UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Análise de um Possível Papel de Biocondutor no Citoesqueleto de Células de Sertoli Tratadas com Retinol (Vitamina A).

Ramatis Birnfeld de Oliveira

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"O mundo que vemos através do filtro da atenção consciente é um fragmento daquele que nos é dado pela visão subliminar".

John Gray

Aos companheiros desta maravilhosa jornada terrena.

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PARTE I

RESUMO

Trabalhos recentes na literatura demonstraram que proteínas do citoesqueleto, mais especificamente os microfilamentos de actina, estão correlacionadas com a promoção e manutenção do estresse oxidativo, bem como modulação de processos mitocondriais. Trabalhos do nosso grupo caracterizaram que o tratamento com retinol (vitamina A) em células de Sertoli cultivadas era capaz de induzir a produção de espécies reativas de oxigênio (ERO), desbalanço na atividade de enzimas antioxidantes, com conseqüente promoção de estresse oxidativo. Radicais livres produzidos pelo tratamento com retinol, também foram capazes de induzir uma transformação pré-neoplásica, modulação de eventos recombinatórios, modulação de enzimas envolvidas na divisão celular como ornitina decarboxilase (ODC), progressão de ciclo celular e apoptose. O tratamento com retinol também foi capaz de, por uma via radical livre dependente, induzir a divisão de células de Sertoli terminalmente diferenciadas e não-proliferativas, com a conseqüente formação de focos proliferativos. Uma vez que o citoesqueleto participa de muitos dos processos relatados (proliferação, transformação e apoptose), e trabalhos recentes na literatura correlacionam estresse oxidativo com proteínas do citoesqueleto, nós decidimos avaliar a influencia do citoesqueleto e suas proteínas na produção e manutenção do estresse oxidativo gerado pelo tratamento com retinol. O primeiro capítulo dessa dissertação caracteriza uma modulação radical livre dependente do citoesqueleto das células de Sertoli tratadas com retinol, onde uma proteção/adaptação do citoesqueleto foi observada frente ao ambiente pró oxidativo gerado pelo retinol. Neste primeiro capítulo nós também sugerimos que a adaptação observada poderia ser relacionada com um possível papel de condução de elétrons pelos filamentos de actina. O segundo capítulo dessa dissertação demonstra uma dependência da correta organização dos filamentos de actina para a produção/manutenção

do estresse oxidativo gerado pelo tratamento com retinol. Juntamente, o segundo capítulo apresenta uma explicação para a teoria de condução de elétrons pelos filamentos de actina, onde é descrito como esse fenômeno poderia ocorrer. Os resultados apresentados nessa dissertação sugerem que os microfilamentos de actina são essenciais para a produção/manutenção do estresse oxidativo gerado pelo tratamento com retinol, e que essa dependência pode ser explicada por uma nova função fisiológica de biocondutor de elétrons.

ABSTRACT

Recent works presented in the literature have shown that cytoskeleton proteins, specifically actin microfilaments, are correlated with promotion and maintenance of oxidative stress, and mitocondrial functions modulations. Works of our research group have characterized that retinol treatment in cultived Sertoli cells was able to induce reactive oxygen species (ROS) production, antioxidant enzymes activity imbalance, resulting in oxidative stress production. Free radicals produced by retinol treatment, were also able to induce pre neoplasic transformation, modulation of recombinatory events, modulation of enzymes involved in cell division such as ornitina decarboxilase (ODC), cell cycle progression and apoptosis. Retinol treatment was also able by a free radical-dependent way, induce the division of terminate differentiated and non proliferative Sertoli cells, resulting in proliferative focus formation. Once cytoskeleton participates of many of the related process (proliferation, transformation and apoptosis), and recent works presented in the literature correlated oxidative stress with cytoskeleton proteins, we decided evaluated the cytoskeleton influence in the production and maintenance of oxidative stress generated by retinol treatment. The first chapter presented in this manuscript characterizes a cytoskeleton modulation free radical-dependent in the Sertoli cells treated with retinol, where a cytoskeleton adaptation/protection was observed in response to a pro oxidative environment produced by retinol. In this first chapter we also suggested that the adaptation observed could be related with a possible function of electrons conductions through actin microfilaments. The second chapter of this manuscript shows an actin microfilaments dependence to production/maintenance of oxidative stress generated by retinol treatment. Together, the second chapter shows an explanation of the electron conduction theory through actin microfilaments, where a description of the electron conduction process is

presented. The results presented in this manuscript suggest that actin microfilaments are essential to production/maintenance of oxidative stress generated by retinol treatment, and that this dependence could be explained by a new physiologic function of electron bioconductor.

LISTA DE ABREVIATURAS

ADH: álcool desidrogenases

ALDH: aldeído desidrogenases

AP-1: proteína ativadora-1

CAT: catalase

Cyt B: citocalasina B

DNA: ácido desoxirribonucléico

GPX: glutationa peroxidase

GSH: glutationa reduzida

GSSG: glutationa oxidada

H_2O_2: peróxido de hidrogênio

MAPK: mitogen activated protein kinase

NAD^+: adenina nicotinamida dinucleotídeo

NADH: adenina nicotinamida dinucleotídeo reduzida

NFkB: fator nuclear kappa B

Nrf2: nuclear factor E2 related factor

NO: óxido nítrico

$O_2^{\bullet -}$: radical superóxido

$^{\bullet}OH$: radical hidroxil

ODC: ornitina decarboxilase

ERO: espécies reativas de oxigênio

SOD: superóxido dismutase

1. INTRODUÇÃO

1.1 O citoesqueleto celular

O citoesqueleto é um sistema de filamentos que possui diversas funções celulares, ele é responsável por organizar as células espacialmente e permitir que elas interajam mecanicamente com seu ambiente. Muitas células também podem mudar sua forma, movimentar-se de um lugar para outro, bem como reorganizar seus componentes internos para dividir, crescer, adaptar e responder a mudanças no ambiente (Alberts *et al.,* 2002).

O citoesqueleto é composto por três grandes grupos protéicos: os *microfilamentos de actina, filamentos intermediários* e os *microtúbulos*. Microfilamentos de actina determinam a forma da superfície celular e são necessários para os movimentos celulares como um todo. Filamentos intermediários fornecem resistência ao estresse mecânico, e protegem o núcleo celular. Microtúbulos determinam as posições de organelas internas e direcionam o transporte intracelular. O citoesqueleto é um sistema dinâmico e adaptável, podendo polimerizar e despolimerizar seus filamentos com rapidez, permitindo que a célula responda a diversos estímulos e altere sua morfologia com incrível eficiência. Porém, sozinho o citoesqueleto seria ineficiente em controlar sua dinâmica e funções devido as suas características intrínsecas de polimerização e estrutura molecular, entretanto as células possuem *proteínas acessórias* que auxiliam e regulam diferentes propriedades do citoesqueleto por interagirem com ele (Alberts *et al.,* 2002).

Proteínas do citoesqueleto remodelam a forma celular através da dinâmica de polimerização. Microtúbulos a microfilamentos de actina adicionam e perdem subunidades somente nas extremidades dos polímeros, com uma extremidade crescendo mais rapidamente do que a outra. Tubulina a actina (que são as subunidades dos microtúbulos e

dos filamentos de actina) hidrolisam seus nucleotídeos trifosfatos quando entram nos polímeros, e essa hidrólise é uma importante característica do comportamento da dinâmica desses filamentos. Dependendo da proteína acessória a qual esses filamentos se associam eles podem desempenhar diferentes funções. Um exemplo é a interação de actina com proteínas da família *miosina* que em células musculares promovem a contração. Outro exemplo é a interação dos microtúbulos com a proteína *dineina* que está envolvida no transporte de vesículas. Embora actina e tubulina sejam extremamente conservados na evolução eucariótica, a família dos filamentos intermediários é extremamente diversa. Existem várias formas tecido específicas de filamentos intermediários, incluindo filamentos de queratina em células epiteliais, neurofilamentos em células nervosas, e filamentos de desmina em células musculares. Em todos esses tipos celulares, a função primária dos filamentos intermediários é fornecer resistência mecânica (Alberts *et al.,* 2002; Kreis e Vale, 1999).

A organização do citoesqueleto permite que uma célula seja uma estrutura polarizada, ou que apresente assimetria na sua organização espacial. Essa característica não significa que uma célula não tenha suas rotas funcionais e estrutura tridimensional mal organizadas, mas sim que a organização espacial dos seus componentes não é assimétrica. Os filamentos de actina, por exemplo, são necessários para o crescimento polarizado em leveduras, onde o alinhamento das suas fibras é um processo essencial para que esse crescimento ocorra. Interações entre actina e microtúbulos reforçam essa polaridade celular.

O citoesqueleto celular é um sistema de filamentos extremamente coordenado, e a interação de seus componentes com proteínas acessórias e proteínas celulares permite à célula manter a ordem interna, remodelar sua forma e superfície, mover organelas, e controlar sua sobrevivência ou morte (Alberts *et al.,* 2002; Kreis e Vale, 1999).

1.2 A vitamina A (Retinol) em células de Sertoli

A vitamina A (all-*trans* retinol) e seus derivados naturais, coletivamente referidos como retinóides, são nutrientes essenciais provenientes da dieta, responsáveis pela regulação de diversos processos fisiológicos tais como: embriogênese, reprodução e visão. A despeito de ter sido um dos primeiros compostos antioxidantes descritos, em 1913, a ampla ação biológica dos retinóides ainda nos dias de hoje permanece desconhecida (Duester, 1996).

A célula de Sertoli apoia sua região basal na lâmina basal e seu ápice livre fica direcionado para a luz dos túbulos seminíferos presentes nos testículos. As superfícies laterais das células de Sertoli se aproximam de tal maneira entre si, que se conectam profundamente por especializações de membrana do tipo ocludente, projetando-se totalmente sobre as espermatogônias e espermatócitos, formando desta maneira uma barreira hemato-testicular. Esse tipo celular é um dos alvos preferenciais dos retinóides em sistemas biológicos. Por sua localização e íntima associação com a progênie de espermatócitos em fase de diferenciação, oferecendo nutrição e apoio mecânico, acredita-se que as células de Sertoli controlem esse processo (Garcia *et al.,* 1991).

Retinol e o seu precursor ácido retinóico estão envolvidos na regulação da função testicular e espermatogênese. Uma vez dentro das células alvo, o retinol é convertido ao ácido retinóico – a forma descrita mais ativa biologicamente – pela ação de duas famílias de álcool desidrogenases. O primeiro passo é a conversão oxidativa reversível de retinol em retinal, mediado pela família de enzimas microssomais álcool desidrogenases (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 desidrogenases (ALDH) e por isoenzimas da família do citocromo P450 (CYP) (Clagett-Damel e DeLuca, 2002). A deficiência de retinol e ácido retinóico

induz a parada da espermatogênese, bem como afeta a produção de testosterona, que é outro fator regulador desse processo. Os retinóides podem exercer ações em três tipos celulares presentes nos testículos: Sertoli, Leydig e germinativas. Aparentemente, o retinol possui funções contrárias na regulação da espermatogênese que o ácido retinóico (Debier e Larondelle, 2005).

As ações biológicas do retinol e do ácido retinóico em culturas de células de Sertoli aparentemente envolvem espécies reativas como segundo mensageiros. Trabalhos recentes na literatura demonstraram que o retinol é capaz de gerar estresse oxidativo em células de Sertoli cultivadas, caracterizado por um aumento no dano oxidativo a biomoléculas (proteínas, lipídios e DNA) e desbalanço na atividade de enzimas antioxidantes (Dal-Pizzol *et al*., 2000). Outros trabalhos demonstraram que células de Sertoli terminalmente diferenciadas e não proliferativas, voltavam a dividir quando suplementadas com retinol em uma rota dependente de radicais livres (Dal-Pizzol *et al*., 2001). Também foram demonstrados efeitos dependentes de radicais livres como transformação pré-neoplasica, modulação de eventos recombinatórios, genotoxicidade e progressão de ciclo celular pelo tratamento com retinol (Klamt *et al*., 2003). A ativação de MAPK (mitogen-activated protein kinase) como ERK1/2 pelo tratamento com retinol em células de Sertoli cultivadas, também demonstrou ser radical livre dependente (Gelain *et al*., 2006). Esses trabalhos sugerem que uma modulação no momento oxidativo celular poderia estar controlando diversas rotas biológicas, e que o retinol poderia estar promovendo efeitos biológicos por produzir um ambiente pró-oxidativo.

Outros trabalhos demonstram que assim como o retinol, o seu principal metabólito ácido retinóico, também pode gerar danos oxidativos e modular a atividade de enzimas

antioxidantes, sugerindo que radicais livres também poderiam atuar como segundo mensageiros nas rotas biológicas deste composto (Frota Jr *et al*., 2006).

1.3 Espécies Reativas

Espécies reativas são compostos altamente reativos que podem trocar elétrons por reações de óxido/redução formando assim radicais livres. Os radicais livres são conceituados 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. Essa é uma definição ampla e é conveniente lembrar que a reatividade dos radicais livres varia grandemente entre estas moléculas ou átomos e o ambiente onde se encontram (Halliwell e Gutteridge, 1999).

