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BRUNA FONTANA THOMAZINI

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Biologia Celular e Estrutural, na Área de Biologia Tecidual

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RESUMO
A isotretinoína é a substância mais usada no tratamento de acne persistente, representando esperança de cura da doença. O tratamento sistêmico é indicado para casos severos e resistentes a outros tratamentos ou com recidivas frequentes. Uma vez que o intestino é o sítio de absorção de medicamentos e o fígado o local de seu metabolismo, torna-se interessante observar o resultado de um tratamento com isotretinoína nestes órgãos. O objetivo deste trabalho foi investigar o uso de três doses diferentes de isotretinoína no fígado, intestino delgado e no perfil bioquímico de enzimas ligadas à função hepática e de estresse oxidativo. 36 ratos machos foram separados em 6 grupos: água; óleo de soja; 1 mg/kg de isotretinoína; 2 grupos recebendo 5mg/kg de isotretinoína, sendo que um deles permaneceu outros 30 dias sem tratamento; 10mg/kg de isotretinoína. O veículo foi o óleo de soja e a solução foi ofertada por gavagem diária. Após 60 dias de experimentação, os ratos foram submetidos à eutanásia e as porções desejadas coletadas e processadas com rotina usual para análise na microscopia de luz e eletrônica. Após outros 30 dias o grupo com tratamento interrompido foi submetido ao mesmo procedimento. No fígado não encontramos alterações estruturais associadas a 1mg/kg da droga, mas elevação nos índices lipídicos, bem semelhantes às já descritas na literatura, apenas no grupo com 10mg/kg. A ultraestrutura mostrou resposta ao tratamento com indícios de maior atividade celular de forma dose dependente com aumento da frequência de mitocôndria e maior área de retículo endoplasmático rugoso. No perfil sérico e enzimático tecidual, notamos que 10mg/kg causou mais alterações com elevação dos níveis de HDL e VLDL. Jejuno e íleo mostraram-se como os mais sensíveis ao tratamento. As células caliciformes sofreram alterações em frequência nas três porções e independente da dose. Notamos que 5 mg/kg trouxe uma tendência a aumento da célula AB+PAS no íleo e redução no jejuno e duodeno, enquanto com a célula PAS+ o inverso foi notado. Essa alteração pode modificar a modulação da microbiota e pH na região, sendo necessários outros testes que viessem a elucidar o resultado para o organismo. Com relação a espessura da parede do órgão, o que poderia indicar uma tendência a desenvolvimento tecidual, vimos que duodeno não sofre alteração, jejuno tem uma tendência a aumento e íleo também. Considerando o grupo mantido após interrupção da droga, houve uma redução da parede do jejuno e um aumento constante no íleo. Interessante foi observar que 1mg/kg, a dose mais recomendada em tratamentos com o princípio ativo, não foi suficiente para alterações pronunciadas. Com o grupo 10mg/kg, notamos redução da área de mucosa ocupada por vilos e criptas no duodeno, que é a área mais sensível nessa dose. Observamos que o organismo mostrou um novo padrão em resposta ao tratamento proposto, o que traz uma nova perspectiva com relação ao princípio ativo. Os resultados desta tese indicam ser interessante o uso de técnicas que viessem a elucidar o possivel efeito que as alterações das células caliciformes podem trazer principalmente ao órgão.

Palavra-chave: isotretinoína, intestino delgado, fígado, histologia, microscopia

ABSTRACT
Isotretinoin is the substance most commonly used to treat persistent acne and it represents a hope to cure the disease. Systemic treatment is indicated for severe cases and those resistant to other treatments or having frequent recurrences. Since the drug absorbed in the intestine and metabolized in the liver, it is interesting to note the result in these organs of an isotretinoin treatment. The objective of this study was to investigate the use of three different doses of isotretinoin in the liver, small intestine and biochemical profile of enzymes linked to liver function and oxidative stress. 36 male rats were separated into 6 groups: water; soybean oil; 1mg/kg of isotretinoin; Two groups received 5mg/kg of isotretinoin, one of which remained another 30 days without treatment; 10mg/kg of isotretinoin. The vehicle was soybean oil and the solution was offered daily by gavage. After 60 days of trial, the mice were euthanized and the desired portions were collected and processed with the usual routine for analysis in light microscopy and electronic transmission one. The group that was maintained another 30 days after the treatment was subjected to the same procedure. Regarding the liver we did not find structural changes associated with 1mg/kg of the drug, but an increase in lipid levels similar to those described in the literature was observed in the group receiving 10mg/kg. The ultrastructural analysis showed increasing, dose dependent activity of the cells, with more frequent mitochondria and greater granular endoplasmic reticulum area. Regarding the serum and tissue enzyme profile, we noted that 10mg/kg group had increased elevation of HDL and VLDL levels. Jejunum and ileum structural features showed a greater sensibility to the treatment. Goblet cells changed their frequency in the three intestinal portions, independently of the isotrentinoin dose. The 5mg/kg group had a tendency to increase the AB+PAS+ cell in the ileum but they are reduced in the duodenum and jejunum, while the contrary was found for PAS+ cells. This change can modify the microflora and pH of the region, requiring further tests to elucidate this condition. Body wall thickness, which could indicate a tendency for tissue development, remained unchanged in the duodenum, while the jejunum and ileum had a tendency to increase their thickness. The group that was maintained for a month after drug discontinuation, reduced the wall thickness of the jejunum but a continued increase of the ileum wall was found. It was interesting to note that 1mg/kg, the recommended dose for treatments with the active ingredient was not sufficient to produce pronounced changes. With 10mg/kg the mucosal area occupied by villi and crypts in the duodenum was reduced, this being the most sensitive area at the highest dose. Our study showed a new standard of response to the treatments proposed, which brings a new perspective regarding the active ingredient of this medicine. The results of this thesis suggest the use of new techniques that could elucidate the effect of the changed pattern of goblet cells regarding organ function.

Keywords: isotretinoin, small intestine, liver, histology, microscopy

SUMÁRIO

1-INTRODUÇÃO

1.1-A Acne

A acne é uma dermatose inflamatória extremamente comum na prática médica. Em levantamento epidemiológico realizado pela Sociedade Brasileira de Dermatologia, a acne foi a causa mais frequente de consultas ao dermatologista, correspondendo a 14% dos atendimentos (MINISTÉRIO DA SAÚDE, 2015; SOCIEDADE BRASILEIRA DE DERMATOLOGIA, 2006). É uma desordem multifatorial que atinge indivíduos de todas as idades. Estudos epidemiológicos mostraram que 80% dos adolescentes e jovens entre 11 e 30 anos estão propensos a apresentar acne. A doença chama atenção quando consideramos seu impacto emocional na rotina do paciente, que pode ser comparado ao de pacientes com doenças sistêmicas como diabetes e epilepsia, o que torna necessária atenção terapêutica eficiente (LAYTON, 2016; PARK & SKOPIT, 2016; MINISTÉRIO DA SAÚDE, 2015; KNUTSEN-LARSON et al, 2012; GOLLNICK et al, 2003).

A acne caracteriza-se por ser uma enfermidade inflamatória dos folículos sebáceos de partes específicas do corpo como face, tórax, ombros e costas. Sua expressão clínica depende de vários fatores e costuma aparecer durante a fase da puberdade, quando a estimulação hormonal androgênica aumenta a secreção de sebo, altera a queratinização do folículo, desencadeia inflamação local e sua colonização por bactérias, sendo a *Propionibacterium acnes* a mais comum durante o ciclo da doença. A acne apresenta diferentes graus, indo do mais leve, onde há presença de poucos comedões (cistos sebáceos dilatados com a presença de sebo, epitélio queratinizado, bactérias e até mesmo leveduras), até as formas mais graves, que podem provocar nódulos e fístulas com grande reação inflamatória, podendo descaracterizar a região ao qual estão localizados (FIFE, 2016; WILLIAMS et al, 2011; LEAL et al, 2008; DINIZ et al, 2002; LOTAN, 1980).

Essa doença geralmente tem um curso prolongado, com recidivas eventuais ou agudas, o que afeta direta e negativamente a vida social e autoestima dos pacientes, em especial os adolescentes. Muitas opções terapêuticas estão disponíveis dependendo da gravidade da doença, sendo que uma boa parcela dos pacientes obtém sucesso com tratamento tópico, como peróxido de benzoíla, antibióticos e retinóides. Entretanto, nos casos severos ou resistentes a tentativas terapêuticas anteriores, a indicação clínica seguinte é o tratamento sistêmico, sendo a isotretinoína a droga mais utilizada para esse fim (LAYTON, 2016; PARK & SKOPIT, 2016; ABALI, 2013).

Fica cada vez mais claro que as cicatrizes na pele dos pacientes causadas pela doença são mais comuns quando o tratamento é iniciado tardiamente, atingindo 95% dos pacientes, com cerca de 1-11% deles mantendo cicatrizes permanentes. Segundo pesquisa apresentada pela Academia

Americana de Dermatologia (LAYTON, 2016), a inflamação típica da doença é essencial para o surgimento das cicatrizes. Isso enfatiza a necessidade de se estabelecer há quanto tempo a doença está presente e quais tratamentos prévios já foram realizados sendo que a escolha do tratamento mais adequado e com menor tempo de duração influenciará positivamente em seu bem-estar (FIFE, 2016; LAYTON, 2016; WILLIAMS et al, 2011).

1.2-A isotretinoína: a revolução no tratamento para a acne

De acordo com a definição da IUPAC e da União Internacional de Bioquímica e Biologia Molecular, retinóides é o termo empregado para uma classe de compostos cuja estrutura química consiste em quatro grupos isoprenóides unidos de modo cabeça-cauda (Figura 1). O metabolismo e o catabolismo dos retinóides provocam o rearranjo de sua estrutura, justificando a existência de vários análogos com efeitos biológicos diversos (DINIZ et al, 2002; IUPAC-IUB, 1983; IUPAC-IUB, 1966).

Em 1934, Wald isolou uma substância da retina e, em 1944, Mortom afirmou que essa substância era, na verdade, um aldeído da vitamina A e a denominou de retinaldeído ou retinal. A partir da descoberta da vitamina A e sua estrutura molecular deu-se início à pesquisa por derivados químicos e sintéticos com ação biológica do retinol. Em 1976, Sporn e seus colaboradores estabeleceram o termo retinóide para todas as substâncias análogas de estrutura natural, bem como para todas as substâncias sintéticas derivadas da vitamina A. Fazendo parte desse grupo, encontram-se: vitamina A, ácido-trans-retinóico (tretinoína), ácido-13-cis-retinóico (isotretinoína), ácido-trans-5,6-epoxi-5,6-diidro-retinóico, ácido-13-cis-4-hidroxi-retinóico, ácido-trans-4-hidroxi-retinóico, ácido-13-cis-4-oxo-retinóico, ácido-trans-4-oxo-retinóico e ácido-13-cis-2-hidroxi-4-oxo-retinóico (CHOONG et al, 2015; DINIZ et al, 2002; SPORN et al, 1976).

A partir da molécula da vitamina A, observou-se uma diferenciação epitelial, mesmo com baixo índice terapêutico. Com isso foi sintetizado o seu isômero, onde se obteve a isotretinoína, apresentando margem terapêutica 2,5 vezes maior que a tretinoína. A isotretinoína foi inicialmente introduzida nos Estados Unidos em 1982 para o tratamento da acne nodular cística e, desde então tem sido o tratamento mais efetivo para a doença (TAN et al, 2016; CAJUEIRO et al, 2014; BETTONI, 2009; CHARAKIDA et al, 2004; DINIZ et al, 2002).

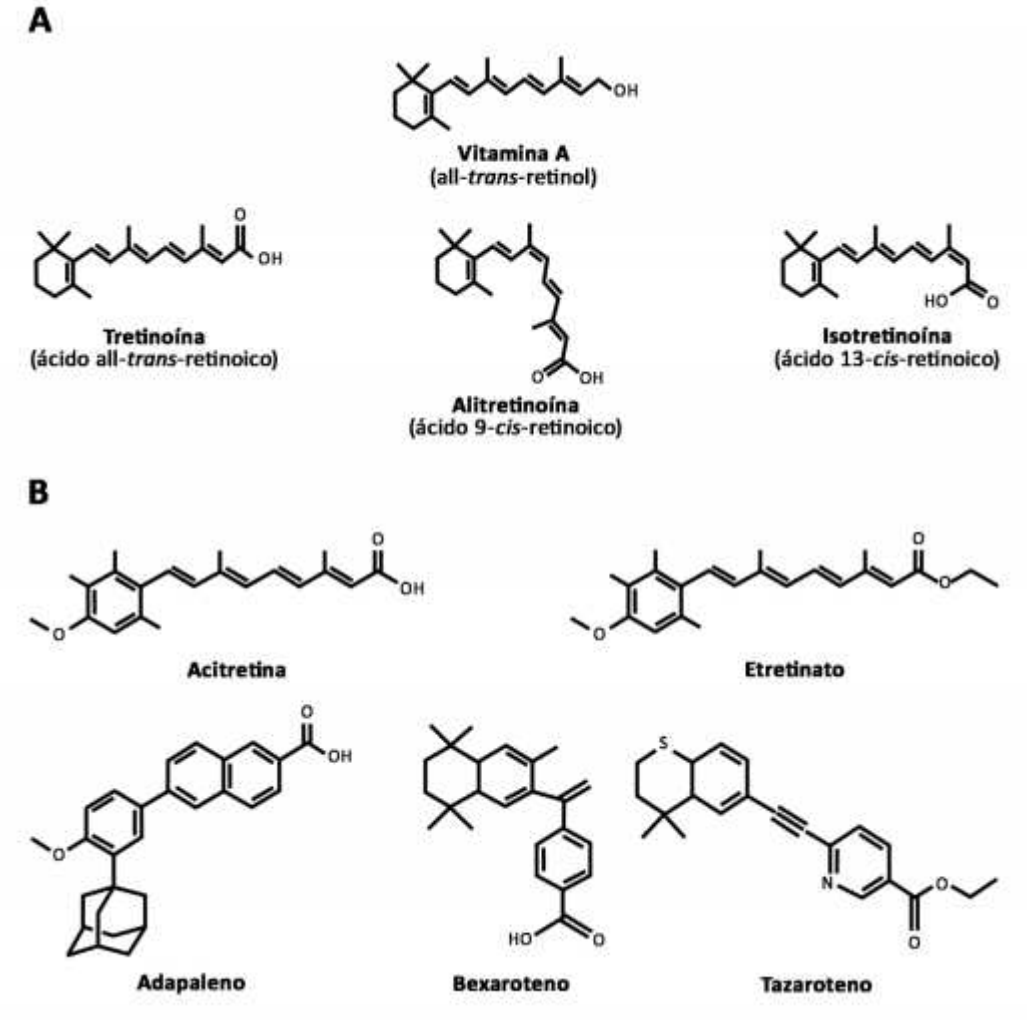

Figura 1: Estrutura química dos retinóides naturais (A) e sintéticos (B) (SILVA et al, 2013).

