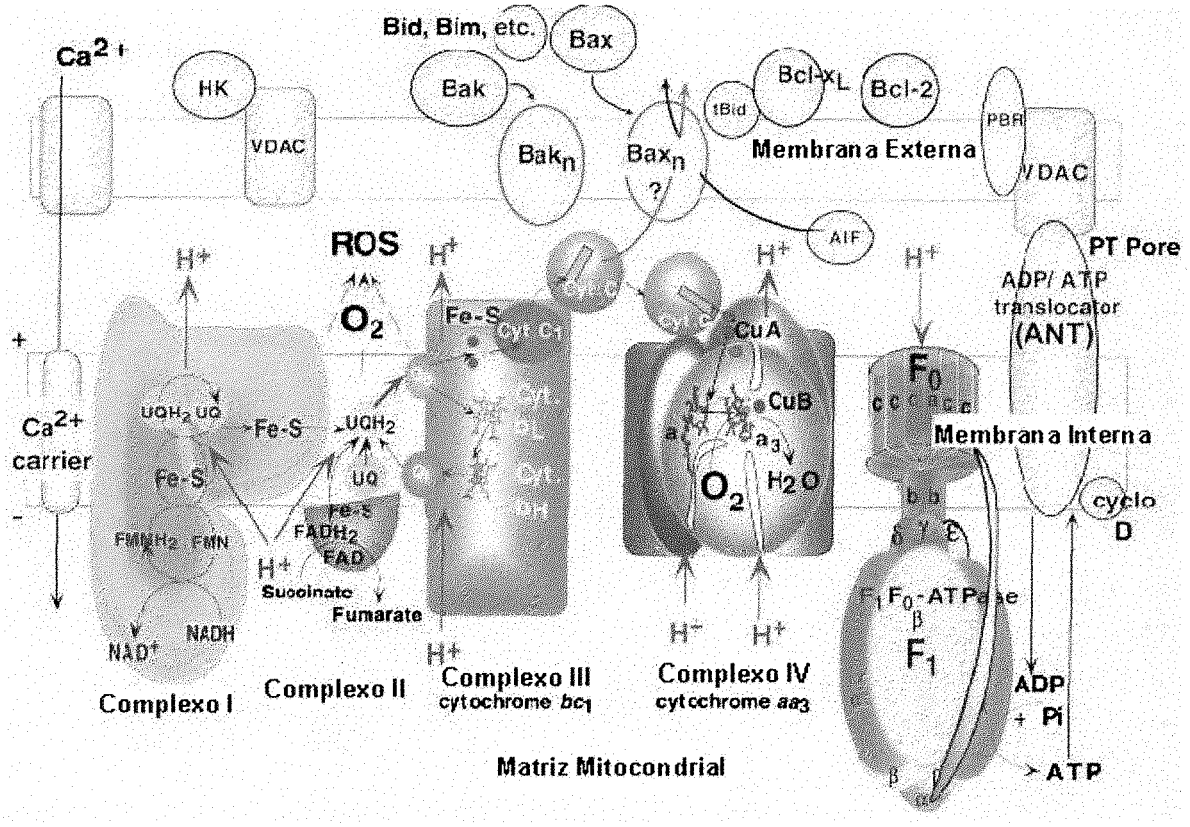

Figura 2: Papel mitocondrial na biosíntese de ATP e na retenção de fatores de indução a apoptose. Estão representados os complexos (I, II, III e IV) da cadeia transportadora de elétrons, a F_1F_O –ATP sintase, os sítios de produção de espécies reativas de oxigênio (ROS), a estrutura do poro de permeabilidade transitória (PTP), e a ação de proteínas pró- e anti-apoptóticas da família Bcl na regulação da liberação dos fatores apoptóticos citocromo *c* (cyt *c*) e AIF. (Modificado de Newmwer, 2003)

surgimento das características morfológicas da morte celular programada, como: condensação da cromatina, fragmentação internucleossomal do DNA, "blebbing" da membrana plasmática e liberação do conteúdo celular envolto por membranas nos chamados "corpos apotóticos". Este tema será melhor abordado nos capítulos VI e VII.

Vários trabalhos demonstram que os radicais livres funcionam como sinalizadores celulares [22]. O exemplo mais clássico disso é o efeito vasodilatador do radical NO, produzido pela enzima NO sintase de células do endotélio. Diferentes rotas de sinalização celular são dependentes da formação de radicas livres e oxidantes ou antioxidantes modulam a expressão gênica.

Para ativação da sinalização mediada pela rota do fator nuclear kapa B (NFκB), por exemplo, há necessidade de um ambiente oxidante mediado pela produção de H_2O_2 e $O_2^{\bullet-}$ pela enzima NAPH-oxidase, onde o inibidor desta rota – o IκB – é oxidado e marcado para degradação via proteossomos, permitindo que o NFκB seja translocado para o núcleo da célula e module a expressão de diversos genes alvos [15].

Mais de 130 genes ou proteínas têm suas atividades moduladas por radicais livres, seja H_2O_2, pela produção de ROS pela radiação UV ou ionizante, e radical superóxido. Entre estes alvos de modulação estão fatores de transcrição (myc, c-jun, c-fos, AP-1, NFκB, p53), proteínas quinases (PKC e B, MAP quinases, tirosina quinase – Syk, ZAP-70), enzimas (ciclooxigenase-2, catalase, ornitina decarboxilase, NO sintase, fosfolipase A, C e D, heme oxigenase, colagenase), receptores (insulina, PDGF, transferrina) e proteína ribossomal S6, HSP-70, FOS e colágeno [14]. O capítulo IV abordará o papel dos radicais livres produzidos pela suplementação com vitamina A na modulação da atividade da enzima ornitina decarboxilase (ODC).

Estas novas descobertas demonstram que os organismos se adaptaram evolutivamente à vida em um ambiente aeróbico, fazendo com que o produto de um metabolismo oxidante – os radicais livres – fossem utilizados como moléculas úteis, sejam como sinalizadores celulares ou moduladores de expressão gênica, demonstrando a importância do estudo destas moléculas na atualidade.

1.4 *Vitamina A e radicais livres*

Ao longo dos últimos anos, diversos estudos clínicos e epidemiológicos buscaram uma correlação entre o consumo de suplementos vitamínicos na dieta com uma diminuição na incidência de diferentes tipos de tumores e doenças cardiovasculares. O primeiro grande estudo relacionando o consumo elevado de β - carotenos e retinol (β -carotene and Retinol Efficacy Trial; CARET), demonstrou um aumento na incidência de câncer de pulmão, o que causou a suspensão do estudo [23]. O aumento de concentração de retinol na dieta de camundongos irradiados com ultravioleta causa uma maior indução de tumores de pele, além de uma redução no tempo de surgimento dos primeiros nódulos tumorais, no entanto os mecanismos que levam a um aumento na incidência de tumores de pele mediado pela suplementação com retinol ainda não foi determinado [24]. A interação da suplementação com retinol e a ultravioleta na produção de radicais livres será melhor abordada no capítulo III.

Atualmente, diferentes estudos descrevem que os retinóides possuem propriedades pró-oxidantes, provavelmente pela presença, em sua estrutura, de ligações duplas

conjugadas. A suplementação com vitamina A causa, em culturas de células de Sertoli, um aumento na captação e armazenamento de Fe^{2+}, principalmente no núcleo e na fração mitocondrial, induzindo a formação do radical hidroxil [25]. Este estresse oxidativo causa um aumento no dano oxidativo a biomoléculas e na atividade de enzimas antioxidantes [26]. O tratamento com retinol aumenta a produção de radical superóxido *in vitro* e em cultivos celulares [27]. Estes resultados demonstram que a suplementação com vitamina A e o uso dos retinóides como coadjuvantes em diferentes intervenções terapêuticas devem levar em consideração a possível participação destes compostos em reações de oxirreduções, buscando minimizar a toxicidade destes compostos, além dos mecanismos de interação bioquímica e metabolização pelos sistemas biológicos. O possível papel genotóxico e carcinogênico do tratamento com retinol é o tema do capítulo V.

1.5 *Vitamina A e a célula de Sertoli*

A célula de Sertoli, utilizada como modelo nesta tese, é uma célula somática localizada na face basal dos tubos seminíferos, cuja função principal é compor o ambiente adequado e dar suporte físico para que ocorra o fenômeno de espermatogênese. A célula de Sertoli possui grande capacidade fagocitária dos debris celulares provenientes da formação dos espermatozóides e forma a chamada barreira hemato-testicular, selecionando e provendo os fatores essenciais que entram em contato com a linhagem germinativa [28]. Como as células da pele, a célula de Sertoli é de origem epitelial e tem a capacidade de responder e armazenar grandes quantidades de retinol, além de possuir um amplo e completo sistema de proteção antioxidante e serem bem caracterizadas morfo e

fisiologicamente. Essas características, em conjunto, fazem com que culturas primárias de células de Sertoli sejam um bom modelo experimental para o estudo do papel das propriedades oxidativas do tratamento com retinol sobre os processos biológicos e bioquímicos regulados por estes compostos.

Capítulo II

Objetivos

2. OBJETIVOS

2.1 *Objetivo Geral*

Tendo em vista que os retinóides são moléculas ativas em reações de oxiredução, com já comprovada capacidade de causar estresse oxidativo em sistemas biológicos e que, tanto dietas suplementadas com retinol quanto a utilização de retinóides no tratamento e prevenção de diversos tipos de tumores e doenças epidérmicas estão em ampla vigência em intervenções clínicas, nos dias de hoje, a presente tese de doutorado teve por objetivo principal a caracterização da possível ação biológica da vitamina A mediada pela produção de espécies reativas do oxigênio.

2.2 *Objetivos Específicos*

Para tal, os seguintes objetivos específicos foram desenvolvidos nos capítulos que seguem:

- Capítulo III: Avaliação do possível efeito da suplementação com vitamina A nos danos oxidativos causados pela irradiação com ultravioleta em cultivo de células de Sertoli;

- Capítulo IV: Avaliação do possível papel das espécies reativas de oxigênio produzidas pela suplementação com vitamina A na modulação da atividade da enzima ornitina decarboxilase (ODC) em cultivo de células de Sertoli;

- Capítulo V: Avaliação da genotoxicidade e tumorogenecidade da vitamina A em diferentes modelos animais, e o possível papel das espécies reativas de oxigênio nestes processos;

- Capítulo VI: Avaliação da influência do tratamento com vitamina A sobre a ativação de vias apoptóticas dependentes da mitocôndria e o possível papel das espécies reativas de oxigênio nestes processos, utilizando-se de mitocôndrias isoladas de fígado;

- Capítulo VII: Avaliação da suplementação com vitamina A na indução à apoptose e o possível papel das espécies reativas de oxigênio nestes processos em cultivo de células de Sertoli.

Capítulo III

Enhanced UV-mediated free radical generation; DNA and mitochondrial damage caused by retinol supplemetation

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Enhanced UV-mediated free radical generation; DNA and mitochondrial damage caused by retinol supplementation

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Retinoid supplementation has been therapeutically used against various human disorders. We and others have demonstrated that retinol treatment causes free radical generation and increased iron uptake, iron storage and oxidative damage, both *in vitro* and *in vivo*. Here, we investigate the possible synergistic effect of retinol on UV-mediated free radical generation, oxidative damage to biomolecules and decreased cellular viability in primary cultured mammalian cells. Retinol treatment (7 μM) resulted in a threefold increase in UV-mediated free radical generation and a 40% increase in lipoperoxidation. DNA fragmentation and mitochondrial oxidative damage also increased significantly in retinol-supplemented UV-irradiated cultured cells as compared to UV-irradiated control cells, which were only treated with the solvent used to deliver the retinol (0.1% ethanol). All measurements were restored to control values when an iron chelator, 1,10-phenanthroline (100 μM), or an $OH^{\bullet}$ scavenger, mannitol (1 mM), was co-administrated. Rather than protecting against free radical generation, retinol seems to enhance UV-mediated oxidative damage and decreases cellular viability in cultured cells. We suggest that retinol-enhanced iron uptake and storage and increased reactive oxygen species generated by the Fenton reaction may act synergistically with UV-irradiation in causing oxidative damage to cells.

Introduction

Vitamin A (all-*trans*-retinol) is an essential nutrient which can either be obtained directly in the diet or by the intake and conversion of pro-vitamin A, *β*-carotenoid. It is well established that retinol acts as a modulator of gene expression *via* nuclear retinoic acid (RAR) and retinoid X (RXR) receptors, and, more recently, it has been shown to act by modulating different PKC isoforms, serine/threonine kinases and cRaf activities.[1] Retinoid supplementation has been widely used therapeutically in the prevention and treatment of several types of diseases, particularly epithelial cancers, including both pre-malignant and malignant tumors.[2] Apart from these beneficial effects, a growing body of evidence from epidemiological and animal studies suggests that retinol supplementation may enhance the development of cancers, specially lung, liver, oral cavity and gastric cancers.[3] Murata and Kawanishi first showed that high doses of retinol induced DNA damage in HL-60 cells *via* superoxide radical generation.[4] Our research group demonstrated that retinol treatment (7 μM) induced free radical generation in cultured rat Sertoli cells.[5,6] The effects of retinoid supplementation in biological systems may be dose dependent. Badr *et al.* demonstrated that high doses of retinol increased chromosomal aberrations in human lymphocyte cultures.[7] The participation of vitamin A in redox reactions appears to be controlled by retinol-binding proteins and transporters.[8] However, supplementation above physiological amounts may disturb key physiological processes.[9] Normal values of retinol in human serum are around 360–1200 μg L^{-1} (1.25–4.1 μM), and it is estimated that the physiological retinol concentration in Sertoli cells is around 5 μM.[10]

We investigated the effect of vitamin A supplementation (7 μM) on UV-irradiated cultured Wistar rat Sertoli cells. The present study shows that supplementation with vitamin A increases UV-mediated lipoperoxidation, mitochondrial oxidative damage and free radical generation. These effects increase DNA fragmentation which, in turn, diminishes cell viability. The involvement of the iron-mediated Fenton reaction in retinol-enhanced UV-mediated free radical generation is proposed.

Materials and methods

1 Chemicals

Type I collagenase, medium 199, HBSS and all-*trans*-retinol were purchased from Sigma (St. Louis, MO, USA). Trypsin was purchased from Difco (Detroit, MI, USA). ^{3}H-Thymidine (3.15 TBq $mmol^{-1}$) was purchased from Amersham Place (Little Chalfont, UK).

2 Cell isolation and culture

Sertoli cells from 15-day-old Wistar rats were prepared and cultured as previously described.[11] 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 3.2×10^5 cells cm^{-2} in medium 199 (pH 7.4) supplemented with 1% fetal bovine serum (v/v) in plastic vessels. Cells were maintained at 34 °C in a humidified atmosphere of 5% CO_2 in air. The

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medium was replaced after 24 h by serum-free medium to remove unattached Sertoli and germinative cells. Experiments were performed on cells treated with 7 μM retinol for 24 or 48 h with or without simultaneous UV irradiation. Control cultures received only the solvent used for retinol delivery (0.1% ethanol, v/v). Previous testing had shown that ethanol alone has no effect in any of the experimental procedures (data not shown). The formation of oxidized retinol metabolites was monitored spectroscopically in the retinol solution before it was used. Cell viability was assessed by Trypan Blue exclusion. All results were normalized against protein content,[12] using bovine serum albumin as the standard.

3 Irradiation of cell cultures

Cell cultures were irradiated with a Philips® UV lamp (256 nm, 20 W, 1 J cm^{-2} s^{-1}; source-to-target distance 100 cm) for 5 s. Before irradiation, the culture medium was removed. Irradiation was carried out at room temperature. This UV dose causes a reduction of 20–25% in the viability of the cells. Cells were incubated for an additional period of 30 min or 24 h at 34 °C in a humidified 5% CO_2 atmosphere.

4 Isolation of mitochondria

Cultured Sertoli cells were homogenized in a medium consisting of 0.23 M mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. Cell homogenates were centrifuged at 700*g* for 10 min to remove nuclei and cell debris. The supernatant was centrifuged at 7000*g* for 10 min. The resulting mitochondria-enriched pellet was used in the assays.[13]

5 Determination of thiobarbituric acid reactive species

Reactive oxygen species (ROS) production was assessed by the formation of thiobarbituric acid reactive species (TBARS) using a sensitive method for measurement of lipid peroxidation.[14] The samples were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid, and heated in a boiling water bath for 15 min. Butanol (2 : 1 v/v) was added and, after centrifugation (800*g*, 5 min), TBARS were determined by monitoring the absorbance at 535 nm. Results are expressed as malondialdehyde (MDA) equivalents [nmol $mg(protein)^{-1}$].

6 Determination of conjugated dienes

The level of lipid peroxidation was estimated by assay of conjugated diene double bonds.[15] Sertoli cells were homogenized in 2 mL of 0.25 M sucrose–5 mM EDTA and centrifuged at 14 500*g* for 15 min at 4 °C. From the post-mitochondrial supernatant, the microsomes were spun down at 105 000*g* for 60 min at 4 °C. Lipid was extracted as described by Folch *et al.*[16] The lipid extract was dried under vacuum at room temperature and then dissolved in 1 mL of cyclohexane. The lipid–cyclohexane solution was immediately scanned for optical density between 220 and 300 nm and the second-derivative spectrum was recorded; this confers greater sensitivity on the diene conjugation method. A first-derivative spectrum plots rate of change of absorbance (A) with wavelength against wavelength (λ). The second-derivative spectrum plots the rate of change of this value ($dA/d\lambda$) as $d\mathbf{A^2}/d\lambda^2$. In the second-derivative spectrum, the conjugated diene signal was characterized by the height of the minimum peak at 233 nm, and is expressed in arbitrary units.

7 *tert*-Butyl hydroperoxide-initiated chemiluminescence

The balance between pro-oxidants and antioxidants in the cells was evaluated by *tert*-butyl hydroperoxide-initiated chemiluminescence.[17] Cell homogenates were diluted to a final concentration of 1 mg(protein) mL^{-1}, 3 mM of *tert*-butyl hydroperoxide was added and chemiluminescence was measured in a scintillation counter as previously described.[17]

8 DNA fragmentation

DNA fragmentation was measured as described by Venable *et al.*[18] Cells were labeled for 24 h with 3 μCi mL^{-1} of ^{3}H-thymidine before retinol treatment and UV irradiation. After treatment, the medium was aspirated and the radioactivity counted. The cells were lysed with PBS containing 1% Triton® X-100 and 2 μM EDTA, and the mixture centrifuged for 15 min at 14 000 rpm in a microcentrifuge. The supernatant and the pellet containing larger DNA fragments were counted separately. DNA fragmentation was calculated by adding the counts in the medium and the supernatant and dividing by the total counts.

9 Statistical analysis

Results are expressed as the mean ± SEM (standard error of the mean). Data were analyzed by one-way analysis of variance (ANOVA), using a Neuman–Keuls test to compare mean values across groups. Differences were considered to be significant when $p < 0.05$.

Results

1 Viability of cultured Sertoli cells treated with retinol and UV

Fig. 1 shows that retinol treatment (7 μM) alone for 24 or 48 h caused a decrease in cell viability by 15 and 31%, respectively, as compared to control cells. When retinol-treated cells were UV-irradiated, cell viability decreased significantly (36% after 24 h and 68% after 48 h) in comparison to control cells, in which the respective reductions in viability were 18 and 49%.

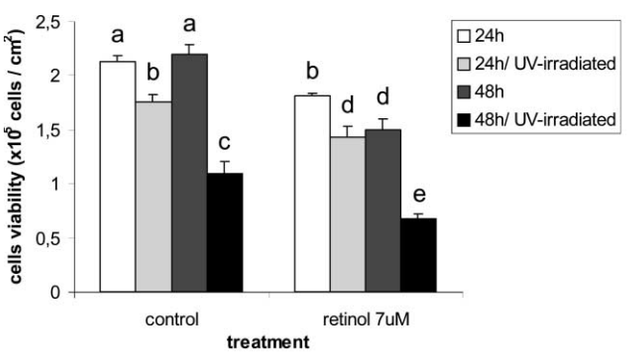

Fig. 1 Effect of retinol treatment on the viability of cultured rat Sertoli cells exposed to UV irradiation. Cultured Sertoli cells were isolated and cultivated as described in the Materials and methods section, treated with 7 μM retinol for 24 or 48 h and UV irradiated (256 nm, 1 J cm^{-2} s^{-1}) for 5 s. Cell viability was determined by Trypan Blue exclusion. Data represent mean ± SEM of triplicate measurements per experiment and four independent experiments. Results labeled with different letters are significantly different ($p < 0.05$) from each other (one-way analysis of variance).

2 ROS formation in cultured Sertoli cells treated with retinol and UV

Retinol treatment increased UV-mediated free radical formation and oxidative damage to lipids (Table 1). UV-irradiated cells treated with 7 μM retinol showed a fourfold increase in ROS (*tert*-butyl hydroperoxide-initiated chemiluminescence) when compared to UV-irradiated control cells. UV-mediated TBARS and conjugated diene formation also increased in retinol-treated cells (Table 1). Control UV-irradiated values were restored when 100 μM of 1,10-phenanthroline (an iron chelator) or 1 mM mannitol (a scavenger of $OH^{\bullet}$ radicals) was co-administered.