Entre todas as classes de espécies reativas duas são as mais estudadas: Espécies Reativas e Oxigênio (ERO) e as Espécies Reativas de Nitrogênio (ERN). O termo ERO abrange coletivamente os radicais livres derivados do oxigênio ($O_2^{\bullet-}$, radical superóxido; $OH^{\bullet}$, radical hidroxil; $RO_2^{\bullet}$, radical peroxil; $RO^{\bullet}$, radical alcoxil) e os derivados não-radicais potencialmente oxidantes (H_2O_2, peróxido de hidrogênio; HOCl, radical hipoclorito; O_3, ozônio e singletos de O_2 – $^1O_2$) (Halliwell e Gutteridge, 1999). O termo ERN corresponde às espécies reativas derivadas do nitrogênio. Essas podem ser radicalares como o radical NO (óxido nítrico) e não radicalares como o $ONOO^-$ (peróxido nitrito) entre outros (Beckman e Koppenol, 1996). As espécies reativas são formadas em diferentes locais celulares sendo a mitocôndria o principal local de formação; durante a respiração celular cerca de 1 a 3% do oxigênio metabolizado gera ERO. Alguns trabalhos também demonstram que ERN também poderiam ser formadas na mitocôndria (Valdez *et al.,* 2005).

Por serem altamente reativas, ERO e ERN podem gerar danos a biomoléculas como lipídios, proteínas e DNA, causando mudanças na estrutura tridimensional, mudança de polaridade e perda de função (Halliwell e Gutteridge, 1999). Essas alterações a biomoléculas estão associadas com muitos processos patológicos como Alzheimer, neoplasias, aterosclerose, etc.

Mesmo sendo causadores de danos e podendo produzir disfunção celular, as espécies reativas também possuem papel fisiológico fundamental em sistemas biológicos, sugerindo uma adaptação dos organismos à presença do oxigênio ao decorrer da evolução. ERO e ERN participam ativamente em diversas rotas fisiológicas tais como: proliferação, diferenciação, transformação, resposta a patógenos, apoptose, modulação da forma celular, ativação de fatores de transcrição, etc. A participação ocorre em diversos níveis, desde sinalizadores celulares até como compostos finais da rota (Finkel, 1998). Um exemplo são moléculas como peróxido de hidrogênio, ácido hipocloroso e superóxido que são potentes microbicidas e são produzidas em grandes quantidades por macrófagos e neutrófilos (Hampton *et al*., 1998).

Para lidar com essas moléculas altamente reativas, mas essenciais para a homeostasia, os sistemas biológicos desenvolveram defesas antioxidantes. Existem dois grupos de defesas antioxidantes: as *enzimáticas*, que abrangem enzimas antioxidantes como Superóxido Dismutase (SOD), Catalase (CAT) e GPX (glutationa peroxidade); e as *não enzimáticas*, que engloba a glutationa (GSH), vitaminas provindas da dieta, ácido úrico, flavonóides, albumina e quelantes de metais. Essas defesas são responsáveis por manter um nível basal de espécies reativas, de maneira que elas não sejam tóxicas para as células, mas ao mesmo tempo não eliminem totalmente a sinalização produzida por essas espécies.

Quando existe um desbalanço entre as defesas antioxidantes e a produção de espécies reativas, as células desenvolvem um estado denominado *Estresse Oxidativo (EO)* (Halliwell e Gutteridge, 1999). Estresse oxidativo é encontrado em muitos processos patológicos, e as células desenvolveram mecanismos de "perceber" uma possível situação de EO, através de moléculas redox-sensíveis, e evitá-lo. Um exemplo de moléculas redox-sensíveis são alguns fatores de transcrição como NFKB (*Nuclear factor-kb*; fator nuclear-kb), AP-1 (*activated protein 1*; proteína ativadora 1), Nrf2 (*nuclear factor E2 related factor*; fator nuclear E2-fator relatado), ou diretamente resíduos de cisteínas presentes em vários tipos de proteínas e enzimas. Estresse oxidativo, portanto, não é uma situação fisiológica normal e há mecanismos celulares específicos para evitá-lo; os fatores que levam a promoção ou manutenção do estresse oxidativo até hoje têm sido amplamente estudados e discutidos.

1.4 Mitocôndria, Microfilamentos de Actina e Espécies Reativas.

A mitocôndria é uma organela celular onde ocorrem muitos processos biológicos fundamentais para a homeostasia dos sistemas biológicos, alguns exemplos são: ciclo do ácido cítrico, b-oxidação, produção de ATP, indução à apoptose, etc (Nelson e Cox, 2000). Outra característica importante é que a mitocôndria é o principal sítio de produção de ERO, e que muitos fatores podem influenciar a quantidade de ERO produzida nas mitocôndrias (Halliwell e Gutteridge, 1999).

Os microfilamentos de actina têm sido descritos como possíveis reguladores de algumas funções mitocondriais em resposta a diferentes rotas biológicas, podendo controlar a produção de ERO (Breitenbach *et al.,* 2005; Rudolf *et al.,* 2005). A ativação de GTPases, que são enzimas capazes de modular a organização dos microfilamentos de actina, foi

capaz de alterar funções mitocondriais como o potencial de membrana e produção de ERO dependente da alteração da organização de actina (Werner e Werb, 2002). Também foi demonstrada uma cooperação entre F-actina e mitocôndria para respostas endoteliais como mecanotransdução mediadas por espécies reativas (Ali *et al*., 2004). Rotas apoptótica independentes de caspases envolvendo TNF-α, aparentemente também dependem de actina e da produção de espécies reativas mediada pela mesma (Li J *et al*., 2004). Alguns estudos em leveduras demonstraram que a diminuição do turnover de actina, leva a acúmulos de grandes agregados de F-actina produzindo um aumento de ERO no citosol (Gourlay *et al*., 2004; Gourlay e Ayscough, 2005). Também em leveduras, a actina recentemente foi associada ao envelhecimento e apoptose por controlar a produção de ERO na mitocôndria (Gourlay e Ayscough, 2005). Trabalhos demonstraram que a arquitetura da F-actina pode controlar a liberação de citocromo C mitocondrial para o citosol, e a proteção da integridade da arquitetura da F-actina pode reverter esse processo (Paul *et al*., 2002). Substâncias que alteram a arquitetura dos microfilamentos de actina tem sido utilizadas como ferramentas para avaliar a influência desta estrutura nas funções mitocondriais. Em células U937 a alteração da arquitetura dos microfilamentos de actina pelo uso de Citocalasina B (Cyt B), uma micotoxina capaz de despolimerizar a F-actina, reverteu a apoptose induzida por residronato (Fujita *et al*., 2005). A reversão da produção de ERO também foi observada pelo uso de outros tipos de citocalasinas, como a Citocalasina D (Cyt D) (Yamamoto et al., 2005).

1.5 Fosforilação em Filamentos de Actina

A fosforilação de actina e proteínas acessórias, juntamente com outras modificações que podem ocorrer nas suas estruturas, estão relacionadas com o controle de sua dinâmica de polimerização e despolimerização (Kreis e Vale, 1999). Muitos estudos associam a fosforilação de actina como um meio alternativo de regulação. Um exemplo, é o estímulo de fatores de crescimento em fibroblastos induzindo a fosforilação de resíduos de serina na actina (van Delft *et al*., 1995). Uma inibição na fosforilação da actina induzida por lipopolisacarídeos, foi associada com uma inibição da produção de citoquinas em monócitos (Hauschild *et al.,* 1997). Em *Physarum polycefalum* foi caracterizada uma quinase específica para actina, chamada de actin-fragmin-kinase (AFK) (Gettemans *et al.,* 1993). Também foi demonstrado que actina pode ser fosforilada no resíduo tirosina 53 de *Dictyostellum*, a fosforilação deste resíduo aparentemente é específica para este organismo, uma vez que a fosforilação desde não foi encontrada em alguns tipos de tumores de mamíferos (Liu *et al.,* 2006). Outro exemplo é a ativação da PAK1 (p21-associated-kinase 1) pela PI3K (phophatidylinositol 3 kinase), que pode fosforilar a actina e promover a dissolução de fibras de estresse com conseqüente rearranjo do citoesqueleto de actina (Papakonstanti e Stournaras 2002).

Evidências recentes demonstram que a actina pode ser substrato para a atividade quinásica da serina/treonina quinase AKT/PKB, onde foi proposto que muitas das funções regulatórias desempenhadas pela AKT/PKB na fisiologia celular, poderiam ser em parte mediada pela interação e subseqüente fosforilação da actina (Vandermoere *et al*., 2006).

1.6 Condução de elétrons em biomoléculas

Embora a condução de elétrons em biomoléculas já tenha sido caracterizada há muito tempo, como por exemplo na cadeia transportadora de elétrons da mitocôndria e nuclear, são recentes os estudos de biocondução em outros tipos de proteínas e moléculas.

Um fluxo de elétrons dentro da estrutura da enzima NADPH Oxidase com conseqüente formação do radical superóxido foi demonstrado em neutrófilos humanos, sugerindo que elétrons poderiam ser conduzidos por proteínas fora da mitocôndria (Schrenzel *et al*., 1998). Trabalhos recentes também demonstraram que proteínas purificadas poderiam ligar e densamente cobrir a superfície de hematita de ferro, reduzir ferro III, e criar um fluxo de elétrons (Xiong et al., 2006). Outras características bioelétricas de proteínas também estão sendo exploradas atualmente. Um exemplo é a característica que microfilamentos de actina possuem de serem o alvo celular de campos eletromagnéticos de baixa potencia (Gartzkel e Lange, 2002). Microfilamentos de actina também estão associados com uma modulação na atividade elétrica e mecânica em miócitos cardíacos (Calaghana *et al*., 2004).

Muito pouco se sabe das propriedades elétricas de biomoléculas. Atualmente este é um campo de estudo que vem crescendo e ganhando considerável espaço na literatura, uma vez que muitos dos processos celulares conhecidos poderiam ser explicados, ou melhor entendidos, por essas propriedades elétricas das biomoléculas.

2. OBJETIVOS

2.1 Objetivo geral

Uma vez que novas evidências demonstram que os microfilamentos de actina estão intimamente relacionados com o controle de processos mitocondriais, e desta maneira, poderiam estar participando da produção de espécies reativas e consequentemente da produção ou manutenção do estresse oxidativo, a presente dissertação teve por objetivo avaliar a participação da arquitetura dos microfilamentos de actina frente ao estresse oxidativo gerado pelo tratamento com retinol em células de Sertoli.

2.2 Objetivos específicos

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

- Capítulo I: Avaliação da modulação do citoesqueleto de células de Sertoli cultivadas frente ao estresse oxidativo produzido pelo tratamento com retinol (vitamina A), e proposta de um novo papel fisiológico para os microfilamentos de actina.

- Capítulo II: Avaliação da necessidade do citoesqueleto de actina para a produção/manutenção do estresse oxidativo produzido pelo tratamento com retinol em células de Sertoli, e apresentação de uma sugestão/hipótese de como elétrons poderiam ser transportados pelos microfilamentos de actina.

PARTE II

CAPÍTULO I

MORPHOLOGICAL AND OXIDATIVE ALTERATIONS ON SERTOLI CELLS CYTOSKELETON DUE TO RETINOL-INDUCED REACTIVE OXYGEN SPECIES

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Morphological and oxidative alterations on Sertoli cells cytoskeleton due to retinol-induced reactive oxygen species

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Abstract

Retinol (vitamin A) is involved in several cellular processes, like cell division, differentiation, transformation and apoptosis. Although it has been shown that retinol is a limitant factor for all these processes, the precise mechanisms by which retinol acts are still unknown. In the present study we hypothesised that alterations in the cytoskeleton of Sertoli cells induced by retinol supplementation could indicate an adaptive maintenance of its functions, since it plays an important role in the transformation process that we observed. Previous results demonstrated that Sertoli cells treated with retinol showed an oxidative imbalance, that leads the cell to two phenotypes: apoptosis or transformation. Our group has identified characteristics of Sertoli cells transformed by retinol which results in normal cell functions modification. In the present study the actin filament fluorescence assay and the deformation coefficient showed a modification in the morphology induced by retinol. We also observed an oxidative alteration in isolated cytoskeleton proteins and did not show alterations when these proteins are analyzed by electrophoreses. Our results showed an increase in mitochondria superoxide production and a decrease in nitric oxide levels. All results were partially or completely reverted by co-treatment of the antioxidant Trolox®. These findings suggest that the cytoskeleton components suffer individual alterations in different levels and that these alterations generate a global phenotype modification and that these processes are probably ROS dependent. We believe that the results from this study indicate an adaptation of the cytoskeleton to oxidative imbalance since there was not a loss of its function. (Mol Cell Biochem **271:** 189–196, 2005)

Key words: cytoskeleton, retinol, Sertoli cells, ROS

Introduction

Retinol (vitamin A) and its metabolites are involved in a number of pathologies [1, 2]. It has been suggested that its effects in several diseases are secondary to their free radical scavenger activity in living systems [3]. However, several authors propose that they are actually pro-oxidants which could lead the cells to carcinogenesis through oxidative damage [4, 5].

It has been demonstrated that the cytoskeleton can be modulated by free radicals [6, 7], and that these phenomena are

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associated with cell transformation [7]. It is also known that the cytoskeleton organization reflects directly on the cell shape and it is, in part determined by cellular interactions with the extracellular matrix [8, 9].