No Brasil, a regulamentação do medicamente tendo como princípio a isotretinoína ocorreu em 2002 e vem sendo usada desde então (OFUCHI, 2010). A portaria do Ministério da Saúde nº1159, de 18 de novembro de 2015 "Aprova o Protocolo de uso da isotretinoína no tratamento da acne grave", e revoga a Portaria nº 143/SAS/MS, de 31 de março de 2010 (MINISTÉRIO DA SAÚDE, 2015).

A isotretinoína demonstra grande eficiência terapêutica relacionada à indução e controle da diferenciação epitelial, nos tecidos secretores de muco ou queratinizantes, à produção de prostaglandinas E2, de colágeno, de precursores da queratina, como os tonofilamentos e tonofibrilas e ao controle da proliferação de *Propionibacterium acnes*. Uma vez que o desenvolvimento das glândulas sebáceas e a exacerbação de sua atividade secretora são fatores essenciais para a ocorrência de lesão inflamatória associada à acne, o bloqueio na produção de sebo provocado pela isotretinoína constitui fator determinante de sua atividade farmacológica no tratamento desta afecção (PREVOST

et al, 2013; RIGOPOULOS et al, 2010; SAMPAIO, 2008; DINIZ et al, 2002; SAURAT, 1997; ALLEN & BLOXHAM, 1989).

O perfil farmacocinético da isotretinoína é análogo ao da vitamina A. Após administração oral, o pico de concentração plasmática da isotretinoína é atingido em cerca de 2 a 4 horas. Aproximadamente 20% da isotretinoína serão absorvidos quando administrados com o estômago vazio, aumentando para 40% quando em presença de alimento (WEBSTER et al, 2013; WHITE, 1999; NANKERVIS et al, 1995). Isotretinoína e tretinoína são interconvertidas *in vivo* e cerca de 20 a 30% da dose de isotretinoína são aparentemente metabolizados nesta rota. É um agonista fraco para os receptores de ácido retinóico, mas é provavelmente convertida intracelularmente para compostos mais ativos. O principal metabólito da isotretinoína é a 4-oxo-isotretinoína, excretada pela bile após ser conjugada com o ácido glicurônico, possuindo meia-vida média de eliminação de 25 horas. Com a administração repetida, a concentração de equilíbrio é estabelecida em 5 a 7 dias. A isotretinoína é excretada pela via urinária, sendo possível detectar na urina a presença de quantidades baixas de isotretinoína não conjugada. A excreção de cerca de 50 a 74% da isotretinoína administrada ocorre nas fezes, como resultado de uma absorção incompleta, eliminação biliar ou recirculação entero-hepática (ARONSON, 2016; DINIZ et al, 2002; NAPOLI, 1999; DUESTER, 1996; ALLEN & BLOXHAM, 1989).

A absorção do fármaco isotretinoína ocorre no intestino, onde os quilomícrons reesterificados com o grupamento retinol são absorvidos pelo sistema linfático. Devido ao caráter lipofílico da isotretinoína, sua absorção é aumentada com a ingestão concomitante de alimento. No sangue, a isotretinoína é transportada ligada à proteína albumina e entra na célula por um processo de difusão passiva (OFUCHI, 2010; TSUKADA et al, 2002).

1.3-Indicações de tratamento e dose recomendada

As indicações convencionais do tratamento com isotretinoína oral são: acne nódulo-cística e acne resistente ao tratamento convencional. A dose diária é calculada de acordo com o peso do paciente e varia de 0,5 a 2mg/kg/dia por 2-10 meses. Para prevenir as recidivas, que já são pouco comuns, é recomendada uma dose cumulativa entre 100 e 150mg/kg, mais comumente recomendada a dosagem de 120mg/kg (MINISTÉRIO DA SAÚDE, 2015; BRITO et al, 2010; SAMPAIO, 2008; CHARAKIDA et al, 2004; DINIZ et al, 2002). As recidivas parecem ser mais frequentes quando as doses diárias ou totais mais baixas são utilizadas, embora outros fatores possam estar envolvidos. As taxas de recidivas variam entre 10 a 60% dependendo da dose e do regime utilizado, sendo que com

doses adequadas o risco de recidiva é inferior a 1% (MORALES-CARDONA, 2013; OFUCHI, 2010; SAMPAIO, 2008; O´REILLY et al, 2006; FERGUSON et al, 2005a; FERGUSON et al, 2005b).

1.4-Efeitos adversos do tratamento com isotretinoína

Cerca de 25% dos pacientes em tratamento com isotretinoína apresentam elevação do nível plasmático de triglicérides o que, em alguns casos, pode estar associado ao aparecimento de pancreatite aguda. A isotretinoína pode provocar ainda uma leve queda da concentração plasmática de colesterol associado à lipoproteína de alta densidade (HDL) e aumento de colesterol associado à lipoproteína de baixa densidade (LDL) e associado à lipoproteína de muito baixa densidade (VLDL). As alterações nos níveis séricos de triglicérides e colesterol são reversíveis com a interrupção do tratamento. A avaliação da função hepática é normalmente feita com testes de rotina como a dosagem de bilirrubina, albumina e presença de lesões hepatocelulares. A análise de rotina das enzimas transaminases hepáticas também é usada para avaliar a função hepática controlando o aparecimento de lesão nos hepatócitos (TANASOV, 2012; FERRIOLI et al, 2011).

O evento adverso relacionado ao uso da isotretinoína que representa maior severidade é sua capacidade teratogênica. Estudos indicam que a exposição a 0,4-1,5mg/kg/dia de isotretinoína durante as primeiras semanas de gravidez causa aborto espontâneo em 22% dos casos e má formação congênita do feto em 18%. A relação entre o tempo de exposição e os efeitos observados ainda não estão totalmente esclarecidos então, considera-se que a exposição a isotretinoína em qualquer momento durante uma gravidez representa perigo ao feto (CHARAKIDA et al, 2004).

As alterações nas membranas mucosas e pele são decorrentes da diminuição da produção de sebo, redução da espessura do estrato córneo e alteração da função de barreira da pele. A maioria dos pacientes desenvolve ressecamento de lábios, pele e mucosas. A secura labial ocorre em 100% dos casos e da mucosa nasal em 50%. Prurido e descamação da pele são frequentes (25%), podendo desenvolver, inclusive, fissuras digitais. A fotossensibilidade ocorre em 40% dos casos, devido à redução das camadas do estrato córneo da pele (TAN et al, 2016; GOULART, 2013; PREVOST et al, 2013; AL-BREIKI et al, 2012; BRITO et al, 2010; WOLVERTON, 2007).

Reações adversas menos frequentes e também reversíveis incluem vômitos, sangramentos gastrointestinais, apendicite, inflamação das gengivas, esofagite, anorexia, perda de peso e colite ulcerativa. Também há indícios que o uso da droga pode agravar os sintomas de doenças inflamatórias, como as colites e pancreatites. Apesar dessa tendência a desenvolver processos inflamatórios, a droga já foi administrada com sucesso em pacientes com doença de Crohn e colite

ulcerativa sem desconforto aos pacientes (PREVOST et al, 2013; AL-BREIKI et al, 2012; BRITO et al, 2010; WOLVERTON, 2007; CHARAKIDA et al., 2004; DINIZ et al, 2002; McCARTER & CHEN, 1992; BIGBY & STERN, 1988).

Alguns dos relatos com relação ao trato gastrointestinal apresentaram melhora ou a resolução completa dos sintomas após a suspensão do tratamento com isotretinoína, tornando a aparecer com nova administração. Outros relataram o início dos sintomas após a conclusão do tratamento (AL-BREIKI et al, 2012; BHARMAL & ANDERSON, 2010; PASSIER & SRIVASTAVA, 2006; REDDY et al, 2006; RENIERS & HOWARD, 2001).

1.5- O intestino delgado e fígado: Estrutura

O aparelho digestório é composto pelo tubo digestivo e glândulas anexas (fígado, pâncreas e glândulas salivares). O tubo digestivo compreende: cavidade oral, esôfago, estômago, intestino delgado e intestino grosso. É no intestino que encontramos a conversão de alimentos ingeridos em pequenas partículas de nutrientes em solução (JUNQUEIRA & CARNEIRO, 2013; BARRET, 2006; D´ANGELO & FATTINI, 1995).

Todas as partes do tubo digestivo apresentam certas características estruturais comuns. O tubo digestivo é composto por uma luz, ou lúmen, cujo diâmetro é variável, circundado por uma parede formada por quatro túnicas: mucosa, submucosa, muscular e serosa. A túnica mucosa é composta por revestimento epitelial apoiado sobre uma lâmina própria de tecido conjuntivo frouxo rico em vasos sanguíneos e linfáticos e células musculares lisas, algumas vezes apresentando também glândulas e tecido linfoide, além de uma delicada túnica de músculo liso constituindo a muscular da mucosa (Figura 2) (JUNQUEIRA & CARNEIRO, 2013).

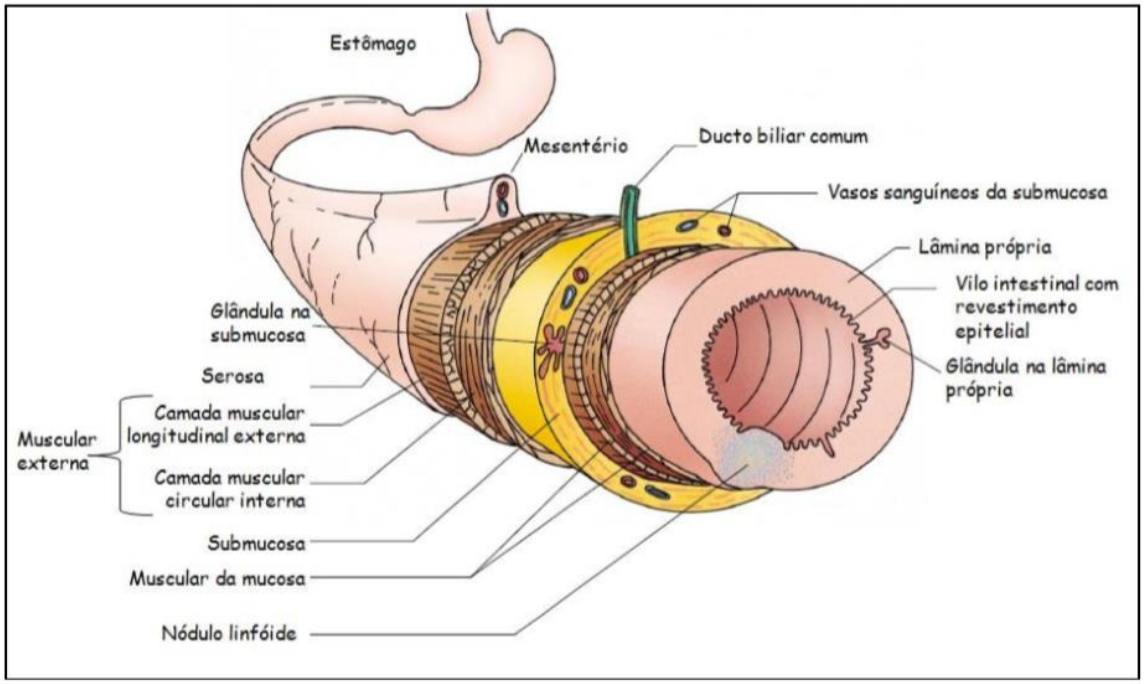

Figura 2: Desenho esquemático da organização da parede do tubo digestivo (Adaptado de GARTER & HIATT, 2002)

A túnica submucosa é composta por tecido conjuntivo denso não modelado, com muitos vasos sanguíneos e linfáticos, e o plexo de Meissner. Esta túnica pode conter também glândulas e tecido linfóide. A túnica muscular contém células musculares lisas orientadas em espiral, divididas em duas subcamadas: na subcamada mais interna (próxima ao lúmen), a orientação é geralmente circular; na subcamada externa, é majoritariamente longitudinal (JUNQUEIRA & CARNEIRO, 2013).

A serosa é formada por uma camada delgada de tecido conjuntivo frouxo, revestida por um epitélio pavimentoso simples denominado mesotélio. Na cavidade abdominal, a serosa que reveste os órgãos é denominada peritônio visceral e está em continuidade com o mesentério, que suporta os intestinos, e com o peritônio parietal. O peritônio parietal, por sua vez, é caracterizado como uma membrana que reveste a parede da cavidade abdominal (JUNQUEIRA & CARNEIRO, 2013).

O intestino delgado corresponde ao órgão do tubo digestivo situado entre o piloro e o ceco e é o sítio terminal da digestão dos alimentos, absorção de nutrientes e secreção endócrina. Os processos de digestão são completados no intestino delgado, onde os nutrientes são absorvidos pelos enterócitos. O intestino delgado é relativamente longo e consiste em três segmentos: duodeno, jejuno e íleo. A presença de pregas, vilosidades e microvilosidades aumentam muito a superfície de revestimento intestinal. Essa é considerada uma característica importante num órgão onde a absorção ocorre tão

intensamente, um aumento de aproximadamente 600 vezes na superfície intestinal (JUNQUEIRA & CARNEIRO, 2013).

O epitélio dos vilos é formado principalmente por células absortivas (enterócitos) e células caliciformes, o qual é contíguo com o epitélio das criptas que, por sua vez, contêm algumas células absortivas, células caliciformes, células enteroendócrinas, células de Paneth e células-tronco (GARTNER & HIATT, 2003; JUNQUEIRA & CARNEIRO, 2013). O percentual de renovação celular é determinado pela atividade das células-tronco/progenitoras multipotentes, localizadas perto da base da cripta de Lieberkühn, próximas as células de Paneth (Figura 3) (JUNQUEIRA & CARNEIRO, 2013; GARCIA-MIRANDA et al, 2010; KARAM, 1999).