Table 1 Additive effect of ultraviolet irradiation and retinol on conjugated diene formation, lipid peroxidation and *tert*-butyl hydroperoxide-initiated chemiluminescence in cultured rat Sertoli cells[a]

Assay	Control cells		7 μM Retinol		7 μM Retinol + 1mM mannitol		7 μM Retinol + 100 μM 1,10-phenanthroline	
	Without UV	With UV	Without UV	With UV	Without UV	With UV	Without UV	With UV
TBARS [nmol mg(protein)$^{-1}$]	1.28 ± 0.12 (a)	1.63 ± 0.03 (b)	1.83 ± 0.21 (b)	2.34 ± 0.12 (c)	1.16 ± 0.26 (a)	1.45 ± 0.3 (a)	1.32 ± 0.07 (a)	1.39 ± 0.12 (a)
Conjugated dienes (arbitrary units)	0.07 ± 0.05 (a)	0.26 ± 0.03 (b)	0.57 ± 0.08 (c)	0.73 ± 0.13 (d)	0.23 ± 0.05 (b)	0.32 ± 0.07 (b)	0.18 ± 0.06 (a)	0.25 ± 0.13 (a)
tert-Butyl hydroperoxide-initiated chemiluminescence [cpm mg(protein)$^{-1}$]	414 ± 40 (a)	400 ± 119 (a)	717 ± 52 (b)	1589 ± 87 (c)	525 ± 32 (a)	751 ± 93 (b)	480 ± 77 (a)	633 ± 87 (b)

[a] Cultured rat Sertoli cells were isolated and cultivated, treated with 7 μM retinol and co-treated with 1mM mannitol or 100 μM 1,10-phenanthroline for 24 h, then UV irradiated (256 nm, 20 W, 1 J cm^2 s^{-1}) for 5 s. Assays were performed as described in the Materials and methods section. Data represent mean ± SEM of at least triplicate measurements per experiment and four independent experiments. Results labeled with different letters are significantly different ($p < 0.01$) from each other (one-way analysis of variance).

3 Mitochondrial damage in cultured Sertoli cells treated with retinol and UV

Fig. 2 shows a 50% increase in the mitochondria TBARS content of Sertoli cells treated with retinol and UV irradiated, as compared to UV-irradiated control cells. When UV-irradiated cultured Sertoli cells were co-treated with retinol and mannitol (1 mM) or 1,10-phenanthroline (100 μM), control irradiated values were restored.

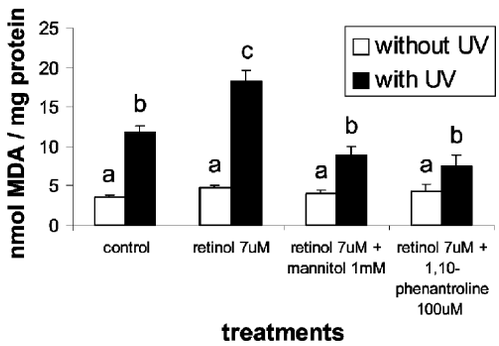

Fig. 2 Mannitol and 1,10-phenanthroline co-treatment decreases retinol-induced lipoperoxidation of isolated mitochondria from cultured rat Sertoli cells exposed to UV irradiation. Cultured Sertoli cells were isolated and cultivated, treated with 7 μM retinol and co-treated with 1mM mannitol or 100 μM 1,10-phenanthroline for 24 h, then UV-irradiated (256 nm, 1 J cm^{-2} s^{-1}) for 5 s. Mitochondria were isolated and the TBARS content was determined. Data represent mean ± SEM of triplicate measurements per experiment and four independent experiments, and expressed as nmol(MDA equivalents) mg(protein)$^{-1}$. Results labeled with different letters are significantly different ($p < 0.05$) from each other (one-way analysis of variance).

4 DNA fragmentation in cultured Sertoli cells treated with retinol and UV

Retinol alone for 24 h induced DNA fragmentation of cultured Sertoli cells by the same amount as UV irradiation (Fig. 3). Retinol treatment increased the UV-mediated DNA fragmentation by 20%; when co-treated with 1 mM mannitol control UV-irradiated values were restored.

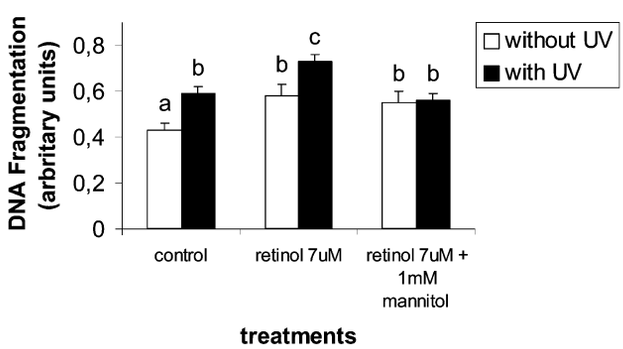

Fig. 3 Mannitol co-treatment decreases retinol-induced DNA fragmentation in cultured rat Sertoli cells exposed to UV irradiation. Cultured Sertoli cells were isolated and cultivated, treated with 7 μM retinol and 1mM mannitol for 24 h, then UV irradiated (256 nm, 1 J cm^{-2} s^{-1}) for 5 s and the DNA fragmentation determined. Data represent mean ± SEM of triplicate measurements per experiment and four independent experiments. Results labeled with different letters are significantly different ($p < 0.01$) from each other (one-way analysis of variance).

Discussion

The generation of free radicals by ultraviolet light irradiation from the sun is the primary causative agent for premature skin aging (photo-aging) and skin cancer, which is the most prevalent form of malignancy in humans. This effect might be caused, at least in part, by iron-mediated free radical generation, which may contribute directly to DNA, lipid and protein damage.[19]

Recent epidemiological studies attempted to correlate high cancer incidence to dietary retinol supplementation. Although several workers have demonstrated the protective effect of pro-vitamin A β-carotenoid supplementation in lipid peroxidation,[2,20] we report here that retinol supplementation causes an increase in UV-mediated free radical generation and cellular damage. The involvement of the iron-mediated Fenton reaction in the retinol-induced increase in UV-mediated free radical generation is proposed, as demonstrated by the reduction in damage on co-treatment with 1,10-phenanthroline (Table 1). Surprisingly, human skin chronically exposed to UV light is known to accumulate iron,[21] and retinol treatment also induces an increase in iron uptake and storage.[22] Our present results are in agreement with previous studies which demonstrate that retinol induces oxidative damage *in vivo* and *in vitro*.[4–6]

When cultured mammalian cells treated with 7 μM retinol were UV irradiated, decreased cell viability was observed (Fig. 1). This decreased viability may be due, at least in part, to free radical generation (Table 1). Co-treatment with 1,10-phenanthroline (100 μM) or mannitol (1 mM) prevented this response. These results strongly suggest that iron may participate in the increased UV-mediated free radical formation in retinol-treated cells. Production of reactive oxygen species by iron is mediated by the Fenton reaction as follows:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \qquad (1)$$

$$Fe^{3+} + NADH \longrightarrow Fe^{2+} + NAD^{\bullet} + H^{+} \qquad (2)$$

Iron(II) donates electrons to H_2O_2, producing the highly reactive hydroxyl radical (eqn. 1). If NADH is present in the system, it may drive the re-use of iron by reducing Fe^{3+} to Fe^{2+}, propagating free radical chain reactions (eqn. 2). Retinol treatment causes an increase in iron uptake and storage in cultured rat Sertoli cells, principally in the nucleus and mitochondria.[22] We observed an enhanced reduced-nucleotide content in retinol-treated Sertoli cells (unpublished data). Recently, it was demonstrated that carotenoids could act as pro-oxidant molecules, producing free radicals in an iron-dependent Fenton reaction.[23] Conjugated bonds in linear repeats or in the ring structures of the retinol molecule absorb short wavelength UV irradiation around 200–250 nm, propagating free radical chain reactions. Our present data support the idea that retinol supplementation increases ROS formation by the Fenton reaction which, in turn, amplifies UV-mediated free radical generation.

Mitochondrial oxidative damage (Fig. 2) and DNA fragmentation (Fig. 3) may participate in the decreased cellular viability induced by retinol treatment and UV irradiation (Fig. 1). High levels of reactive oxygen species trigger apoptosis, or programmed cell death, by two major routes. The first is mitochondria-dependent and acts by increasing the basal ROS production and liberating multiple death-promoting factors such as cytochrome *c* and Smac/DIABLO, leading to the activation of procaspase-9 (called apoptosome) and caspase-3. This route could also be activated by DNA damage in a p53/Bax-dependent manner.[24] The second route acts by activating cytoplasmic and nuclear oxidative response elements, such as associating protein 1 (AP-1), c-Jun NH_2-terminal kinases (JNKs), Ras, cRAf and nuclear factor kappa B (NFκB).[1,25] UV irradiation activates a group of related serine/threonine c-Jun NH_2-terminal kinases.[26]

Retinol treatment increased UV-mediated mitochondrial oxidative damage, probably by iron-mediated hydroxyl radical formation—suggested by the reversion on mannitol/1,10-phenanthroline co-treatment (Fig. 2)—which, in turn, could activate programmed cell death. DNA damage caused by UV irradiation involves reactive oxygen species.[27,28] Single and double strand breaks are a direct consequence of the attack of hydroxyl radicals on deoxyribose in the DNA molecule.[14] The addition of mannitol restored the retinol increased UV-mediated DNA fragmentation to UV-irradiated control values, suggesting a relationship between iron-mediated hydroxyl radical formation and DNA damage caused by retinol treatment (Fig. 3).[28]

Skin cells contain high retinol concentrations.[8] Although there are numerous published works demonstrating the overall beneficial effects of systemic and topical retinol supplementation, in hairless mice, dietary supplementation with retinol enhanced skin photocarcinogenesis induced by ultraviolet irradiation.[29] Wang *et al.* demonstrated that UV irradiation substantially reduced nuclear retinoid receptors, causing a functional vitamin A deficiency that may have deleterious effects on skin physiology, leading to an upregulation in AP-1 activity, contributing to skin photo-aging and carcinogenesis.[30] Our results demonstrate that retinol supplementation directly enhances UV-mediated free radical generation, which could be responsible for the activation of oxidative response elements, like AP-1.

In conclusion, the present results show that retinol supplementation amplifies UV-mediated cellular oxidative stress, probably through the Fenton reaction, leading to mitochondrial damage and DNA fragmentation, which, in turn, alter cultured Sertoli cell viability. Although these results strongly suggest a probable mechanism by which retinol may enhance photodamage, the role of retinol in skin photocarcinogenesis remains to be fully established.

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Capítulo IV

Retinol-induced elevation of ornithine decarboxylase activity in cultured rat Sertoli cells is attenuated by free radical scavenger and by iron chelator

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Retinol-induced elevation of ornithine decarboxylase activity in cultured rat Sertoli cells is attenuated by free radical scavenger and by iron chelator

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Abstract

We investigated retinol effects in ornithine decarboxylase activity in Sertoli cells. We also tested the hypothesis that free radical scavengers and iron chelators may attenuate the effect of retinol. Sertoli cells isolated from 15-day-old Wistar rats were previously cultured for 48 h and then treated with retinol by 24 h with or without mannitol (1 mM) or 1,10 phenanthroline (100 μM). We measured ornithine decarboxylase and catalase activities and malondialdehyde concentrations in response to retinol treatment. In response to 7 μM retinol treatment ornithine decarboxylase activity increased 30%. Retinol-induced ornithine decarboxylase activity was significantly decreased by addition of free radical scavenger (mannitol) or iron chelator (1,10 phenanthroline). In addition the same effect was observed in catalase increased activity and in malondialdehyde concentrations. These results suggest that retinol treatment induced ornithine decarboxylase and catalase activity and increased malondialdehyde concentration. These effects appear to be mediate by ROS. (Mol Cell Biochem **208**: 71–76, 2000)

Key words: retinol, ornithine decarboxylase, reactive oxygen species, iron chelator, catalase

Introduction

Ornithine decarboxylase (ODC) is the rate limiting enzyme in the biosynthesis of the positively charged polyamines which plays key role in DNA, RNA and proteins synthesis. The naturally occurring polyamines are biologic polycations which have been implicated in many growth and differentiation process [1]. The intracellular levels of polyamines are elevated during growth process and lowered when growth is arrested. Thus, polyamines and diamines accumulate in regenerating liver, in embryonic tissues and in tumor cells. Changes in levels and activity of ODC therefore precedes changes in polyamine levels. ODC activity declines in slow-growing systems and rises during rapid proliferation including malignant transformed cells [2]. ODC is induced by UVB-irradiation and oxidative stress. On the other hand, inhibition of ODC makes cells more sensitive to radiation damage. The antioxidant effect of spermine (polyamine) may be due to metal chelation [3]. In addition, Fe-NTA treatment enhances ODC activity, [H-3] thymidine incorporation into DNA, lipid peroxidation level and hydrogen peroxide (H_2O_2) generation in liver and kidney. These effects were attenuated in animals which received a pre-treatment with antioxidants [4, 5]. Saito *et al*. [6] have shown, both *in vivo* [7, 8] and *in vitro* [9], that

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hypoxia increases ODC activity and polyamine concentration in fetal and neonatal rat brain. It is not known, however, to what extent this hypoxic-associated increase in ODC activity is mediated by reactive oxygen species (ROS).

Sertoli cells are somatic cells in the testicular seminiferous tubules within which spermatic cells differentiate. Shubhada and Tsai [10] demonstrated that the whole testis and isolated Sertoli cells exhibited similar patterns of ODC, as well as AdoMetDC activities, during testicular maturation [10]. These data suggested that Sertoli cells may be the major cell type contributing to total testicular enzyme activities. The measurable enzyme activities of the germ cells and interstitial cells constituted only a very small portion of the total testicular activity.

Sertoli cells are target cells for retinol, FSH and testosterone, which are essential for initiation and completion of spermatogenesis. Retinol modulates many functions of Sertoli cells [10, 11]. Our previous works demonstrated that retinol treatment (7 μM/24 h) increased the Sertoli cells chromatin sensitivity to DNAse I [12], increased the histones phosphorylation level [13], decreased HMGs (high mobility group proteins) phosphorylation level [13] and increased [H-3]-thymidine incorporation into DNA [14]. These effects were attenuated by 100 μM 1,10 phenanthroline (iron chelator) indicating that is mediated by Fe(II) ions and probably involving a Fenton reaction.

The aim of this study was to test the hypothesis that retinol treatment may induce ornithine decarboxylase activity in rat Sertoli cells, and that this induction is mediated by ROS.

Materials and methods

All-trans-retinol, medium 199 and other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO, USA.

Cell culture

Sertoli cells were obtained from 15-day old Wistar rats from our breeding stock. Pregnant rats were housed individually in plexiglass cages. Litters were restricted to eight pups each. The animals were maintained on a 12 h light/dark cycle at a constant temperature of 23°C, with free access to commercial food and water.

The animals were killed by ether asphyxiation, testes were removed and Sertoli cells isolated and cultured as previously described [12]. After 24 h of culture with medium 199 supplemented with 1% fetal bovine serum, the cellular monolayer were washed with phosphate-buffered saline (PBS) and cultured for another 24 h in serum free medium before the addition of retinol. A retinol stock solution was prepared in ethanol and the final concentration of ethanol in medium was 0.1%. Control cultures received only ethanol. We observed no significant differences between Sertoli cells treated only with 199 medium and with medium supplemented with ethanol 0.1% (data not shown).

Treatment and assays

Retinol-induced both enzymes activities and lipid peroxidation was measured 24 h after the beginning of treatment. The cells were washed with PBS and scraped in ice cold appropriate medium to assay. Experiments were performed on cells treated with retinol with or without 1 mM mannitol (free radical scavenger) or 100 μM 1,10 phenanthroline (iron chelator) simultaneously [15].

Ornithine decarboxylase and catalase activity

We measured ODC activity by decarboxylation of [1-^{14}C] L-ornithine as described by Russel and Snyder [16]. Catalase (CAT) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance at 240 nm [17].

Malondialdehyde determination

As an index of ROS production we used the formation of a thiobarbituric acid-malondialdehyde (TBA-MDA) complex during an acid-heating reaction, which is widely adopted as a sensitive method for measurement of lipid peroxidation, as previously described [18]. In brief, the samples were mixed with 1 ml of 10% trichloroacetic acid (TCA) and 1 ml of 0.67% thiobarbituric acid (TBA), then heated in a boiling water bath for 15 min. Butanol (2:1 v / v) was added to extract the TBA-MDA complex from the cloudiness solution. After a centrifugation (800 × g/5 min) thiobarbituric acid reactive species (TBARS) were determined by the absorbance at 535 nm.

Protein quantification

All the results were standardized with the protein content [19].

Statistical analysis

ODC and catalase activities and MDA concentrations for each time point represent mean ± S.E.M values of separate experiments. Statistical analysis were performed only on the raw data ANOVA, with Duncan's *post-hoc* test, was used for analysis. Differences were considered to be significant when $p < 0.01$.

Results

ODC is the rate-limiting enzyme in the polyamine biosynthetic pathway. ODC activation is associated with cell growth, differentiation and proliferation. To examine the Sertoli cells ODC activity as a function of retinol treatment, we measured ODC activity after 24 h of retinol treatment. Figure 1 shows that the effect of retinol low doses (0.1, 1, and 5 μM) do not induce an increase in ODC activity in Sertoli cells. Although, retinol doses higher than 5 μM ODC activity increase 28% (7 μM) and 30% (20 μM) as compared with ODC activity in non-treated Sertoli cells. We observed no increase in ODC activity in cells treated with both mannitol or 1,10 phenanthroline and retinol (7 or 20 μM). In both situations ODC activities were slightly below control values (Fig. 2), suggesting that Fe(II) chelator and ROS scavengers can attenuate retinol effects in ODC activity.

Since ODC activation was inhibited by free radical scavenger and/or iron chelator we decided investigated the effect of retinol treatment and ROS scavenger on lipid peroxidation. We measured malondialdehyde concentration in cultured Sertoli cells treated by 24 h with retinol (Fig. 3). It also may be seen that MDA increase 47% after 7 μM retinol treatment. However, in the presence of 1 mM mannitol the retinol-induced-increase in MDA was significantly attenuated. These results suggest that the treatment with retinol induced oxidative damage in Sertoli cells.

In order to investigate changes in antioxidant defenses we measured the CAT activity in retinol treated and non-treated Sertoli cells. In Fig. 4, at may be seen that CAT activity increase 20% after 7 μM retinol treatment. As occur on MDA concentration, retinol treatment simultaneously with free radical scavenger attenuated the retinol induced-increase in catalase activity.

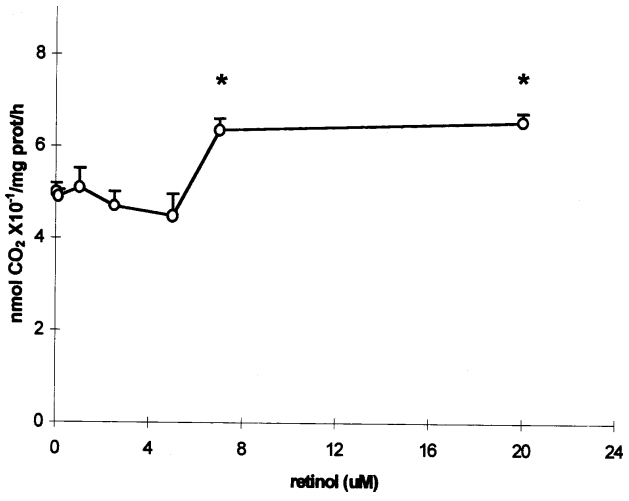

Fig. 1. Effect of different retinol doses on ODC activity (nmol CO_2/mg prot/h) after 24 h treatment. Data represent mean ± S.E.M. of separate experiments. *different from control, $p < 0.01$.

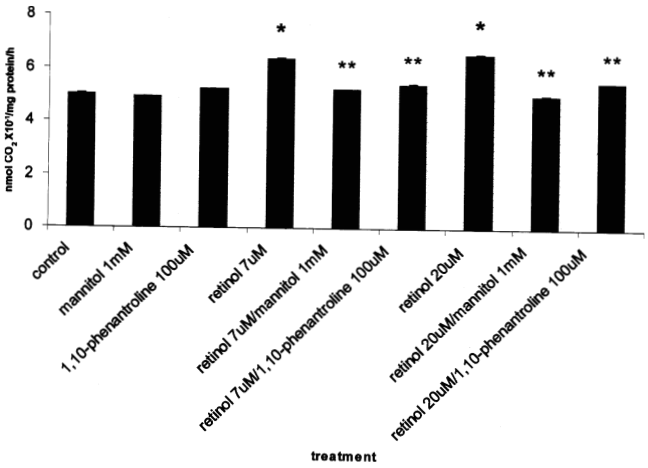

Fig. 2. ODC activity (nmol CO_2/mg prot/h) after 24 h treatment with retinol (7 or 20 μM) with or without mannitol (1 mM) or 1,10 phenanthroline (100 μM). Data represent mean ± S.E.M. of separate experiments. *different from control, $p < 0.01$; **different from retinol 7 and 20 μM, $p < 0.01$.

Discussion

In brain, ODC activity is a sensitive indicator of stress, being significantly increased after thermal, physical, chemical or metabolic stress [6]. The same basic pattern of ODC response is observed in many different cell types. It has been shown that iron overload induced cutaneous ODC activity and [H-3] thymidine incorporation into DNA. Similar to other oxidant tumor promoters, iron overload enhanced cutaneous lipid peroxidation [20]. Besides this, different iron uptake and

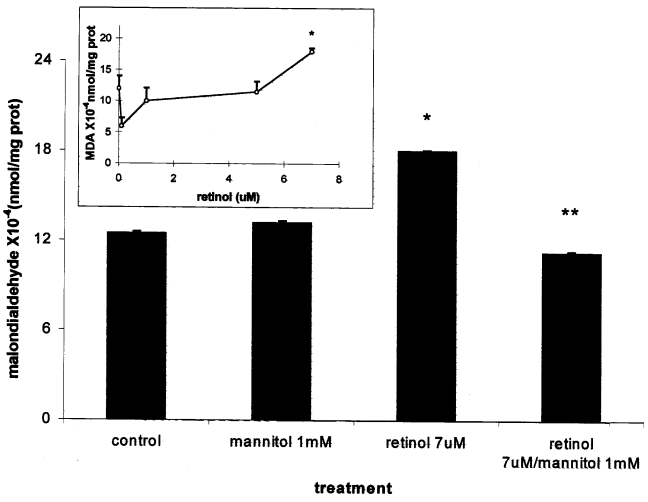

Fig. 3. Effect of retinol treatment (7 μM) with or without mannitol (1 mM) on malondialdehyde concentration after 24 h treatment. Data represent mean ± S.E.M. of separate experiments. Insert box: Effect of different retinol doses on malondialdehyde concentration after 24 h treatment; *different from control, $p < 0.01$; **different from retinol 7 μM, $p < 0.01$.