There are few works studying retinol effects in the cytoskeleton. Mermelstein et al. [10], demonstrated that retinol influences the reorganization of hepatic stellate cells GRX cytoskeleton, leading to phenotype transformation. Previous studies from our group showed that retinol treatment leads to an increase in ornitine decarboxilase activity in Sertoli cells. This increase is attenuated by the co-treatment with either iron chelator or free radicals scavengers [11], suggesting that this effect is mediated by reactive oxygen species (ROS). Ornitine decarboxilase is involved in normal cellular processes, neoplasic growth and invasive process *in vitro* [12].

Our laboratory demonstrated that retinol treatment induces alterations in the phosphorylation levels of nuclear proteins [13], which can indicate an effect in gene regulation. In addition, we demonstrated that retinol treatment induces a mitotic signaling through superoxide radical production, which leads to cell proliferation and focus formation in terminal-differentiated rat Sertoli cells [14]. Increased activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), were also observed as a result of retinol 7 μM supplementation in rat Sertoli cells [15]. We have also demonstrated a retinol-mediated increase in iron internalization [16], which can increase iron availability for Fenton reaction generating hydroxyl radical [17]. These results suggest that some of retinol effects in biological systems are mediated by free radical production, since the addition of iron chelator or free radical scavengers has attenuated these effects.

Our group has previously shown that 7 μM retinol (but not 5 μM) treatment can lead to two different cellular phenomena: apoptosis and transformation. In addition, Klamt et al. [18] demonstrated that retinol supplementation induces genotoxicity, and leads the cells to changes in their cell cycle phase. An increase in DNA single and double-strand breaks, and a mutagenic potential were also caused by retinol treatment [18]. Intriguingly, the retinol concentration which causes these deleterious effects (7 μM) is close to the basal levels of retinol in Sertoli cells (5 μM) [19]. It is well-known that the cytoskeleton modulation is involved in the regulation of these processes [20], which could indicate that retinol is able to influence cell morphology.

In this regard, the present work aims to evaluate the effect of retinol on the cytoskeleton of the retinol-transformed population of rat Sertoli cells which did not enter apoptosis. We believe that the retinol treatment possibly generates an oxidative imbalance and subsequently cell transformation and, at the same time, could lead to adaptive/preservative alterations in the cytoskeleton through an unknown mechanism. This adaptive/preservative alteration in the cytoskeleton probably occurs because the cytoskeleton is an essential component for the cell integrity and fundamental for the cell transformation process. As we have previously described the physiologic concentration of retinol 5 μM was not pro-oxidant and toxic, while the concentration of 7 μM was strongly deleterious [14–18], in this work we compared the effects of both retinol concentrations on the cytoskeleton of Sertoli cells.

Materials and methods

Chemicals and animals

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. Rhodamine-phalloidin was purchased from Molecular Probes Inc. (Eugene, OR). Pregnant wistar rats were housed individually in plexiglass cages. 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.

Sertoli cells culture and treatment

Sertoli cells from 15-day-old Wistar rats were isolated and cultured as previously described [13]. Briefly, animals were killed by cervical dislocation, testes were removed and washed in saline pH 7.4; Sertoli cells were isolated by enzymatic digestion of uncapsulated 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 37°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 (vitamin A) 5 μM, 7 μM, 7 μM + 0.1 mM Trolox®, 0.1 mM Trolox® and vehicle (ethanol 0.1% v/v) for 24 h. Trolox® is a hydrophilic analogue of vitamin E, and was used as a standard antioxidant.

Obtaining of submitochondrial particles (SMP)

Sertoli cell cultures were washed with saline buffer (pH 7.4) and homogenized in a medium containing 230 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 700 × g for 10 min to eliminate nuclei and cell debris and the pellet was washed to enrich the supernatant; the supernatant was centrifuged at 7000 × g for 10 min. The pellet was washed and resuspended in the same buffer, consisting mainly of intact mitochondria able to carry out oxidative phosphorylation.

The operations were carried out at 0–2°C. Submitochondrial particles (SMP) were obtained by freezing and thawing (three times) the isolated mitochondria. For superoxide production measurements, SMP were washed twice with 140 mM KCl, 20 mM Tris-HCl (pH 7.4) and suspended in the same medium.

Superoxide production in Sertoli cells SMP

Superoxide production was determined in washed SMP using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37°C (E480 nm$\frac{1}{4}$4.0/mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl (pH 7.4), SMP (0.3–1.0 mg protein/mL), 0.1 mM catalase, and 1 mM epinephrine. Succinate (7 mM) was used as substrate. Superoxide dismutase was used at 0.1–0.3 mM final concentration to give assay specificity.

Cytoskeleton proteins isolation

After isolation and treatment with different doses of retinol and Trolox®, Sertoli cells cytoskeleton proteins were isolated. Cell cultures were washed with phosphate-buffered saline (136.5 mM NaCl, 0.27 mM Na_2HPO_4, 1,10 mM KH_2PO_4) and extracted with ice-cold buffer containing 1% Triton X-100, 600 mM KCl, 5 mM EDTA, 2 mM EGTA, 2 mM PMSF, 50 mM Tris-HCl (pH: 6,8) and protease inhibitor cocktail (p-8340, Sigma). Cell lysates were centrifuged (13000 × g, 4°C, 15 min). Pellet was treated with DNAse-I (0.5 mg/mL), centrifuged (13000 × g, 4°C, 5 min) and the new pellet washed with 5% trichloroacetic acid (TCA). After centrifugation (13000 × g, 4°C, 5 min), insoluble fraction was treated with ethanol and acetone respectively. The isolated cytoskeletal proteins were solubilized in specific buffer for each assay. Total proteins resulted were measured by Bradford [21].

Protein oxidative damage (carbonyl)

As index of protein oxidative damage, the carbonyl groups were determined according to Levine et al. [22]. Isolated cytoskeletal proteins were divided into four aliquots of 200 μL (∼0.2 mg of protein). Proteins were precipitated by the addition of 100 μL 20% TCA for 5 min on ice, and centrifuged at 4000 × g for 5 min. The pellet was dissolved in 100 μL of NaOH 200 mM, and 100 μL of HCl 2M or 10 mM of 2,4-dinitrophenylhydrazine (DNPH) in HCl 2M was added to duplicate aliquots for blanks or for derivatizing of carbonyl groups respectively. Samples were maintained for 30 min at room temperature. Proteins were precipitated with TCA, and washed three times with 500 μL 1:1 ethanol:ethyl acetate with 15 min standing periods to remove excess of DNPH. Samples were dissolved in 200 μL 6M guanidine in 20 mM KH_2PO_4, pH 2.3 and the absorbance was read at 370 nm. The carbonyl content (nmol/mg protein) was calculated using a molar extinction coefficient of 22000/M/cm at 370 nm after subtraction of the blank absorbance. The results were normalized by total protein, assayed by Bradford [21].

Electrophoresis assay

Isolated cytoskeleton proteins were dissolved in 1% sodium dodecyl sulfate (SDS). Sample buffer (0.0625M Tris-HCl, 10% Glycerol, 3% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue, pH: 6.8) was used 1:1 (v/v) with cytoskeleton proteins solution. The samples (35 μg of protein per lane) were subjected to 10% SDS-polyacrylamide gel (SDS-PAGE) electrophoresis (120 V, 60 mA). Resulting gel was washed three times with distillated water, and incubated with comassie blue (50% methanol, 10% acetic acid, 0.1% comassie blue R, q.s.p. water) for 1 h. The gel was then discolored by descorant solution (1:3:6 acetic acid/methanol/water) for 1 h. Discolored gel was fixed with cellophane paper, and dried for 24 h. Resulting lanes were analyzed by densitometric assay.

Morphologic structure of actin filaments

To evaluate a possible disassembly of actin filaments induced by retinol treatment-produced ROS, we used rhodamine-marked phalloidin. Cells were cultured in glass coverslips in similar densities. After 24 h of retinol treatment, cells were fixed with 4% paraformaldehyde (PFA), and washed three times with PBS. The excess aldehyde groups were blocked by glycine solution incubation (20 mM Tris, 150 mM glycine, pH: 8.0) during 15 min. The fixed cells were permeabilized with 0.3% Triton X-100, and blocked with 3% albumin for 1 h in room temperature. Rhodamine-phalloidin in 3% albumin incubation (45 min, 37°C in dark) was performed. The cells were washed with water, and coverslips were fixed with fluorsafe and incubated for 30 min in room temperature. Actin filaments were then visualized by fluorescence microscopy.

Deformation coefficient D mensuration

Deformation coefficient D measurement was carried by protocol adaptation [23]. Briefly, the morphological relation between spreading and confluent cells was measured, which gives an estimate of cell plasticity. The increase in the

difference between spread and confluent cells implies an increase of coefficient D. Data were resampled with replacement (bootstrapped) 1000× to estimate sample error [24]. Morphometrical measurements were obtained by NIH-image program analysis of the scanned phase contrast photomicrographs of cells plated as dispersed and confluent densities. At least 35 cells from each experimental group in three independent experiments were measured to estimate shape parameters of each cell. Data are reported as mean ± SEM, with the level of significance set at $P < 0.05$.

Nitric oxide levels

Nitric oxide levels were measured by total amount of nitrite present in cultured Sertoli cells medium. We used a Griess reagent kit for nitrite determination *G-7921* (Molecular Probes). The medium was collected after treatments and centrifuged (2000 × g, 4°C, 15 min) to eliminate cellular *debris*. Griess reagent was used (Griess reagent 1:3 medium) as the color reagent. The samples were incubated in the dark for 30 min in room temperature, and analyzed in 548 nm in spectrophotometer. Retinol and Trolox® absorbance were deducted to total values. The results were normalized by total protein, assayed by Bradford [21].

Statistic analysis

Results are expressed as the mean ± StandardError(S.D.). Data were analyzed by one-way analysis of variance (ANOVA), using an Independent T-test to compare mean values across groups. Differences were considered to be significant when $p < 0.05$. Densitometric analysis are performed by ImageJ® software.

Results

Retinol treatment enhanced superoxide radical production

Retinol treatment at 7 μM leads to an increase in mitochondrial superoxide production, and Trolox® co-treatment reversed this effect. Cells receiving the vehicle alone or 5 μM retinol did not show significant differences in mitochondrial superoxide production compared to control cells (Fig. 1).

Oxidative damage to isolated cytoskeleton proteins

In contrast to the results on superoxide production, after 24 h 7 μM retinol led to a decrease in oxidative damage of cytoskeleton proteins as determined by carbonyl assay. Trolox® co-treatment and Trolox® alone led to a decrease in carbonyl levels (Fig. 2). Previous results demonstrated that retinol 7 μM treatment increased Sertoli cells homogenate oxidative stress levels [15]. These findings suggest that cytoskeleton proteins have adapted themselves to the increase in free radical production. We believe this may explain why they were not damaged by oxidative stress.

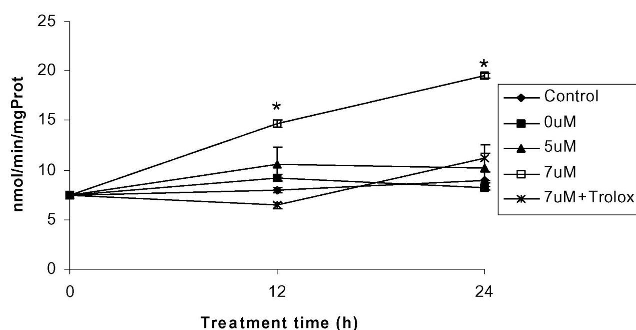

Fig. 1. Increased production of superoxide radical by submitochondrial particles after retinol treatment. Superoxide production was determined using a spectrophotometric assay based on superoxide-dependent oxidation of epinephrine to adrenochrome at 37°C (E480 nm$\frac{1}{4}$4.0/mM/cm). *different from control; $p < 0.05$; $n = 3$.

SDS-PAGE electrophoresis of cytoskeleton proteins are not affected by retinol treatment

Cytoskeleton proteins were fractionated by 10% SDS-polyacrilamide gel electrophoresis. Neither retinol nor Trolox® caused any difference on the total amount of each cytoskeletal protein fraction compared to the vehicle alone or control, as analyzed by dry gel densitometry (Fig. 3).

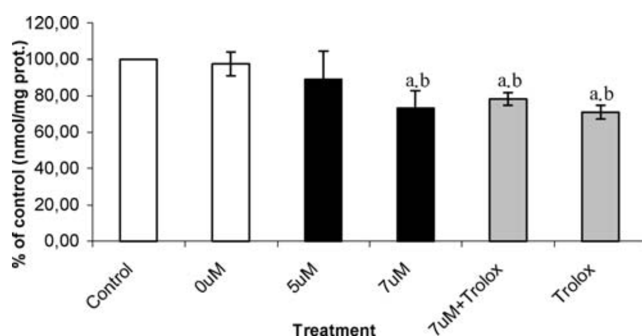

Fig. 2. Protein oxidative damage index (carbonyl levels). Retinol produced a decrease in oxidative damage to isolated cytoskeleton proteins. All treatments were carried out for 24 h in 37°C humidified atmosphere with 5% CO_2. [a]different from control; [b]different from vehicle (ethanol). Data are reported as mean ± SE, $p < 0.05$, $n = 3$.