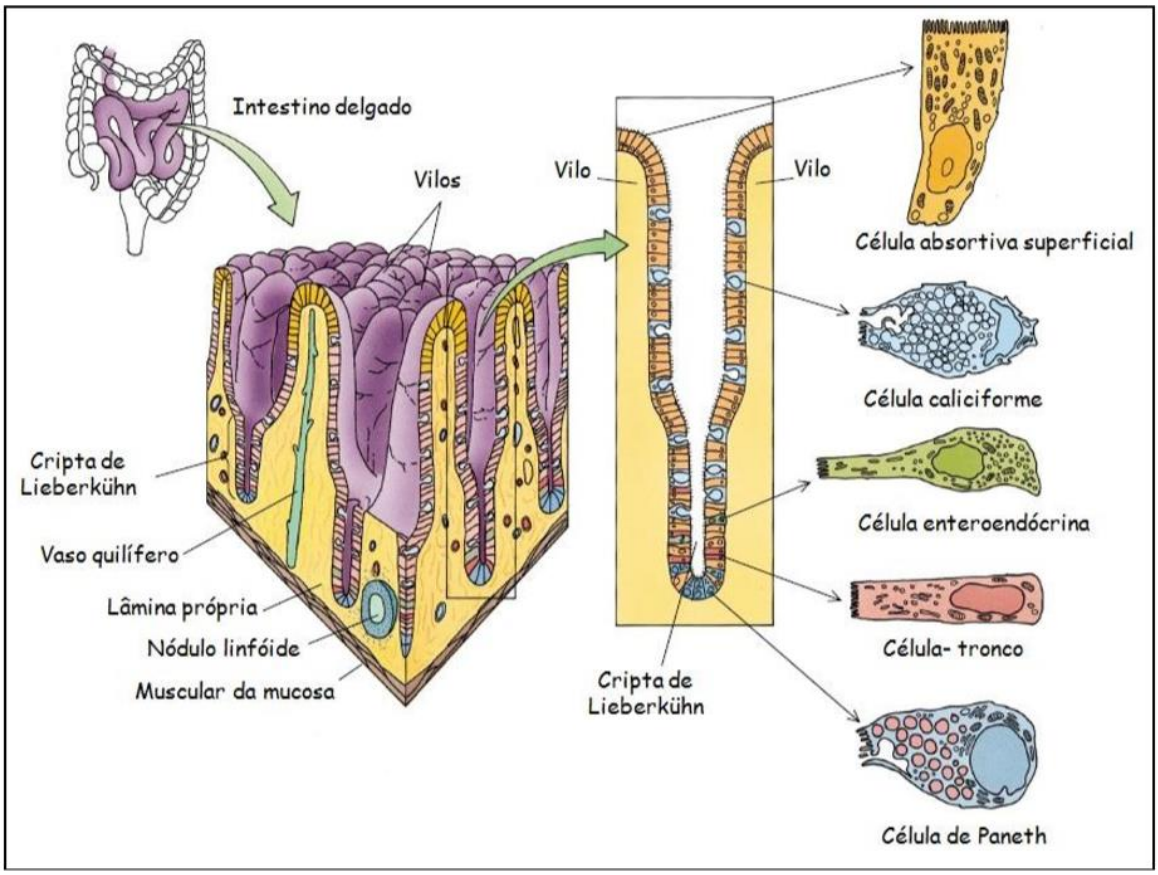

Figura 3: Diagrama esquemático da mucosa, vilos, criptas de Lieberkühn, e componentes celulares do intestino delgado de humanos (Adaptado de GARTNET & HIATT, 2002).

O fígado é o segundo maior órgão do corpo e considerado também a maior glândula. Nele os nutrientes absorvidos no intestino delgado são processados e armazenados para utilização por outros órgãos. O componente estrutural básico do fígado é a célula hepática ou hepatócito. Sua posição no

sistema circulatório é ideal para captar, transformar e acumular metabólitos bem como para neutralizar e eliminar toxinas pela bile (JUNQUEIRA & CARNEIRO, 2013).

Histologicamente, o fígado é formado pelo parênquima lobular sendo esse constituído pelas células (hepatócitos, Kupffer e Ito); pelos capilares sinusóides e perissinusoidais (espaços de Disse); e pelos ductos biliares. O parênquima não lobular é formado pelos espaços portais (ducto biliar, vasos linfáticos e vasos sanguíneos), veias centro-lobulares e ramos de vasos sanguíneos. O parênquima lobular compreende a maior parte do tecido hepático. O fígado é considerado um importante alvo de toxicidade devido à sua localização, entre o local de absorção e o sistema circulatório, e devido ao fato de ser o principal local de metabolização e eliminação de substâncias estranhas ao organismo (KIERSZENBAUM & TRES, 2016; JUNQUEIRA & CARNEIRO, 2013; SILVA, 2013; RUSSMANN et al, 2009; WEIBEL et al, 1969).

2-JUSTIFICATIVA

A isotretinoína possui sucesso já comprovado e inegável na cura da acne, uma doença que atinge todas as idades e que pode trazer graves consequências psicossociais. É um princípio ativo muito usado na clínica médica mas com fortes indícios de efeito agravante nas enzimas hepáticas e índices de estresse oxidativo e em colite e pancreatite. Os efeitos já descritos ocorrem em longos períodos de tratamento e tendem a voltar ao nível basal ao final dele. A escassez de trabalhos experimentais envolvendo o uso da substância chama atenção e justifica a realização deste trabalho com cunho experimental e voltado para a prática histológica envolvendo um curto tempo de tratamento.

3-OBJETIVOS

3.1-Geral

Observar os resultados de um tratamento com medicamento, tendo a isotretinoína como princípio ativo, no protocolo similar ao aplicado em humanos, sobre a estrutura do intestino delgado e fígado de ratos machos Wistar.

3.2-Específicos

-Acompanhar o consumo alimentar e variação de massa corporal dos grupos ao longo das semanas,
-Realizar avaliação bioquímica do soro sanguíneo dos animais após o tratamento,
-Realizar avaliação bioquímica tecidual hepática,

-Realizar avaliação estrutural e ultraestrutural de componentes da mucosa e submucosa do intestino delgado, nas três porções; duodeno, jejuno e íleo;

-Realizar avaliação estrutural e ultraestrutural do fígado;

-Comparar diferentes dosagens e observar os mesmos parâmetros observando um período pós tratamento.

4-MATERIAIS E MÉTODOS

4.1-Animais e protocolo experimental

No experimento foram utilizados 36 ratos Wistar machos (*Rattus norvegicus*), divididos em seis grupos. Todos os ratos passaram pelo desmame padrão aos 21 dias de idade, após o qual foram aleatoriamente divididos nos grupos experimentais. O tratamento teve início quando os ratos atingiram a idade de 54 dias.

Controles:

- C (n=6): gavagem diária de água,
- D0 (n=6): gavagem diária de óleo de soja,

Tratamento 1:

- D1 (n=6): gavagem diária de dose referente a 1mg/kg,
-D10 (n=6): gavagem diária de dose referente a 10mg/kg,

Tratamento 2:

- D5a (n=6): gavagem diária de dose referente a 5mg/kg,
- D5b (n=6): gavagem diária de dose referente a 5mg/kg por 60 dias acrescidos de outros 30 dias pós tratamento.

Os animais foram obtidos no Centro Multidisciplinar de Investigação Biológica (CEMIB), Universidade Estadual de Campinas (Unicamp) e mantidos em gaiolas coletivas contendo 6 animais em cada uma. Foram alojados no Biotério do Departamento de Biologia Celular e Estrutural/ Unicamp com controle de ciclos de luminosidade de 12 horas e temperatura de 22±1°C. Os animais tiveram livre acesso a ração padrão para roedores e água.

As doses da substância foram definidas com base na literatura. Segundo os autores, a dose diária recomendada para humanos é calculada de acordo com o peso do paciente e varia de 0,5 a 1mg/kg/dia por 2-10 meses (MINISTÉRIO DA SAÚDE, 2015; BRITO et al, 2010; SAMPAIO, 2008; CHARAKIDA et al, 2004; DINIZ et al, 2002). O experimento contou com ratos recebendo dosagem acima (Grupo D10) e outros com dosagem próxima da recomendada para humanos (Grupos D1, D5a

e D5b). O tratamento teve duração de 60 dias, uma vez que esta é a duração mínima do tratamento em humanos. Os ratos que foram mantidos mais 30 dias sem tratamento (Grupo D5b) (metade do período de tratamento) foram avaliados para verificar como os órgãos se comportam após o final do tratamento.

A escolha de animais juvenis tenta reproduzir a sugestão de tratamento que também pode ser feita em adolescente. A quantidade de animais requerida segue os princípios éticos estabelecidos, pela Sociedade Brasileira de Ciência em Animais de Laboratório e este projeto foi avaliado e aprovado pelo Comitê de Ética no Uso de Animais da Unicamp (protocolo nº 2831-1).

As cápsulas de Roacutan® (Laboratório Roche, São Paulo, SP, Brasil) de 20mg foram mantidas em temperatura ambiente (15 a 30°C), nas embalagens originais e protegidas da luz e umidade. As cápsulas foram cuidadosamente abertas e todo o conteúdo líquido foi retirado e transferido para um frasco âmbar. As diluições seguiram as proporções para as doses de cada tratamento, ajustadas a cada 20 dias de acordo com a massa média do grupo, em protocolo similar a experimento prévio (GOULART, 2013). A diluição foi preparada em local protegido de luz e estocada em frascos âmbares, para proteger da fotoisomerização, em local com umidade e temperatura controladas (FERGUSON et al, 2006). A substância foi dissolvida em óleo de soja, de forma a produzir as doses desejadas para o teste *in vivo* (NANKERVIS et al, 1995).

4.2-Consumo alimentar e ganho de massa corporal

Os ratos foram identificados individualmente e pesados toda semana. A ingestão alimentar e de água foram definidas a partir da quantidade ofertada e do que sobrou no comedouro e no bebedouro da gaiola. Esse dado foi obtido duas vezes por semana.

4.3-Eutanásia, coleta de sangue e determinação de níveis bioquímico sérico

No final do período experimental, os animais dos grupos controle (C e D0) e tratado (D1, D10, D5a) foram submetidos a jejum de 12 horas. Após outros 30 dias, o grupo D5b passou pelo mesmo procedimento. Os animais foram pesados e anestesiados com injeção intramuscular de Xilazina e Cetamina (5 e 80mg/kg de peso corporal, respectivamente). O sangue foi coletado por punção cardíaca no ventrículo esquerdo, utilizando tubos Vacuette® contendo acelerador de coágulo e centrifugado a 3500rpm por 10 minutos, a 4ºC.

O soro obtido foi utilizado para a determinação dos seguintes perfis bioquímicos:

-perfil hepático: alanina transaminase, aspartato aminotransferase, bilirrubina total e frações, fosfatase alcalina, gama-glutamil transferase;
-perfil lipídico: colesterol total e frações, triglicerídeos;
-perfil protéico: proteína total, albumina, globulina.
-avaliação da peroxidação lipídica: dosagem do malodialdeído

Os testes foram feitos em laboratório especializado VetPat©- Laboratório de Análises Veterinárias (CNPJ: 08.730.717/0001-09) e indicaram se as dosagens bioquímicas, no período experimental sugerido foram capazes de levar a alterações da função hepática, a dislipidemias e a alterações na homeostase de proteínas séricas que tem suas concentrações influenciadas pelo estado nutricional hepático, renal e erros metabólicos.

4.4-Coleta e processamento do material biológico

a)Determinação de níveis bioquímicos teciduais hepático e análise do nível de estresse oxidativo

Após a coleta do sangue, o fígado foi retirado, pesado e, na sequência, porções do duodeno, jejuno e íleo. Parte do fígado foi congelado a -80°C para a determinação dos seguintes níveis: Superóxido dismutase *(*Superoxide Dismutase Assay Kit Cayman Chemicals® Cat #706002), Catalase (Catalase Assay kit- Sigma Aldrich® Cat #100-1KT), Fosfatase alcalina (Fosfatase Alcalina Bioclin® Cat #K019), Glutationa total (Kit Sigma Aldrich Glutathione Assay® Cat#CS0260- 1KT), Gama-Glutamil Transferase (Gama Glutamil Transferase Laborlab® Cat#09900), Aspartato Aminotransferase (Transaminase Oxalacética InVitro® Cat #015), Alanina Transaminase (Transaminase Pirúvica InVitro® Cat #016), Determinação de proteínas pelo ensaio de Bradford (Bio-Rad Protein Assay® Cat #500-0006) e peroxidação lipídica pelo método de determinação de Malondialdeído *(*TBars Assay Kit Cayman Chemicals® Cat #10009055). Os testes foram realizados seguindo o protocolo do Kit com alguma modificação.

b)Preparo da amostra para avaliação estrutural e ultraestrutural

Fragmentos do fígado e das porções do intestino foram coletados para avaliação estrutural e ultraestrutural e seguiram a rotina usual. Logo após a coleta, foram lavados em solução salina seguido de imersão em solução fixadora de glutaraldeído a 2,5% com paraformaldeído a 4% (solução fixadora de Karnovsky modificado) (KARNOVSKY, 1965) em tampão fosfato pH7,2. O processamento para inclusão em parafina do fígado e intestino seguiu o protocolo com desidratação em solução crescente

de álcool e diafanização em xilol para posterior inclusão. Desses blocos foram obtidos cortes com 5µm de espessura. A inclusão do fígado em resina glicolmetacrilato Leica® seguiu o protocolo do Kit e foram obtidos cortes com 2µm de espessura. Do intestino delgado foram produzidas lâminas contendo 8 cortes com 50µm de intervalo entre cortes. Do fígado foram produzidas lâminas com 8 cortes com 30µm de intervalo entre os cortes.

Os fragmentos com inclusão em parafina foram submetidos a coloração pelo Tricrômico de Masson (Weigert´s Iron Hematoxilin Set®, Sigma-Aldrich e Masson Trichrome Stain Kit®, Sigma-Aldrich) e aos testes histoquímicos: Ácido Periódico de Schiff combinado com Alcian Blue pH2,5 (Alcian Blue pH 2.5-PAS®, EasyPath) e Reticulina (Reticulina®, EasyPath).