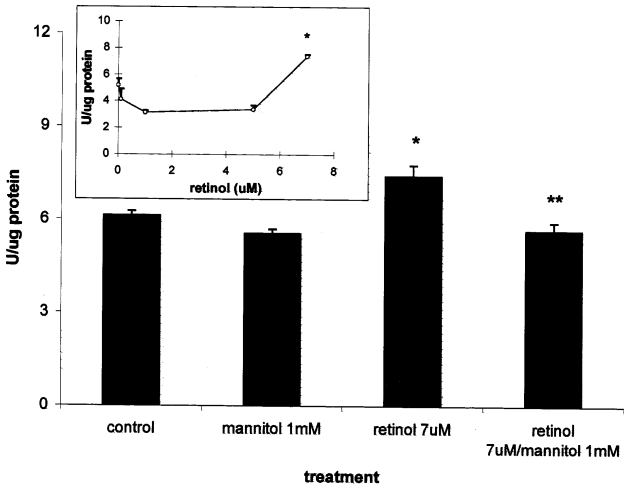

Fig. 4. Effect of retinol treatment (7 μM) with or without mannitol (1 mM) by 24 h on catalase activity. Data represent mean ± S.E.M. of separate experiments. Insert box: Effect of different retinol doses on catalase activity after 24 h treatment. *different from control, $p < 0.01$; **different from retinol 7 μM, $p < 0.01$.

cellular iron distribution in mouse L-cells induced pro-oxidant conditions with DNA damage [21]. Fe-NTA is a potent inducer of renal ODC activity and DNA synthesis. In addition, Fe-NTA augmented renal microsomal lipid peroxidation [4]. These effects were alleviated in rats who received a pretreatment with an antioxidant [5, 22]. Similar to Fe-NTA, Cu-NTA has an ability to induce hepatic ODC activity and [H-3] thymidine incorporation dose-dependently [23].

The ·OH radical is probably the most potent of the ROS, and the probable initiator of the reaction which form lipid peroxides and organic radicals. One of the most important sources of ·OH is the decomposition of H_2O_2 by reduced metals, as iron and copper, by the Fenton reaction [24]. Our previous studies demonstrated that 7 μM retinol induced a increased chromatin sensitivity to DNAse I and this increase was significantly attenuated by 1,10 phenanthroline, an iron quelator, suggesting the involvement of Fenton chemistry reaction in this retinol-induced increase sensitivity [12].

As shown in Fig. 1, in response to 24 h crescent dose retinol treatment, ODC activity increased around 30%, this increase could be indicative that retinol in doses up to 7 μM may induce oxidative stress. The estimated physiologic retinol concentration in Sertoli cells is around 5 μM [25]. As a result of simultaneous treatment with retinol and mannitol, ODC activity was significantly decreased as compared with ODC activity from retinol treated Sertoli cells (Fig. 2). In addition, 1,10 phenanthroline also had similar effect, implying that the effect of retinol on ODC activity is probably mediated by ROS production and associated with Fenton reaction (Fig. 2). Mello-Filho and Meneghini (1991) demonstrated that 1,10-phenanthroline prevents the DNA damage caused by oxidative stress via a Fenton reaction [26]. There was no significant statistical difference between doses of 7 and 20 μM (Fig. 1). Cells treated with 20 μM retinol presented significant increase in membrane damage determined by Trypan blue exclusion (data not shown). We believe that the present difference between 7 and 20 μM could be explained by high cellular inviability.

Reactive oxygen species are by-products of aerobic metabolism which are normally generated in small amounts. In normal conditions, there is a steady-state balance between the production of ROS and their destruction by the cellular antioxidant systems. However, the balance can be broken either by increasing the ROS production or by decreasing the defense system. The hydrogen peroxide (potent ROS) causes damage in living system often because it can give rise to the formation of ·OH radicals. It is therefore biologically advantages for cells to control the amount H_2O_2 that is allowed to accumulate. In turn, H_2O_2 is catabolized by catalase or glutation-peroxidase to oxygen and water. As an index of ROS production we used the formation of a TBA-MDA complex, which is widely adopted as a sensitive method for measurement of lipid peroxidation [15]. To measure the defense system we used the CAT activity [17].

As shown in Fig. 3, Sertoli cells malondialdehyde concentration shows slight decrease after 0.1–1 μM retinol treatment and increased, 47%, after 7 μM retinol treatment. This increase was significantly inhibited by mannitol. This data suggest that ROS, specially ·OH, are implicated as mediator of retinol-induced increased MDA concentration and probably ODC and CAT activities in rat Sertoli cells. The exact mechanism by which retinol increases ROS production is still not completely understood.

Activation of the lipid peroxidase enzyme itself has also been considered to be one of the causes of brain damage in oxidative stress. Some reports have suggested that poly-amines may inhibit lipid peroxidase activity [27–29].

In response to retinol 24 h treatment CAT activity increase 22% after 7 μM treatment (Fig. 4). As a result of mannitol/retinol simultaneous treatment by 24 h, CAT activity significantly decrease. These results suggest that retinol-induced-increase CAT activity may be associated with free radicals induction. Some authors demonstrated that CAT is a free radical induced enzyme [30]. We do not know if the increased in antioxidant enzyme activity are due to induction of mRNA or protein activation, and these point needs further investigation. Retinol induction of ODC activity that we believe is mediated by free radical was not reverted by the associated increased CAT activity but is significantly attenuated by mannitol, a potent free radical scavenger. Both CAT and ODC activities activation induced by retinol declines sharply when associated with mannitol (Figs 2 and 4).

Saito *et al*. [6] observed that free radical-induced ODC activity was attenuated by extracellular catalase, superoxide dismutase and glutation-peroxidase suggesting that $^{\bullet}OH$ may be involved in the free radical-induced ODC activity in rat brain slices. Similarly, studies involving asbestos exposure in hamster tracheal epithelial cells suggest that H_2O_2 may be the predominant inductor of ODC activity [31]. Our data suggest that retinol-induced free radical probably plays a mayor role in ODC retinol-induced activity, in Sertoli cells. It is well known that the recurrent intake of retinol 13 μM for an infant or 35 μM for an adult could induce chronic toxicity in humans. A long-term daily intake of 26 μM during early pregnancy can induce spontaneous abortions or major fetal malformations [32]. The World Health Organization (WHO) recommend a dietary retinol intake of 300 UI (approximately 1 μM), and consider a safe level of intake 600 UI. It is important to note that the intake of retinol 7 μM could readily be achieved with oral supplementation [32]. Thus, ours results indicating that treatment with large retinol concentrations could induce oxidative stress in this experimental model, and this effect could be, in part, responsible for the adverse effects of retinol supplementation.

Because ODC is the rate-limiting enzyme in polyamine biosynthesis, we speculate that the retinol-induced increase in ODC activity and consequent polyamine concentration may be linked to limiting cellular damage. Spermine has been identified as a potent antioxidant and anti-inflammatory agent. The spermine may act as metal chelator and/or radical scavenger [3, 33].

Our results showed that 24 h retinol-treatment increased ODC and CAT activities as well as MDA concentration. Retinol-induced effects in Sertoli cells, observed in this work, are significantly attenuated or reverted by ROS scavenger, suggesting that at least in part some of the retinol-induced effects are mediated via ROS. These studies also suggest that polyamines may play a protective role in retinol-induced associated ROS damage.

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Capítulo V

Genotoxicity, recombinogenicity and cellular preneoplasic transformation induced by vitamin A supplementation

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Genotoxicity, recombinogenicity and cellular preneoplasic transformation induced by Vitamin a supplementation

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Abstract

In spite of being one of the first vitamins to be discovered, the full range of biological activities of Vitamin A remains incomplete. A growing body of evidence has demonstrated an apparent enhancement of carcinogenesis, induced by dietary retinol. Since DNA damage is a well-recognized inducer of carcinogenesis, the aim of this study was to test the possible genotoxic effect of dietary retinol, using different types of bioassays. Retinol caused an increased recombinogenic activity in *Drosophila melanogaster* larvae as measured by the SMART test. In mammalian cell cultures, retinol supplementation-induced DNA double-strands breaks (DSB) and single-strands breaks (SSB), cell cycle progression and proliferative focus formation in terminal-differentiated rat Sertoli cells and increased DNA fragmentation in Chinese hamster lung fibroblasts (V79 cells), as measured by the comet assay. Altogether, our results suggest that retinol causes DNA damage and chromosomal rearrangements, which may disturbs key physiological processes and lead to cell cycle progression and preneoplasic transformation of terminal-differentiated mammalian cells.

Keywords: Vitamin A; DNA damage; Comet assay; Cell cycle progression

1. Introduction

Several epidemiological studies have attempted to explain the role of dietary antioxidant Vitamins A, C and E in protection against various types of human cancers and cardiovascular diseases [1]. It is usually suggested that the activity of these compound is related to their ability to scavenge toxic forms of oxygen and other free radicals in living systems [2].

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Oxidative DNA damage may be important in mutagenic, carcinogenic and aging processes [3]. Although antioxidant vitamins may reduce oxidative DNA damage, evidence from human studies has been sparse and inconsistent. The α-tocopherol, β-carotene (ATBC) cancer prevention study group and the β-carotene and retinol efficacy trial (CARET) failed to show any protective effect of the vitamins against cancer [4]. An increase in the risk of lung cancer among smokers and asbestos workers was observed in the treatment group, which caused a premature end to these studies. This emphasizes the importance of animal bioassay studies which take into account the influence of dose, treatment, timing and sex in the development of chemopreventive regimens for human trials. Attempts to use retinoids and carotenoids for cancer prevention and therapy are, nevertheless, ongoing.

Vitamin A (all-*trans* retinol) and its derivatives, the retinoids, are recognized as key regulators of vertebrate development, cell growth, and differentiation processes. Retinoid action is mediated by specific nuclear retinoic acid receptors (RARs) and retinoid receptors (RXRs) belonging to the steroid/thyroid superfamily of transcription factors [5]. Recent work suggests that retinol may also act directly on the modulation of different PKC isoforms, serine/threonine kinases and cRaf activities [6].

We have been studying the effects of retinol treatment on chromatin structure and DNA repair processes, as well as on reactive oxygen species metabolism. In cultured rat Sertoli cells, retinol supplementation changes the phosphorylation pattern of histones and the high mobility group (HMG) of proteins, altering the organization and function of chromatin, which in turn modulates the switching on and off of transcriptionally active regions of DNA [7]. Increased chromatin sensitivity to DNAse I, an effect reversed by pre-treatment with 1,10-phenanthroline, has also been observed in Sertoli cells treated with retinol [8].

A growing body of evidence has demonstrated that pro-Vitamin A, β-carotene, retinol and retinoids have pro-oxidant properties, which might lead to cell oxidative damage and carcinogenesis [9,10]. In primary cultured Sertoli cells, retinol treatment (7 μM, 24 h) caused an enhancement in antioxidant enzyme activities and oxidative damage to biomolecules, probably due to a retinol-mediated increase in iron uptake and storage, which in turn led to highly reactive $^{\bullet}$OH radical generation by Fenton chemistry [11,12].

In this report, we use different approaches to clarify the possible genotoxic features of retinol supplementation in biological systems. We employed the wing spot test in *Drosophila melanogaster*, which is an efficient and sensitive assay for the detection of somatic mutation and recombination (SMART) [13]. In addition, we tested retinol-mediated DNA fragmentation in cultured Chinese hamster lung fibroblasts (V79 cells) and in rat Sertoli cells. We observed that retinol induces a significant increase in mitotic recombination in the SMART assay, at the higher of the two dose used, as well as fragmentation in both cell culture models. Retinol also stimulates cell cycle progression of differentiated Sertoli cells and the generation of clonal proliferative foci. We provide evidence for a genotoxic effect of retinol supplementation in various ex vivo and in vivo bioassays.

2. Materials and methods

2.1. Chemicals

Type I collagenase, medium 199, HBSS and all-*trans* retinol 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.

2.2. Cell cultures

Sertoli cells from 15-day-old Wistar rats were isolated and cultured as previously described [14]. In brief, 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 and grown at a plating density of 3.2×10^5 cells/cm^2 in medium 199 (pH 7.4) supplemented with 1% fetal bovine serum (v/v). 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. Experiments were performed on cells treated with retinol at a dose level of 7 μM for 24 h, based on previous results [12].

Chinese hamster lung fibroblasts were cultivated under standard conditions in minimum essential medium (MEM) with Earle's salts, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics [15]. Cells were maintained in tissue culture flasks (Nunc, 25 cm^2) at 37 °C, in a humidified atmosphere containing 5% CO_2 and were harvested by treatment with 0.15% trypsin and 0.008% EDTA in phosphate-buffered saline (PBS). The 2×10^5 cells were seeded into each flask and cultured for 1 day prior to treatment with the test substances. The cells were treated for 24 h with all *trans*-retinol (5, 7 μM), dissolved in 1% ethanol, with and without the antioxidant agent TROLOX (0.1 mM).

The culture flasks were protected from direct light during treatment with retinol. Control cultures received only the solvent of retinol (0.1% ethanol in culture medium (v/v)). In all experimental procedures, a group without the addition of ethanol was also included and no significant differences were observed between ethanol and no ethanol control groups (data not shown). Before use, all retinol solutions were monitored by spectroscopy for the formation of oxidized retinol metabolites. Cell viability was assessed by trypan blue exclusion. All results were normalized against protein content [16], using bovine serum albumin as standard.

2.3. DNA single-strand breaks (SSB)

DNA single-strand breaks (SSB) were determined basically as described by Olive [17]. In brief, 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 10 mM Tris, 10 mM EDTA, 2% SDS and 50 mM NaOH pH 12.4. They were then treated with KCl 120 mM, incubated for 10 min at 65 °C and centrifuged at 2400 × *g* for 10 min at 4 °C. Radioactivity in the supernatant and the pellet was measured. SSB were calculated by dividing the counts in the supernatant by the total counts.

2.4. DNA double-strand breaks (DSB)

DNA double-strand breaks (DSB) were determined basically as described by Venable et al. [18]. Cells were labeled for 24 h with 3 μCi/ml of [^{3}H]thymidine before retinol treatment. After treatment, the medium was aspirated and counted. The cells were lysed with PBS containing 1% Triton® X-100 and 2 μM EDTA. They were centrifuged for 15 min at 14,000 rpm in a microcentrifuge. Both the supernatant and the remaining pellet containing larger DNA fragments were counted. The DNA fragmentation was calculated by adding the counts in the medium and the supernatant and dividing by the total counts.

2.5. SMART test

To confirm possible genotoxic role of retinol, the wing somatic mutation and recombination test in somatic cells of *D. melanogaster* was used. This test allows the detection of different genetic end-points, including point mutation, chromosome deletion, and mitotic recombination, using two different cell strains, which carry specific genetic markers (multiple wing hair (*mwh*)and flair (*flr*^{3})) on the left arm of chromosome 3. The standard cross was used as described by Graf et al. [19]. Virgin females were collected from the *flr*^{3}*/In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} fly stock and crossed with *mwh/mwh* males. These flies were allowed to lay eggs for 8 h in culture bottles containing a solid agar base (3% (w/v)) completely covered with a layer of live fermenting yeast supplemented with sucrose. Approximately, 72 h after the end of the egg-laying stage, larvae were collected and distributed into plastic vials containing 1.5 g of Drosophila Instant Medium (Carolina Biological Supply, Burlington, NC, USA) re-hydrated with 5 ml of the test solutions at two different concentrations. The larvae fed on this medium until the end of their development. The experiment was carried out at 25 °C and 65% relative humidity. After hatching, adult flies were gathered and preserved in ethanol 70%. Wings were mounted on slides with Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g and water 50 ml). The dorsal and ventral faces were analyzed microscopically for unique or double spots at 400× magnification. On marker-heterozygous wings (*mwh/flr*^{3}), it is possible to record three different categories of spots: (i) small single spots (one to two cells in size) and (ii) large single spots (more than two cells), expressing either *mwh* or *flr*^{3} phenotype, as well as (iii) twin spots, consisting of both *mwh* and *flr*^{3} subclones. On balancer-heterozygous wings

(*mwh/TM3*) only *mwh* single spots can be observed, as the inverted *TM3* balancer chromosome does not carry *flr*^{3} or any other suitable marker mutation. While in *mwh/flr*^{3} flies mutant clones can originate from somatic point mutation, chromosome aberration and/or mitotic recombination, in the *mwh/TM3* genotype this last genotoxic event is suppressed due to the multiple inversions present in the *TM3* balancer chromosome. Comparing these two genotypes it was possible to quantify the recombinogenic action of the tested drug. The frequencies of mutant clones per fly of a treated series were compared to its concurrent negative control series using the conditional binomial test according to Kastembaum and Bowman.

2.6. Comet assay

The alkaline comet assay was performed as described by Singh et al. [20] with minor modifications [21]. Fully frosted microscope slides were coated with 200 μl of 0.5% normal-melting-point agarose (NMPA) in calcium- and magnesium free phosphate-buffered saline and covered with a coverslip. Slides were stored at 4 °C in a dark, humid box until use (within 24 h). After incubation, 10 μl of cell suspension was added to 100 μl of 0.5% low-melting-point agarose (LMPA, at 35 °C) in physiological saline and layered over the NMPA layer. After agarose polymerization (5 min on a metal tray over ice), a final layer of 80 μl of LMPA in PBS was added. Following agarose solidification, the coverslips were removed and slides lowered into freshly made lysing solution (10% DMSO, 1% Triton X-100, 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, at pH 10) for at least 1 week at 4 °C. Slides were rinsed in distilled water, placed on a horizontal gel electrophoresis tray, and covered with freshly made electrophoresis buffer (0.0075 M, 1 mM EDTA, pH 13) for 20 min to allow the DNA to unwind. Electrophoresis was carried out at 25 V, 300 mA for 15 min. Slides were removed, placed on a staining tray and covered with neutralizing solution (0.4 Tris, pH 7.5) for 5 min (repeated three times). Slides were drained, 50 μl of filtered (0.2 μM) ethidium bromide (20 μg/ml) was added and a coverslip placed on top. Slides were placed in a humid dark box at 4 °C until analysis was possible (within 24 h). The alkylating agent MMS, at final concentration of 1.6×10^{-4} M, was used as positive control. The presence of comets was examined in cells from the V79 cell suspension using a microscope equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm. All slides were coded and randomly scanned, with 100 cells per incubation quantified and scored visually according to tail intensity into five classes (from undamaged (0) to maximally damaged (4)). Thus, the damage score for each sample can range from 0 (completely undamaged: 100 cells × 0) to 400 (maximum damaged: 100 cells × 4). Apoptotic cells were also observed but not evaluated. Comet image lengths (nuclear region plus tail) were also measured using a calibrated eyepiece graticule.

2.7. Analysis of DNA content using flow cytometry (FACS)

The retinol treated Sertoli cells were harvested with trypsin-EDTA, washed twice with PBS containing 5 mM EDTA, and fixed in 70% ethanol at −30 °C. After 24 h, cells were collected by centrifugation and resuspended in PBS containing 5 mM EDTA. After pre-treatment with 50 μg/ml RNAse A, cells were stained with 50 μg/ml propidium iodide (PI; Sigma) solution for at least 30 min on ice and in the dark. Stained cells were dispersed with a syringe and single cells were analyzed by flow cytometry (FACS calibur system, Becton and Dickinson, San Jose, CA, USA). Ten thousand cells were analyzed per sample.

2.8. 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.

2.9. Statistical analysis

Results are expressed as the mean ± S.E. Data were analyzed by one-way analysis of variance (ANOVA), using a Neuman–Keuls test to compare mean values across groups. Differences were considered to be significant when $P < 0.05$. Differences in the extent

of DNA strand breakage between controls and treatments, determined by the comet assay in dose response studies, were tested for significance using the ANOVA analysis of variance with Dunnett's multiple comparison test and Pearson's correlation.

3. Results

The bioassays demonstrated various genotoxic effects of retinol.

The SMART test with *D. melanogaster* gave a positive effect in the marker-heterozygous flies, producing statistically significant increases in the frequencies of total spots, indicating that retinol at 96 μM is active in this test system. The data also indicated that genotoxic effects were related to increases in the frequencies of small and large single spots. Twin clone frequencies, on the other hand, were not influenced by the action of the drug. In fact, the absence of increments in the frequencies of twin spots is not an indication that the drug has no recombination action, since small and large single spots could be produced by recombinagenic events. Trying to confirm the no recombinagenic action of retinol it was analysed the balancer-heterozygous genotype, which express only mutagenic events. Considering that in this genotype no increments in the frequencies of spots were observed, we could infer that de positive response observed for the 96 μm concentration—in the *trans*-heterozygous flies—is due exclusively to mitotic recombination. (Table 1).

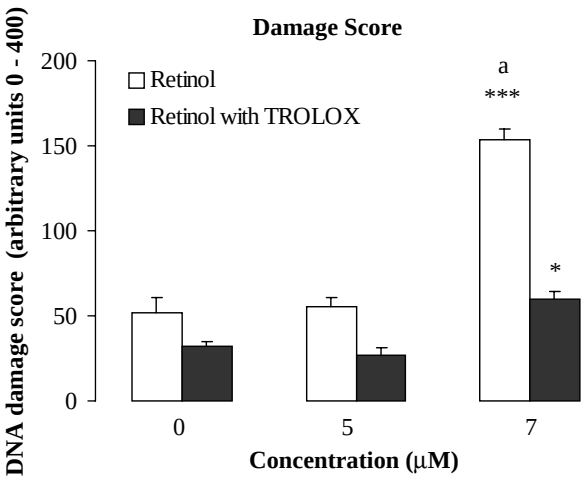

Fig. 1. Use of the comet assay for the evaluation of genotoxic effect of retinol in V79 cells in vitro, and the influence of co-treatment with TROLOX®. Each bar represents the mean value ± S.E. for three individual experiments. *Significantly different from the control value $P < 0.05$; ***significantly different from the control value $P < 0.001$; [a]significantly different from the co-treatment value $P < 0.001$.