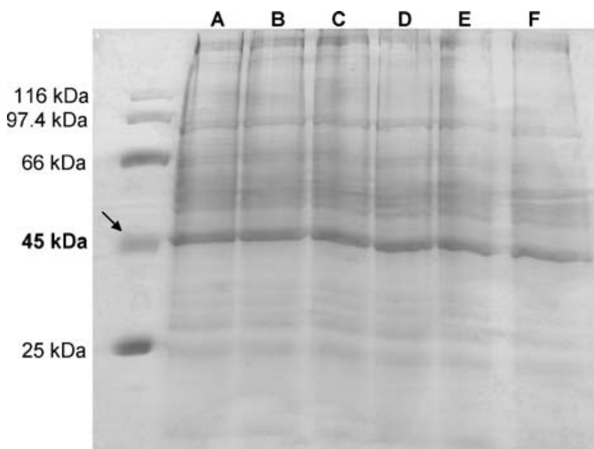

Fig. 3. Electrophoresis assay of cytoskeleton proteins. After isolation of cultured Sertoli cells cytoskeleton proteins, the samples were subjected to 10% SDS-polyacrylamide gel (SDS-PAGE) electrophoresis. The densitometry analysis of dry gel are performed by ImageJ® (data not shown). No differences were found in cells treated with retinol or Trolox®. A) control; B) vehicle; C) 5 μM retinol; D) 7 μM retinol; E) 7 μM retinol + 0.1 mM Trolox®; F) Trolox® 0.1 mM. Black arrow indicates α-actin lane (45 kDa) in the protein molecular weight pattern. Data representative of three independent experiments, $n = 3$.

Actin filament changes promoted by retinol treatment is reactive oxygen species dependent

Actin fibers observation by florescence demonstrated that the treatments lead to different fiber organizations. The cells treated with retinol 7 μM (Fig. 4D) showed more elongated actin fibers than the control cells and the vehicle (Fig. 4A and 4B). Retinol 5 μM (Fig. 4C) present larger and more spread actin fibers when compared to control cells and with cells that received the vehicle alone or retinol 7 μM. Cells that were co-treated with retinol 7 μM + Trolox® 0.1 mM (Fig. 4E) showed the same phenotype of the cells treated with retinol 5 μM. The vehicle alone did not induce any phenotype changes when compared to control cells. We did not observe any stress fiber formation following the treatments. These results show that retinol supplementation modifies the actin fibers and that there probably is an involvement of reactive oxygen species in this phenomenon.

ROS-dependent phenotypic changes

Retinol induced a phenotypic alteration in rat cultured Sertoli cells at disperse and confluent densities. Retinol 7 μM presented the greatest morphological variability index (Table 1). Trolox® co-treatment reversed retinol effects, which reinforces the hypothesis that the effect of retinol is mediated by ROS. Phenotype modification by retinol suggests that retinol 7 μM induces a global cellular alteration.

Nitric oxide levels

Reactive nitrogen species (RNS) can modulate the cytoskeleton components [25]. For that reason we investigated the influence of retinol on nitric oxide levels by total amount

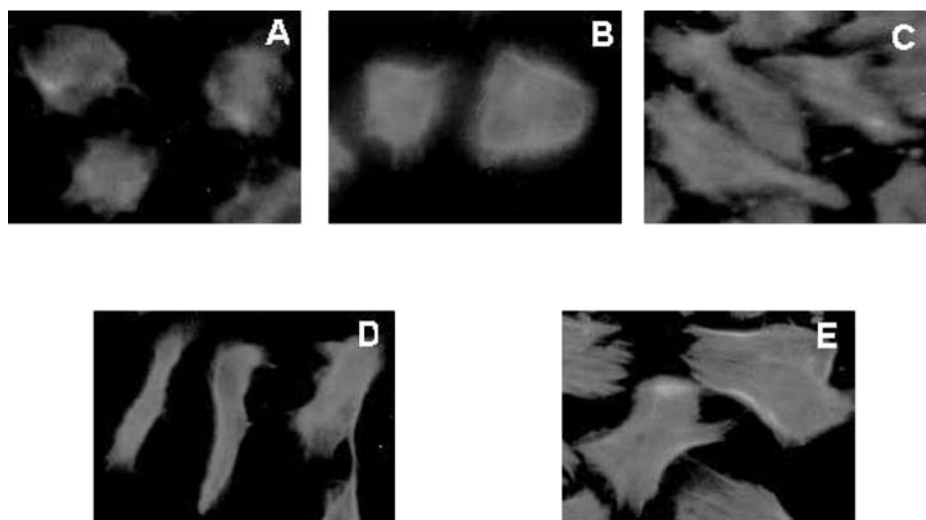

Fig. 4. Actin fibers fluorescence. Morphological structure of actin filaments was assayed by fluorecence of rhodamine-phalloidin. Cells were cultured in glass coverslips in similar densities. After retinol treatment cells were fixed with 4% paraformaldehyde (PFA). Finally, the cells were incubated with rhodamine-phalloidin and actin fibers were visualized by fluorecence microscopy. A) control; B) vehicle; C) retinol 5 μM; D) retinol 7 μM; E) retinol 7 μM + Trolox® 0.1 mM. Representative data from three independent experiments, $n = 3$.

Table 1. Deformation coefficient *D* in rat cultured Sertoli cells treated with retinol

	n	*D*
Control	35	2,4238 ± 0,2557
Vehicle	35	2,4248 ± 0,3201
5 μM	32	2,7424 ± 0,3232
7 μM	41	3,4543 ± 0,3947*
7 μM + Trolox®	42	2,6733 ± 0,2480
Trolox®	43	2,5242 ± 0,2343

Note. Morphometrical measurements were obtained by NIH-image program analysis of the scanned phase contrast photomicrographs of cells plated at dispersed and confluent densities. *Different from control. $p < 0.05$.

of nitrite present in Sertoli cells medium. Cells treated with retinol 5 μM and 7 μM showed a decrease of nitric oxide levels compared to control cells. Cells co-treated with Trolox® showed the same level of control cells (Fig. 5).

Discussion

In this work we demonstrated that retinol treatment at 7 μM lead to a global cellular morphological alteration. Since retinol treatment has been related to an increase in oxidative damage to biomolecules [15], cytoskeleton dysfunction was expected. In contrast, we did not observe any indication of loss function. We also demonstrated that retinol treatment induces an oxidative imbalance in the cell and, at the same time, protects the cytoskeleton from the high levels of ROS through an unknown mechanism. We believe that the decrease in the carbonyl levels could be a necessary adaptation of the cytoskeleton to a new cellular state which would be a consequence of the retinol treatment.

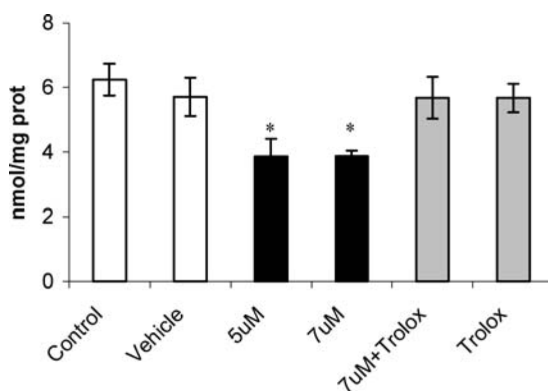

Fig. 5. Nitric oxide levels. Retinol treatment produced changes in nitric oxide levels in cultured cells. The medium was collected and assayed spectrophotometrically by the method of Griess. *Different from control. Data are reported as mean±S.E., with the level of significance set at $p < 0.05, n = 3$.

Analyzing actin filaments, we observed a ROS-dependent modification in its organization (Fig. 4). It has been described that free radicals could induce actin fibers to produce stress fibers as an adaptation to oxidative stress [26]. It was also described that free radicals may induce cytoskeleton filaments to lose its function by modifying its structure [27]. In the present study we did not find any of these alterations.

The deformation coefficient *D* [23], which is an indicator for phenotype transformation that usually occurs in neoplasia, was increased by 7 μM retinol treatment (Fig. 4). These results agree with previous data from our group and confirm that retinol treatment promotes cell transformation [18] which leads to cell proliferation and focus formation [14]. The phenotype transformation and consequent cytoskeletal modification could indicate that the cytoskeleton is adapting to maintain its general functions.

Our group characterized the involvement of free radicals in the mechanisms of retinol action [11, 13, 16, 18]. Analyzing the superoxide production by submitochondrial particles (SMP) we found that retinol 7 μM induced an increase in superoxide production (Fig. 1). In addition, we observed that retinol treatment decreases nitric oxide levels, through a ROS-dependent mechanism, since the addition of an antioxidant Trolox® prevents the decrease in nitric oxide levels induced by retinol (Fig. 5). This negative modulation is probably caused by mechanism different from peroxynitrite formation by the reaction of nitric oxide with superoxide. Retinol 5 μM, differently from retinol 7 μM, did not increase mitochondrial superoxide production, but decreased nitric oxide levels (Fig. 5), as well as the morphological shape of Sertoli cells (Fig. 4). We suggest here that these changes could be associated to an adaptive response of Sertoli cells to the oxidative stress induced by retinol treatment. Clearly, more studies are necessary for the complete understanding of the role of RNS in retinol biological effects, since it is possible that retinol 5 μM could induce superoxide production among other cellular compartments beside the mitochondria. It seems that retinol modulates nitric oxide levels to preserve the cytoskeleton, since RNS can be extremely damaging to these proteins.

To explain how the retinol treatment leads to cell transformation through a ROS-dependent mechanism and simultaneously causes adaptive/preservative morphological alterations, we propose that the cytoskeleton may be able to conduct electrical charges, which are presumably necessary for the transformation process. The theory supporting this idea was first presented by Cavelier et al. [28], suggesting that an electronic transportation is carried out by actin filaments during the cell transformation. According to this theory, the superoxide radical would act as an electron donator-acceptor

in this process. It is well established that actin filaments form a net connecting the mitochondria membranes to the plasmatic membrane [29]. It is also known that these filaments are involved in mitochondria transportation [30]. However, further studies are necessary to address this issue.

We here demonstrated that retinol increases superoxide production by SMP (Fig. 1). Superoxide would generate an eletronical flux, necessary for the transformation process, and the cytoskeleton would adapt and maintain its functions by acting as a bioconductor that transports the superoxide-generated eletronical flux. Biophysical theories predicted that coherent endogenous high frequency-electric fields can play a significant role in organization, and it is possible that there are coherent vibrations in cytoskeleton components in particular in the microtubules [31]. Other studies showed that human phagocyte NADPH oxidase could generate an eletronical flux by superoxide radical production [32].

Since the electronical flux would perform a possible important physiological role for normal cell functioning, we believe that the cytoskeleton could be preserved because it is possibly a bioconductor which is necessary to transport these electrons. We also believe that this eletronical flux could be involved with the natural cellular process, being responsible for important physiological functions. Evidence that can help to confirm this proposal comes from previous results from cultured rat Sertoli cells showing that superoxide is causing mitotic signaling induced by retinol treatment [14]. We have proposed that the increase in oxidative damage to proteins, lipids and DNA observed in previous studies could be resulting from increased hydroxyl radical production through Fenton reaction since retinol leads to iron accumulation [17].

In conclusion, 7 μM retinol treatment caused ROS dependent alterations to rat Sertoli cells cytoskeleton and these alterations may cause a morphological cell change. We believe that the cytoskeleton undergoes an adaptive/preservative modification since it plays an essential role as a bioconductor in the transformation process, induced by retinol, to take place.

Acknowledgments

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CAPÍTULO II

CAN ELECTRONS TRAVEL THROUGH ACTIN MICROFILAMENTS AND GENERATE OXIDATIVE STRESS IN RETINOL TREATED SERTOLI CELL?

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Can electrons travel through actin microfilaments and generate oxidative stress in retinol treated Sertoli cell?

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Abstract In early reports our research group has demonstrated that 7 μM retinol (vitamin A) treatment leads to many changes in Sertoli cell metabolism, such as up-regulation of antioxidant enzyme activities, increase in damage to biomolecules, abnormal cellular division, pre-neoplasic transformation, and cytoskeleton conformational changes. These effects were observed to be dependent on the production of reactive oxygen species (ROS), suggesting extra-nuclear (non-genomic) effects of retinol metabolism. Besides 7 μM retinol treatment causing oxidative stress, we have demonstrated that changes observed in cytoskeleton of Sertoli cells under these conditions were protective, and seem to be an adaptive phenomenon against a pro-oxidant environment resulting from retinol treatment. We have hypothesized that the cytoskeleton can conduct electrons through actin microfilaments, which would be a natural process necessary for cell homeostasis. In the present study we demonstrate results correlating retinol metabolism, actin architecture, mitochondria physiology and ROS, in order to demonstrate that the electron conduction through actin microfilaments might explain our results. We believe that electrons produced by retinol metabolism are dislocated through actin microfilaments to mitochondria, and are transferred to electron transport chain to produce water. When mitochondria capacity to receive electrons is overloaded, superoxide radical production is increased and the oxidative stress process starts. Our results suggested that actin cytoskeleton is essential to oxidative stress production induced by retinol treatment, and electrons conduction through actin microfilaments can be the key of this correlation.