A técnica combinada de Ácido Periódico Schiff e Alcian Blue pH2,5 (AB+PAS) foi empregada nos cortes histológicos do intestino uma vez que pode evidenciar as mucinas secretadas pelas células caliciformes de acordo com seu grupamento dominante. Assim, foi possível estimar a frequência de células secretoras de mucina básica (PAS^+), ácida (AB^+) e de uma mistura dessas (AB^+PAS^+). Quando consideramos o fígado, esta técnica detecta acúmulos de carboidratos, sendo o principal encontrado, o glicogênio. A técnica de Reticulina evidencia as fibras reticulares, no tecido conjuntivo adjacente. Essas fibras se organizam formando uma trama mantendo a organização geral dos tecidos. O Tricrômico de Masson foi escolhido pois diferencia perfeitamente porções de tecido muscular (cor vermelho) e tecido conjuntivo (em azul). Caso estivessem presentes, regiões de fibrose poderiam ser evidenciadas.

Cortes de intestino incluídos em parafina e do fígado incluído em resina foram utilizados para as determinações morfométricas e estereológicas após coloração com Hematoxilina e Eosina. Essas avaliações estão descritas no tópico a seguir.

Outras porções do fígado foram processados para observação de sua ultraestrutura por Microscopia Eletrônica de Transmissão. Após coleta, pequenos fragmentos foram lavados com solução salina e imediatamente imersos em solução fixadora de glutaraldeído 2,5%. A pós-fixação foi em solução de tetróxido de ósmio a 1% durante 2 horas, à temperatura ambiente, seguida de desidratação com soluções de acetona com concentração crescente. A inclusão foi feita em resina Epon®. Cortes com 70nm de espessura foram obtidos por ultramicrotomia e a contrastação seguiu a rotina com Acetato de Uranila (2%) por 30min, seguido de incubação com solução de Citrato de Chumbo (0,2%) por 5min.

Outras porções do duodeno, jejuno e íleo foram avaliados por Microscopia Eletrônica de Varredura a fim de evidenciar possíveis alterações estruturais mascaradas nos cortes em duas

dimensões utilizados na avaliação da microscopia de luz. Neste caso, porções do intestino delgado foram totalmente desidratados (série crescente de etanol de 70 a 100%) logo após a fixação usual com solução modificada de Karnovsky (KARNOVSKY, 1965). Na sequência, passaram pelo Ponto Crítico (CPD-030) e deposição de ouro (Sputter Coater SCD-050). As avaliações na Microscopia Eletrônica de Transmissão (LEO 906) e na Microscopia Eletrônica de Varredura (JSM 5800LV) foram feitas no Laboratório de Microscopia Eletrônica (LME), do Instituto de Biologia/Unicamp.

c)Análises morfométricas

Todas as análises morfométricas foram realizadas no programa de análise *Image Pro Plus* (versão 6.3 para Windows®), a partir de imagens capturadas das preparações histológicas com o mesmo *software* usando microscópio Olympus BX41 com câmera acoplada QColor 3. No intestino delgado foram avaliadas as regiões da mucosa e submucosa (THOMAZINI, 2011) em material corado com Hematoxilina-Eosina. Para isso foram consideradas 30 imagens por animal amostrando todos os cortes da lâmina. A morfometria foi realizada considerando todas as imagens obtidas.

a) Morfometria dos vilos: determinação da altura de 15 vilos/animal em aumento de 100x.

b) Morfometria da cripta de Lieberkühn: determinação da altura de 15 criptas/animal em aumento de 200x.

c) Avaliação da relação entre altura dos vilos com altura das criptas: a partir das médias obtidas nos itens a e b.

d) Morfometria da mucosa: determinação das espessuras da parede do órgão em 10 pontos diferentes/animal em aumento de 100x.

e) Obtenção da superfície de absorção (SA) do intestino delgado:

Para obtenção destes valores, será utilizada a seguinte fórmula (HARDIN et al, 1999):

$$SA\ (\mu m^2) = \text{Altura do vilo } (\mu m) \times \text{Largura a 50\% de altura do vilo } (\mu m)$$

Em material submetido à técnica combinada de Alcian Blue e PAS foi feita a quantificação das células caliciformes por mm² de mucosa: em dez imagens/animal em aumento de 100x foi determinada a área da mucosa a ser analisada limitando com uma linha no próprio *software*. Nesta área, foram contadas manualmente todas as células caliciformes, de acordo com a coloração do muco evidenciado pela técnica. Os valores foram convertidos para uma área de 1mm² de mucosa a fim de

padronizar os dados obtidos. Para as amostras submetidas à técnica histoquímica Reticulina e a coloração com Tricrômico de Masson foram feitas descrição qualitativa.

No tecido hepático, a avaliação realizada foi (THOMAZINI, 2011):

a) Em dez imagens diferentes/animal com aumento de 400x foi aplicada uma grade com 266 intersecções, totalizando 2.660 pontos/ animal. A partir disso, foram consideradas as interseções em: hepatócito binucleado, hepatócito mononucleado, citoplasma do hepatócito, sinusóide, células do tecido conjuntivo frouxo no espaço intercelular, gotícula lipídica no citoplasma do hepatócito, necrose e vaso sanguíneo.

b) Nas mesmas imagens anteriores foram obtidos o diâmetro celular e nuclear de 30 hepatócitos aleatoriamente entre as imagens.

Nas amostras submetidas à combinação de Alcian Blue e PAS, Reticulina e coradas com Tricrômico de Masson foi feita uma descrição qualitativa.

d)Forma de análise dos resultados - A análise estatística

A comparação das médias entre os grupos controle e tratados foi feita utilizando-se o teste de Kruskal-Wallis seguido pelo pós-teste de Dunn. Os testes foram realizados considerando nível de significância igual a 5% ($p<0,05$) e os resultados de morfometria foram apresentados em tabelas na forma de média±desvio padrão.

5-ESTRUTURA DA TESE

Os resultados dessa tese estão organizados em quatro capítulos, cada um referente a um artigo. Os artigos estão aqui apresentados nas normas das revistas as quais foram submetidos a publicação na época de defesa desta tese.

Capítulo 1: ”Safety of Isotretinoin Treatment as Measured by Stress Oxidative Parameters, Liver Structure and Ultrastructure.”

Este trabalho trata do experimento 1 e dos resultados no fígado. O objetivo foi traçar uma investigação dose dependente do tratamento, considerando sua morfometria, histoquímica e avaliação.

Capítulo 2: “Liver morphology is not altered after 60 and 90 days of isotretinoin treatment: a study considering a recovery period.”

Este trabalho traz os resultados da investigação da dose de 5mg/kg e o comportamento do fígado no período pós tratamento.

Capítulo 3: "Dose dependent treatment with isotretinoin showed that ileum is more susceptible to the treatment in relation to duodenum and jejunum in young rats."

Este é o primeiro capítulo sobre intestino delgado e traz os efeitos encontrados no tratamento de dose dependência entre 1mg/kg e 10mg/kg. Foi feita uma explanação sobre o duodeno, jejuno e íleo, apontando os resultados morfométricos e da microscopia eletrônica de varredura.

Capítulo 4: "Small intestine struture after 60 days of treatment with isotretinoin and a rest period."

Nesta parte da tese foi abordado como a estrutura do duodeno, jejuno e íleo se comportam após 60 dias de tratamento com 5mg/kg de isotretinoína e outros 30 dias pós tratamento.

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Capítulo 1:

Safety of Isotretinoin Treatment as Measured by the Hepatic Level of Stress Oxidative Related Enzymes, Liver Structure and Ultrastructure.

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ABSTRACT

Acne is the most common skin disorder and can affect directly the patients' self-esteem and social life. Systemic treatment with isotretinoin has been indicated for nodular, cystic or persistent acne. It is an analogue of vitamin A and by suppressing the sebaceous glands the disease can be controlled. The treatment in humans is carried out during two to ten months. This study was designed to investigate the liver structure, hepatic enzyme levels and the stress oxidative parameter after treatment with isotretinoin for a short period and using the dosage of 1mg/kg and another one of 10mg/Kg in young male Wistar rats. We have analyzed the blood serum biochemical levels to determine hepatic function and lipid peroxidation, hepatic tissue levels of hepatic enzymes, histology and ultrastructure. The groups receiving 1mg/kg were not altered after treatment. Their ultrastruture showed a more metabolically active organ after treatment with 10mg/kg. The group receiving 10mg/kg showed an increase of alkaline phosphatase, a decrease in high density lipoprotein and low density lipoprotein. The changes observed with the 10mg/kg dose were not conclusive for liver damages. We confirm that the proposed protocol with 1mg/kg or 10mg/kg isotretinoin is safe for male Wistar rats.

Key words: liver, isotretinoin, stress oxidative, liver damage

INTRODUCTION

Isotretinoin is chemically known as 13-cis retinoic acid and is part of a broad group of compounds related to vitamin A. This substance was first introduced in the United States in 1982 for the treatment of nodular cystic acne and others types of persistent acne (Vieira *et al*., 2012; Rigopoulos *et al*., 2010; Charakida *et al*., 2004; Diniz *et al*., 2002). Isotretinoin is one of the most effective drugs for the acne treatment and has a positive effect when we consider that less than 1% of patients have a relapse after one cycle employing the appropriate dosage (Owen, 2014; Sampaio, 2008). The determination of this dosage depends on various factors but the usual dosage recommended is 0.5-1.2mg/kg/day for two to ten months. Four weeks treatment is the minimum accepted for the first signs of acne improvement. The total dosage indicated is between 100 to 150mg/kg to avoid relapse (Brito *et al*., 2010; Sampaio, 2008; Charakida *et al*., 2004; Diniz *et al*., 2002).

Isotretinoin shows great therapeutic efficiency in the control of *Propionibacterium acnes* proliferation. It also induces epithelial differentiation of mucus secreting tissue and since the development of the sebaceous glands and the exacerbation of their secretory activity are essential factors for the occurrence of inflammatory lesions associated with acne, the inhibition of sebum production by isotretinoin is a determining factor for its pharmacological activity in the treatment (Ali & Yorulmaz, 2014; Kizlyel *et al*., 2014; Sampaio, 2008; Diniz *et al*., 2002; Saurat, 1997; Allen and Bloxham, 1989).

It is well known that the treatment with retinoids, including isotretinoin, may lead to alterations of liver enzyme and lipid levels. These may include increased serum levels of liver enzymes, increased triglycerides, total cholesterol, low-density lipoprotein cholesterol and reduced high density lipoprotein cholesterol levels. According to previous authors, the increase in plasma triglyceride levels in humans, due to the use of retinoids, is accompanied by an increase in apolipoprotein C-III levels, which may contribute, at least in part, to the hypertriglyceridemia induced by retinoids (Kızılyel *et al*., 2014; Owen, 2014; Blasiak *et al*., 2013; Vieira *et al*., 2012; Nankervis *et al*., 1995; White, 1999).

The effects of isotretinoin treatment, showing higher serum enzyme and lipid profiles, has been linked with liver injuries, as is well described in the literature. However, not much information is available about structural modifications due to this treatment. So, the aim of this study was to investigate the effects in blood serum and liver biochemical analysis, and also in the liver structure after treatment with two dosages of isotretinoin in young male Wistar rats.

MATERIAL AND METHODS

Experimental groups

Wistar rats (*Rattus novergicus*) (n=24) initiated the experimental aged 21 days and were randomly allocated into four experimental groups: C (control with water); D0 (control with soybean oil); D1 (1mg/kg of isotretinoin) and D10 (10mg/kg of isotretinoin). The experimental protocol began with daily gavage of the solutions when rats reached 54 days of age. The group D0 received only the dilution vehicle; 1mg/kg is the recommended dosage for humans and 10mg/kg was selected because is a higher dosage but not a toxic one. The drug was diluted in soybean oil and offered for 60 days. A previous study (Nankervis *et al*., 1995) demonstrated that soybean oil is a good vehicle for such studies with this substance since the dilution in oil improves the distribution and uptake by the lymphatic system resulting in a good rate of drug absorption.

The experiment followed the established ethical standards in accordance with the Brazilian animal protection laws (CEUA/Unicamp/Brazil protocol #2831-1). The rats had free access to rodent food and water and the animal house had controlled luminosity with 12 hours of light/dark and temperature at about 22±1°C.

The experimental solution

To guarantee stability of the substance, the solutions were prepared every week in the dark and kept in an amber bottle for manipulation. Isotretinoin was incorporated in soybean oil to improve absorption since the drug is better absorbed with food.

Blood extraction and plasma biochemistry

After the 60 days of treatment the rats were euthanized with a mixture of 10mg/kg of ketamine and 80mg/Kg of xylazin solutions. After this procedure, the blood was collected from the right ventricle using a vaccuette. The blood was immediately centrifuged for 10min at a speed of 3500RPM in 4°C. The supernatant plasma was collected and frozen at -20°C. The plasma was processed as routine samples using standard laboratory procedures and used to determine the level of the following components: aspartate aminotransferase, alanine aminotransferase, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, gamma-glutamyl transferase, total cholesterol, total triglycerides, high density lipoprotein cholesterol, low density lipoprotein cholesterol, very-low-density lipoprotein cholesterol, total protein, albumin, globulin and malondialdehyde.

Liver extraction: Tissue Biochemistry, light and electron microscopies

Immediately after, the liver was collected, the organ mass measured and smaller sections were prepared. Some fragments were frozen at -80°C, others were processed for light microscopy and transmission electron microscopy routines.

Light microcopy routines were the usual ones for paraffin and glycol methacrylate Leica® embedding. The samples embedded in glycol methacrylate were sectioned with 2µm thickness and stained with Hematoxilin-Eosin, for morphometry data. The samples embedded in paraffin were sectioned at 5µm thickness stained with Masson Trichrome Technique, to observe the distribution of connective tissue, epithelium and muscle, or with a combination of Periodic Acid Schiff (PAS) with Alcian Blue (AB) pH 2.5 (PAS+AB) (Alcian Blue pH 2.5-PAS®, EasyPath) to show areas with mucin deposition, or with Reticulin (Reticulina®, EasyPath) to reveal reticulin fiber distribution and structure. For transmission electron microscopy, the usual routine for Epon® embedding was employed. This tissue was sectioned at a thickness of 70nm and was typically stained with uranyl acetate followed by lead citrate.