Fig. 1 shows the distribution of DNA damage among cells treated with retinol and retinol with Trolox, estimated by the alkaline single-cell gel (comet) assay with V79 cells. Significantly increased DNA damage was observed at 7 μM concentration, and this was reduced by co-treatment with Trolox. Fig. 2 shows the frequency of different types of DNA damage (classes 0–4) in cells treated with various

Table 1
Fly spot data obtained after exposure of marker and balancer-heterozygous larvae of *D. melanogaster* to retinol

Genotypes	Retinol concentration (μM)	No. of Flies (*N*)	Spots per fly (no. of spots) statistical diagnosis[a]				Total *mwh* clones[b] (*n*)
			Small single spots (1–2 cells)[c] $m = 2$	Large single spots (>2 cells)[c] $m = 5$	Twin spots $m = 5$	Total spots $m = 2$	
mwh/flr^3	0	100	1.10 (110)	0.15 (15)	0.00 (0)	1.25 (125)	125
	48	100	1.11 (111) −	0.25 (25) i	0.02 (2) i	1.38 (138) −	130
	96	100	1.84 (184) +	0.40 (40) +	0.03 (3) i	2.27 (227) +	220
mwh/TM3	0	100	1.0 (10)	0.05 (5)	[d]	1.50 (15)	15
	96	100	0.90 (11) i	0.10 (10) i	[d]	2.1 (21) i	21

[a] Statistical diagnoses: (+) positive; (−) negative; (i) inconclusive; (*m*) multiplication factor for the assessment of significantly negative results. Significance levels $\alpha = \beta = 0.05$.

[b] Considering *mwh* clones from *mwh* single spots and from twin spots.

[c] Including rare *flr*^{3} spots.

[d] Only *mwh* single spots can be observed in *mwh/TM3* heterozygotes as the balancer chromosome *TM3* does not carry the *flr*^{3} mutation.

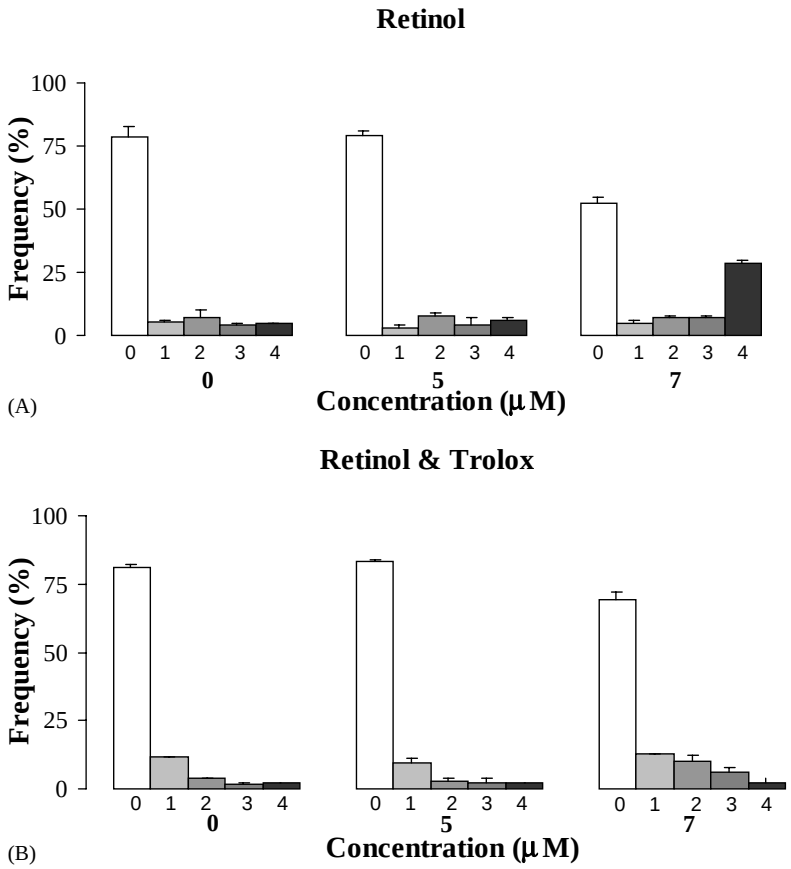

Fig. 2. Distribution of DNA damage observed in V79 cells exposed to a range of retinol concentrations for 24 h without or with co-treatment with TROLOX® (A and B, respectively). Each bar represents the mean value ± S.E. for three individual experiments.

concentrations of retinol (A). At 7 μM, the frequency of class 4 damage was over 25% (A), but this decreased when cells were co-treated with an antioxidant agent (B).

In Wistar rat Sertoli cell cultures, the genotoxic effect of 7 μM retinol led to a significant enhancement in single and double DNA strand breaks after 24 h treatment (Table 2). DNA fragmentation was also detected in retinol treated cells by FACS analysis, where an increased hypodiploid cell population was found (Fig. 3). The 24 h treatment caused a 48% increase in the *S* phase population and a three-fold increase in *G2-M* cells (Fig. 3). This induction in cell cycle progression was confirmed by the increase in proliferative focus formation in differentiated Sertoli cells supplemented with retinol (Fig. 4). This is an index of preneoplasic transformation.

Table 2
DNA single- and double-strand breaks in cultured rat Sertoli cells treated for 24 h with retinol 7 μM

Assay	Groups	
	Control cells	Retinol (7 μM)
DNA single-strand breaks (% control)	0.00 ± 17.24	86.20 ± 24.13*
DNA double-strand breaks (arbitrary units)	0.43 ± 0.03	0.58 ± 0.05*

Sertoli cells were treated with retinol 7 μM for 24 h and DNA single- and double-strands breaks were determined as described in Section 2. Data are expressed as mean ± S.E. of triplicate samples ($n = 4$).

* Statistically different from respective control cell group, $P < 0.01$ (one-way analysis of variance (ANOVA)).

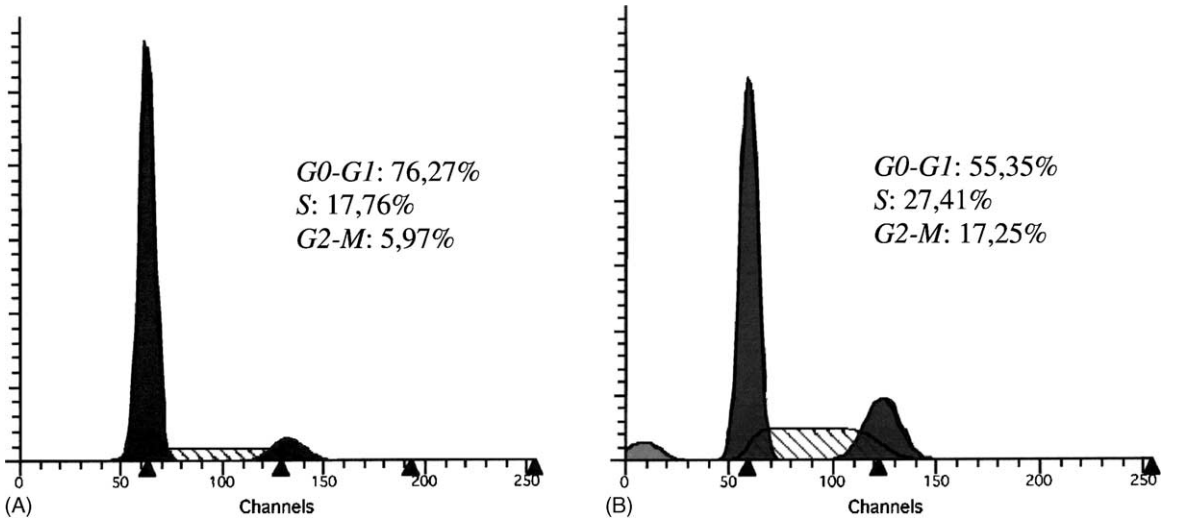

Fig. 3. Effect of retinol supplementation on cell cycle distribution of cultured rat Sertoli cells. FACS analysis of Sertoli cells following treatment with 7 μM retinol for 36 h. (A) Control cells; (B) retinol treated. Figure shows a representative experiment of at least four independent assays ($n = 4$).

4. Discussion

In spite of being one of the first vitamins to be discovered, the full range of biological activities for Vitamin A remains to be fully defined. Evidence from epidemiological studies has shown that a high consumption of compounds rich in pro-Vitamin A carotenoids is associated with a low risk of cancer and heart disease [2]. However, a growing body of evidence has demonstrated that carotenoids and retinoids, especially at doses that exceed the normal dietary intake or in conditions of enhanced oxidative stress, may act as propagators of free radicals, leading to increased incidence of various types of cancer. An apparent enhancement of carcinogenesis by dietary retinol, particularly for cancer of the esophagus, oral cavity, pharynx, larynx, stomach, colon, and rectum, could be related to an interaction between ethanol and retinol, and/or to a mechanism involving pro-oxidant activity [22]. These contradictory findings require further research to elucidate the mechanism(s) by which retinoids may alternate from pro- to anti-DNA-damage inducers.

We report here that retinol supplementation has a genotoxic effect in various animal models, including increased mitotic recombination in the SMART assay with *D. melanogaster* (Table 1). Retinol also induced DNA fragmentation in Chinese hamster lung fibroblasts in the comet assay (Figs. 1 and 2) and single- and double-strand breaks in primary Wistar rat Sertoli cell culture (Table 2 and Fig. 3).

All in all, our data are in agreement with previous reports that show retinol induces chromosomal aberrations in human lymphocyte cultures [23] and DNA damage in HL-60 cells via superoxide radical generation [24]. Oxidative cellular damage induced by retinol seems to be related to iron metabolism and highly reactive hydroxyl radical generation [11,12]. Single- and double-strands breaks (Table 2) are a direct consequence of the attack of hydroxyl radicals on deoxyribose in the DNA molecule [25]. This last observation is reinforced by the demonstration that DNA fragmentation induced by retinol in V79 cells, as determined by the comet assay, was reduced by co-treatment with trolox, an analog of Vitamin E (Figs. 1 and 2).

Oxidative stress may cause two distinct phenomena; cell adaptation or cell death. Free radicals have been related to physiological functions, regulating signal transduction pathways and cellular proliferation [26]. Sertoli cells, one of the most important physiological targets of retinol in the body, are somatic cells of the testis, which physically forms and regulates the environment milieu where spermatogenesis occurs

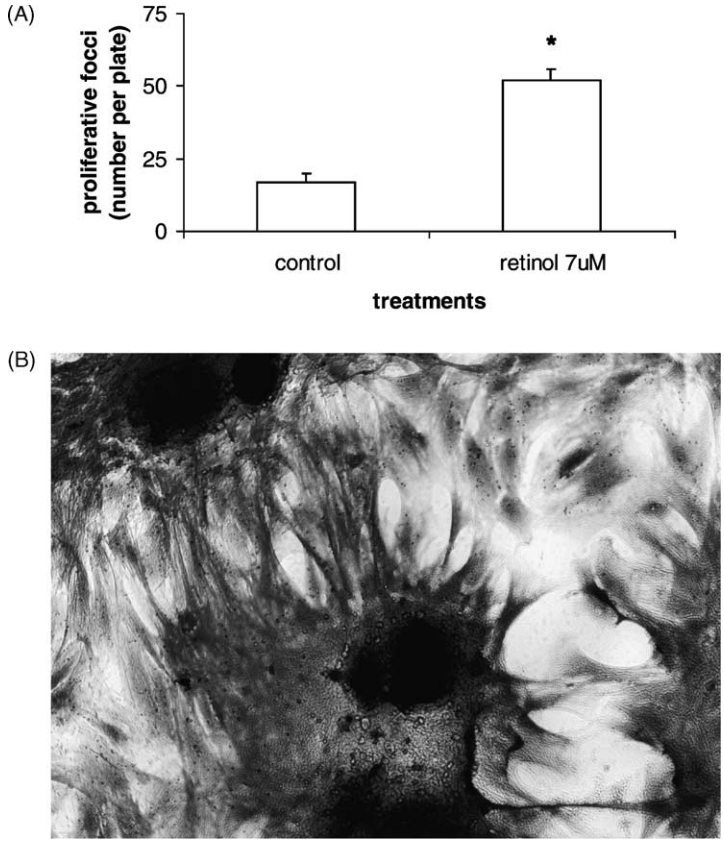

Fig. 4. Retinol produced focus formation in Sertoli cells. Cells were treated as described in Section 2 and maintained in medium 199 pH 7.4 supplemented with 10% fetal bovine serum for 15 days. (A) Morphology of treated cells was examined under a light microscope; (B) a representative photograph (400×) of at least four independent experiments is shown.

[27]. In terminal-differentiated Sertoli cells, retinol supplementation-induced cell cycle progression by a mitogenic signal, observed as the increased *S* and *G2-M* phase populations in treated cells (Fig. 3). Dierov et al. [28] showed that, in human adult T-cell leukemia cells, a brief retinoid exposure-induced proliferation, as demonstrated here for retinol. Retinol treatment induced a mitogenic signaling mediated by superoxide anion generation, which is reversed by the addition of exogenous superoxide dismutase [14]. We have previously observed that the oxidative signal mediated by retinol treatment increased ornithine decarboxylase activity in Sertoli cells, a key enzyme in the growth process [29]. Retinoids seem to be linked to the expression of a variety of protooncogenes, which function as transcriptional regulators during normal cell growth and as contributors to cell transformation [30]. Proliferation of terminal-differentiated cells is an indicator of cell transformation. Sertoli cells treated with retinol for 24 h and maintained in culture for 15 days showed increased numbers of proliferative foci (Fig. 4). This assay has been widely adopted as an indicator of cell preneoplasic transformation.

Our results show that retinol supplementation caused an enhancement in DNA damage in various biological systems, probably by a pro-oxidant effect. This genotoxicity may be related to cell instability, cell cycle progression and preneoplasic transformation of terminal-differentiated Sertoli cells. Although these results strongly suggest a probable mechanism by which retinol may enhance cell transformation, the correlation between retinol-induced DNA damage and mutagenesis with tumorigenesis remains to be fully established.

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Capítulo VI

Retinol induces Permeability transition and loss of membrane potential, increases lipoperoxidation, superoxide generation and enhances cytochrome c release from rat liver mitochondria

Manuscrito submetido para publicação

RETINOL INDUCES PERMEABILITY TRANSITION AND LOSS OF MEMBRANE POTENTIAL, INCREASES LIPOPEROXIDATION, SUPEROXIDE GENERATION AND ENHANCES CYTOCHROME C RELEASE FROM RAT LIVER MITOCHONDRIA

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Abstract

Biological actions of retinoids on modulation of cellular gene expression by nuclear receptors have been widely known. Recently, extra-nuclear effects of retinoids - such as modulation of PKC and Raf activities, inhibition of thioredoxin redutase, and interference in mitochondria homeostasis - have been proposed, but remain to be better elucidated. Considering that retinoids induce apoptosis in tumor cells by an unknown mechanism, and that mitochondria play a key role in controlling apoptosis via cytochrome c (cyt c) release and caspase-3 activation, we decided to investigate the possible effects of exposure of 3-month old Wistar rat liver mitochondria to different concentrations of retinol (vitamin A). We measured: lipoperoxidation (TBARS method); superoxide generation (adrenochrome formation); loss of membrane potential (rhodamine 123 fluorometric determination); mitochondrial swelling, and cyt c liberation (spectrophotometric determination). Results showed that retinol causes lipoperoxidation, as well as an increase in mitochondria swelling, which leads to a loss of membrane potential, in a dose dependent manner. As a consequence, there is cyt c release and an enhance in superoxide production by the electron transport chain. In view of these findings, our results demonstrate that retinol interferes with mitochondrial metabolism, promoting cyt c release, thus suggesting a putative mechanism of induction of apoptosis by retinoids.

Key words: vitamin A, retinol, mitochondria, cytochrome c, permeability transition.

Introduction

In spite of being one of the first vitamins to be discovered, the full range of biological activities of vitamin A (all-trans retinol) remains incomplete. Retinol and its derivatives, the retinoids, are recognized as key regulators of vertebrate development, cell growth, and differentiation processes. Retinoid action is mediated by specific nuclear retinoic acid receptors (RARs) and retinoid receptors (RXRs) belonging to the steroid / thyroid super-family of transcription factors [1]. In addiction to these classical mechanisms, recent work suggested that retinoids may act directly on the modulation of different PKC isoforms, serine / threonine kinase and cRaf activities [2]. Retinoid supplementation has been widely used therapeutically in the prevention and treatment of several types of diseases, particularly epithelial cancers and leukemia, including both pre-malignant and malignant tumors [3]. There is substantial evidence that retinoids exert their effects through the induction of apoptosis in different tumor cell lines, but the precise mechanism leading to cell death remains to be fully established [4]. Moreover, retinoids inhibit Bcl-2 expression, a mitochondria-associated key factor that increases cell resistance to apoptosis [5].

Apoptosis is regarded as an active and organized form of cell death, triggered in response to physiologic or pathologic stimuli. Mitochondria play a central role in the initiation of apoptosis. Specifically, the release of different proteins that are usually present in the intermembrane space of these organelles has been observed during early stages of apoptotic cell death. Cytochrome c (cyt c), an essential constituent of the respiratory chain, is often released from mitochondria and, once in the cytosol, interacts with this adaptor molecule – Apaf-1 – resulting in the processing and activation of pro-caspase-9 in the

presence of dATP. Caspase-9, in turn, cleaves and activates effectors pro-caspase-3 and -7; being responsible for the biochemical and morphological features characteristic of apoptosis [6].

Although cyt c release activates the death machinery in many experimental models of apoptosis, the mechanism of translocation across the outer mitochondrial membrane remains unclear. It is generally accepted that increased mitochondrial permeability transition (mPT) by the open of the permeability transition pore (PTP), which accompanies a loss of transmembrane potential ($\Delta\psi_m$), and mitochondrial swelling, leads to cyt c release from mitochondria [7]. This process is induced by Ca^{2+}, oligomerization of pro-apoptotic proteins like Bax or Bid, and inhibited by cyclosporin A (CsA) and Bcl-2 protein. Besides that, oxidative damage to mitochondria by pro-oxidant or thiol-reactive agents induces mPT, and releases cyt c from its loosely bound phospholipid cardiolipin [8].

We have been studying the effect of retinol treatment on chromatin structure and DNA repair processes, as well as on reactive oxygen species metabolism. We and others authors demonstrated that retinol treatment caused an enhancement in antioxidant enzyme activities and oxidative damage to biomolecules, probably due to a retinol-mediated increase in iron uptake and storage, which in turn leads to highly reactive $^{\bullet}OH$ radical generation by Fenton chemistry [9,10]. Previously, it has been observed that retinoic acid and its derivatives are able to induce swelling and release of cytochrome c in isolated mitochondria [11].

In this report, we decided to investigate the possible feature of retinol in the induction of membrane permeability transition and cytochrome c release from isolated mitochondria. Our results provide evidences for a possible mitochondria-mediated

mechanism of apoptosis induction by retinol. The data are discussed with reference to the use of retinol supplementation during therapeutic interventions.

Material and Methods

Chemicals

Vitamin A (all-trans retinol), fatty acid-free bovine serum albumin, rotenone, rhodamine 123, EGTA, and HEPES were purchased from Sigma, St. Louis, MO, USA.

Isolation of mitochondria

Mitochondria from fresh rat liver were isolated as described elsewhere [12]. Briefly, liver of Wistar rats suspended in ice-cold isolation buffer A (220 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 1 mM EGTA, and 0.5 mg / mL fatty acid-acid free bovine serum albumin) was gently homogenized with a glass-homogenizer and centrifuged at 2000X*g* for 10 min at 0 °C. Approximately three-quarter of the supernatant was further centrifuged at 10000X*g* for 10 min at 0 °C in a new tube. The fluffy layer of the pellet was removed by gently shaking with buffer A and the firmly packet sediment was resuspended in the same buffer without EGTA and centrifuged at 10000X*g* for 10 min at 0 °C. Mitochondria pellet was resuspended in buffer B (210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4,2 mM succinate, 0,5 mM KH_2PO_4, and 4 μg / mL rotenone). This procedure, which was designed to isolate intact mitochondria rather that to recover all of the mitochondria present in the liver, yielded about 20 mg of mitochondrial protein / g

of liver. Mitochondria protein was determined by the Bradford method with bovine serum albumin as standard. In some experiments 6 μM CsA were also added.

Transmission electron microscopy analysis

Isolated mitochondria (0,25 mg) for TEM were fixed in 2.5% glutaraldehyde/1% paraformaldehyde (pH 7,2) for approximately 4 h. Samples were allowed to settle out by gravity and rinsed twice with 0,2 M phosphate buffer. The rinsed samples were centrifuged at 12500g for 10 min. Following centrifugation, samples were postfixed in 1% OsO4 for 1 h, rinsed through a graded acetone series, and infiltrated with and embedded in epoxy resin. Ultrathin sections were cut and stained with uranyl acetate.

Measurement of the mPT by swelling of the mitochondria

The PTP opening was assayed essentially as described by Petronilli *et al.* (1993) [13]. Opening of the PTP causes mitochondria swelling that is conveniently assayed as a decrease in the light scattering (and thus absorbance) of a mitochondria suspension. Mitochondria (0.25 mg protein / mL) were suspended in cuvettes in buffer B. After 2-min equilibration period, different concentrations of retinol were added, and mitochondrial swelling was followed spectrophotometrically by the decrease in absorbance at 540 nm, at 25 °C, for 10 min.