Keywords Retinol · Actin · Cytoskeleton · Electron flux · Conduction theory · Morphology · Architecture · Sertoli cells · ROS · Oxidative stress · Superoxide radical · Mitochondria

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Introduction

Retinol (vitamin A) is an essential nutrient that plays a very important role in numerous biological processes such as vision, immunity, reproduction, growth, and development [1–4]. Our group has been demonstrating that retinol supplementation in cultured Sertoli cells could modulate a series of cellular pathways in a free radical-dependent way, suggesting non-nuclear (non-genomic) effects in retinol actions. Our initial works have shown that retinol treatment was able to increase [methyl-H-3] thymidine incorporation [5] and DNAse

I sensitivity [6], and could modulate the phosphorylation level of histones and HMGs [7]. We soon associated retinol actions in cultured Sertoli cells with free radicals [8, 9], and discovered that above a 7 μM dose, that is very close to the physiologic concentration range of retinol in Sertoli cells (2–5 μM) [10], Sertoli cells had undergone oxidative stress. This oxidative stress was characterized by an increase in antioxidant enzymes activities such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) [11], increase in oxidative damage in lipids, proteins and DNA, and modulation of iron turnover [12, 13]. We have also demonstrated that superoxide radical produced by retinol treatment was able to induce an abnormal mitogenic signal in terminally-differentiated Sertoli cells, characterized by proliferative focus formation [14]. We have used different bioassays to show preneoplasic transformation, chromosomal rearrangements, and DNA damage induced by pro-oxidant effects of retinol treatment [15]. Since cytoskeleton participates in many processes related above, we have investigated the influence of this structure in retinol treated Sertoli cells. In a recent work, we have curiously found that besides an increased oxidative damage on cellular biomolecules, retinol caused the cytoskeleton to change its phenotype and decreased its levels of molecular oxidative damage, appearing to be protected from the pro-oxidative environment [16]. Retinol modulation in cytoskeleton and all other changes found in Sertoli cells were free-radical dependent, since co-treatment with antioxidants reversed these effects. Some works already related the importance of oxidative stress in the integrity and function of cytoskeleton proteins [17, 18]; therefore we have proposed that the protection observed may be related with a possible capacity of cytoskeleton actin to conduct electrons inside its structure [16].

Architecture of actin cytoskeleton is essential to control many processes. In U937 cells cytochalasin B (Cyt B), a mycotoxin that disrupts actin cytoskeleton, is able to suppress apoptosis induced by residronate [19]. Damage to actin cytoskeleton was associated to cytocrome C release and this effect is negatively regulated by the ability of Hsp27 to protect F-actin network integrity [20]. A direct link between architecture of actin and ROS production has been demonstrated with osteoblast-like HT-3 cells when disruption of actin abolished the stress induced by ROS synthesis [21]. Other evidences correlate actin architecture, ROS production, and mitochondria physiology with tumor cell metabolism [22]. We believe that actin architecture is essential to electron conduction, and changes on its normal physiology can be harmful to mitochondrial metabolism provoking ROS overproduction. There is an actin microfilament network formed by actin and accessory proteins—for example Fodrin—which connects cellular proteins and extracellular matrix to outer mitochondria membrane [23–25]. We give a new interpretation to this subject and hypothesize that actin network can also act as an electron conductor to mitochondria. In the present work we have added 0.5 μM Cyt B 10 min before retinol treatment to study the effects of actin architecture on oxidative stress induced by retinol. Literature has related that Cyt B had effect on culture models in 3 min of treatment, and 0.5 μM concentration had the best F-actin depolimerization effect related to all different types of cytochalasins [26]. In the present approach we showed that treatment with 0.5 μM Cyt B was able to disrupt the morphology of actin microfilaments according to the literature.

In this paper we showed evidences that may suggest the occurrence of electron conduction through actin cytoskeleton to mitochondria, and the necessity of the actin cytoskeleton functional architecture to retinol-induced oxidative stress. We suggest an ‘‘electron conduction theory’’ to elucidate how electrons can flow in actin microfilament based on the phosphorylated state and molecular structure of actin, when the cleft and nucleotide present in the single subunit of actin are essential to occur.

Materials and methods

Chemicals and animals

Type I collagenase, medium 199, HBSS, Cytochalasin B, and all-*trans* retinol were purchased from Sigma, St. Louis, MO, USA. Trypsin was purchased from Difco, Detroid, MI, USA. Pregnant Wistar rats were housed individually in Plexiglas cages. The animals were maintained in a 12 h light/dark cycle at a constant temperature of 23°C, with free access to commercial food and water.

Sertoli cell culture and treatment

Sertoli cells from 15-day-old Wistar rats were isolated and cultured as previously described [5]. In short, animals were killed by cervical dislocation, testes were removed and washed in saline pH 7.4, Sertoli cells were isolated by enzymatic digestion of uncapsulated testes with trypsin and type I collagenase. Isolated cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air, and grown in a plating

density of 3.2×10^5 cells/cm^2 in medium 199 (pH 7.4) supplemented with 1% fetal bovine serum (v/v) in the first 24 h. After that, the medium was replaced by serum free medium and cells were maintained for more 24 h. Experiments were performed on cells treated with retinol (vitamin A), Trolox®, and retinol vehicle (ethanol 0.1% v/v) for 24 h; Cytochalasin B was added 10 min before each treatment. Trolox® is a hydrophilic analogue of vitamin E and was used as a standard antioxidant. Retinol and Cytochalasin B treatments were performed in a light-protected environment.

Phosphorylation of cytoskeleton proteins (Incorporation of [^{32}P]orthophosphate)

[^{32}P]orthophosphate was added to cultured Sertoli cells 6 h before the end of treatments. Cytoskeleton proteins isolation and 10% polyacrylamide SDS-PAGE electrophoresis were performed according de Oliveira et al. [16]. Around 10% polyacrylamide SDS-PAGE electrophoresis resulting lanes of isolated cystoskeleton proteins was removed from the gel and dissolved with 8.8 M hydrogen peroxide. Positively actin band was determined by Immunoblot analysis with anti β-actin (Sigma). The radiation was measured in a liquid scintillation counter (Wallac 1409), and the result was expressed by the ratio of scintillation and densitometry analysis of each lane.

Protein oxidative damage (carbonyl)

As index of protein oxidative damage, the carbonyl groups were determined according to Levine et al. [27]. Homogenized cultures were divided into four aliquots of 200 μl (~0.2 mg of protein). Proteins were precipitated by the addition of 100 μl 20% TCA for 5 min on ice, and centrifuged at $4000 \times g$ for 5 min. The pellet was dissolved in 100 μl of NaOH 200 mM, and 100 μl of HCl 2 M, or 10 mM of 2,4-dinitrophenylhydrazine (DNPH) in HCl 2 M was added to duplicate aliquots for blanks or to measure of carbonyl groups. Samples were maintained for 30 min at room temperature. Proteins were precipitated with 20% TCA, and washed three times with 500 μl 1:1 ethanol:ethyl acetate with 15 min standing periods to remove excess of DNPH. Samples were dissolved in 200 μl 6 M guanidine in 20 mM KH_2PO_4, pH 2.3, and the absorbance was read at 370 nm. The carbonyl content (nmol/mg protein) was calculated using a molar extinction coefficient of 22,000/M/cm at 370 nm after subtraction of the blank absorbance. The results were normalized by total protein assayed by Bradford [28].

Index of lipid peroxidation (TBARS)

Thiobarbituric acid reactive substances (TBARS) were measured [29]. After the assay, reaction was stopped by removing the incubation medium. The cells were scraped off, mixed with ice-cold Tris–HCl 15 mM (pH 7.4) and an equal volume of 40% trichloroacetic acid (TCA), followed by an addition of 0.67% TBA. Samples were then heated in a boiling water bath for 25 min. After cooling, they were centrifuged (750 g/10 min), and the absorbance of the supernatant was read at 535 nm. An absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} was used to calculate the amount of TBARS.

Determination of sulfhydryl groups (–SH)

Total cellular and isolated cytoskeleton sulfhydryl groups were carried by protocol adaptation [30]. In brief, after treatments cells were scraped with PBS (136.5 mM NaCl, 0.27 mM Na_2HPO_4, 1,10 mM KH_2PO_4, pH 7.4) or the cytoskeleton was isolated with ice-cold buffer containing 1% Triton X-100, 600 mM KCl, 5 mM EDTA, 2 mM EGTA, 2 mM PMSF, 50 mM Tris–HCl (pH: 6,8), and protease inhibitor cocktail (p-8340, Sigma) according de Oliveira et al. [16]. The samples were incubated for 15 min with 30 mM DTNB at room temperature and the absorbance was read at 412 nm.

Cell viability measurements

Lactate dehydrogenase (LDH) activity in the incubation medium was measured after 24 h treatments with a commercial kit (Sigma). The MTT colorimetric assay was used to estimate mitochondrial viability as described by Carmichael et al. [31]. MTT solution (sterile stock solution of 5 mg/ml in phenol red-free HBSS in the dark) was added to the medium in the wells at a final concentration of 0.2 mg/ml. The cells were left for 45 min at 37°C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm (reference).

Superoxide production in sertoli cells submitochondrial particles

Superoxide production was determined in submitochondrial particles (SMP) according to de Oliveira et al. [16]. This spectrophotometer assay is based on

superoxide-dependent oxidation of epinephrine to adrenochrome at 37°C (E480 nm¼4.0/mM/cm). The reaction medium consisted of 230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl (pH 7.4), SMP (0.3–1.0mg protein/ml), 0.1 mM catalase, and 1 mM epinephrine. Succinate (7 mM) was used as substrate. Superoxide dismutase was used at 0.1–0.3 mM final concentration to give assay specificity.

Chemiluminescent assay for free radical quantification

To evaluate the cellular oxidant status we measured the *tert*-butyl hydroperoxide-initiated chemiluminescence. This method measures the balance between pro-oxidants and cell antioxidant defenses in the cellular environment. Briefly, cell homogenates were diluted to a final concentration of 1 mg/ml protein before adding *tert*-butyl hydroperoxide to a final concentration of 3 mM. Chemiluminescence was measured at 30°C in a scintillation counter as previously described [32]. Values are expressed as percentage of counts/min/mg^{-1} relative to control cells.

Mitochondrial potential membrane $\Delta\psi$m (JC-1)

Mitochondrial potential membrane was evaluated by protocol adaptation [33]. About 30 min before the end of the treatments lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenz imidazolcarbocyanine iodide (JC-1) (Molecular Probes) (5 μg/ml in complete cell culture media) was added to culture, followed by washing the cells twice with HBSS. The samples were photographed at room temperature in a darkened room with a Nikon inverted microscope using a TE-FM Epi-Fluorescence accessory, using a Zeiss barrier 515–565 nm and exciter 450–490 for green fluorescence, and a Zeiss barrier LP 590 nm and exciter BP 546/12 nm for red fluorescence. JC-1 assay shows two fluorescence intensity colors in living cultured cells, the red intensity color means high mitochondrial potential membrane that correspond to JC-1 aggregates, and the green intensity color means low mitochondrial potential membrane. Pictures fluorescence intensity was determined by ImageJ® software, and these intensity figures were showed by the ratio of the red intensity picture/green intensity picture.

Cellular non-enzymatic redox state (TRAP)

The cellular non-enzymatic redox state of the cultured Sertoli cells was estimated by the total radical-trapping antioxidant parameter (TRAP). The principle of TRAP measurement has been previously described [34]. Briefly, the reaction was initiated by injecting luminol and AAPH—a free radical source that produces peroxyl radical at a constant rate—in 0.1 M glycine buffer (pH 8.6) that resulted in steady luminescence emission. The addition of the homogenate samples decreases or facilitates the luminescence emission proportionally to its redox state. The luminescence emission was followed for 25 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).

Sertoli cells glucose content

Sertoli cells glucose content was determined using a commercial kit Glucose PAP (Labtest). After treatment cultured Sertoli cells were washed twice with PBS (136.5 mM NaCl, 0.27 mM Na_2HPO_4, 1,10 mM KH_2PO_4, pH 7.4) and scraped in this buffer. Samples were incubated for 15 min 37°C with Reagent 1 and the absorbance was measured in spectrophotometer at 505 nm. Glucose values were compared with a glucose standard curve.

Morphologic structure of actin filaments

To check if the treatment with Cyt B effectively affects actin microfilament we used rhodamine marked phalloidin according de Oliveira et al. [16]. Cells were cultured in glass coverslips in similar densities. After 24 h of retinol treatment, cells were fixed with 4% paraformaldehyde (PFA), and washed three times with PBS (136.5 mM NaCl, 0.27 mM Na_2HPO_4, 1,10 mM KH_2PO_4, pH 7.4). The excess aldehyde groups were blocked by glycine solution incubation (20 mM Tris, 150 Mm glycine, pH: 8.0) for 15 min. The fixed cells were permeated with 0.3% Triton X-100, and blocked with 3% albumin for 1 h in room temperature. Rhodamine-phalloidin in 3% albumin incubation (45 min, 37°C in dark) was performed. The cells were washed with water, and coverslips were fixed with fluorsafe and incubated for 30 min in room temperature. Actin filaments were then visualized by fluorescence microscopy.

Statistic analysis

Results were expressed as the mean ± Standard Error (SE). Data were analyzed by SPSS 8.0 for Windows®, one-way analysis of variance (ANOVA) using Duncan test to compare mean values across groups. Densitometry and fluorescence intensity analysis have

been performed by ImageJ® software. Differences were considered to be significant when $p < 0.05$.