To investigate the oxidative stress level and liver integrity we measured the following enzyme or protein levels in liver tissue samples: Superoxide Dismutase *(*Superoxide Dismutase Assay Kit Cayman Chemicals® Cat #706002), Catalase (Catalase Assay kit- Sigma Aldrich® Cat #100-1KT), Alkaline Phosphatase (Fosfatase Alcalina Bioclin® Cat #K019), Total Glutathione (Kit Sigma Aldrich Glutathione Assay® Cat#CS0260- 1KT), Gamma-Glutamyl Transferase (Gama Glutamil Transferase Laborlab® Cat#09900), Aspartate Aminotransferase (Transaminase Oxalacética InVitro® Cat #015), Alanine Transaminase (Transaminase Pirúvica InVitro® Cat #016), Bradford protein assay (Bio-Rad Protein Assay® Cat #500-0006) and Malondialdehyde *(*TBars Assay Kit Cayman Chemicals® Cat #10009055).

Qualitative and quantitative observations

Images of samples stained with Hematoxilin and Eosin were captured at 400x magnification and 10 aleatory sites were selected. Using the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29), we measured the cell height and nuclear diameter of 30 hepatocytes. In the same areas, we applied a grid mask with 266 intersections, with a total of 2660 intersections per animal. With this method, the frequency of the following parameters was determined: binuclear and mononuclear hepatocytes, cytoplasm area, connective tissue, sinusoid vessels, connective tissue cells, lipid droplets and blood vessels.

In the samples stained with Masson´s Trichrome, AB+PAS and Reticulin, we performed a qualitative evaluation observing the tissue structure and organization. Using transmission electron microscopy, we performed a more specific evaluation observing the connective tissue components as well as the cytoplasmic organelles such as endoplasmic reticulum distribution and frequency, mitochondria morphology, frequency and distribution, Golgi complex, chromatin distribution as well as other characteristics typical of the liver.

Statistical Analysis

For multiple comparison, we applied the Kruskal-Wallis test followed by Dunn's post test. The level of significance applied was $p < 0.05$. We used Minitab® 16 program (LEAD Technologies, Inc. Charlotte, North Carolina) and the data are presented as mean ± standard deviation in the tables.

RESULTS

There were no significant macroscopic alterations in the liver, observed after euthanasia (Table 1). The morphometrical parameters of hepatocytes showed no difference after the treatment protocol (Table 1 and Figure 1). The frequency of hepatocytes, cytoplasm areas, connective tissue, blood vessels, connective tissue cells and lipids droplets showed no difference among the groups (Table 1, Figure 1). The biochemical evaluation of liver tissue (Table 2) showed a decrease in Superoxide Dismutase in the D1 group in relation to C group, and an increase in Alkaline phosphatase in the D10 group in relation to the D0 control. The other enzymes, Total Glutathione, Gamma-Glutamyl Transferase, Aspartate Aminotransferase, Alanine Transaminase showed no difference. Protein quantification based on the Bradford method showed no variation, as was also found for lipid peroxidation based on malonodialdehyde determination.

Serum biochemical analysis (Table 2) showed changes related to the D10 group. We observed an increased triglyceride and very low density lipoprotein levels in relation to the D0 control. A decrease in high density lipoprotein and low density lipoprotein levels in D10 in relation to the controls C and D0 was also observed. For plasma protein, a decrease in the D10 group of total proteins and albumin occurred in relation to the control D0. The D1 group showed fewer modifications and they are related to an increase of alkaline phosphatase and a decrease of gamma glutamyl transferase in relation to the control C.

The stain analyses showed no tissue organization difference among the groups. We observed the liver histology as described in the literature. The hepatocytes were organized around the central

vein, with connective tissue between the hepatocyte cords. The organization of hepatocytes and reticulin fibers providing support were unaltered and a small amount of connective tissue with blood vessels, connective tissue cells were observed (Figure 1).

Transmission electron microscopy showed the expected distribution of cytoplasmic elements and some changes related to the treatment. A higher area of rough endoplasmic reticulum and mitochondria was observed as being dose dependent, increasing in D10 group in relation to D1 group. Lipid droplet distribution and the area they occupied, tight junctions, defense cells, and connective tissue elements showed no qualitative difference in morphology or distribution among the groups (Figure 2).

DISCUSSION

In general, the treatment with isotretinoin has been claimed to cause alterations in blood serum levels of liver enzymes and lipid particles. So, isotretinoin treatment may increase serum levels of liver enzymes, triglycerides, total cholesterol and low-density lipoprotein, and it may reduce the level of high-density lipoprotein (Owen, 2014; Beneret *et al*, 2009; Tallab *et al*., 2004; Shalita *et al*., 1983). In our study we did not observe modification in the liver enzymes and parameters which could indicate liver damage. The maintenance of protein parameters and lipid peroxidation indicates that the protocol of this study was not sufficient to cause biochemical liver injuries. It is important that this occurred with both dosages, so even a higher dosage was not enough to cause this kind of injury. On the other hand, increased alkaline phosphatase and a decrease of gamma-glutamyl transferase were observed for D1 in relation to C. The increase of alkaline phosphatase levels can indicate canalicular liver damage and liver inflammation, although not in a linear scale. We found a decrease in tissue level of gamma-glutamyl transferase in D1 in relation to the C group.

Previous authors demonstrated that the increase in triglyceride levels in patients being treated with oral isotretinoin may be related to a reduction in the removal rate of these lipids from the plasma. According to these authors, it also appears to be influenced by the increase in gene expression for Apo E (de Marchi *et al*., 2006; Rodondi *et al*., 2002; Sedova *et al*., 2004; Vu-Dac N *et al*., 1998; Vieira *et al*., 2012). The increase of triglyceride levels in the D10 group is in accordance with the literature that affirms this tendency. It is interesting to observe that the most commonly suggested dosage of 1g/kg did not alter any lipid levels in male Wistar rats. Saied and Hamza (2014) treated rats with isotretinoin and selenium and observed that the exposure to isotretinoin increased serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, cholesterol,

triglycerides, and high-density lipids content. The authors also showed a significant rise in thiobarbituric acid reacting substance and nitric oxide content with concomitant decrease in reduced glutathione and the antioxidant enzyme activities of superoxide dismutase and catalase in liver tissue after daily isotretinoin exposure at the dosage of 7.5mg/kg for 28 days.

The increase of triglyceride levels in D10 was expected, based on the literature but the decrease of low density lipoprotein and high density lipoprotein are not in accordance with what is known for the retinoid treatments. The very low density lipoprotein level was found to increase. We believe that this higher concentration is associated to the total triglyceride elevation considering that this protein is related with triglyceride transport in the blood. Despite the many studies confirming that retinoid treatment alters lipid concentrations, some authors contest this result.

Previous authors (Kaymak *et al*., 2006; Bershad *et al*., 1985) reported an increase in low density lipoprotein and triglycerides but a decrease in high density lipoprotein during isotretinoin therapy. These changes in lipid profile also appeared to be transient and returned to baseline level two months following the end of treatment. In another study (Vieira *et al*., 2012) with 130 patients who were treated with isotretinoin, the authors noted an increase in aspartate aminotransferase, alanine aminotransferase, and triglyceride levels. Most of the studies in the literature that reported effects of isotretinoin on liver enzymes and lipids suggested that the effects were reversible and the patterns followed those already presented in this discussion.

A previous study with 150 participants, the authors found no statistically significant changes in liver transaminase and lipid levels following usual treatment with isotretinoin (Brito *et al*., 2010). In another study with 30 participants, the authors reported that triglycerides, low density lipoprotein or high density lipoprotein levels showed no difference with the treatment (Baxter *et al*., 2003). In another study with 1292 participants, the authors found no increase in serum levels of liver enzymes occurred up to the end of the treatment (Alcalay *et al*., 2001). Another study found that liver enzymes were less affected than lipids in patients who underwent treatment with isotretinoin (Kızılyel *et al*., 2014). A study of seven patients with severe rosacea observed increases in serum triglycerides, cholesterol, triglycerides associated with very low density lipoprotein, low density lipoprotein, high density lipoprotein and aspartate aminotransferase. These authors also observed a decrease in bilirubin levels (Marsden *et al*., 1984). The lack of significant differences of the liver enzyme level and lipid parameters found for this study are in accordance with the literature that indicates variable effects with the isotretinoin treatment.

The histological analyses of liver sections showed no detectable alteration due to the treatment. Reticulin fibers were distributed as usual in the connective tissue, providing support for the organ. Masson´s Trichrome showed no signs of fibrosis and no sign of mucin accumulation was established by the combination technique of Alcian Blue pH2.5 and PAS. Some previous studies indicated that retinoids suppressed fibrosis induced by CCL4 (Wang *et al*., 2007; Okuno *et al*., 2003; Blomhoff, 1997). The absence of fibrosis among the groups is in accordance with the literature (Wang *et al*., 2007; Okuno *et al*., 2003; Blomhoff, 1997) and the lack of histological differences in agreement with the other results we registered.

The samples submitted to transmission electron microscopy showed evidence of higher liver activity, as the increased number of mitochondria and of rough endoplasmic reticulum area. The connective tissue components showed no difference, in agreement with previous authors that showed that retinoids do not appear to produce consistent toxic liver abnormalities (Roenigk, 1988). Lipid droplets were also present but we found no difference in this parameter. Compiling serum and tissue biochemistry, that show no hepatotoxity signs, we have demonstrated that isotretinoin in these two dosages did not cause lasting liver alterations in young male Wistar rats.

The lack of alterations, as assessed by morphometry, is in accordance with the biochemical and ultrastructural results. The frequency of binuclear and mononuclear hepatocytes, cytoplasm area, defense cells, vessels, are not different among the groups. This reinforces the finding that the treatment with isotretinoin proposed in this protocol does not alter liver structure.

Alterations in the proportion and area occupied by cytoplasmic organelles, necrotic areas, and abnormal frequency of binuclear or mononuclear hepatocytes depend on the kind of treatment (Areshidze *et al*., 2013; Petrovova *et al*., 2013; Weibel *et al*., 1969). In our study, we found no clear signs of alterations of the parameters, as also found by previous authors, showing that retinoids do not appear to produce consistent toxic liver abnormalities (Roenigk, 1988).

CONCLUSIONS

The treatment with 1mg/kg and 10mg/kg of isotretinoin can be considered safe, in that it did not alter significantly the liver tissue and blood serum biochemical parameters related to liver injuries, protein levels and stress oxidative levels. Liver structure did not show differences among the groups. We confirm that the proposed treatment protocol with isotretinoin does not appear to produce consistent liver abnormalities in young male Wistar rats.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Table 1: Morphometry and stereology of Wistar rat livers treated with 1mg/kg and 10mg/kg of isotretinoin for 60 days.

Groups	**C- control with water**	**D0- control with soybean oil**	**D1-1mg/Kg of isotretinoin**	**D10- 10mg/kg of isotretinoin**
Initial body weight (g)	192, 82±13,96	193,5±10,17	193,46±15,24	192,78±3,8
Final body weight (g)	468,43±40,15	459,44±53,34	466,68±22,54	474,25±41,73
Liver mass (g)	12,89±0,97	12,82±1,44	12,97±1,41	14,21±1,65
Hepatocyte morphometry				
Hepatocyte diameter	22,25±8,11	21,54±1,44	21,33±0,31	22,51±1,13
Hepatocyte nuclear diameter	9,39±3,34[a]	7,18±0,58[ab]	6,85±0,53[b]	7,63±0,68[ab]
Binuclear hepatocyte (%)	0,3±0,25	0,20±0,10	0,17±0,09	0,08±0,10
Mononuclear hepatocyte (%)	5,83±0,66	5,43±0,61	6,05±1,36	4,80±0,63
Citoplasmic (%)	90,53±1,84	90,03±3,45	91,18±1,3	92,28±2,25
Connective tissue (%)	1,44±0,85	2,23±2,00	1,01±0,35	0,95±0,75
Sinusoid vessel (%)	0,86±0,50	1,00±0,55	0,86±0,56	0,80±0,74
Connective tissue cell (%)	0,22±0,09	0,14±0,11	0,21±0,12	0,24±0,13
Lipid droplets (%)	0,06±0,09	0,23±0,24	0,09±0,12	0,02±0,05
Blood vessel (%)	0,75±0,98	0,74±0,78	0,43±0,59	0,82±1,00

Mean ± standard deviation. Averages in the same row followed by different letters differ statistically by the Kruskal-Wallis test followed by Dunn's post test at a 5% significance level.

Table 2: Liver tissue and blood serum biochemical analysis in male Wistar rats after 60 days of treatment with 1mg/kg and 10mg/kg of isotretinoin.