Measurement of mitochondria membrane potential ($\Delta\psi_m$)

The $\Delta\psi_m$ was estimated by measurement of the fluorescence change of Rho 123 (0.1 μM) as described [12]. Mitochondria (1 mg protein / mL) were incubated at 25 °C in buffer B containing 4 μg / mL of rotenone, and $\Delta\psi_m$ was assessed by spectrophotometer

(RF-540) with excitation at 505 nm and recording at 534 nm after addition of 0.1 μM Rho 123.

Measurement of cytochrome *c*

Cytochrome *c* release from rat liver mitochondria was assayed according to Borutaite *et al.* (1999) [14]. Mitochondria (1 mg / mL) were incubated with different concentrations of retinol for 10 min at 37 °C, and then centrifuged at 12000 rpm for 10 min in an eppendorf centrifuge. Supernatants were used for spectrophotometrically measurement of cytochrome *c* release from mitochondria by using the absorption difference of the wavelengths pair 550/540 nm for ascorbate-reduced minus ferricyanide-oxidised cyt *c* (ε = 18.5 mM^{-1} cm^{-1}). The total amount of mitochondrial cyt *c* were determined in the pellets obtained after retinol treatment, and was measured spectrophotometrically as the second derivative ($d^2A/d\lambda^2$) of the absorption spectra, after extraction with *n*-butanol [11].

Thiobarbituric Acid Reactive Species (TBARS)

Lipid peroxidation were measurement by the formation of TBARS during an acid-heating reaction, which is widely adopted as an index of ROS production as previously described [15]. 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 x g / 5 min) thiobarbituric acid reactive species (TBARS) were determined by the absorbance at 535 nm. Results are expressed as MDA (malondialdehyde) equivalents (nmol / mg protein).

Mitochondrial Superoxide production

Superoxide production was determined in washed sub-mitochondrial particle (SMP) using a spectrophotometric assays based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37 °C (E_{480nm} = 4.0 mM^{-1} cm^{-1}) [16]. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), SMP (1.0 mg protein / mL), 0.1 μM catalase, and 1 mM epinephrine. Succinate (7 mM) was used as substrate. Superoxide dismutase (E.C. 1.15.1.1.) was used at 0.1-0.3 μM final concentration to give assay specificity.

Mitochondrial viability

Isolated liver rat mitochondria (1 mg / mL) supported with 5 mM succinate, was treated with different concentrations of retinol and incubated in buffer B with 0.25 mg / mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) at 37 °C / 30 min. After centrifugation in an eppendorf microcentrifuge (12000 rpm / 5 min), pellets were solubilized in 1 mL DMSO and MTT reduction was determined using the absorbance of the wavelength pair $A_{570-630}$ nm. It is widely assumed that MTT is reduced by active mitochondria [17].

Statistical Analysis

Results are expressed as the mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA), using a Neuman-Keuls test to compare means values across groups. Differences were considered to be significant when $p < .05$.

Results

Induction of mPT opening and loss of mitochondria membrane potential by retinol

In isolated rat liver mitochondria, incubation with different concentrations of retinol caused an abrupt swelling in a dose dependence pattern (FIG. 1 and 2). Retinol in the concentration of 20 and 40 μM induces mitochondria permeability transition in the same degree as $CaCl_2$ 150 μM, a classical mPT inducer (FIG. 2). As a consequence of the mPT opening, retinol caused a dose dependent loss of mitochondrial membrane potential ($\Delta\psi_m$) (FIG. 3).

Mitochondrial lipoperoxidation and increased superoxide radical generation by retinol

Figure 4 shows a significant increase in oxidative damage to mitochondrial lipids (A.), and superoxide radical generation (B.), mediated by retinol 20 and 40 μM ($p< .05$ and $p< .01$, respectively), as compared to vehicle alone.

Cytochrome *c* release and decreased mitochondrial viability by retinol

To determine the influence of retinol in the release of cyt *c*, we directly measured the presence of cyt *c* in the supernatants of mitochondria incubated under different conditions (Table 1), and in the total amount of mitochondrial cytochrome *c* (FIG. 5). Retinol caused cytochrome c release from mitochondria in a dose dependence pattern (Table 1). Retinol-mediated cyt *c* release was reverted to vehicle values when trolox 0.1 μM – an α-tocopherol analog – was co-administered, suggesting a possible involvement

of free radical (Table 1). As a positive control, $CaCl_2$ 150 μM was administered. Retinol treatment caused a decrease in the total amount of mitochondrial cytochrome *c* (FIG. 5) and in mitochondrial viability, determined by MTT reduction (Table 1).

Discussion

Natural and synthetic derivatives of vitamin A show significant efficacy in the treatment of experimental breast cancers, acute promyelocytic leukemia and they play a fundamental role in chemoprevention of epithelial carcinogenesis and in differentiation therapy [18]. Although these outcomes support the possible use of retinoids in the prevention and the therapy of different types of tumors, the biochemical and cellular mechanisms by which retinoids may block (or promote) cancer progression are largely unknown. In terminal-differentiated mammalian cells, retinol supplementation causes oxidative DNA damage, leading to cell cycle progression and preneoplasic transformation [19]. Recent work demonstrated an enhancement in oxidative damage caused by ultraviolet irradiation mediated by vitamin A [20]. These contradictory findings require further research to elucidate the mechanism(s) by which retinoids may alternate from pro- to anti-DNA-damage inducers.

An increase in mitochondrial permeability is now established as an important route by which stimuli can activate apoptosis in mammalian cells [21]. There is, therefore, much interest in the development of suitable drugs for therapeutic intervention that can regulate the opening of such permeability transitions in tumor cells. Retinoic acid and its

derivatives are able to induce swelling and release of cytochrome c in isolated mitochondria [11]. Moreover, retinoids downregulate the expression of Bcl-X_L, one antiapoptotic member of Bcl-family, and have demonstrated an altered subcellular distribution of Bcl-2 [22, 23].

Exposing of isolated rat liver mitochondria to different concentration of retinol, the natural precursor of retinoic acid synthesis, caused mitochondrial permeability transition, loss of membrane potential and cytochrome *c* release in a concentration dependent pattern (Fig. 1, 2, 3, 5 and Table 1). When trolox is co-administered, no differences in the cyt *c* release is detected, as compared to control (Table 1), suggesting that this effect is not simply attributed to a general membrane disrupting action of retinol, as previously observed [24], but related to the oxidative damage generated by retinol supplementation (Fig. 3) [9,10,19,20]. Intense mitochondrial lipoperoxidation may disrupt the loose bound of cyt *c* from its membrane anchoring lipid, cardiolopin, permitting the release of cytochrome *c* to extramitochondrial environment (Fig. 4; Table 1) [8]. Recent experimental data suggest that several of classical biological effects of retinol supplementation, like well described elevation of ornithine decarboxilase (ODC) activity, is caused by its pro-oxidant feature [25].

In conclusion we demonstrated, in the present report, that retinol administration is able to induce permeability transition and cytochrome *c* release in isolated rat liver mitochondria and that this effect is, at least in part, related to the recent pro-oxidant feature of retinoids in biological system. These results suggest that, in addiction to the use of combined therapies based on both maturation of neoplasic cells by retinoids and cell death induced by others chemotherapeutic drugs, new therapies exploring the direct influence of retinol in the activation of apoptotic machinery in tumor cells could be developed as well.

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Table 1: Release of cytochrome *c* and decrease in mitochondrial viability induced by retinol treatment.

	Assays	
treatments	cytochrome *c* released (pmol / mg protein)	MTT reduction (A.U.)
control	1.34 ± 0.10	0.28 ± 0.05
vehicle 3 μM	1.18 ± 0.38	0.20 ± 0.02
vehicle 40 μM	1.95 ± 0.16	-- --
retinol 3 μM	1.97 ± 0.16	0.29 ± 0.03
retinol 7 μM	2.80 ± 0.56	0.20 ± 0.02
retinol 20 μM	2.85 ± 0.15 *	0.18 ± 0.01*
retinol 40 μM	5.24 ± 0.66**	0.16 ± 0.01*
ret 40μM + trolox® 0.1μM	1.86 ± 0.29#	-- --
Ca^{2+} 150 μM	6.08 ± 1.57	-- --
1 % tritonX100®	10.53 ± 1.54	-- --

1 mg of rat liver mitochondrial protein in 1 mL of buffer B (210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4,2 mM succinate, 0,5 mM KH_2PO_4, and 4 μg / mL rotenone) were treated by 10 min at 25 °C with different concentration of retinol and cyt *c* release were determined by the difference in $A_{550/540}$ of ascorbate-reduced minus ferricianide oxided supernadant (10000g / 5 min) (ε = 18,5 mM^{-1} cm^{-1}). Mitochondrial viability was spectrophotometric determined by MTT reduction at $A_{570\text{-}630}$ nm. Data represent means ± SEM (n = 6). * different from respective control group (p<.05); **(p<.01); #Different from ret 40 μM (p<.01) (one way analysis of variance).

Figure Legend:

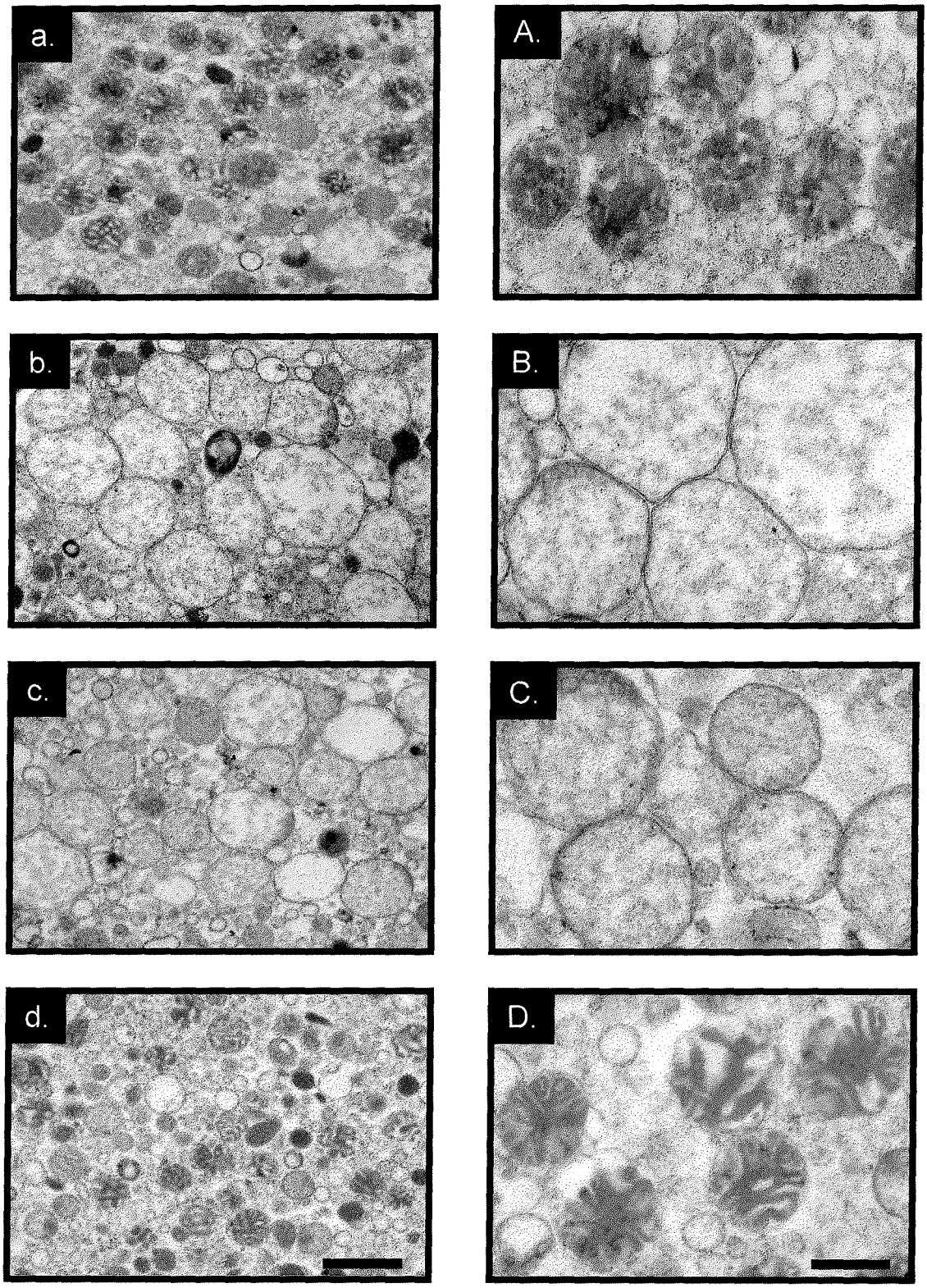
FIG. 1: Ultrastructure of mitochondria treated with retinol. Mitochondria (0.25 mg/ mL) were exposed to different treatments followed a 10 min incubation in buffer B and prepared for TEM analysis as described in Materials & Methods. Legend: (a) untreated; (b) 150 μM of $CaCl_2$; (c) retinol 40 μM; (d) retinol 40 μM + 6 μM CsA. Magnification = 10000X; Bar = 0,5 μm. The right column is the same treatments, but magnification = 25000X; Bar = 0,2 μm.

FIG. 2: Mitochondrial permeability transition mediated by retinol. Rat liver mitochondria (0.25 mg / mL) were incubated at 25 °C in buffer B. Swelling was triggered by the addition (at the arrow) of retinol at the following concentrations: untreated (a), 20 μM (b), 40 μM (c). As a positive control, 150 μM of $CaCl_2$ were also added (d). The involvement of mPTP opening by retinol was assayed by co-administrating 6 μM CsA with retinol 40 μM (e). Representative of at least five independent experiment (n = 5).

FIG. 3: Loss of mitochondrial membrane potential ($\Delta\psi_m$) by retinol. Rat liver mitochondria (1 mg / mL) were incubated at 25 °C in buffer B with Rh 123 (0.1 μM). Energization of mitochondria was obtained by the addition of 4,2 mM succinate. Loss of $\Delta\psi_m$ was triggered by the addition (at the arrow) of retinol at the following concentrations: untreated (dashed line), 10 μM (a), 20 μM (b), 40 μM (c). The involvement of mPTP opening by retinol was assayed by co-administrating 6 μM CsA with retinol 40 μM (d). Representative of at least five independent experiment (n = 5).

FIG. 4: Increased mitochondrial lipoperoxidation and superoxide radical generation by retinol. Rat liver mitochondria (1 mg / mL) were incubated at 25 °C by 10 min with different concentrations of retinol in 210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH (pH 7.4), 4,2 mM succinate, 0,5 mM KH_2PO_4, and 4 μg / mL rotenone. Lipoperoxidation were determined by TBARS methods (A.), and superoxide radical generation by adrenochrome formation (B.). Data represent means ± SEM (n = 6). * different from respective control group ($p<.05$); **($p<.01$), one way analysis of variance.