Results

Increase on actin cytoskeleton phosphorylation

[^{32}P]orthophosphate incorporation demonstrated that 7 μM retinol treatment has increased phosphorylation levels of all isolated cytoskeleton proteins and the actin fraction has shown an increase greater than all other cytoskeleton fractions (Fig. 1). Co-treatment with 100 μM Trolox® has partially reversed cytoskeleton phosphorylation increase, demonstrating a free radical-dependent pathway in retinol action mechanism (Fig. 1). Treatment with only 100 μM Trolox® also presents an effect in the phosphorylation levels. Bands 1, 2, 4, 5, and 6 presented an increase in the incorporation of [^{32}P]orthophosphate, but bands 3 and 7 presented a decrease when compared to control and 7 μM retinal + 100 μM Trolox® (Fig. 1). We believe there are two possible explanations for that: (1) Phosphorylation produces an electric isolation that prevents electron leak and leads electrons into the F-actin. (2) Phosphorylation produces a negative electric field surrounding actin and protecting the cytoskeleton architecture against oxidants. The effect of Trolox may indicate a specific phosphorylation action of this compound in cultured cells; this is supported by the different response between cytoskeleton fractions, suggesting different effects in different cytoskeleton components.

Decrease in oxidative stress markers by Cyt B in retinol treated cells is not associated with cellular death

In this paper we have demonstrated that 0.5 μM Cyt B has reversed oxidative stress indicators in retinol-treated Sertoli (Fig. 2). Protein carbonyl and TBARS content, indicators of protein oxidative damage and lipoperoxidation, respectively, were decreased by 0.5 μM Cyt B co-treatment (Fig. 2A and C). Retinol 7 μM did not change the content of sulfhydrylated proteins in total cellular fractions (Fig. 2B *right*), but in isolated cytoskeleton they were found to be increased (Fig. 2B *left*). Around 0.5 μM Cyt B co-treatment reverts the increase produced by retinol and 0.5 μM Cyt B treatment alone presents a decrease of sulfhydryl content when compared to all other treatments (Fig. 2B *left*). Reversion of oxidative stress markers is not caused by cellular death (Fig. 3A and B). Although Cyt B may be cytotoxic when administered in high concentrations (Fig 3 B), we used a dose (0.5 μM) that did not affect cell viability (Fig 3 A and B), thus indicating that its effects on retinol-treated cells were on the level of actin architecture disruption only, and not related to cell death.

Cytochalasin B pre-treatment reverts ROS production and modulates mitochondrial membrane potential ($\Delta\psi$m) in retinol treated Sertoli cells

Unbalance in ROS production is the major cause leading to oxidative stress [35], and the signaling produced by superoxide radical may lead cells to abnormal

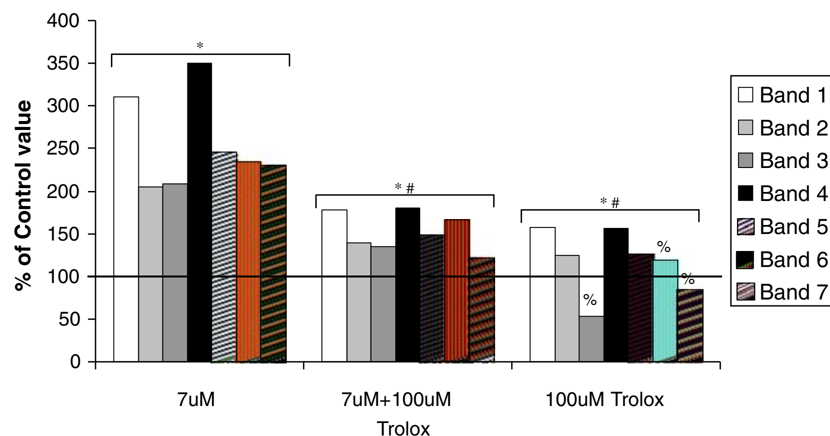


Fig. 1 Phosphorylation of cytoskeleton proteins: 7 μM retinol treatment increase incorporation of [^{32}P]orthophosphate of all cytoskeleton fractions and co-treatment with 100 μM Trolox® revert partially. Band 1 ≈116 KDa; Band 2 ≈97.4 KDa; Band 3 ≈66 KDa; Band 4 Actin 43KDa (immunoblot analisys); Band 5 ≈35 KDa; Band 6 ≈25 KDa; Band 7 less than 25 KDa. Trolox® was added as antioxidant control to demonstrate that retinol effects are free radical-dependent. Control value was used as 100% of orthophosphate incorporation, and the results are expressed in % of values. * different of Control; % different of 7 μM retinal + 100 μM Trolox®. Representative of three individual experiments ($n = 3$)

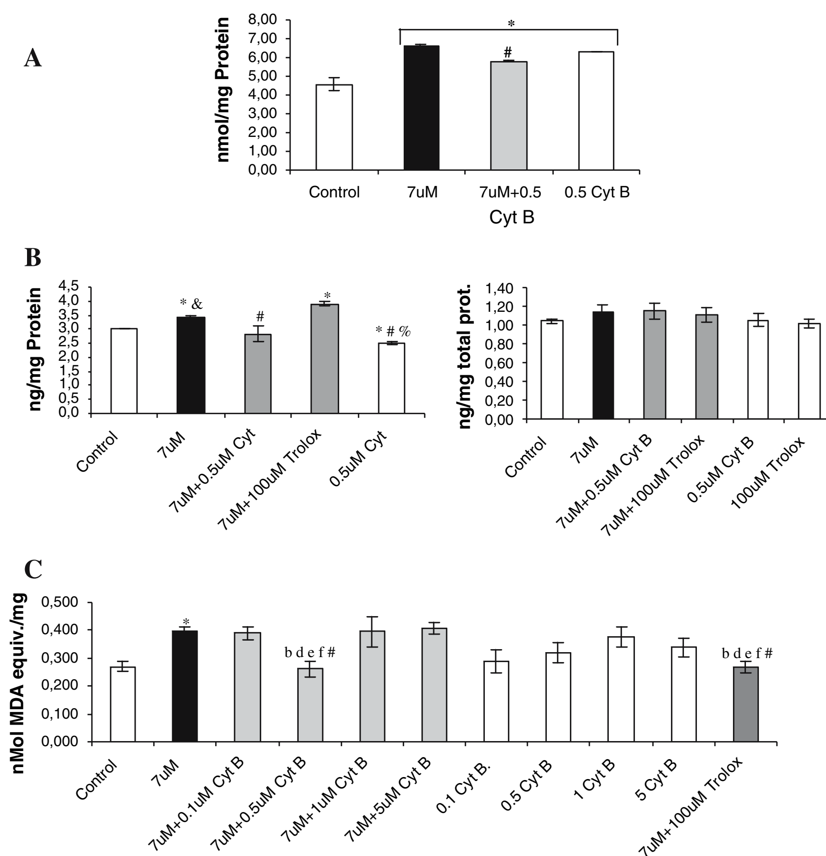


Fig. 2 Oxidative stress markers: Co-treatment with 0.5 μM Cyt B revert total or partially the increase generated by 7 μM retinol. (**A**) Total carbonyl content. (**B**) *Left* sulfhydryl content of isolated cytoskeleton proteins, *right* total cellular sulfhydryl content. (**C**) TBARS content in cellular homogenate. * different of control; # different of 7 μM retinol; & different of 7 μM +0.5 μM Cyt B; % different of 7 μM retinol+100 μM Trolox®; b different of 7 μM retinol+0.1 μM Cyt B; d different of 7 μM retinol+1 μM Cyt B; e different of 7 μM retinol+5 μM Cyt B; f different of 1 μM Cyt B. Representative of four individual experiments ($n = 4$)

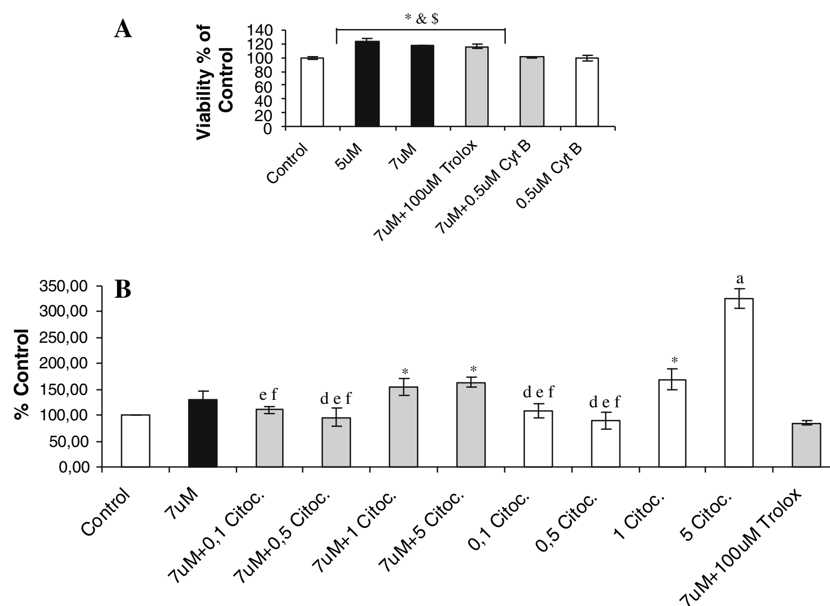


Fig. 3 Cell viability measurements: The experiments performed demonstrated that the reversion of oxidative stress generated by retinol treatment was not by cellular death. (**A**) MTT assay; (**B**) Extra cellular activity of LDH; * different of control; & different of 7 μM +0.5 μM Cyt B; $ different of 0.5 μM Cyt B; a different of all other treatments; d different of 7 μM retinol+1 μM Cyt B; e different of 7 μM retinol+5 μM Cyt B; f different of 1 μM Cyt B. Representative of four individual experiments ($n = 4$)

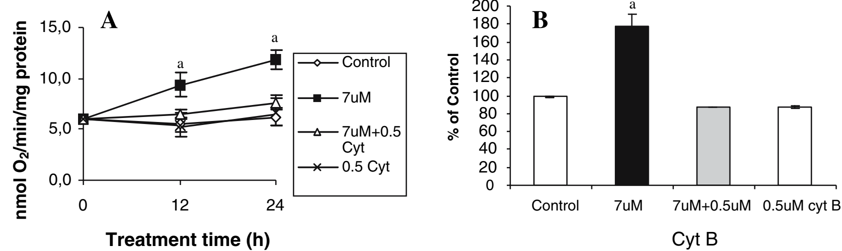


Fig. 4 ROS production in treated Sertoli cells: 7 μM retinol treatment produces increase in mitochondrial and total cellular homogenate ROS production; 0.5 μM Cyt B co-treatment revert ROS increase induced by retinol in both experiments. (**A**) Mitochondrial superoxide production in SMP; (**B**) Chemiluminescent assay in total cellular homogenate. a different of all other treatments. Representative of three individual experiments (n = 3)

events as transformation [15]. Our group has already demonstrated increase in superoxide production by retinol [16], and that this radical causes mitogenic signals in Sertoli cells [14]. In the present work we have demonstrated that architecture of actin cytoskeleton is essential to superoxide production by mitochondria and in total cell homogenate (Fig. 4A and B). Co-treatment with the antioxidant Trolox® (100 μM) was able to decrease ROS levels in both assays, and was used like experimental control (Fig. 4A and B). JC-1 assay shows that retinol causes loss of mitochondrial membrane potential, which was reversed by Cyt B (Fig. 5A and B) indicating an intrinsic relationship between actin cytoskeleton, ROS production and mitochondrial functions. Modulation in mitochondrial membrane potential was associated to changes in ROS production and can lead cells to apoptosis [36] or necrosis [37]. Our results have suggested a direct interconnection between cytoskeleton, actin architecture, and mitochondrial metabolism, leading us to believe that there is a probable electron flux through actin cytoskeleton to mitochondria, and consequently the superoxide production may be the answer to this relationship.

Cellular non-enzymatic redox state depends on cytoskeleton sulfhydryl groups (–SH) integrity

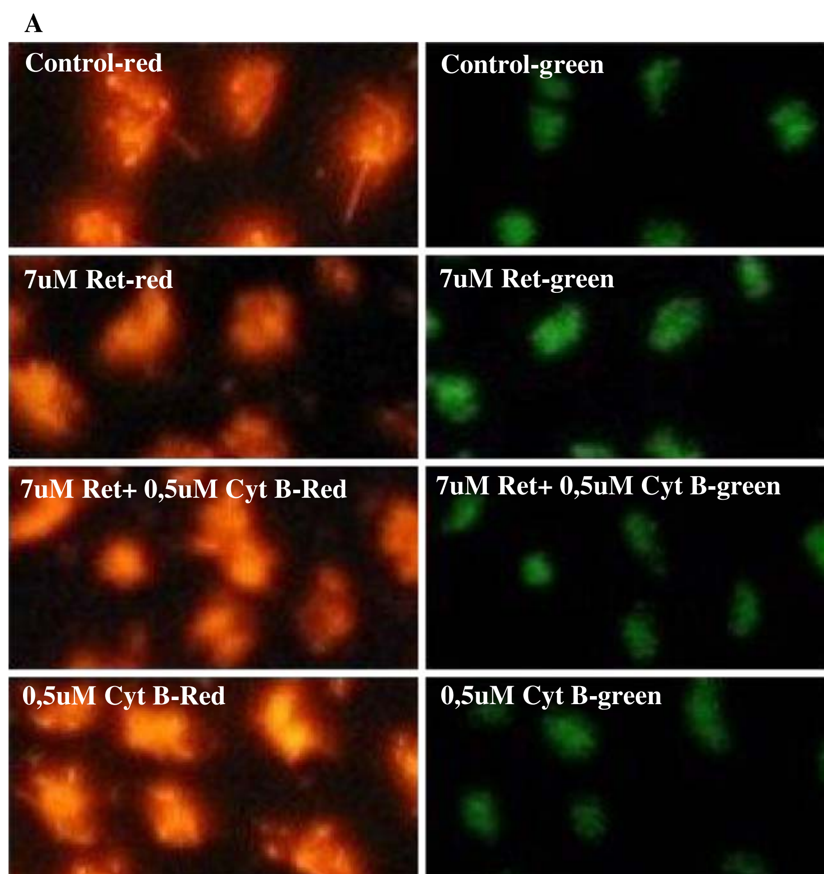
In this work we have shown a relationship between cellular redox status and the integrity of cytoskeleton –SH groups. TRAP assay has demonstrated a pro-oxidant cellular environment in cells treated only with 0.5 μM Cyt B (Fig. 6), and at the same time a decrease in cytoskeleton –SH groups content in cells that received the same treatment (Fig. 2B *left*). We believe the correlation between these two results indicate cellular necessity to deliver electrons through actin cytoskeleton, protecting the cell against an oxidative burst. Haarer et al. [38], have discovered an enzyme responsible to reduce actin disulfide bonds, which can control the plasticity of cytoskeleton. We believe that in Sertoli cells we can have a similar mechanism, where actin –SH groups maybe receive electrons from normal cell metabolism and conduct them to mitochondria. Loss of a reducing actin –SH groups mechanism, induced by Cyt B, for example, may indicate a loss in the electron conduction through actin microfilaments, resulting in a decrease in the cellular ROS cleaning capacity.