Groups	**C- control with water**	**D0- control with soybean oil**	**D1-1mg/Kg of isotretinoin**	**D10- 10mg/kg of isotretinoin**
Liver tissue biochemistry				
AF (U/L)	73,27±12,96^{ab}	64,87±8,32^{b}	77,09±11,00^{ab}	101,26±16,33^{b}
ALT (U/mL)	63,52±7,65	62,88±21,7	85,05±25,37	50,33±28,99
AST (U/mL)	94,41±13,31^{a}	75,90±7,32^{b}	86,57±7,76^{ab}	87,81±3,32^{ab}
Brad (µgptn/µl)	0,24±0,04	0,27±0,03	0,26±0,04	0,28±0,03
CAT (U/mL)	31077±2999	29931±2129	29988±3068	28478±2384
GGT (U/L)	48,89±4,98	49,79±5,73	40,40±3,98	36,02±12,11
GLUT (nmoles/mL)	27,46±543,25	2813±1115	2928±986	3394±1044
MDH	27,14±8,09	23,1±1,01	19,38±4,22	21,03±2,78
SOD (U/mL)	0,14±0,03^{a}	0,09±0,03^{ab}	0,03±0,05^{b}	0,08±0,04^{ab}
Blood serum biochemistry				
AF (U/L)	146,8±59,22^{a}	157,2±63,31^{a}	293,6±84,57^{b}	162,6±64,35^{ab}
Alb (g/dL)	3,48±0,54^{ab}	3,86±0,87^{a}	3,48±0,31^{ab}	2,94±0,17^{b}
ALT (U/L)	37,4±8,44	48±13,30	52,4±10,78	41,2±9,86
AST (U/L)	176,6±66,55	169,8±19,46	225,2±63,21	211,8±73,66
COL (mg/dL)	68,6±11,91	67,6±16,14	68,40±13,45	55,8±8,04
DB (mg/dL)	0,028±0,027	0,028±0,016	0,036±0,03	0,016±0,027
GGT (U/L)	4,86±2,20^{a}	2,22±1,31^{b}	2,48±0,88^{b}	3,74±0,84^{ab}
GLOB (g/dL)	4,52±0,95	5,50±1,18	4,26±0,64	4,04±0,48
HDL (mg/dL)	25±5,15^{ab}	29±8,66^{b}	23±5,24^{ab}	18,8±3,42^{a}
IB (mg/dL)	0,064±0,043^{a}	0,044±0,036^{a}	0,034±0,017^{a}	0,042±0,013^{a}
LDL (mg/dL)	23,56±11,31^{a}	24,32±6,51^{a}	19±8,55^{ab}	10,1±5,99^{b}
MDH	16,05±9,61	17,71±9,09	15,80±2,12	13,38±4,93
TB (mg/dL)	0,092±0,07	0,072±0,04	0,07±0,045	0,058±0,03
TRIG (mg/dL)	100,2±50,99^{ab}	71,4±21,49^{b}	132±42,50^{ab}	145±60,67^{a}
TP (g/dL)	7,99±1,34^{ab}	9,36±2,03^{a}	7,74±0,79^{ab}	6,98±0,64^{b}
VLDL (mg/dL)	20,04±10,20^{ab}	14,28±4,30^{a}	26,4±8,50^{ab}	29±12,13^{b}

Mean ± standard deviation. Averages in the same row followed by different letters differ by the Kruskal-Wallis test followed by Dunn's post test at 5 % significance level. AF: Alkaline Phosphatase*;* Alb: albumin; ALT: Alanine Transaminase; AST: Aspartate Aminotransferase; Brad: Bradford protein assay; *CAT:* Catalase; Col: total cholesterol; DB: direct bilirubin; GGT: Gamma-Glutamyl Transferase*;* Glob: globulin*;* Glut: Total Glutathione; HDL: high density lipoprotein cholesterol; IB: indirect bilirubin; LDL: low density lipoprotein cholesterol; MDH: Malondialdehyde*;* SOD*:* Superoxide Dismutase*;* TB: total bilirrubin*;* TP: total protein; Trig: total triglycerides; VLDL: very low density lipoprotein.

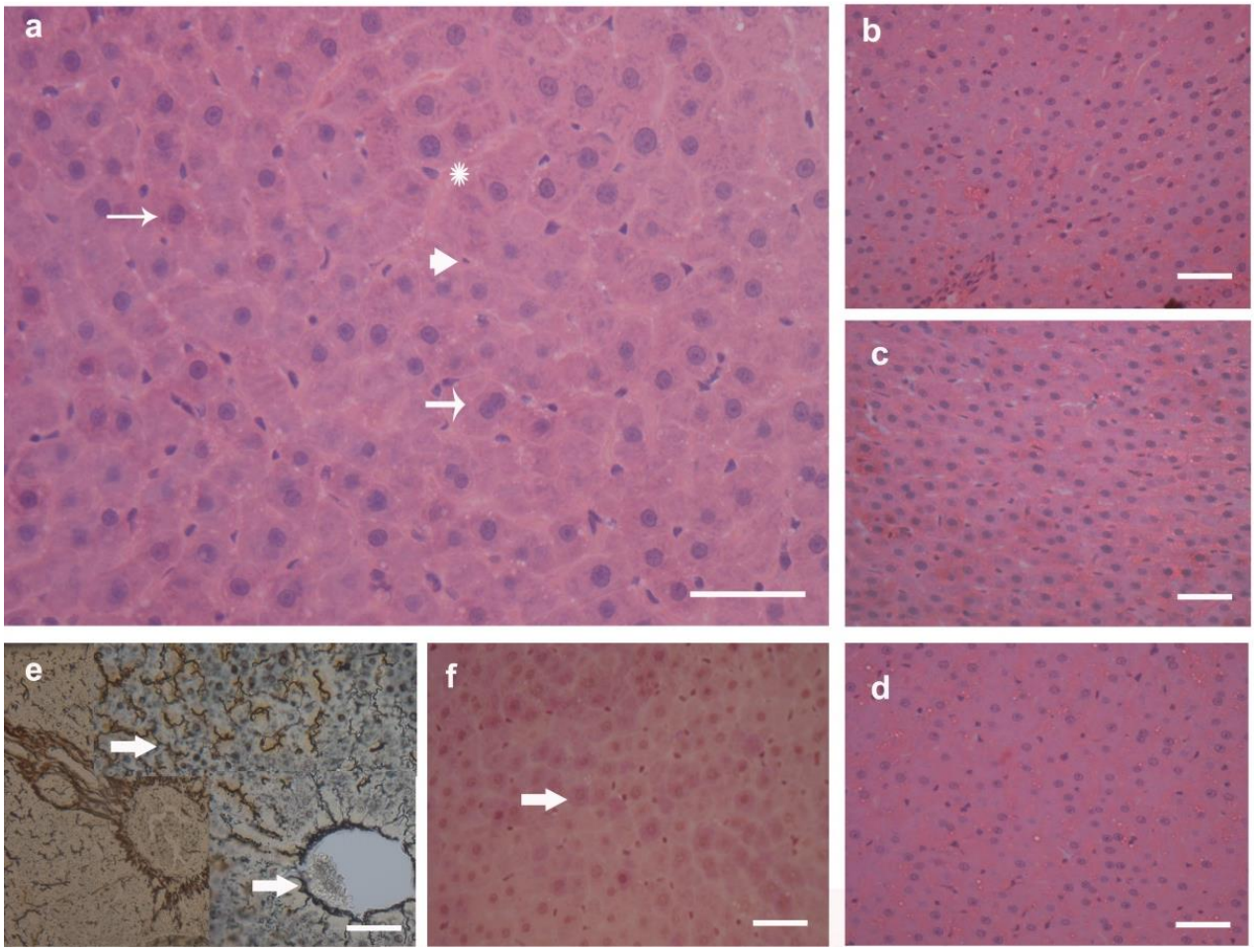

Figure 1: Light microscopy images of the liver. **a-d**: Hematoxilin-Eosin staining. In image **a,** we have the control group, chosen as an example to show the usual histological structures of liver. The thin arrow indicates a mononuclear hepatocyte; the thicker arrow indicates a binuclear hepatocyte, the arrowhead indicates a defense cell in connective tissue, the asterisk (*) indicates the lymphatic space. **b**: group D0; **c**: group D1; **d**: Group D10. **e**: Reticulin in the control group. The arrows indicate the fibers providing support for the tissue and around the central vein of the liver lobule. **f**: combination technique of PAS+AB the in control group. The arrow indicates the usual glycogen deposit in hepatocyte cytoplasm. Bar: 50µm.

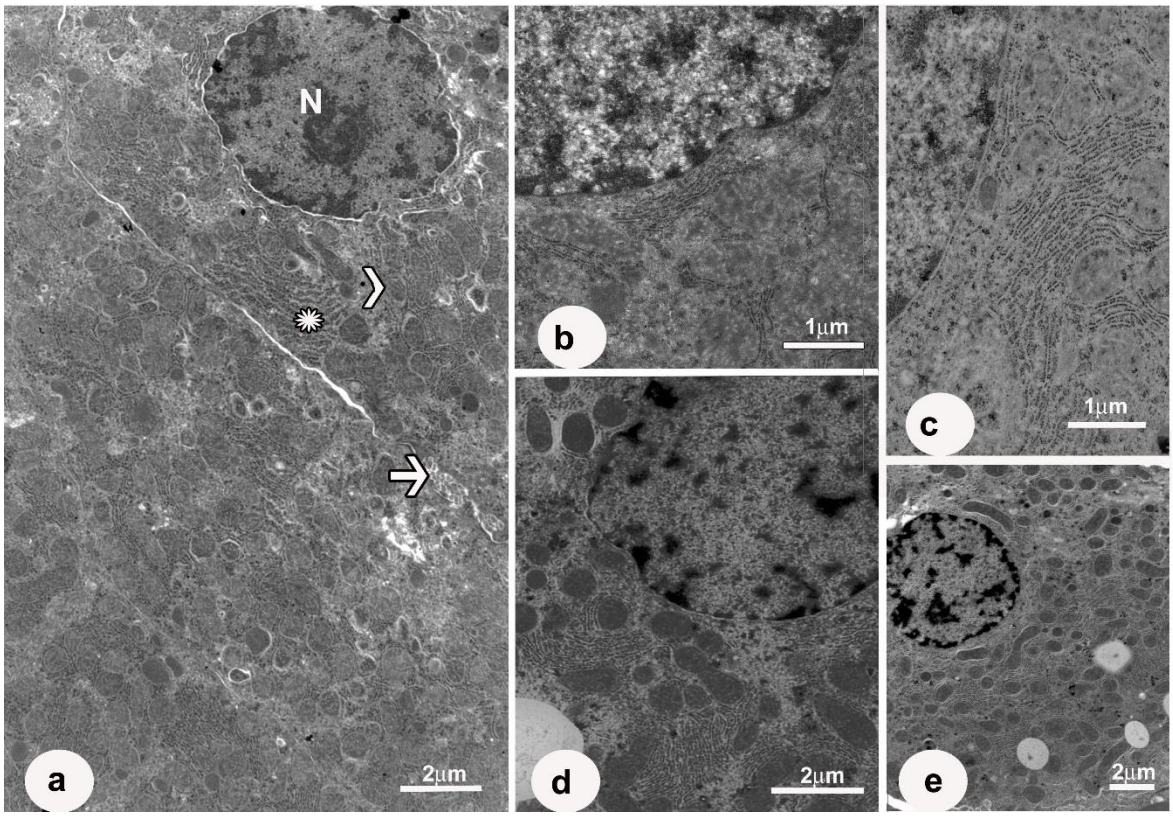

Figure2: Transmission Electron Microscopy. **a**: group C (control group with water); **b**: group D1(1mg/kg of isotretinoin); **c**: group D0 (control group with soybean oil); **d-e**: group D10 (10 mg/kg of isotretinoin). In the control group image, the arrow indicates the bile canalicular duct; the arrowhead indicates mitochondria; the asterisk (*) indicates an area with rough endoplasmic reticulum; N: nucleus. The dose dependent increase of mitochondria and rough endoplasmic reticulum frequency can be observed.

Capítulo 2:

Liver morphology is not altered after 60 and 90 days of isotretinoin treatment: a study considering a rest period

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ABSTRACT

Isotretinoin is a retinoid widely used in cases of nodular and persistent acne. The disease can occur independent of age and gender, and is more common in women aged 25 years and in adolescents. Acne can directly affect the patients' self-esteem and social life and this systemic treatment represents a possible cure. The liver is the major site of retinoid production, metabolism and storage. This research was designed to add information about liver activity following treatment with isotretinoin and a recovery period. The model chosen was young male Wistar rats with aged 54 days receiving 5mg/kg of isotretinoin for 60 days, and one group maintained another 30 days as a possible recovery period. Using blood serum and liver tissue biochemical analyses, stereology, morphometry, histochemical techniques and transmission electron microscopy of the liver we evaluated the treatment in relation to oxidative stress parameters and hepatic structure. The usual lipid alterations due this treatment were found, and some enzymes increased after the recovery period. We showed that a period of 30 days after treatment interruption was sufficient to recover most of the biochemical parameters, but other alterations seemed to appear even after treatment termination. No sign of marked morphological change was found.

Keywords: isotretinoin, recovery, liver, Wistar rats.

INTRODUCTION

Acne is multifactorial and the treatment depends on each patient. It affects about 85% of individuals at any age, particularly adolescents, echoing directly on their social life and self-esteem. The treatment involves topical medicines and in some cases systemic drugs. At first, the dermatologist usually prescribes an oral antibiotic with anti-inflammatory properties and if the response is not satisfactory, the next most common treatment suggested is with isotretinoin (1-5). It has since been used for treatment of severe acne with nodular and cystic formations and resistant acne not responding to other types of treatment. Isotretinoin is a synthetic analogue of vitamin A and it is effective for all the four steps of pathogenesis involving acne vulgaris: follicular epidermis, hyperproliferation, increased sebum production, inflammation and *Propionibacterium acnes* activation. As a consequence, isotretinoin has a positive effect on the patient´s skin and, therefore, his life quality (1-7).

It has been established that retinoids are better absorbed with food and lipophilic vehicles, and since isotretinoin is part of the broad group of retinoids related to vitamin A, it is also better absorbed with food. After absorption in the small intestine, the substance reaches the liver by hepatic circulation (8-10).

Vitamin A metabolism involves storage in the liver. Hypervitaminosis A results in liver abnormalities, including fibrosis and cirrhosis (11, 12). Many different effects in the liver are described in the literature including increased liver transaminase, triglycerides and cholesterol. (13). The aim of this research was to observe the effects of 5mg/kg isotretinoin administration during 60 days followed by 30 days without the treatment.

MATERIAL AND METHODS

Experimental groups

Wistar rats (*Rattus novergicus*) (n=24) initiated the experimental protocol with 54 days of age and were randomly allocated into four experimental groups:

C: control with water;

D0: 0mg/kg of isotretinoin, considered a control with soybean oil;

D5a: 5mg/kg of isotretinoin in soybean oil

D5b: 5mg/kg of isotretinoin for 60 days, followed by 30 days without drug exposure.

The drug was diluted in soybean oil and offered daily by gavage for 60 days. After this period, the animals of groups C, D0 and D5a were euthanized. Group D5b was maintained for another 30

days without isotretinoin. A previous study (14) demonstrated that soybean oil is a good vehicle for studies with this substance. To guarantee stability of the substance, the solutions were prepared every week in a dark laboratory and kept in an amber bottle for manipulation. Isotretinoin was incorporated in soybean oil to improve absorption, since the drug is better absorbed with food.