FIG. 5: Decrease in mitochondrial cytochrome *c* content by retinol. Rat liver mitochondria (1 mg / mL) were incubated at 25 °C by 10 min with different concentrations of retinol in buffer B. Cytochrome *c* were extracted from mitochondrial pellet with *n*-butanol, and spectrophotometric determined by the second derivative of the absorption spectra ($d^2A/d\lambda^2$). The resulting values were compared to a standard curve obtained using horse heart cytochrome *c*; the readings are linear in the range 0.1-1.5 μM cyt *c*. Data represent means ± SEM (n = 6). * different from respective control group ($p<.05$); (one way analysis of variance).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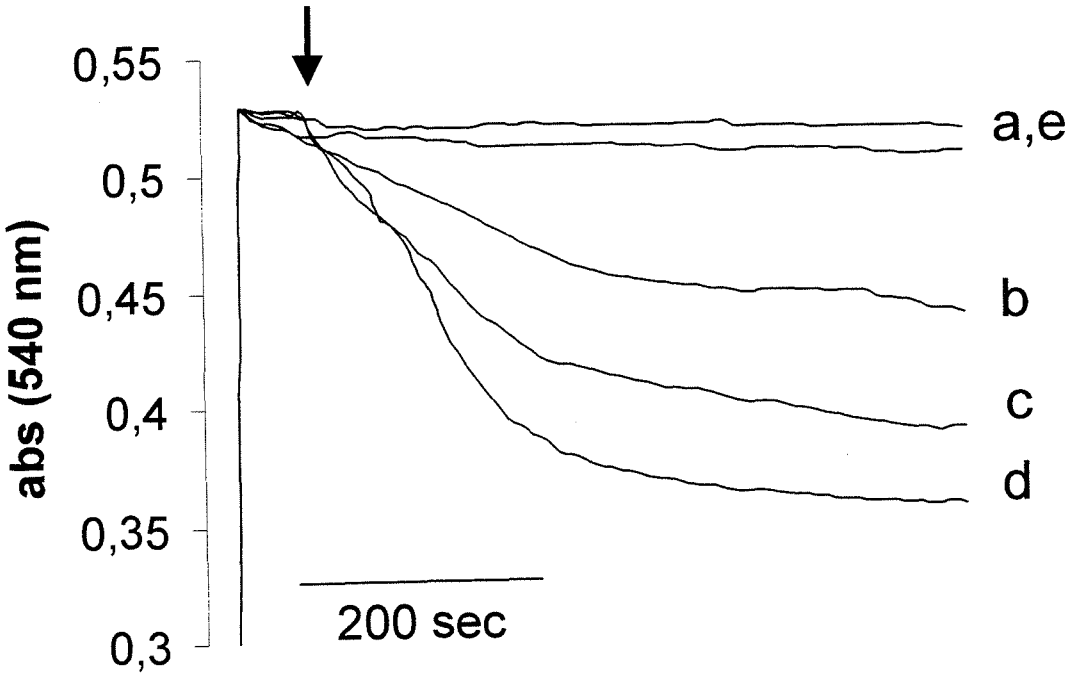
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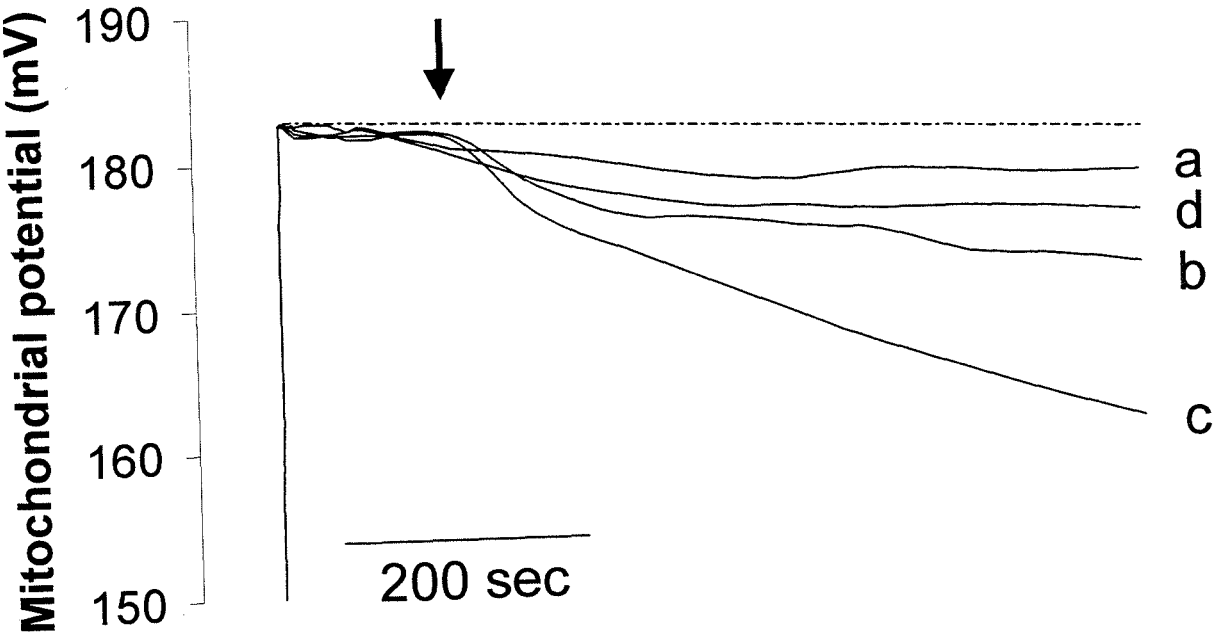
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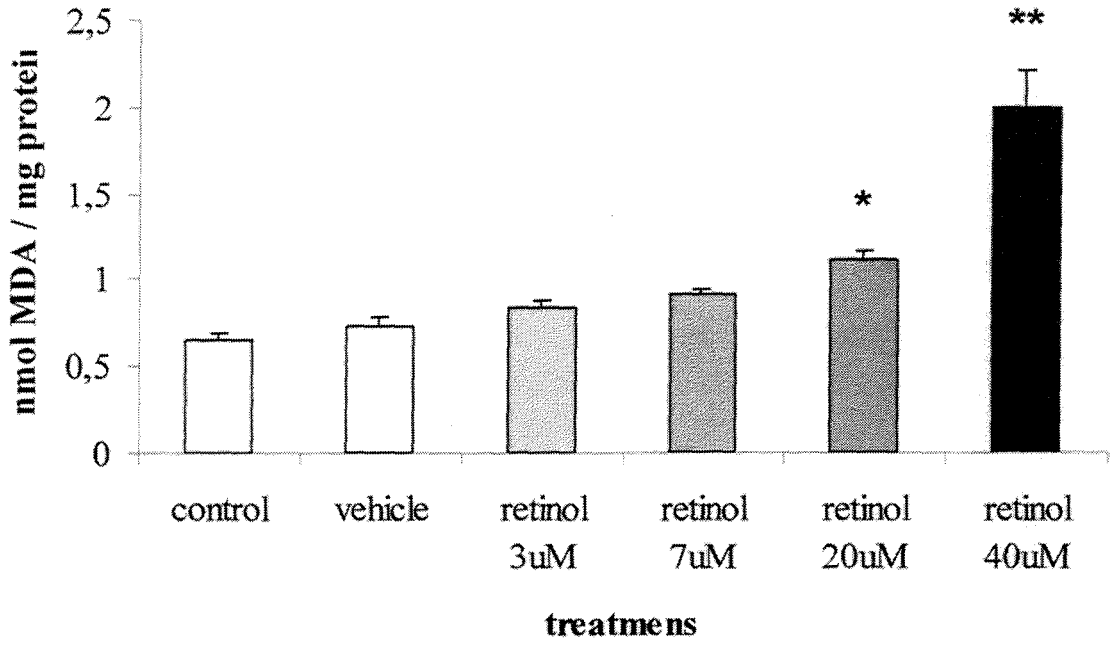
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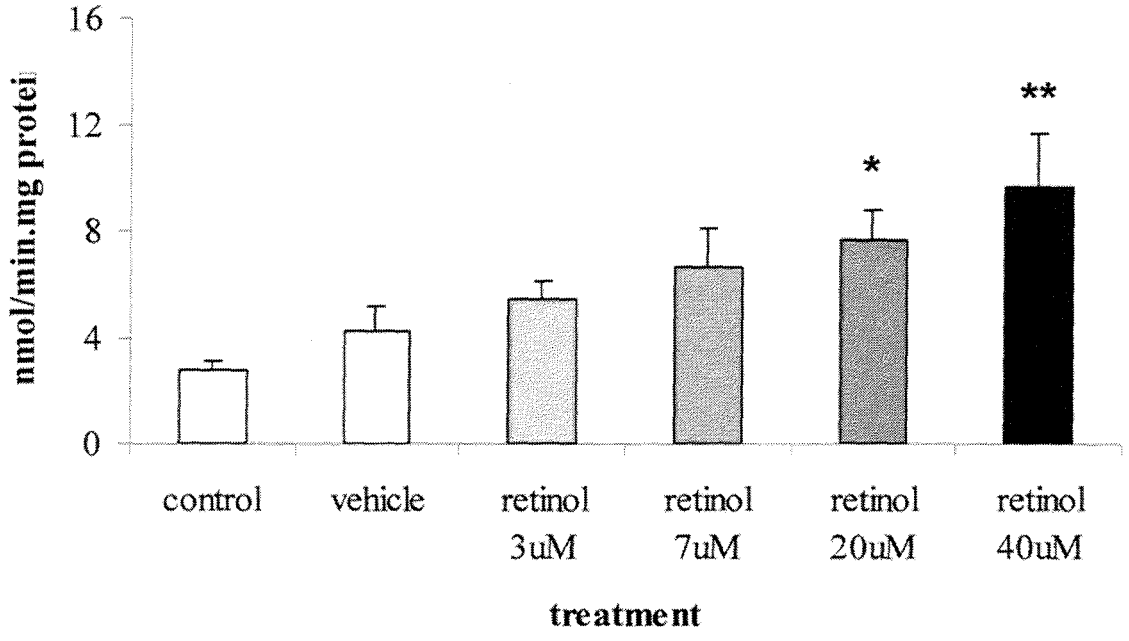
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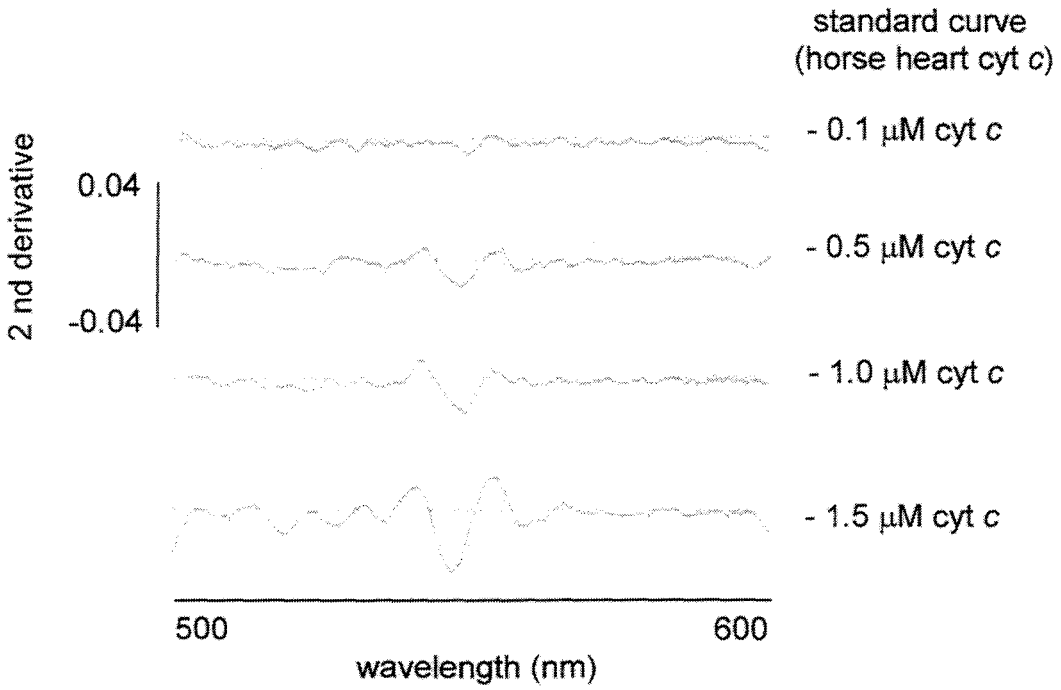
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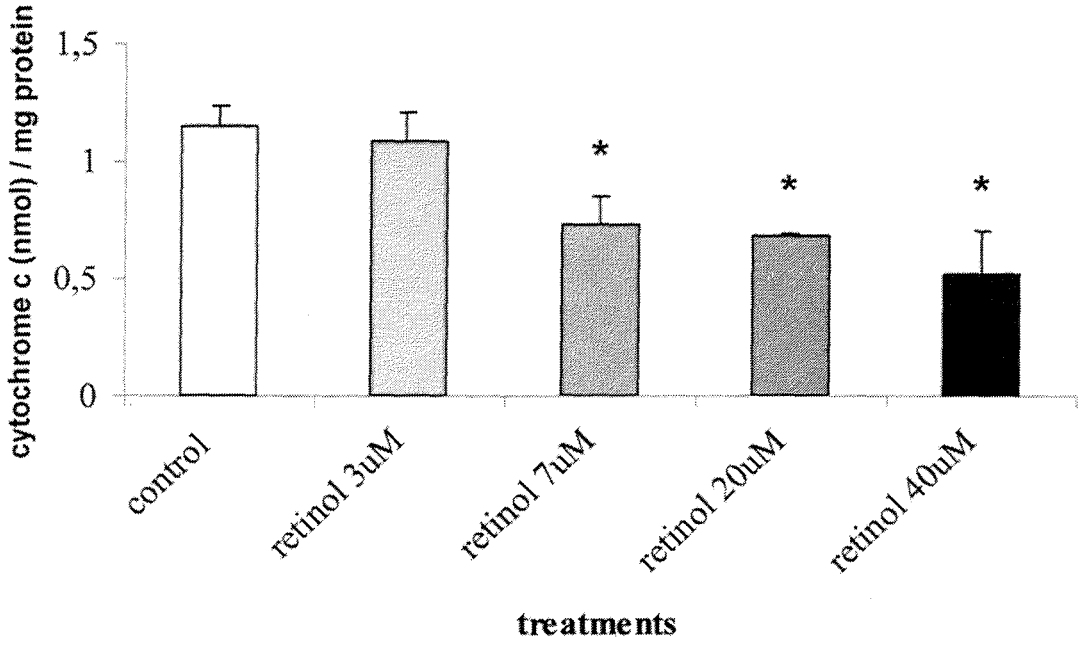
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treatments

Capítulo VII

Vitamin A supplementation induce mitochondrial superoxide radical generation and cytochrome c release, leading to caspase-3-dependent programmed cell death

Manuscrito submetido para publicação

VITAMIN A SUPPLEMENTATION INDUCE MITOCHONDRIAL SUPEROXIDE RADICAL GENERATION AND CYTOCHROME *C* RELEASE, LEADING TO CASPASE-3-DEPENDENT PROGRAMMED CELL DEATH

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Abstract

There are several reports suggesting that retinoid treatment leads to apoptosis in tumor cells, but the precise mechanism remains to be determined. Mitochondrial cytochrome *c* (cyt *c*) release and caspase-3 activation are a well described route of apoptosis induction. We previously described that retinol caused an increased free radical generation, which may be related with mitochondrial damage and apoptosis in cultured rat Sertoli cells. In this work, we investigate the effect of retinol (7 μM) treatment, a natural retinoic acid precursor, in total cellular nonenzymatic antioxidant status, mitochondrial superoxide production and cyt *c* release, and caspase-3 activation in cultured 15 day-old Wistar rat Sertoli cells. Apoptosis cell death mediated by retinol treatment was evaluated by nuclear TUNEL staining and conventional agarose gel eletrophoresis for detection of internucleosomal DNA fragmentation. Our results showed that retinol treatment caused a rapid and temporary cellular oxidative pulse, which may be related to a decreased mitochondrial viability and an enhancement in superoxide radical generation by electron transport chain, both leading to cyt *c* release. In consequence, there is a substantial activation of caspase-3, which in turn induces Sertoli cells internucleossomal DNA fragmentation and positive nuclear staining to TUNEL assay. We also demonstrated that retinol treatment maintain cellular ATP levels probably by PARP cleavage, and a redox permissive environment to caspase-3 activity. In view of these findings, our data establish a putative mechanism in which retinol exert their effect through the induction of apoptosis in different tumor cell lines.

Key-words: retinol, oxidative stress, mitochondria, apoptosis, cytochrome c, Sertoli cells.

Introduction

Vitamin A (all-*trans* retinol) is an essential nutrient which can either be obtained directly in the diet or by the intake and conversion of pro-vitamin A β-carotenoid. Retinoids, the derivatives of vitamin A, have a broad range of effects on biological systems, regulating embryogenesis, neoplasia, and the maintenance of normal tissue homeostasis. The wide spectrum of physiological and pharmacological retinoids effects are attributed to both receptor-dependent and receptor-independent mechanisms [1]. Transcriptional regulation of target genes by retinoids is generally mediated by nuclear receptors: retinoic acid receptors (RARα, -β, and -γ) and retinoid X receptors (RXRα, -β, and -γ) [2]. Once bound to ligand, these receptors homodimerises or heterodimerises, and the complex formed exerts its biological effects via binding to a particular *cis* element, the retinoic acid response element (RARE). Recently, several report demonstrate that retinol could directly modulate serine / threonine kinases, different PKCs isoforms, and cRaf activities by its electrons transfer capacity, functioning as a tag to enable the efficient and direct redox activation of these proteins [3,4].

Retinoid supplementation has been widely used therapeutically in the prevention and treatment of several types of diseases, particularly epithelial cancers and leukemia. A number of retinoids have now been described which induce programmed cell death, in what appears to be an RAR- and RXR-independent mechanism [5]. Apoptosis is regarded as an active and organized form of cell death, triggered in response to physiologic or pathologic stimuli. It is characterized by a number of well-defined features, which include condensation and fragmentation of the chromatin, internucleosomal DNA cleavage,

membrane blebbling, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane and liberation of the so called "apoptotic bodies" [6]. Mitochondria play a central role in the initiation of apoptosis. Specifically, the release of different proteins that are usually present in the intermembrane space of these organelles has been observed during early stages of apoptotic cell death. Cytochrome c (cyt c), an essential constituent of the respiratory chain, is often released from mitochondria and, once in the cytosol, interacts with this adaptor molecule – Apaf-1 – resulting in the processing and activation of pro-caspase-9 in the presence of dATP. Caspase-9, in turn, cleaves and activates effectors pro-caspase-3 and -7; being responsible for the biochemical and morphological features characteristic of apoptosis [7].

Oxidants (reactive oxygen species) are generated during normal cellular metabolism and in high levels by activated phagocytes in inflammatory tissues, and are thought to mediate apoptosis. These oxidants might arise from leakage of electrons from mitochondria, from chemotherapeutic drug metabolism, or from activation of oxidases such as NAPH oxidase. There is substantial evidence that retinoids exert their effects through the induction of apoptosis in different tumor cell lines, but the precise mechanism leading to cell death remains to be fully established [8]. A growing body of evidence has demonstrated that retinoids have pro-oxidant proprieties, which might lead to cell oxidative damage and carcinogenesis [9]. In primary cultured Sertoli cells, retinol treatment (7 μM, 24 h) caused an enhancement in antioxidant enzyme activities and oxidative damage to DNA, lipids and proteins, probably due to a retinol-mediated increase in iron uptake and storage, which in turn led to highly reactive $^{\bullet}OH$ radical generation by Fenton chemistry [10, 11]. We also demonstrated an enhance in 8-oxo 7,8 dihydro-2`-deoxyguanosine (8-

oxodG) in Sertoli cells treated with retinol [10]. These findings suggest a putative role for oxidative stress in retinol-induced apoptosis in mammalian cell lines.

In this report, we investigate and characterize the molecular mechanism involved in the decrease in viability of cultured rat Sertoli cells treated with retinol. Ours result suggest that retinol supplementation causes a rapid and transient oxidative pulse that might cause cellular and mitochondrial injury, leading to the activation of a caspase-3-dependent programmed cell death. The data are discussed with reference to the use of retinol supplementation during therapeutic interventions.

Materials and Methods

Chemicals

Type I collagenase, medium 199, HBSS, trolox, and all-trans retinol were purchased from Sigma, St. Louis, MO, USA. Trypsin was purchased from Difco, Detroit, MI, USA.

Cell isolation and culture

Sertoli cells from 15-day-old Wistar rats were prepared and cultured as previously described [12]. Animals were killed by either 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 3.2×10^5 cells / cm^2 in medium 199 (pH 7.4) supplemented with 1% fetal bovine serum (v/v) in plastic vessels. 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 germinative cells. Experiments were performed on cells treated with retinol 7 μM for 24 h. Control cultures received only the retinol solvent (0.1% ethanol, v/v). Previous testing had shown that ethanol alone had no effect in any of the experimental procedures (data not shown). The formation of oxidized retinol metabolites was monitored by spectroscopy of all retinol solution before its use. Cell viability was assessed by trypan blue exclusion. All results were normalized against protein content (Lowry et al, 1951), using bovine serum albumin as standard.

Conventional agarose gel eletrophoresis for detection of internucleosomal DNA fragmentation

Low-molecular-weight apoptotic DNA fragments was isolated by the procedure of Herrmann et al [13]. DNA fragments (equivalent to ~ 3 X 10^6 cells) were separated by 2% agarose eletrophoresis at 80 V for 2 h, and was performed using 1 X TBE.

Terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining

Apoptotic cell death was also examined by TUNEL staining. The cultured Sertoli cells were incubated for 12 and 24 hs with or without 7 μM retinol, then fixed with 4% paraformaldehyde for 10 min at room temperature. According to the method of a TUNEL staining kit (*in situ* Cell death Detection Kit, Boehringen), the cells were treated with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min at 4 °C and labeled with terminal deoxyribonucleotidyl transferase for 30 min at 37 °C [14].

Assay of caspase-3 activity

Caspase-3 activity was measured as described previously [15]. Cell extracts were incubated for 30 min at 37 °C with 100 μM DEVD-AFC (7-amino-4-trifluoro-methylcoumarin) peptide substrate in a total volume of 200 μL adjusted with ICE buffer (50 mM Hepes buffer, pH 7.5, 10 % sucrose, 0.1 % Triton X-100), DTT (4 mM) was added to the reaction mixture. The fluorescence of cleaved AFC was measured at excitation and emission wavelengths of 400 and 505 nm, respectively. Although caspase-3 may be the major enzyme to cleave at DEVD sequences, other caspases can also recognize this sequence. Hence, DEVDase activity is referred to as "caspase-3-like".

Measurement of mitochondrial superoxide generation

Submitochondrial particles (SMP) were isolated from Sertoli cells by differential centrifugation as previously described [16]. Superoxide production was determined in washed sub-mitochondrial particle (SMP) using a spectrophotometric assays based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37 °C (E_{480nm} = 4.0 mM^{-1} cm^{-1}) [16]. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris-HCl (pH 7.4), SMP (1.0 mg protein / mL), 0.1 μM catalase, and 1 mM epinephrine. Succinate (7 mM) was used as substrate. Superoxide dismutase (E.C. 1.15.1.1.) was used at 0.1-0.3 μM final concentration to give assay specificity.

Mitochondrial viability

Cultured Sertoli cells treated with retinol and incubated in buffer B with 0.25 mg / mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) at 37 °C / 30 min. After centrifugation in a eppendorf microcentrifuge (12000 rpm / 5 min), pellets were solubilized in 1 mL DMSO and MTT reduction was determined using the absorbance of the

wavelength pair $A_{570-630}$ nm. It is widely assumed that MTT is reduced by active mitochondria [17].

Total reactive antioxidant potential (TRAP)

The antioxidant potential of the cultured Sertoli cells was estimated by the total radical-trapping antioxidant parameter (TRAP). The principle of TRAP measurement has been previously described [18]. Briefly, the reaction was initiated by injecting luminol and AAPH – a free radical source that produces peroxyl radical at in a constant rate – in glycine buffer that resulted in steady luminescence emission. The addition of the samples homogenates decreases the luminescence proportionally to its antioxidant potential. The protein content of samples homogenates was determined (Lowry et al., 1951). The luminescence emission was followed for 60 min after the addition of the samples homogenates (150 μg of protein). Chemiluminescence was read in a liquid scintillation counter (Wallace 1409) as counts for minutes (cpm).

ATP assay

Intracellular ATP levels were determined using luciferin/luciferase [19]. Briefly, cells (3 X 10^6 cells) that have been treated with 7 μM retinol for different times were washed with PBS, resuspended in 250 μL of 10 mM KH_2PO_4, 4 mM $MgSO_4$, pH 7.4, heated at 98 °C for 4 min, and placed on ice. At the time of the assay, a 50 μL sample was added to 100 μL of 50 mM $NaAsO_2$, 20 mM $MgSO_4$, pH 7.4, and 80 μg of luciferin/luciferase. Light emission was quantified in Dynatech ML 3000 microtiter plate luminometer (Chantilly, VA, USA). Standard curves were rum in all experiment and were linear in the range of 5 – 2500 nM

ATP. Stock ATP concentrations were measured spectrophotometrically at 259 nm using a molar extinction coefficient of 15,400.

Western blot analysis

Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membranes as described previously [20]. After blocking with 5% milk, membranes were incubated with mouse monoclonal anti-PARP – poly(ADP-ribose) polymerase – or anti-cytochrome c followed by horseradish peroxidase-cojugated secondary antibodies. Bands were visualized by chemiluminescence using the ECL kit from NEM (Boston, MA, USA).

Statistical Analysis

Results are expressed as the mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA), using a Neuman-Keuls test to compare mean values across groups. Differences were considered to be significant when $p<.05$.

Results

Nuclear TUNEL staining and cellular viability

Retinol (7 μM, 24 h) treatment causes a decreases in Sertoli cells viability as demonstrated by the increased percentage of non-attached cells, as visualized by light microscopy (Fig. 1; D, black arrow). When these cells were analyzed by TUNEL assay, we observed a positive

nuclear staining with the morphological features of apoptotic cells (Fig. 1; B, white arrow). When trolox 0.1 μM, an analog synthetic of α-tocopherol, were co-administered there is a substantial decrease in nuclear TUNEL staining and a maintenance of cultured rat Sertoli cells viability (Fig. 1; C and E). There are no differences observed between groups during 12 h of retinol treatment (data not show).

Agarose Gel Eletrophoresis for detection of Internucleosomal DNA Fragmentation

Figure 2 demonstrates internucleossomal DNA fragments (ladder pattern) caused by 12 h of retinol 7 μM treatment (B), which is widely adopted as a fingerprint of apoptotic process. Trolox 0.1 μM co-administration inhibits DNA fragmentation induced by retinol (Fig. 2; C). No DNA fragmentation was observed in control untreated cells (A), or in Sertoli cells treated with vehicle alone (ethanol 0.1%) (data not show).

Cellular Redox environment

To verify a possible alteration on cellular redox environment caused by retinol, the total non-enzymatic antioxidant status (TRAP) of cultured Sertoli cells were determined during different times of treatment. Retinol caused a rapid and temporary oxidative pulse during the first 30 min of treatment, reverted by trolox 0.1 μM co-administration. After that, untreated control cells values were maintained for at least 24 h of retinol treatment (Fig. 3).

Mitochondrial Metabolism

Retinol supplementation (7 μM, 1 h) led to a temporary decrease in mitochondrial viability (Fig. 4; A). Indeed, retinol caused an abruptly increase in mitochondrial superoxide radical

generation (Fig. 4; B). Both effects were prevented by trolox 0.1 μM co-administration (Fig. 4; A and B).

Cytochrome *c* release and caspase-3 activation

Western blot analysis demonstrate that retinol treatment causes elevated cyt *c* release from mitochondria (Fig. 5; A). As a consequence, there is an increase in caspase-3 activity in cultured rat Sertoli cells treated with retinol (Fig.5; B). Caspase-3 activity increases at 4 h of retinol treatment and is maintained for at least 24 h of treatment, and is partially prevented by trolox 0.1 μM co-administration (Fig. 5; B).

PARP cleavage and ATP levels

To verify if there is a favorable conditions to apoptosis occurs in retinol treated Sertoli cells, we determined the pattern of poly(ADP-ribose) polymerase (PARP) cleavage and the cellular ATP levels. Figure 6 (A) demonstrates that PARP protein is cleaved in all groups analyzed. Indeed, there is a reversible decrease in ATP content in the initial firsts hours of retinol treatment, which is re-established to control values during the incubation period (Fig. 6; B).