Sertoli cells glucose content and Cyt B effect in actin microfilament

Cyt B has a secondary effect of glucose transport blocking [39]. Other works have shown that in Sertoli cells this effect can also occur [40]. We observed that glucose transport blocking effect does not occur in our culture model, since the total glucose content present in Sertoli cells does not change with Cyt B treatment (Fig. 7). This negative blockage can be attributed with the low Cyt B concentration used (0.5 μM), since other works that demonstrated glucose transport blocking used higher concentrations. We also have shown that treatment with 0.5 μM Cyt B effectively caused morphology changes in treated cells (Fig. 8). Figure 8B has shown Cyt B effect in 10 min of treatment, exactly the time when retinol is added together in the culture; this result shows that actin cytoskeleton was already disrupt before retinol exerts its actions. Both results have shown that our findings in this paper were related with disruption of actin cytoskeleton and not with another effect of Cyt B.

Discussion

F-actin has regular clefts and nucleotides in its structure [41]. These adenosine biphosfate nucleotides



B	Red/Green Fluorescence
Control	15,2
7µM Ret	12,2*
7µM Ret+0.5µM Cyt B	14,0
0.5µM Cyt B	15,2

Fig. 5 Mitochondrial Potential Membrane $\Delta\psi m$ (JC-1): 7 μM retinol treatment produced a loss of mitochondrial potential membrane and co-treatment with 0.5 μM Cyt B reverted this effect until control levels. (**A**) JC-1 red and green fluorescence photography demonstrated by microscopy in cultured Sertoli cells; (**B**) Ratio of red intensity picture/green intensity picture fluorescence measured by ImageJ® software. * different of control. Representative of four individual experiments (n = 4)

(ADP) are positioned in the final portion of the cleft (Fig. 9A) bound to amino acids residues. We believe that an unknown protein put an electron in the actin cleft space opposite to ADP, when it occurs the actin cleft gets negatively charged. The new negatively-charged cleft pushes by repulsion one electron present in ADP molecule to the next actin cleft present in the next subunit of actin. The moved electron turns the new cleft negatively charged and pushes one new electron from the next ADP molecule to the next cleft, generating a one-directional electron flux (Fig. 9B). We believe that these electrons are provided to F-actin from natural cells metabolism and are conducted to mitochondria to generate water. It may be a cleaning mechanism where cells can prevent oxidative damage to its components.

Some works have already demonstrated the relationship between actin and ROS production in mitochondria. Dynamics of actin cytoskeleton controls the ROS production in yeast [42], and it is also suggested

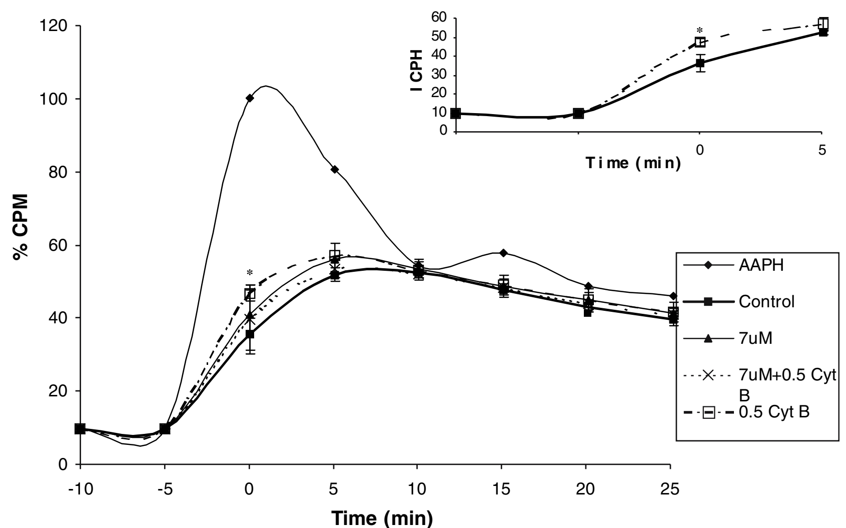


Fig. 6 TRAP assay: 7 μM retinol treatment and 0.5 μM Cyt B co-treatments did not present changes in cellular non-enzymatic redox state, but 0.5 μM Cyt B treatment alone presented a pro-oxidative environment (demonstrated in the insert graphic). This result can be correlated with the decrease on cytoskeleton—SH levels and increase in Carbonyl content induced by the same treatment. * different of control. Representative of three individual experiments ($n = 3$)

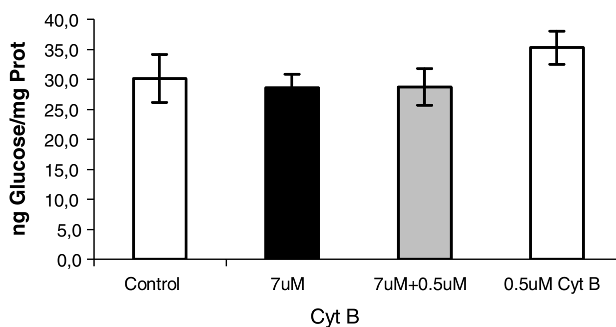


Fig. 7 Sertoli cells glucose content: Culture glucose content was determinate by commercial kit Glucose PAP (Labtest), all treatments did not present any changes at the end of 24 h. The secondary Cyt B effect of blocking glucose transportation was not present in this paper, probably by the low concentration used (0.5 μM). Representative of three individual experiments ($n = 3$)

that yeast actin cytoskeleton has a role in regulating apoptosis and ageing via interaction with mitochondria [43]. Actin also plays an important role in the transduction of cell death signal to mitochondria in TNF-treated L929 cells [44]. Based in many literature evidences and in the results that our group has presented, we suggest that F-actin is able to conduct electrons, and it participates in the normal metabolism of the cells as a way to send electrons to mitochondria. When these electrons reach the mitochondria they are used to form water through electron respiratory chain. Superoxide overproduction occurs when the mitochondrial capacity of receiving electrons is surpassed. Other strong evidences that reinforces our electron conduction theory is the fact that a number of phospholipases and lipid kinases, including PI3K, phospholipase C (PLC), and cytosolic phospholipase A ($cPLA_2$) [45], besides the Src family of non-receptor tyrosine kinases [45, 46] and serine threonine protein kinases [47, 48], and other enzymes, are known to translocate to cytoskeletal fractions upon stimulation, and are regulated by cytoskeleton. These protein

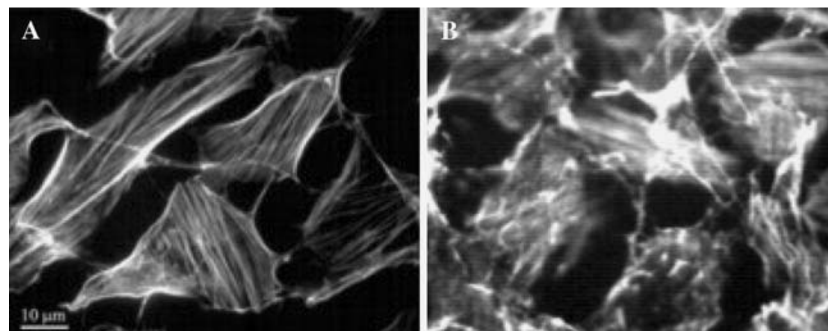


Fig. 8 Cyt B effect in morphology of actin microfilaments: Actin microfilaments were visualized by rhodamine marked phalloidin assay. Around 0.5 μM Cyt B was able to change morphology of actin microfilaments in 10 min of treatment; this result shows that Cyt B already changed actin morphology when retinol was added together in the culture. (**A**) Control; (**B**) 10 min of 0.5 μM Cyt B treatment. Representative of three individual experiments ($n = 3$)

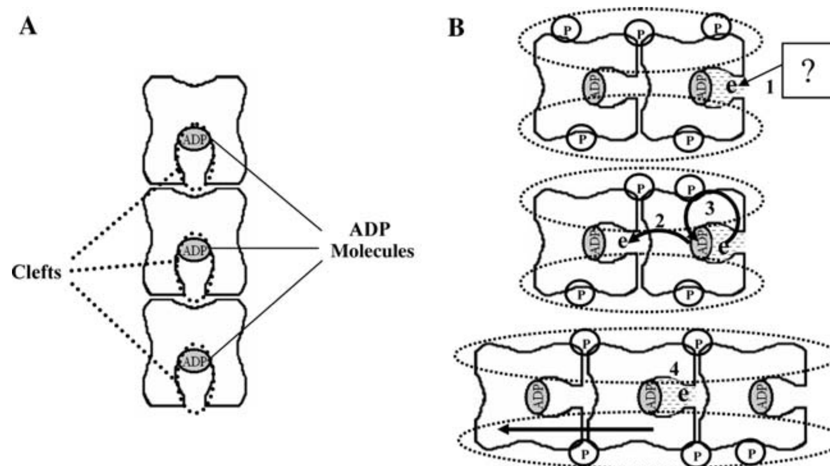


Fig. 9 Electron conduction theory into F-actin: (**A**) F-actin presents regular clefts and bonded ADP in its structure; (**B**) Electron conduction occurs when an unknown protein (?) puts an electron in the ADP opposite space of the cleft (1 *top*), this electron generates a negatively charged cleft and makes an electron present in ADP molecule pass to the next cleft of the next actin subunit (2 *middle*), when ADP gives an electron to the next actin subunit (2 *middle*), it (ADP) receives the electron that was in the first cleft (3 *middle*). Moment 4 (*below*) is when this cycle restarts in the second actin subunit. P symbolizes phosphorylation on F-actin surface, this phosphorylation created a negative electric field that drives the electrons in the right way into the actin, and prevents electron leaks of F-actin

translocations/activations may indicate that cytoskeleton can act like a component that anchored them when they are active, and in case of electron escaping from their reactions, these electrons could be safely conducted to mitochondria to form water, avoiding oxidative damage to the cell. We can find an example of this supposition when actin cytoskeleton is disrupted by cytochalasin, and then other sources of ROS such as NADPH oxidase increase superoxide production [49]. It may mean that electrons ''leak'' their alternative/natural way to mitochondria and produce superoxide.

A special condition is necessary for electron conduction to occur: phosphorylation on F-actin surface. We think that a leak of electrons similar to the respiratory chain of mitochondria may be possible [50]. Electrons would be lost from their conduction inside of the F-actin chain cleft and generate superoxide out of mitochondria. This loss would not be a normal way of conduction. To avoid the problem the surface of F-actin should be phosphorylated. F-actin surface phosphorylation will produce an electrical isolation preventing leak and maintaining the electron in the F-actin chain cleft (Fig. 9B). Actin phosphorylation results in dissolution of actin stress fibers and reorganization of actin cytoskeleton [51]; mitochondria oxidative phosphorylation [52] and the intracellular levels of ATP [53] were associated with the F-actin phosphorylation and rearrangement. T'Jampens et al. [54], characterized an enzyme called Actin-Fragmin Kinase that is responsible for phosphorylating actin. We believe that some parts of the total F-actin content in living cells are in the phosphorylated state, and this occurs as an attempt to conduct electrons generated by cellular processes to mitochondria in a very fast way avoiding oxidative damage.

Some works have given literary support for constructing our electron conduction theory. The first was presented by Cavelier, et al. [55], where they presented the idea of a possible electron conduction through actin in tumor cells. It was a hypothesis article where he said that superoxide could act like a donator-acceptor of electron. We bought this idea, applied it in our culture model, and found the results showed in the present article. We changed the conduction idea and created an explanation for electron conduction through actin based in its molecular structure and phosphorylated state. Other evidence that gave support for us was the fact that human phagocyte NADPH oxidase could generate an electron flux inside its structure and produce superoxide radical [56]. A recent work has also shown that 85 kDa outer membrane decaheme cytochrome (Omc) purified protein was able to bind and densely cover the surface of hematite, reduce Fe (III), and create an electron flux [57]. Other recent works also demonstrated that actin can be a cellular target of weak magnetic fields [58], and play an important role in modulation of electrical and mechanic activity in cardiac myocytes [59]. It was also demonstrated that actin microfilaments regulate different mitochondrial functions such as respiratory burst in chemoattractant-stimulated neutrophils [60], apoptosis [61, 62], movement [63], and other pathways [64]. All these works

demonstrate an intrinsic relationship between mitochondria and actin cytoskeleton in different pathways; we believe that electron conduction through actin to mitochondria can be a key of this relationship.