The experiment followed the established ethical standards in accordance with the Brazilian animal protection laws (CEUA/Unicamp/Brazil protocol #2831-1). The rats had free access to rodent food and water and the animal house had controlled luminosity with 12 hours of light/dark and temperature at about 22±1°C.

Blood collection and plasma biochemical analysis

After the 60 days treatment the rats were euthanized with a mixture of 10mg/kg of ketamin and 80mg/Kg of xylazine solution. After this procedure, blood was collected from the right ventricle using a vaccuette tube. The blood was immediately centrifuged for 10min at a speed of 3500 RPM in 4°C. The supernatant plasma was collected and frozen at -20°C. It was used to determine the level of the following components: aspartate aminotransferase, alanine aminotransferase, total bilirubin, direct bilirubin, indirect bilirubin, alkaline phosphatase, gamma-glutamyl transferase, total cholesterol, total triglycerides, high density lipoprotein, low density lipoprotein, very-low-density lipoprotein, total protein, albumin, globulin, malondialdehyde as an oxidative stress parameter.

Tissue biochemical analysis, light and transmission electron microscopy routines

The entire liver was collected to obtain its mass and prepare samples for tissue biochemical analysis and structural evaluation. The samples for tissue biochemical procedures were frozen at -80°C. The samples for morphometry, histochemistry and ultrastructural observations were fixed in Karnovsky's modified (15) solution for 48 hours. After this, the light microcopy routine followed the usual procedure for paraffin and glycol-methacrylate Leica® embedding. These samples were sectioned with 2µm thickness and stained with Hematoxilin-Eosin, for morphometry data. The samples embedded in paraffin were sectioned with 5µm thickness and stained with Masson Trichrome Technique to observe the distribution of connective tissue or with a combination of Periodic Acid Schiff (PAS) with Alcian Blue (AB) pH 2.5 (PAS+AB) (Alcian Blue pH 2.5-PAS®, EasyPath) to show areas with mucin deposition, or alternately with Reticulin (Reticulina®, EasyPath) to reveal the reticulin fiber distribution and structure.

The routine for transmission electron microscopy followed the usual routine for Epon® embedding. After the 48 hours in Karnovsky's modified solution, the samples stayed another 24 hours in glutaraldehyde solution before been embedding in Epon®. These samples were sectioned at a thickness of 70nm and were typically stained with uranyl acetate followed by lead citrate.

Tissue biochemical analysis was directed to evaluate liver integrity and we used the following enzyme or protein levels in the samples: Superoxide Dismutase (Superoxide Dismutase Assay Kit Cayman Chemicals® Cat #706002), Catalase (Catalase Assay kit- Sigma Aldrich® Cat #100-1KT), Alkaline Phosphatase (Fosfatase Alcalina Bioclin® Cat #K019), Total Glutathione (Kit Sigma Aldrich Glutathione Assay® Cat #CS0260- 1KT), Gamma-Glutamyl Transferase (Gama Glutamil Transferase Laborlab® Cat #09900), Aspartate Aminotransferase (Transaminase Oxalacética InVitro® Cat #015), Alanine Transaminase (Transaminase Pirúvica InVitro® Cat #016), Bradford protein assay (Bio-Rad Protein Assay® Cat #500-0006) and Malondialdehyde (TBars Assay Kit Cayman Chemicals® Cat #10009055).

Structural observations

Images of the samples stained with Hematoxilin and Eosin were captured at 400x magnification and 10 aleatory images were selected. Using the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29), we obtained the cell height and nuclear diameter of 30 hepatocytes. In the same areas, we applied a grid mask with 266 intersections, with a total of 2660 intersections per animal. We determined the frequency of the following structures: binuclear and mononuclear hepatocytes, cytoplasmic area, connective tissue, sinusoid vessels, connective tissue cells, lipid droplets in hepatocyte cytoplasm and blood vessels.

In the samples stained with Massons's Trichrome, Reticulin and PAS+AB we performed a qualitative evaluation observing tissue structure and organization.

Using transmission electron microscopy, we observed, in detail, the connective tissue components and considered the cytoplasmic organelles, mitochondria and rough endoplasmic reticulum distribution and frequency.

Statistical Analysis

For multiple comparison we applied the Kruskal-Wallis test followed by Dunn's post test. The tests were carried out considering the significance level at $p < 0.05$. We used Minitab® 16 program

(LEAD Technologies, Inc. Charlotte, North Carolina) and the morphometry data are presented as mean ± standard deviation.

RESULTS

We observed no alteration of body mass, liver mass and hepatossomatic index (Table 1). The morphometry and stereology showed a decrease in binuclear and mononuclear hepatocytes, and an increase of cytoplasmic area in the group D5a in relation to C. In the D5b group, we observed an increase in nuclear diameter and fewer lipid droplets. The other parameters studied with morphometry showed no difference among the groups (Table 1, Figure 1).

Ultrastructure was similar among the groups. We observed an increase in mitochondria frequency in the D5a group in relation to C, but comparing the two treated groups, they appear very similar (Figure 2). Even after the 30 days interruption of the treatment, the organism appears to be still recovering from the treatment, needing more time to complete this.

The histochemical qualitative observations showed reticulin fibers as expected and no mucin accumulation. With the Masson's Trichrome we observed no fibrotic areas. The hepatocytes, blood vessels and general organization followed the accepted structure known for liver tissue (Figure 1).

The enzyme levels revealed increased liver activity, plasma protein concentration, lipid peroxidation and lipoprotein levels. In the D5a group we observed an increase of triglycerides and very low density lipoprotein in relation to the control, and a decrease in high density lipoprotein and in low density lipoprotein levels, also in comparison to the control group. In D5b, the alkaline phosphatase, gama-glutalyl transferase and globulin decreased in relation to the control group (Table 2). Liver tissue biochemical analysis showed an increase in alkaline phosphatase and protein quantification with the Bradford method applied to group D5a in relation to the control group. For D5b, we found a decrease in gama-glutamyl transferase, and an increase in aspartate aminotransferase and alanine transaminase in relation to the control group (Table 2).

DISCUSSION

Isotretinoin has been used since 1982 and many research projects showed that side effects of isotretinoin are mostly dose related and are not always trivial (16,19). It is accepted that isotretinoin treatment may increase serum levels of liver enzymes, triglycerides, and low-density lipoprotein cholesterol, and reduce the level of high-density lipoprotein cholesterol (20-22). Despite many

alterations described for isotretinoin treatment, most of the studies in the literature suggest that the collateral effects are reversible (21, 23).

Our biochemical results indicate that only 60 days of drug use was not long enough to alter some of the enzyme levels, since the alteration appeared only 90 days after the beginning of the treatment, in group D5b, after what was considered a “recovery period” of 30 days. The recovery in lipid levels was confirmed for the D5b group and can also be corroborated by morphometric results, since we observed the diminished frequency of lipid droplets in hepatocyte cytoplasm of this group in relation to the control. Previous research showed that liver enzymes were less affected than the lipid balance in patients who underwent treatment with isotretinoin (21). In a previous study with 150 participants (24), the authors showed that systemic clinical effects were much less common.

It is interesting to notice that blood serum biochemical analyses revealed no alteration in liver transaminase enzymes, but in tissue determinations we found an increase of both aspartate aminotransferase and alanine transaminase in the D5b group compared to the controls. These enzymes, when verified by blood serum routine, are the most important ones to indicate liver injury (25). We support the hypothesis that the organism was still recovering from the treatment and the enzyme levels were not high enough to be detected in serum examination. Liver disease with damage to the bile ducts causes elevation of transaminase enzymes, gama-glutamyl transferase and alkaline phosphatase (25). In our study we found a decrease in gama-glutamyl transferase. The increase of the other three enzymes can indicate damage to liver canalicular organization, and 30 days were not enough to normalize all these parameters.

Hepatocytes play an indispensable role in the uptake and processing of dietary retinoids in the liver, and in the synthesis and secretion of retinol-binding protein, which is required for mobilizing hepatic retinoid stores (26). The biochemical data could not be correlated with structure. In morphometric evaluation, we found no signs of damage or liver injury. We found a higher frequency of cytoplasmic area in the D5a group, as compared with the control. This could indicate an increase of organelles related to detoxification metabolism. The liver is the main site of metabolism and during the treatment it would be expected to show increased signs of higher metabolism. Transmission electron transmission microscopy investigated this possibility, but both treated groups seemed to have a similar structure considering mitochondrial frequency and granular endoplasmic reticulum area.

The information obtained with this research indicates higher hepatic activity, with few harmful alterations, which were not enough to alter histological structure. The morphometrically analyzed samples and histochemical data contributed to these results since no signs of necrosis, fibrosis or

altered organization could be found. We observed that isotretinoin does not appear to produce consistent toxic liver abnormalities, as some previous authors have indicated (12, 21, 27, 28).

CONCLUSIONS

The proposed 30 days after interruption of the treatment seems to be enough to normalize the parameters altered during the 60 days of treatment with 5mg/kg of isotretinoin but this period showed that enzyme levels can be altered even after the end of the treatment. We conclude that the proposed protocol seems to be safe for young male Wistar rats, considering the lack of notable alterations in hepatic parameters.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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Table 1: Morphometry of male Wistar rat livers treated with 5mg/kg of isotretinoin for 60 and 90 days.

Groups	**C**	**D0**	**D5a**	**D5b**
Initial body weight (g)	192, 82±13,96	193,5±10,17	193,52±21,53	193,52±7,31
Final body weight (g)	468,43±40,15	459,44±53,34	461,85±42,13	463,07±38,31
Liver mass (g)	12,89±0,97	12,82±1,44	13,85±1,3	11,99±1,57
Hepatocyte morphometry				
Hepatocyte diameter	22,25±8,11	21,54±1,44	19,79±2,99	22,41±2,05
Hepatocyte nuclear diameter	9,39±3,34^{ab}	7,18±0,58^{a}	7,5±0,61^{ab}	8,32±0,35^{b}
Binuclear hepatocyte (%)	0,3±0,25^{a}	0,20±0,10^{ab}	0,045±0,02^{b}	0,16±0,11^{ab}
Mononuclear hepatocyte (%)	5,83±0,66^{a}	5,43±0,61^{ab}	4,27±0,74^{b}	5,52±0,99^{ab}
Citoplasmic (%)	90,53±1,84^{ac}	90,03±3,45^{ac}	93,73±1,18^{b}	91,87±1,97^{abc}
Connective tissue (%)	1,44±0,85	2,23±2,00	0,81±0,38	0,88±0,59
Sinusoid vessel (%)	0,86±0,50	1,00±0,55	0,92±0,49	0,49±0,30
Connective tissue cell (%)	0,22±0,09	0,14±0,11	0,12±0,11	0,23±0,10
Lipid droplets (%)	0,06±0,09^{ab}	0,23±0,24^{a}	0,01±0,03^{ab}	0,00±0,00^{b}
Blood vessel (%)	0,75±0,98	0,74±0,78	0,08±0,18	0,84±0,67

Groups with 60 days of treatment: C (control with water); D0 (control with soybean oil); D5a (5mg/kg of isotretinoin). Group D5b received 5mg/kg of isotretinoin for 60 days and were maintained another 30 days without treatment as a possible recovery period. Mean ± standard deviation. Averages in the same row followed by a different letter are statistically different by Kruskal-Wallis followed by Dunn post test at a 5% significance level.

Table 2: Liver tissue and blood serum biochemical analysis of male Wistar rat livers treated with 5mg/kg of isotretinoin for 60 and 90 days.

Groups	**C**	**D0**	**D5a**	**D5b**
Liver tissue biochemistry				
AF (U/L)	73,27±12,96^{abc}	64,87±8,32^{ac}	107,21±26,10^{b}	67,58±18,86^{c}
ALT (U/mL)	63,52±7,65^{ac}	62,88±21,7^{abc}	66,05±17,17^{c}	85,93±10,25^{b}
AST (U/mL)	94,41±13,31^{ab}	75,90±7,32^{a}	82,76±6,96^{ab}	102,89±11,2^{b}
Brad (µgptn/µl)	0,24±0,04^{ac}	0,27±0,03^{ac}	0,31±0,02^{b}	0,24±0,057^{c}
CAT (U/mL)	31077±2999	29931±2129	30409±2819	31269±2883
GGT (U/L)	48,89±4,98^{a}	49,79±5,73^{a}	40,92±4,5^{ab}	31,65±2,17^{b}
GLUT (nmoles/mL)	2746±543,25	2813±1115	3602±716	2388±969
MDH	27,14±8,09	23,1±1,01	22,33±5,5	25,3±10,95
SOD (U/mL)	0,14±0,03^{a}	0,09±0,03^{b}	0,13±0,03^{ab}	0,11±0,03^{ab}
Blood serum biochemistry				
AF (U/L)	146,8±59,22^{ab}	157,2±63,31^{ab}	191±38,38^{a}	101,2±28,76^{b}
ALB (g/dL)	3,48±0,54	3,86±0,87	3,23±0,31	3,29±0,35
ALT (U/L)	37,4±8,44	48±13,30	45,2±11,12	43±4,95
AST (U/L)	176,6±66,55	169,8±19,46	149±47,58	167,2±53,54
COL (mg/dL)	68,6±11,91	67,6±16,14	52,6±3,97	67,8±10,5
DB (mg/dL)	0,028±0,027	0,028±0,016	0,028±0,05	0,042±0,029
GGT (U/L)	4,86±2,20^{ac}	2,22±1,31^{b}	3,9±1,52^{ac}	2,2±1,036^{b}
GLOB (g/dL)	4,52±0,95^{ab}	5,50±1,18^{a}	4,44±0,76^{ab}	3,83±0,30^{b}
HDL (mg/dL)	25±5,15^{ab}	29±8,66^{a}	19,6±1,52^{b}	20,6±4,72^{ab}
IB (mg/dL)	0,064±0,043	0,044±0,036	0,068±0,054	0,07±0,046
LDL (mg/dL)	23,56±11,31^{a}	24,32±6,51^{a}	7,2±5,67^{b}	21,6±11,80^{ab}
MDH	16,05±9,61	17,71±9,09	19,98±9,03	25,47±13,24
TB (mg/dL)	0,092±0,07	0,072±0,04	0.096±0,061	0,112±0,03
TP (g/dL)	7,99±1,34	9,36±2,03	7,67±1,07	7,12±0,53
TRIG (mg/dL)	100,2±50,99^{ab}	71,4±21,49^{a}	129±26,42^{b}	128±59,05^{ab}
VLDL (mg/dL)	20,04±10,20^{ab}	14,28±4,30^{a}	25,8±5,28^{b}	25,6±11,80^{ab}

Groups with 60 days of treatment: C (control with water); D0 (control with soybean oil); D5a (5mg/kf of isotretinoin). Group D5b received 5mg/kg of isotretinoin for 60 days and was maintained another 30 days without treatment for a possible recovery period. Mean ± standard deviation. Averages in the same row followed by a different letter are statistically different by Kruskal-Wallis followed by Dunn post test at a 5% significance level. AF: Alkaline Phosphatase*;* ALB: albumin; ALT: Alanine Transaminase; AST: Aspartate Aminotransferase; BRAD: Bradford protein assay*;* CAT*:* Catalase; COL: total cholesterol; DB: direct bilirubin; GGT: Gamma-Glutamyl Transferase; GLOB: globulin*;* GLUT: Total Glutathione; HDL: high density lipoprotein cholesterol; IB: indirect bilirubin; LDL: low density lipoprotein cholesterol; MDH: Malondialdehyde; SOD: Superoxide Dismutase; TB: total bilirubin; TP: total protein; TRIG: total triglycerids; VLDL: very low density lipoprotein.