Discussion

Several studies previously demonstrated oxidative stress generation by natural and synthetic retinoids [9-12, 21, 22], but the precise biochemical mechanisms underlining the

fate of retinoid chemotherapeutic intervention in the induction of programmed cell death by the activation of mitochondrial pathway remains uncertainly. In isolated rat liver mitochondria we demonstrated that retinol administration induces permeability transition and loss of membrane potential, increases lipoperoxidation, superoxide generation and enhances cytochrome *c* release (submitted). In this context, this report examines and suggest the mechanism involving the induction of programmed cell death mediated by the pro-oxidant effect of retinol supplementation. In cultured rat Sertoli cells vitamin A induces an oxidant-dependent apoptosis, as suggested by the reversion mediated by co-administration of trolox in the positive cellular TUNEL staining and in "ladder pattern" of DNA fragmentation observed in retinol treated cells (Fig. 1 and 2). The presence of the polyene conjugated double bounds characteristic of all natural retinoids could be the source for the pro- and antioxidant properties of the molecule. Moreover, the Fenton-mediated high reactive $^{\bullet}OH$ generation by the interaction of H_2O_2 with the increased cellular iron uptake and storage induced by retinol is another mechanism to explain prooxidant properties of vitamin A supplementation in biological systems [10].

Cellular administration of pro-vitamin A β-carotene cause an increase in subcellular concentration of this compound, principally in the mitochondrial fraction [24]. Retinol pro-oxidative effect caused a decreased mitochondrial viability and cytochrome *c* release (Fig. 4 and 5). Increased mitochondrial lipoperoxidation induces cyt *c* release by the oxidation of cardiolipin [25]. The release of cyt *c* to the extramitochondrial environment by retinol administration (Fig. 5) might lead to induce two processes: *i*) increased mitochondrial superoxide radical production (Fig. 4) [23]; *ii*) activation of the "apoptosome" complex and the effector caspase-3 (Fig. 5) [7]. Mitochondrial role on the activation of apoptotic

machinery may be limited by cellular redox environment [26], and the maintenance of ATP levels [27]. Because caspases are cysteine-dependent enzymes, their activity are sensitive to redox active agent such retinol. Even the redox status of cyt *c* will determine it capacity to activates caspases (Radi, 2003; personal communication). In fact, high oxidative environment change the pattern of cellular death from apoptosis to necrosis [27]. Retinol treatment is able to induces a cellular oxidative environment during the first half hour of incubation, but when cyt c is expected to be released to the cytoplasm – after 4 hour of incubation (Fig. 5) – the cellular redox environment is restored to control cells values (Fig. 3), permitting caspase-3 activation (Fig. 5).

Another limiting situation to activate apoptosis machinery is the intracellular ATP levels. During intensive oxidative biomolecule damage, with high DNA fragmentation, cellular PARP becomes activated. PARP activity causes a rapid depletion of NAD^+ content, leading to ATP depletion, which is required for apoptosis occurs [15]. Sertoli cells treated with retinol suffer intense DNA oxidative damage, including the formation of single and double strands fragment [10]. Surprisingly, PARP protein, a key substrate of cellular caspase-3, is in the cleaved state in control and retinol treated cells (Fig. 6). Apparently, the maintenance of cellular ATP levels during mitochondrial-mediated apoptosis in Sertoli cells could be mediated by cleavage-dependent-PARP diminished activity (Fig. 6).

In the view of the present results, we suggest that retinol is able to induces a prooxidant mitochondrial-dependent activation of the apoptosis machinery, inducing Sertoli cells to die by programmed cell death. Additional studies are required to understand the significance of retinol supplementation in models of apoptosis induction in malignant cells and the intracellular pathways responsible for these retinol effects.

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Figure Legend

Figure 1: Morphologic change of Sertoli cells during retinol treatment. Cells were treated for 24 h, harvested, stained with TUNEL and examined by fluorescence microscopy as described under "Materials & Methods" (original magnification, X400) (A-C) or directly analyzed by light microscopy (original magnification, X200) (D-F). Cells were treated with: *A* and *D*, no treatment; *B* and *E*, retinol 7 μM; *C* and *F*, retinol 7 μM + trolox 0.1 μM. The data are from a representative study that was repeated at least three times.

Figure 2: Effect of retinol on apoptotic internucleossomal DNA fragmentation. Sertoli cells were treated by 12 h, harvested and DNA fragmentation were analyzed by conventional agarose gel eletrophoresis as described under "Materials & Methods". Cells were treated with: *A*, no treatment; *B*, retinol 7 μM; *C*, retinol 7 μM + trolox 0.1 μM. The data are from a representative study that was repeated at least three times. M.W.; Molecular weight standard.

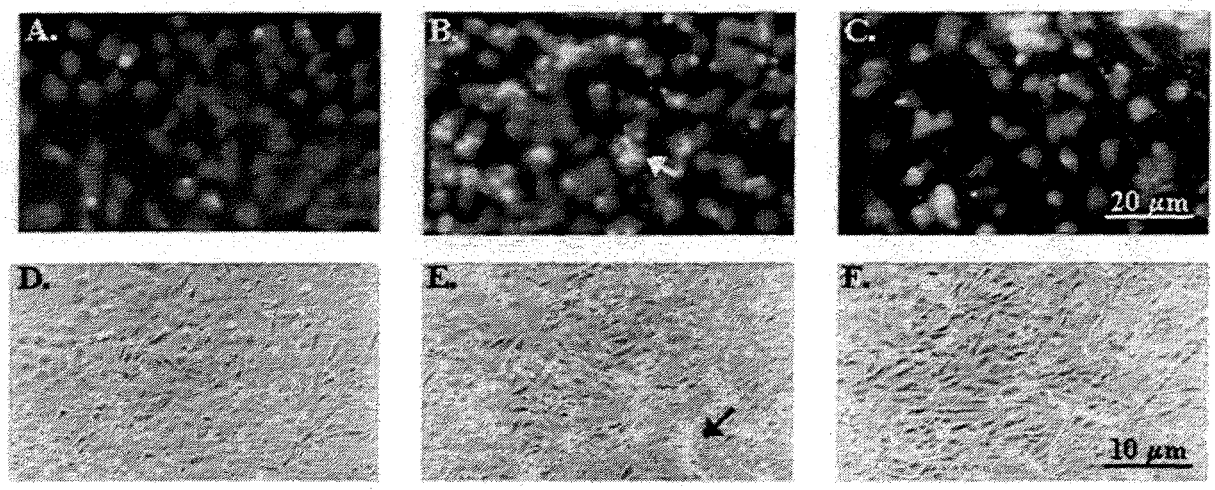
Figure 3: Inhibition of retinol-mediated change in Sertoli cells redox status by trolox co-administration. Cells were treated by different times and total radical-trapping antioxidant parameter (TRAP) was determined as described under "Materials & Methods". Cells were treated with: (■), no treatment; (O), retinol 7 μM; (▲), retinol 7 μM + trolox 0.1 μM. The data are from a representative study that was repeated at least three times.** Different from control cells ($p < .01$) (ANOVA).

Figure 4: Inhibition of the time course retinol-mediated increased superoxide radical generation and decreased mitochondrial viability in Sertoli cells by trolox co-administration. Cells were treated by different times and MTT reduction (A.) and mitochondrial $O_2^{\cdot-}$ production (B.) were determined as described under "Materials & Methods". Cells were treated with: (■), no treatment; (O), retinol 7 μM; (▲), retinol 7 μM + trolox 0.1 μM. Data represent mean ± SEM of triplicates per experiment and four independent experiments (n = 4).** Different from control cells ($p< .01$); * ($p< .05$) (one-way analysis of variance).

Figure 5: Time course of cytochrome *c* release and caspase-3-like activation in Sertoli cells treated with retinol. (A.) Western blot imunoassays for cyt *c* in cells permeabilized with digitonin and treated with retinol, as described under "Materials & Methods". Similar results were obtained in three separate experiments. (B.) At the times indicated, cell lysates were prepared and assayed for caspase-3-like activity using DEVD-AFC as substrate. Cells were treated with: (■), no treatment; (O), retinol 7 μM; (▲), retinol 7 μM + trolox 0.1 μM. Data represent mean ± SEM of triplicates per experiment and four independent experiments (n = 4).** Different from control cells ($p< .01$) (one-way analysis of variance).

Figure 6: PARP status and ATP levels in Sertoli cells after exposure to retinol. Cells were treated with retinol, lysed, and (A.) analyzed for cleavage of PARP by Western blot immunoassay after 12 h (similar results were obtained in three separate experiments), and (B.) assayed for ATP content. Cells were treated with: (■), no treatment; (O), retinol 7 μM; (Δ), retinol 7 μM + trolox 0.1 μM. Data represent mean ± SEM of triplicates per

experiment and four independent experiments (n = 4).* Different from control cells ($p<$ .05) (one-way analysis of variance).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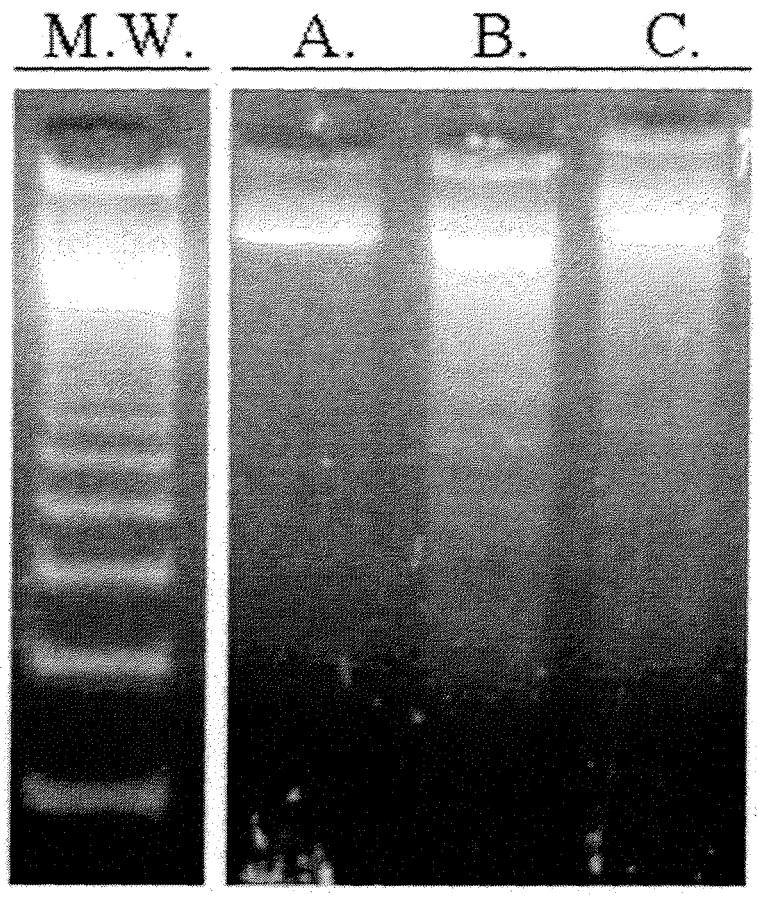
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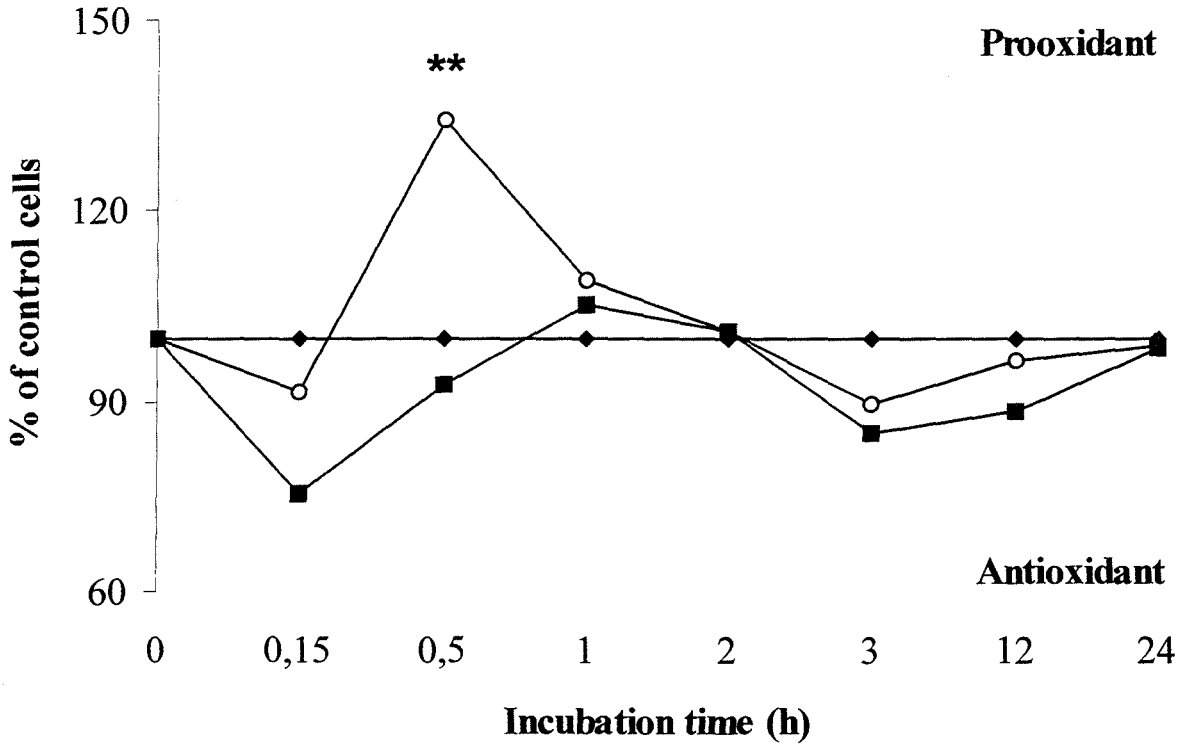
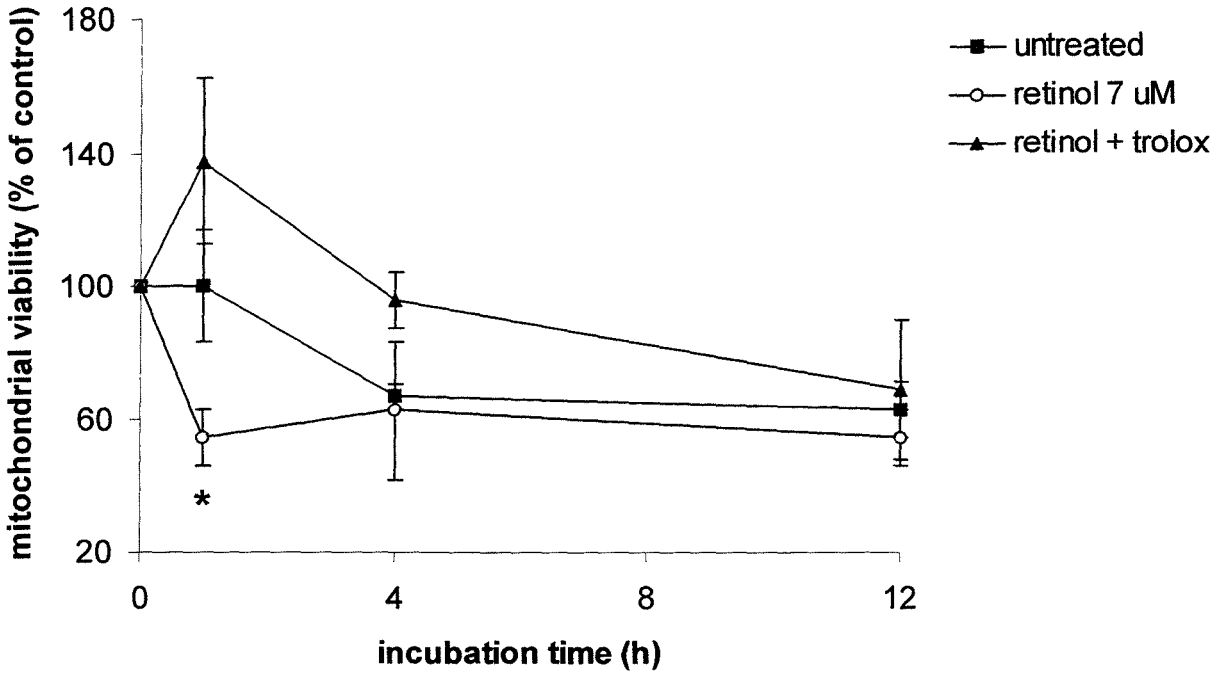
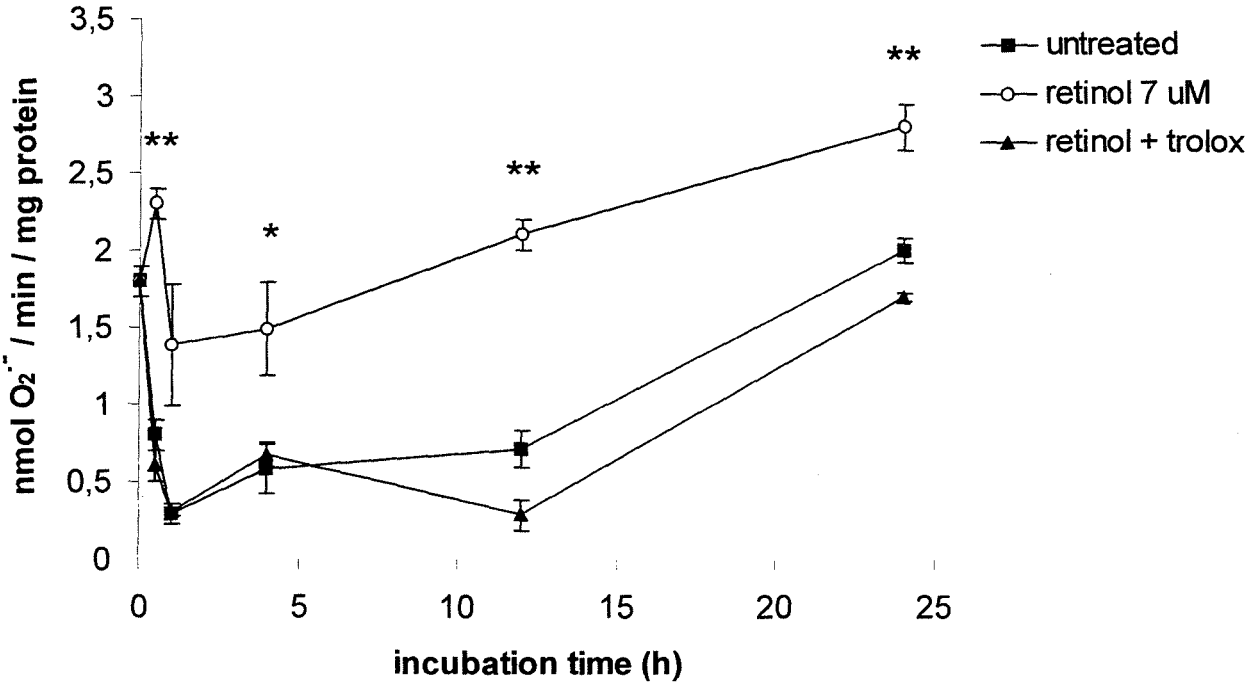

150
120
90
60
% of control cells
**
Prooxidant
Antioxidant
0
0,15
0,5
1
2
3
12
24
Incubation time (h)

A.


mitochondrial viability (% of control)
180
140
100
60
20
0
4
8
12
incubation time (h)
*
untreated
retinol 7 uM
retinol + trolox

B.


nmol $O_2^{\cdot-}$ / min / mg protein
3,5
3
2,5
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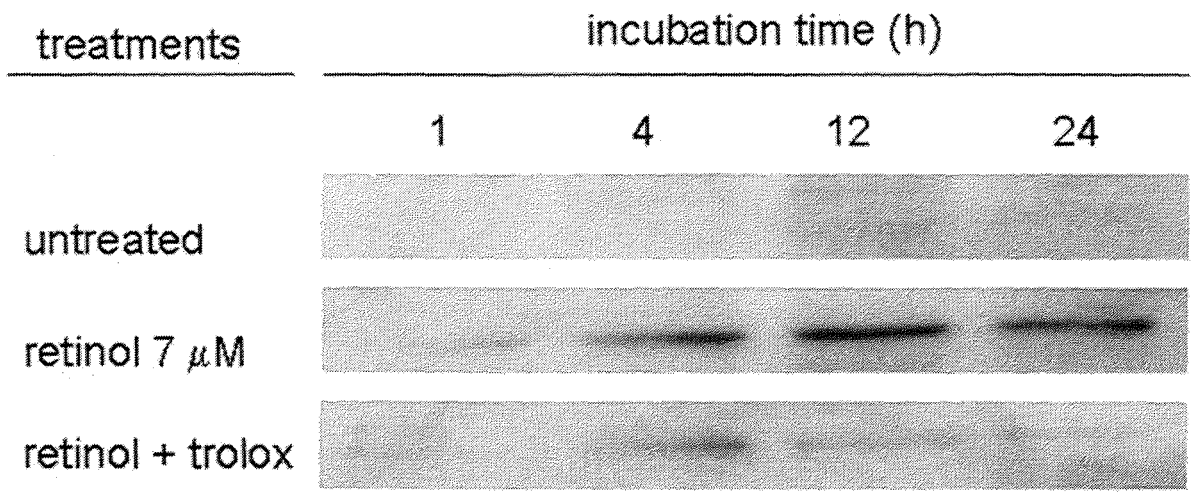
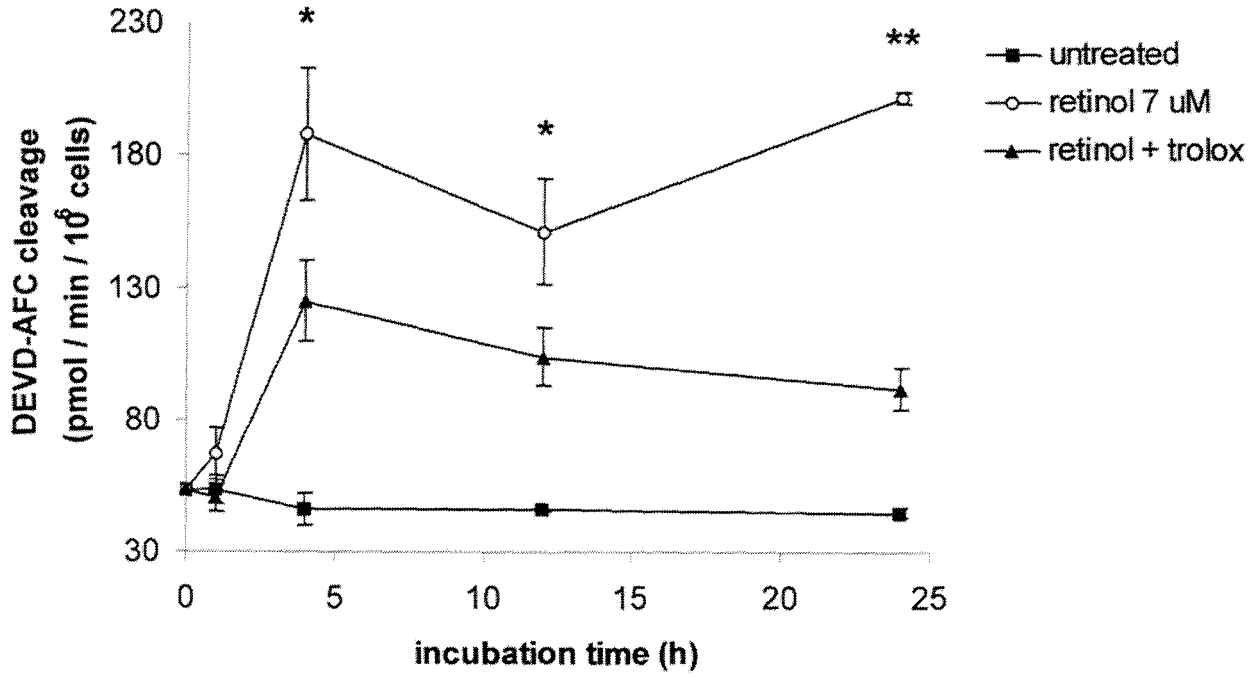
untreated
retinol 7 uM
retinol + trolox