Our group has demonstrated that Sertoli cells treated with 7 μM retinol develop oxidative stress, and the cytoskeleton is modulated in a ROS-dependent way avoiding oxidative stress [16]. In the present work we have demonstrated a fundamental role of actin cytoskeleton architecture in generating this oxidative stress. All indexes of oxidative stress generated by 7 μM retinol treatment were partially or totally reverted by 0.5 μM Cyt B (Figs. 2, 4, 5, 6). We do not know all the pathways in which 7 μM retinol increases superoxide production and causes oxidative stress, but our group has exhaustively demonstrated that this phenomenon is a consequence of many metabolic alterations and modulations in cellular pathways [5–9, 11–16]. We postulated in this paper that electrons produced by 7 μM retinol metabolism are conducted by F-actin microfilaments to mitochondria, and at a certain point the capacity of mitochondria respiratory chain is overloaded, and superoxide radical is overproduced. When we co-treated cell cultures with 7 μM retinol and 0.5 μM Cyt B the F-actin pathway was broken and electrons did not reach mitochondria, resulting in a decrease in superoxide production (Fig. 4A and B). Actin phosphorylation was increased in cells treated with 7 μM retinol (Fig. 1), attending the necessity of more "ways" to conduct exceeding electrons from retinol metabolism. One complementary explanation for phosphorylation to increase is the negative electric protective field on cytoskeleton surface against the pro-oxidative environment created by retinol treatment; this result can explain our previous findings when decrease in oxidative damage in isolated cytoskeleton proteins occurred (see reference [16]). A direct way connecting Sertoli cells actin cytoskeleton and mitochondria is shown by JC-1 experiments in Fig. 5A and B, where 7 μM retinol treatment decreases the ratio of red intensity/green intensity and it's reverted by 0.5 μM Cyt B co-treatment. This result can be explained by exceeding electrons generated by retinol metabolism that arrive in mitochondrial leading superoxide formation, where electrons not all travel to mitochondrial complexes and prevent H^+ pass to intermembrane space, leading a decrease in mitochondrial potential membrane. Cyt B disrupt prevents the electrons that arrive in mitochondria by actin way, thereby fewer electrons generated in retinol metabolism arrive in mitochondria and superoxide formation is not favored. When cytoskeleton is disrupted by Cyt B, the decrease of oxidative stress indexes (Fig. 2A and C) in 0.5 μM Cyt B treated cells are not associated to death or decrease in the cell number (Fig. 3A and B). Actin cytoskeleton integrity can also be associated with non-enzymatic cellular redox regulation as it can be seen in Fig. 6, where a pro-oxidant environment is generated in cell cultures pre-treated only with 0.5 μM Cyt B. This result can be correlated with a decrease in sulfhydryl groups (Fig. 2B *left*) and the increase in carbonyl assay (Fig. 2A) that have received the same treatment (0.5 μM Cyt B alone). We believe that this effect occurred because without F-actin cables to conduct electrons to mitochondria from normal cell metabolism, we can have a lack of this cell cleaning mechanism and an increase in some oxidative markers can occur. In 7 μM retinol co-treated with 0.5 μM Cyt B we have a specific case, where the loss of the F-actin cables is more advantageous to the cell than their loss. It is because 7 μM retinol produces more electrons in treated cells than the normal cell metabolism, so the loss of F-actin cables in this specific case can prevent oxidative stress generation and cellular pathways modulation by exceeding electrons. We have also shown that our pre-treatment with 0.5 μM Cyt B was able to disrupt the actin morphology in treated cells, and when retinol is added together to the cells actin microfilaments were already changed (Fig. 8). Our results are independent from the block transport glucose parallel effect of Cyt B as shown in Fig. 7, once the total glucose content did not change during the experiment.

In conclusion, our results have suggested that in cultured Sertoli cell treated with 7 μM retinol well established oxidative stress model, the actin cytoskeleton architecture is necessary to generate oxidative stress, and it may be through electron conduction to mitochondria. We have proposed a theory trying to explain how actin cytoskeleton can act as an electron conductor inside its structure, and shown results that suggested this new approach. More studies need to be performed to clarify this new approach, but we believe that this theory can help to explain many questions about the importance of the correlation among cytoskeleton proteins, mitochondria, and oxidative stress.

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PARTE III

3. Discussão

Os resultados apresentados nesta dissertação tiveram como objetivo demonstrar o estreito envolvimento da arquitetura do citoesqueleto, mais detalhadamente dos microfilamentos de actina, na produção e manutenção do estresse oxidativo gerado pelo tratamento com retinol em células de Sertoli cultivadas. Mais do que isso, essa dissertação propôe uma teoria que sugere um novo papel fisiológico para os microfilamentos de actina como biocondutores de elétrons.

No capítulo I, demonstramos que o tratamento com retinol aumentava a produção mitocondrial do radical superóxido (Figura 1), resultado que corrobora com trabalhos prévios do grupo que demonstraram a indução de estresse oxidativo por esse tratamento (Dal-Pizzol *et al*., 2000; Dal-Pizzol *et al*., 2001; Klamt *et al*., 2003; Gelain *et al*., 2006). O nível de dano oxidativo em proteínas do citoesqueleto isoladas também foi demonstrado (Figura 2), onde surpreendentemente, as proteínas do citoesqueleto isoladas apresentaram um menor nível de dano oxidativo quando comparadas às proteínas totais da célula e as de citoesqueleto isoladas de células controle. Este resultado sugeriu que o citoesqueleto das células tratadas estava sendo preservado do ambiente pró-oxidativo produzido pelo tratamento com retinol. Embora o nível de oxidação das proteínas do citoesqueleto tenha sido alterado pelo estresse oxidativo gerado pelo tratamento com retinol, o padrão eletroforético destas proteínas não demonstrou alteração frente a este tratamento (Figura 3). A arquitetura dos microfilamentos de actina também foi modulada pelo ambiente pró-oxidativo gerado pelo tratamento com retinol (Figura 4). Um fato importante de ser elucidado, é que a morfologia mesmo alterada em relação as células controle, não apresentou nenhum indicativo de dano ou disfunção. Esse resultado indica que por alguma razão a arquitetura do citoesqueleto de actina é alterada em resposta a um ambiente pró-

oxidativo, e esta alteração pode indicar uma adaptação desta estrutura frente a esse ambiente. Para avaliar se a alteração na organização dos microfilamentos de actina representava uma alteração na morfologia total celular, nós aplicamos uma fórmula matemática denominada *Coeficiente D*. Este coeficiente é aplicado usualmente para prever o crescimento e mudança morfológica em tumores, e é calculado a partir de fotografias de culturas em diferentes tempos e em diferentes densidades de plaqueamento. Quanto maior o valor do *Coeficiente D*, maior a deformação morfológica sofrida pela célula. Nós demonstramos que o tratamento com retinol alterava a morfologia celular de uma maneira radical livre dependente evidenciada pela determinação do coeficiente (Tabela 1). Este resultado indica que não só a morfologia dos microfilamentos de actina é alterada pelo tratamento com retinol, mas também a morfologia da célula como um todo também. Como resultado complementar nós também avaliamos a participação de espécies reativas de nitrogênio no estresse oxidativo gerado pelo tratamento com retinol. Espécies reativas de nitrogênio são associadas com disfunção de proteínas do citoesqueleto em várias situações patológicas (Banan *et al*., 2004). Nós encontramos uma diminuição nos níveis de óxido nítrico em células tratadas com retinol (Figura 5). Nós sugerimos que as células de alguma maneira estavam protegendo o citoesqueleto, uma vez que espécies reativas potencialmente danosas como as de nitrogênio, estavam sendo evitadas de serem produzidas. Todos esses resultados sugerem que o tratamento com retinol induziu uma modulação na arquitetura do citoesqueleto de células de Sertoli, e aparentemente essa modulação é uma resposta celular de adaptação desta estrutura ao ambiente pró-oxidativo gerado por esse tratamento. A hipótese de biocondução de elétrons foi apresentada como possível explicação do fenômeno observado.

No capítulo II, nós usamos a micotoxina Citocalasina B (Cyt B), que é capaz de despolimerizar os microfilamentos de actina, para demonstramos que a arquitetura dos microfilamentos de actina é essencial para a produção e manutenção do estresse oxidativo gerado pelo retinol. O primeiro resultado demonstrou que a arquitetura dos microfilamentos de actina é essencial para a indução de dano oxidativo a biomoléculas, uma vez que o co-tratamento com Cyt B reverteu os danos oxidativos gerados pelo tratamento com retinol (Figura 2). Esta reversão observada não era decorrente de morte ou inviabilidade celular. Nós apresentamos dois resultados que comprovam que o tratamento com Cyt B na dose utilizada não causava morte celular (Figura 3). De alguma maneira os microfilamentos de actina participam da propagação do dano oxidativo gerado pelo tratamento com retinol, quando a arquitetura normal desses filamentos foi alterada, o tratamento com retinol não foi capaz de gerar danos às biomoléculas. Recentemente na literatura trabalhos sugeriram que os microfilamentos de actina poderiam modular funções mitocondriais como a produção do radical superóxido e o potencial de membrana (Motrescu *et al.*, 2005; Yamamoto *et al.*, 2005). Nós investigamos se a reversão do dano oxidativo a biomoléculas pelo co-tratamento com Cyt B poderia estar relacionada com eventos mitocondriais. O co-tratamento com Cyt B foi capaz de atenuar o aumento na produção do radical superóxido mitocondrial e também a produção celular total de espécies reativas induzidas pelo tratamento com retinol (Figura 4). Nós também observamos que o tratamento com retinol modulava o potencial de membrana mitocondrial, causando uma diminuição do mesmo. O co-tratamento com Cyt B foi capaz de reverter essa diminuição do potencial de membrana mitocondrial observada (Figura 5). Esses resultados sugerem que a reversão do dano oxidativo à biomoléculas observada pelo co-tratamento com Cyt B, pode ser relacionada com a diminuição da produção de espécies reativas. Esses resultados reforçam os dados

existentes na literatura, onde funções mitocondriais poderiam ser controladas pelos microfilamentos de actina (Motrescu *et al*., 2005; Yamamoto *et al*., 2005). Nós também demonstramos que o ambiente redox celular não estava alterado no fim do tratamento (Figura 6), apenas um ambiente pró-oxidante foi encontrado em células que receberam somente o tratamento com Cyt B (Figura 6, insert). Nós correlacionamos este resultado com uma diminuição de grupamentos –SH de proteínas do citoesqueleto isoladas em células que receberam o mesmo tratamento. Citocalasina B possui um outro efeito descrito que é bloquear o transporte de glicose (Drewes *et al*., 1977). Neste trabalho esse efeito não foi observado uma vez que as concentrações celulares de glicose não variaram durante o tratamento (Figura 7). Nós também demonstramos a efetividade da concentração de Cyt B utilizada para alterar/despolimerizar os microfilamentos de actina (Figura 8).

Tendo como base os resultados apresentados, nós sugerimos que elétrons poderiam estar sendo conduzidos pelos microfilamentos de actina até a mitocôndria, e essa possível função seria parte da fisiologia normal celular. Essa sugestão/hipótese baseia-se na estrutura molecular e no estado de fosforilação da actina. Nós sugerimos que o tratamento com retinol induz um aumento nos níveis de fosforilação de actina (Figura 1), resultado este que corrobora com os pré-requisitos de condução de elétrons pelos microfilamentos apresentados nessa dissertação. O tratamento com retinol geraria um grande número de elétrons excedentes que seriam conduzidos pelos microfilamentos até a mitocôndria. Esses elétrons sobrecarregariam a capacidade de metabolização da cadeia respiratória mitocondrial, e acabariam gerando o radical superóxido. O co-tratamento com Cyt B despolimerizaria os filamentos de actina, impedindo assim que esses elétrons excedentes atingissem a mitocôndria e produzissem o radical superóxido, evitando assim, o estresse oxidativo gerado pelo retinol. Uma vez evitada a produção do radical superóxido, a

sinalização e o dano oxidativo desencadeado pelo mesmo também seriam evitados, possibilitando que as células respondam de maneira mais adequada ao tratamento com retinol.

4. Conclusão

Como conclusão geral, nós demonstramos que o tratamento com retinol em células de Sertoli cultivadas modula o citoesqueleto de uma forma radical livre dependente. Essa modulação foi associada com uma possível adaptação desta estrutura frente ao ambiente pró-oxidante. Nós sugerimos que a condução de elétrons pelos microfilamentos de actina, poderia explicar a correlação entre a organização desses filamentos, estresse oxidativo e funções mitocondriais. Nós supomos que esta possível nova função dos microfilamentos de actina seja parte integrante do metabolismo celular normal, e que ela poderia ajudar no entendimento de muitos processos celulares ainda não bem esclarecidos.

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