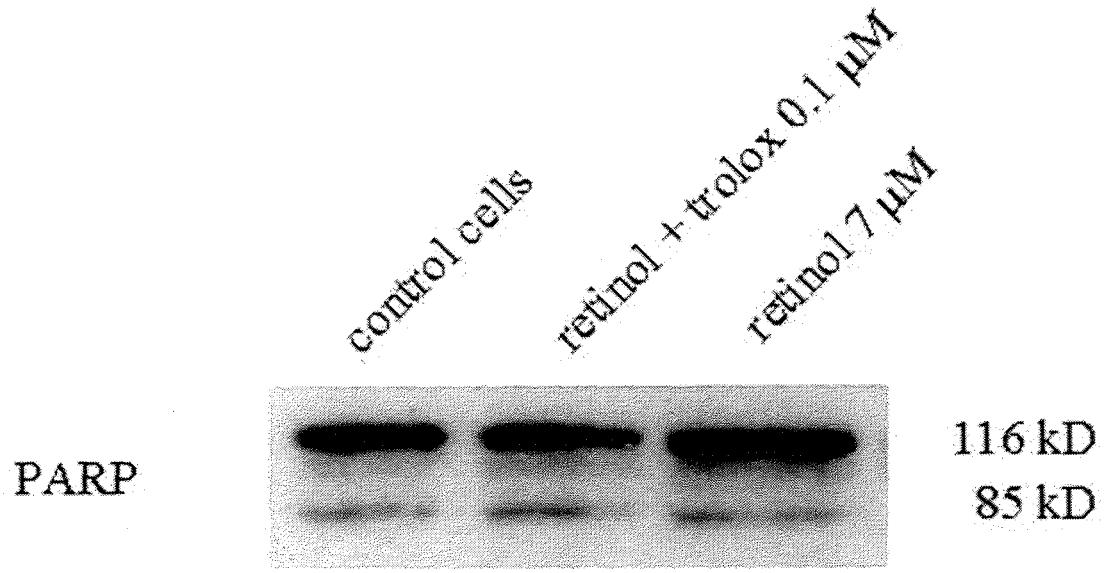
A.


treatments
incubation time (h)
1
4
12
24
untreated
retinol 7 μM
retinol + trolox

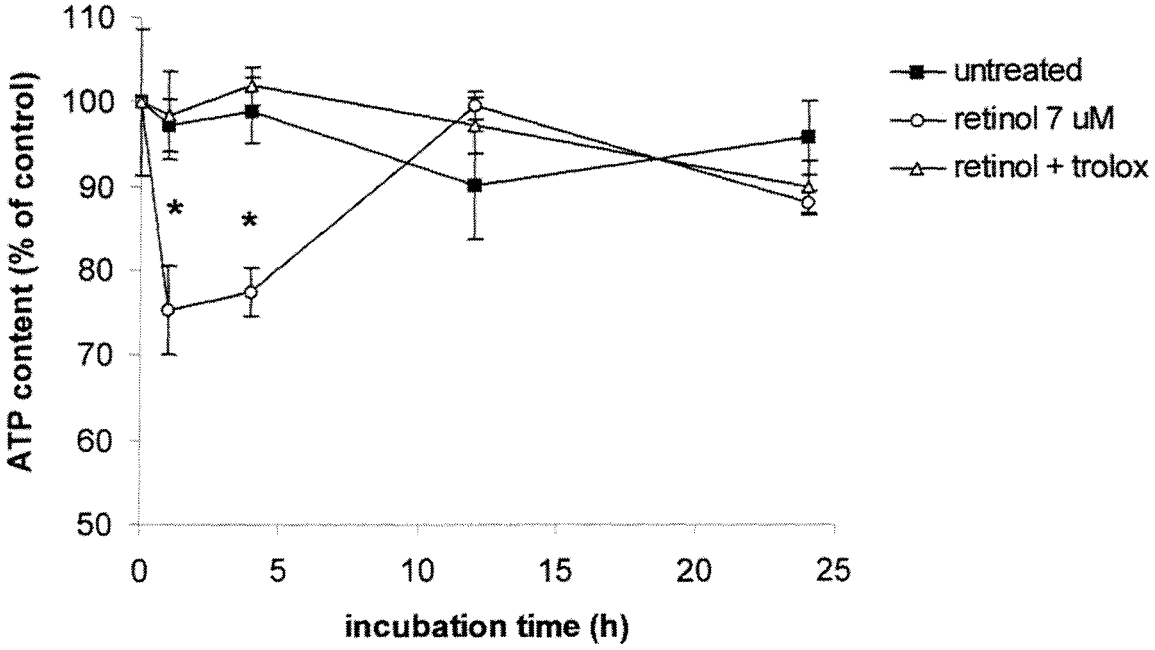
B.


DEVD-AFC cleavage
(pmol / min / 10^6 cells)
230
180
130
80
30
0
5
10
15
20
25
incubation time (h)
*
*
**
untreated
retinol 7 uM
retinol + trolox

A.


control cells
retinol + trolox 0.1 μM
retinol 7 μM
PARP
116 kD
85 kD

B.


ATP content (% of control)
110
100
90
80
70
60
50
0
5
10
15
20
25
incubation time (h)
*
*
untreated
retinol 7 uM
retinol + trolox

Capítulo VIII

Discussão Geral

8. DISCUSSÃO

Os resultados presentes nos capítulos III, IV, V, VI e VII tiveram como objetivo principal a busca da caracterização de uma possível ação biológica da vitamina A mediada pela produção de espécies reativas do oxigênio.

Quando submetemos cultivo de células de Sertoli, células estas um dos principais alvos fisiológicos da vitamina A, ao tratamento com retinol, verificamos uma diminuição na viabilidade celular na mesma intensidade que o efeito de uma dose de irradiação ultravioleta (Cap. III, Fig. 1). Tanto em 24 h quanto 48 h de tratamento, a suplementação com retinol causou um aumento na morte celular mediada pela UV. Este mesmo efeito, de intensificar o dano causado pela irradicação com UV, foi verificado quando analisamos tanto a lipoperoxidação total e níveis de produção de radicais livres, quanto a oxidação de lipídeos da fração mitocondrial e fragmentação de DNA, danos estes provavelmente responsáveis pela diminuição da viabilidade (Cap. III; Tabela 1, Fig. 2 e 3). Todos estes efeitos da suplementação com vitamina A foram revertidos aos níveis das células não-tratadas quando foi co-administrado um "scavenger" de radicais hidroxil, o manitol, ou 1,10-fenantrolina, um quelante de ferro.

Estes resultados apresentados no capítulo III nos sugerem que a vitamina A causou um aumento nos danos oxidativos causados pela irradiação com ultravioleta em cultivo de células de Sertoli, e que este fenômeno é dependente da formação de radicais hidroxil, provavelmente pela chamada reação de Fenton [10]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$$

$$Fe^{3+} + NADH \rightarrow Fe^{2+} + NAD^{\bullet} + H^{+}$$

Estes resultados corroboram trabalhos prévios do nosso grupo que demonstraram uma aumentada captação e armazenamento de ferro, principalmento na fração nuclear e mitocondrial de células de Sertoli, além de que a metabolização dos retinóides pelas dehidrogenases aumenta os níveis de NADH celular (Cap. I; fig 1), permitindo assim a formação de radical hidroxil ($^{\bullet}OH$) [25]. Como o dano oxidativo está relacionado com a iniciação e promoção de carcinogênese, nós sugerimos um possível mecanismo de ação do retinol, envolvendo um efeito pró-oxidante, no aumento da formação de tumores em camundongos irradiados com UV que receberam uma dieta rica em vitamina A [24].

O dano oxidativo mediado pela suplementação com retinol é dose dependente. No capítulo IV, nós demonstramos que doses de retinol entre 0,1 e 5 μM não induzem a lipoperoxidação nem aumentam a atividade da catalase, enzima esta chave na decomposição do peróxido de hidrogênio (Cap. IV; Fig. 2 e 3). Não obstante, tratamento com doses de 7 μM ou mais causaram dano oxidativo, revertido novamente pela co-administração de quelantes de metais ou scavenger de radicais $^{\bullet}OH$. A célula de Sertoli possui em torno de 5 μM de retinol, como sua concentração fisiológica.

Um dos marcadores mais utilizados para proliferação celular e promoção tumoral é a atividade da enzima ornitina decarboxilase (ODC), enzima chave na síntese de poliaminas. A atividade da ODC serve como um marcador de passagem de fase do ciclo celular, da fase G1 para S e, recentemente, demonstrou-se que sua expressão é induzida por radicais livres (Cap. IV). A ativação da ODC pelo tratamento com retinol é dependente do efeito pró-oxidante do retinol em células de Sertoli (Cap. IV; Fig. 1 e 2). Estes resultados

sugerem que a suplementação com retinol causa um dano oxidativo pela produção de espécies reativas do oxigênio e que, além do dano oxidativo, diversos genes responsivos a radicais livres são ativados, entre eles o gene de promoção tumoral ODC [14].

Como os retinóides são amplamente utilizados na prevenção e tratamento de diversas doenças epidérmicas e diferentes tipos de tumores [5] e, levando-se em conta os resultados obtidos nos capítulos III e IV, o próximo passo foi determinar se os radicais livres produzidos pelo tratamento com retinol possuiam um papel importante em uma provável genotoxicidade e promoção tumoral. No capítulo V estão agrupados os resultados que demonstram que a suplementação com vitamina A induz a quebra de fita simples e dupla de DNA, tanto em células de Sertoli quanto em linhagem de fibroblasto de pulmão (Cap. V; tabela 2, fig.1 e 2). Em um ensaio de genotoxicidade, utilizando-se do padrão de pelagem de moscas alimentadas com vitamina A, nós verificamos que o reitnol possui uma capacidade de induzir a fenômenos de recombinação gênica (Cap. V; tabela 1).

O intenso dano oxidativo ao DNA, somado ao evento de recombinação, são fortes promotores de instabilidade genômica, causando assim três processos biológicos distintos: a adaptação celular, a transformação celular e a morte celular. Previamente, nosso grupo demonstrou que o tratamento com retinol aumenta a atividade das enzimas de defesa antioxidantes, um evento claro de adaptação celular [26]. Mas estes mesmos resultados demonstram que o tratamento com retinol causa um aumento de três vezes na atividade da enzima superóxido dismutase (SOD) e apenas 20 % na atividade da catalase (CAT), quando comparadas às células controles. Este desbalanço leva a uma grande taxa de dismutação do radical superóxido, gerando o peróxido de hidrogênio, e uma ineficaz detoxificação deste que, ao acumular dentro da célula, reage com a grande quantidade de ferro presente formando, via reação de Fenton, o radical hidroxil [10, 25, 26]. Esta produção de espécies

reativas de oxigênio, em células de Sertoli cultivadas, servem como sinal mitogênico [28], levando à ativação de enzimas de passagem de fase de ciclo celular (ODC) (Cap. IV) e à proliferação celular. As células de Sertoli tratadas com retinol, quando analiasadas em citometria de fluxo, possuem uma distribuição alterada no ciclo celular, aumentando a população de células na fase S e na fase G2-M, demonstrando claramente a proliferação celular induzida por retinol. Além disso, há um aumento na população de células na fase hipodiplóide, sugerindo o processo de apoptose (Cap.V; fig. 3). Quando as células de Sertoli tratadas com retinol são mantidas em cultivo por 15 dias, evidenciamos a formação de focos proliferativos, um dos primeiros indícios de transformação celular pré-neoplásica (Cap. IV; fig 4). Estes resultados somados sugerem um mecanismo de ação pró-oxidativo da vitamina A estando relacionado com um aumento na incidência de tumores, como demonstrados em estudos prévios [23,24].

O dano oxidativo induz a morte celular, tanto via necrose quanto via apoptose, dependendo de que tipo de radical formado, de sua concentração e do tempo de exposição. No capítulo III (fig.1) nós verificamos uma diminuição na viabilidade celular mediada pelo tratamento com vitamina A e, no capítulo V, um aumento na população de células em fase hipodiplóide (fig. 3). Ambos resultados sugerem um aumento na morte celular mediada pelo retinol. Os retinóides são amplamentes utilizados na clínica como agentes quimioterápicos no combate e na prevenção de tumores, principalmente pela capacidade de indução de diferenciação celular (maturação) e morte celular programada (apoptose) mas, ainda hoje, o mecanismo de ação destes compostos não é bem conhecido.

O capítulo VI descreve uma possível via de ativação de apoptose mediada pela vitamina A sobre o metabolismo mitocondrial. A mitocôndria está relacionada com a ativação da maquinaria apoptótica, isto é, caspase-9 e caspase-3, pela liberação de fatores

pró-apoptóticos, principalmente o citocromo c. Apesar de bem conhecido o papel do citocromo c na ativação caspases, o mecanismo que induz a liberação de citocromo c pela mitocôndria e os agentes que modulam este processo ainda não estão bem determinados (Cap. VI).

O tratamento com retinol causa uma lipoperoxidação mitocondrial dose dependente, em mitocôndrias isoladas de fígado (Cap. VI; fig 4). Esta oxidação está relacionada com a perda de cardiolipina, lipídio de membrana que ancora fracamente o citocromo c na membrana interna da mitocôndria, causando a liberação deste para o espaço intermembrana (Cap. I; fig 2). A liberação para o citoplasma deve-se à perda transitória da permeabilidade mitocondrial (mPT), devido à abertura do chamado poro de permeabilidade transitória (PTP), ou a formação de canais na membrana externa mediada pela oligomerização de proteínas da família Bcl, como Bak e Bax (Cap. I; fig 2). Este processo, em última análise, leva à perda do potencial mitocondrial ($\Delta\psi m$) e ao "swelling". As figuras 1, 2 e 3 do capítulo VI demonstra um efeito dose dependente do tratamento com retinol nos processos de "swelling" e perda do potencial de membrana mitocondrial. Nós verificamos também um aumento na liberação de citocromo c (Cap. VI; tabela 1) e uma conseqüente diminuição no conteúdo total de citocromo c em mitocôndrias tratadas (Cap. VI; fig 5) e este aumento foi revertido pelo co-tratamento com trolox, um análogo sintético da vitamina E, sugerindo a participação do efeito pró-oxidante do retinol neste fenômeno. A liberação de citocromo c pelo tratamento com retinol provavelmente induz, na mitocôndria, um aumento na produção de radical superóxido pela cadeia transportadora de elétrons e diminuição da respiração celular, evidenciado pela diminuição na oxidação da sonda MTT, de uma maneira dose dependente.

Uma vez estabelecida, em mitocôndrias isoladas de fígado, uma possível via de ativação de morte celular programada pelo tratamento com retinol mitocôndria-dependente, o próximo passo foi de testar este modelo experimental em cultivo de células de Sertoli, e de determinar o possível papel do efeito pró-oxidante da suplementação com retinol neste processo.

Culturas de células de Sertoli foram tratadas com retinol 7 μM por 24 horas e co-tratadas com trolox e a apoptose foi determinada pela análise morfológica do padrão de marcação de núcleos pela técnica de TUNEL (Cap. VII; fig 1). Os resultados demonstram que a vitamina A foi capaz de induzir a apoptose, e que este efeito foi dependente da formação de radicais livres, já que o co-tratamento com antioxidante reverteu o processo. A fragmentação internucleossomal de DNA pelas endonucleases ativadas durante a apoptose gerou um padrão de escada (ladder pattern), quando estes fragmentos forão analizados em eletroforese em gel de agarose. Em 12 horas de tratamento já foi possível observar a fragmentação apoptótica do DNA de células tratadas com retinol, fragmentação esta revertida pelo tratamento com trolox (Cap. VII; fig. 2).

Uma vez determinado que os radicais livres produzidos pelos tratamento com retinol induziam apoptose, o papel da mitocôndria neste processo foi determinado. O tratamento com retinol causa um pulso oxidante temporário na célula (Cap.VII; fig. 3), que induz a diminuição temporária da viabilidade mitocondrial e leva a uma queda nos níveis de ATP (Cap.VII; fig. 4, 6). Todos estes processos ocorrem na primeira hora de tratamento, e podem ser revertidos pelo tratamento com antioxidante. Como conseqüência, há um aumento na produção de radicais superóxido pela cadeia transportadora de elétrons e uma liberação de citocromo c. A liberação de citocromo c, que ocorre em torno da quarta hora

de tratamento, leva a uma ativação da caspase-3, uma das enzimas efetoras na morte celular programada (Cap.VII; fig. 5). Para que ocorra a apoptose é necessário que exista, na célula, um ambiente favorável para a atividade das caspases, como manutenção dos níveis basais de ATP e um ambiente redutor. A figura 3 demonstra que o ambiente redox celular, no momento da liberação mitocondrial de citocromo c, se encontra propício para a a atividade das caspases, além de que os níveis de ATP não apresentaram diferenças com os das células controles (Cap. VII; fig 5). Um dos principais depletadores dos níveis de ATP durante o processo de morte celular é a enzima PARP. Esta enzima é ativada durante grande dano oxidativo ao DNA, evidenciado, em nosso modelo, no capítulo V (tabela 2). Em células de Sertoli a PARP apresenta-se na forma clivada e, consequentemente, inativada em todos os tratamentos, como demonstra a figura 5 do capítulo VII, permitindo que os níveis de ATP não variem durante a ativação e execução da morte celular programada. Estes resultados descrevem um mecanismo espacial e temporal ne ativação e indução da maquinaria apoptótica pelos radicais livres produzidos pela suplementação com vitamina A.

Capítulo IX

Conclusão

9. CONCLUSÃO

9.1 *Conlusões Específicas*

- O tratamento com retinol (7 μM, 24 h) amplificou os danos oxidativos causados pela irradiação ultravioleta em cultivos de células de Sertoli, provavelmente pela formação, via reação de Fenton, do radical hidroxil;

- O tratamento com retinol aumentou a atividade da enzima ornitina decarboxilase (ODC), e este aumento é mediado pela produção de espécies reativas de oxigênio;

- O tratamento com retinol causou quebra de fita simples e dupla de DNA, e recombinação mitótica. Este efeito genotóxico está relacionado com a progressão no ciclo celular e transformação pré-neoplásica, em diferentes modelos experimentais;

- O tratamento com retinol alterou o metabolismo de mitocôndrias isoladas de fígado, levando a um aumento na lipoperoxidação, produção de radical superóxido e liberação de citocromo c, induzindo a permeabilidade transitória mitocondrial e perda do potencial de membrana, de uma forma dose dependente;

- Em cultivo de células de Sertoli, o tratamento com retinol causou ativação da maquinaria apoptótica pela liberação mitocondrial de citocromo c e ativação de caspase-3, em um processo dependente da produção de radicais livres.

9.2 *Conclusão Geral*

Os presentes resultados, tomados em conjunto, sugerem que alguns fenômenos biológicos modulados pelo retinol, como o aumento na citotoxicidade da irradiação UV, a ativação de expressão gênica (ODC), a proliferação celular, a transformação pré-neoplásica, bem como a morte celular, são relacionados ao menos parcialmente à produção de espécies reativas de oxigênio.

Dada a ampla utilização dos retinóides em formulações, suplementações dietéticas e como co-adjuvante em intervensões terapêuticas, este trabalho nos possibilita evidenciar que, ainda nos dias de hoje, a comunidade médica e científica ainda não possui a real dimensão do amplo espectro de fenômenos influenciados pelo tratamento com vitamina A, além do papel dos radicais livres na modulação destes processos. A grande necessidade da busca e caracterização dos processos mediados pelos radicais livres produzidos pelo retinol serve de base para o futuro desenvolvimento de protocolos de utilização dos retinóides, permitindo assim uma maior eficácia e segurança, e, ao mesmo tempo, miminizando possíveis efeitos colaterais mediados pelo seu uso para a saúde pública.

Capítulo X

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