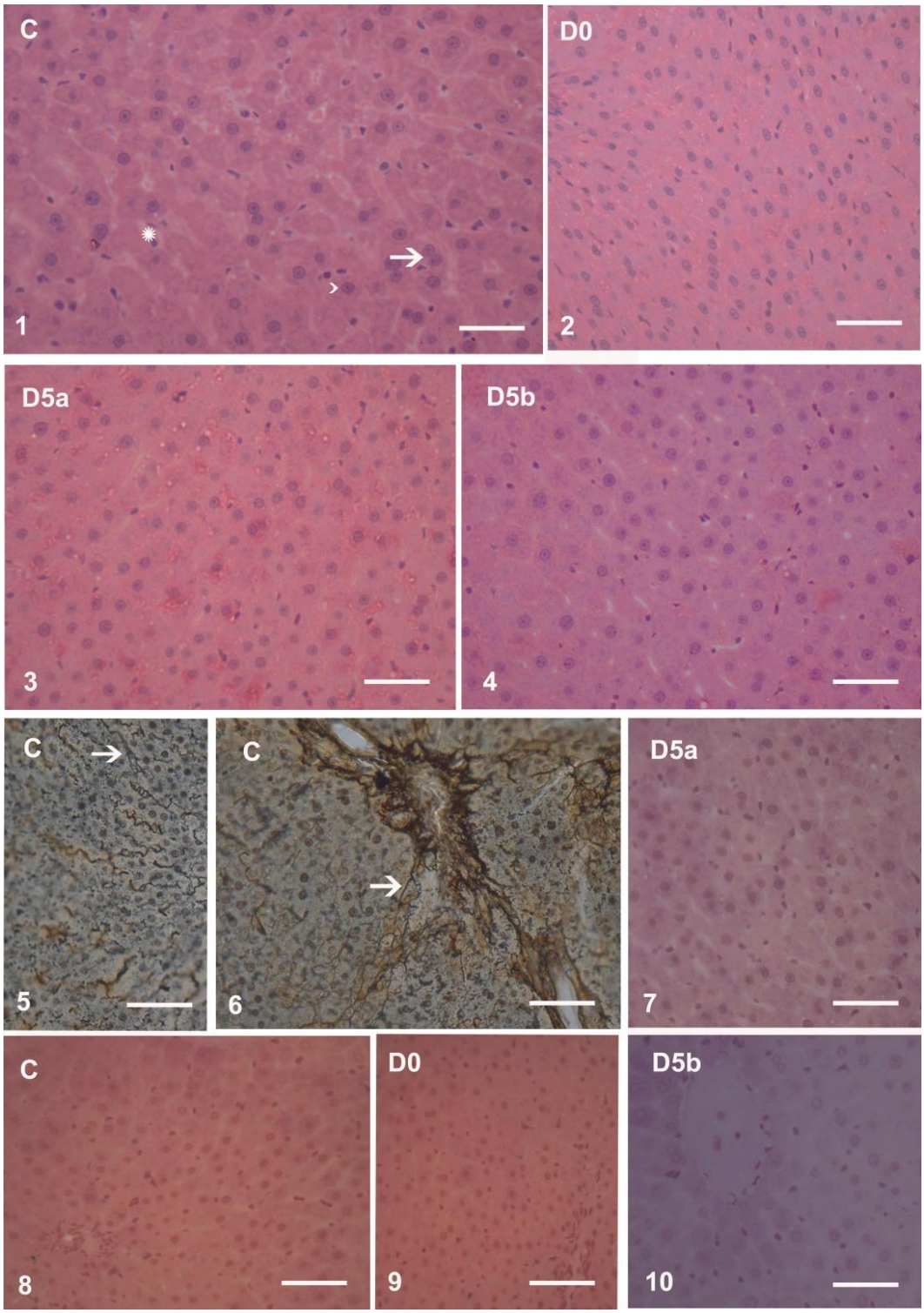

Figure 1: Light microscopy images of liver. 1-4: Hematoxilin-Eosin staining. C: control group; D0: 0mg/kg of isotretinoin; D5a: 5 mg/kg of isotretinoin; D5b: 5 mg/kg of isotretinoin for 60 days and 30 days after the treatment interruption. Images 1-4: Hematoxilin-Eosin staining; 5-6: Reticulin histochemical stain of control group; 7-10: AB+PAS histochemical stain. In image 1: arrow: binuclear hepatocyte; arrowhead: mononuclear hepatocyte; *: connective tissue space. In images 5 and 6: arrow: reticulin fibers. The structural pattern was not altered among the groups after treatment and we have no signs of necrosis or fibrosis. Bar: 40µm.

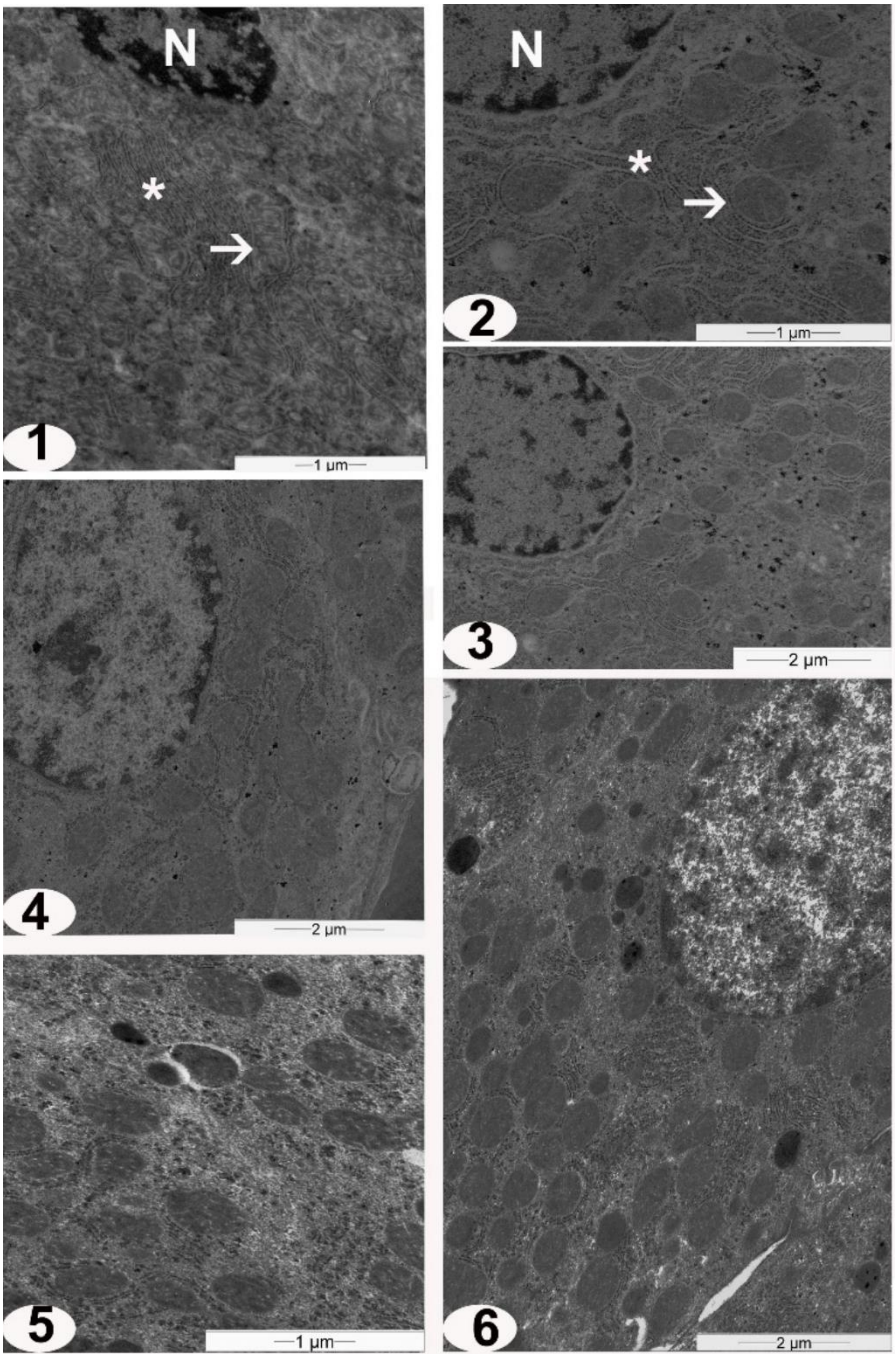

Figure 2: Transmission Electron Microscopy. **1**: control group with water (C); **2-3**: control group with soybean oil (D0); **4**: 5mg/kg of isotretinoin (D5a); **5-6**: 5mg/kg of isotretinoin for 60 days and 30 drug-free days (D5b). Observing images **1** and **2**: N- nucleous; * indicates endoplasmic reticulum and the arrow indicates mitochondria. This pattern is repeated for, all images. Observe that the endoplasmic reticulum and mitochondria frequency and morphology are similar in both treated groups. Notice also that chromatin organization is similar among the groups.

Capítulo 3

Dose Dependent Treatment with Isotretinoin Induces Changes in the Ileum Mucosa but No Structural Alteration of Duodenum and Jejunum, in Wistar Rats.

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ABSTRACT

Acne is the most common skin disorder and can directly affect the patients' self-esteem and social life. Systemic treatment has been indicated for nodular, cystic or persistent acne rather than another type of treatment, such as a topic one. Isotretinoin is an analogue of vitamin A and by suppressing the sebaceous glands the disease can be controlled. This study was designed to mimic the treatment performed in young patients using the dosage of 1mg/kg, and a higher dose of 10mg/Kg, for 60 days in young male Wistar rats. Using a morphometry tool and histochemical techniques we evaluated the villus, intestinal crypts, and goblet cells to find signs of possible alterations of the duodenum, jejunum and ileum segments of the small intestine. We found no signs of changes in the jejunum mucosa after 8 weeks of treatment with 1mg/kg and 10mg/kg. The duodenum is also less affected, whereas significant modifications were found in the ileum. The goblet cell frequency was altered, indicating a proliferative potential for the substance, but no important alterations were found with this protocol.

Keywords: small intestine, morphometry, 13-cis-retinoic acid, acne, dermatology

INTRODUCTION

Isotretinoin has been available for the last thirty years for the treatment of cystic acne that is severe and resistant to other types of treatment. Systemically administered, this substance has proved to be the most significant advance in acne therapy. Some side effects have been reported, including mucosal dryness, psychiatric reactions and some teratogenic effects (Ünal et al 2013; Raza et al 2012; Schaffer et al, 2009, Leyden 1998,). Relapse rates after the treatment of acne patients, using all methods, vary between 10% and 60% depending on the dosage regimen used, the length of follow-up, and characteristics of the population study (Morales-Cardona &Sánchez-Vanegas, 2013).

Isotreinois is the most effective treatment targeting all four mechanisms of acne formation. Emerging research suggests that lower doses of isotretinoin initiated during the mild to moderate stges can yield early and equally effective long-term results with a safer side-effect profile (Studer & Colella, 2014). Acne vulgaris is a multifactorial condition often conferring significant psychosocial morbidity, especially in young adults. The pathophysiology of the condition is still not fully known, but it is believed to be related in part to excess sebum production, follicular hyperkeratinization, microbial colonization by *Propionibacterium acnes*, and inflammation (Olutunmbi et al, 2008). Retinoids might be considered as the ideal agents for treating acne because they are able to interfere with most of the pathogenic aspects, having anti-inflammatory effects. Isotretinoin regulates keratinocyte proliferation leading to a reduction of comedone formation. It also suppresses sebaceous gland activity with a subsequent reduction of sebum production, reducing *P. acnes* colonization (Chicozzi et al 2013).

The pharmacokinetic profile of isotretinoin is analogous to vitamin A. Following oral administration, peak plasma concentrations of isotretinoin is reached in about 2 to 4 hours. Approximately 20% of isotretinoin is absorbed when administered with an empty stomach, increasing to 40% when in the presence of food (Diniz et al, 2002; White, 1999; Nankervis et al, 1995). Isotretinoin is excreted through the urinary tract, and the presence of low amounts of unconjugated isotretinoin can be detected in the urine. However, the excretion of about 53 to 74% of the isotretinoin administered occurs through the faeces, as a result of incomplete absorption, biliary excretion or enterohepatic recirculation (Diniz et al, 2002; Allen & Bloxham, 1989).

A dose of 0.5–1.0 mg/kg/day dramatically reduces sebum excretion by the order of 90% within 6 weeks. Unlike tretinoin (all-trans retinoic acid), isotretinoin has little or no ability to bind to cellular retinol-binding proteins or retinoic acid nuclear receptors (RARs and RXRs) but may act as a pro-drug that is converted intracellularly to metabolites that are agonists for RAR and RXR nuclear

receptors. Isotretinoin has at least five biologically important metabolites: 13-cis-4-oxo-retinoic acid (4-oxo-isotretinoin), all-trans-RA (tretinoin), all-trans-4-oxo-retinoic acid (4-oxo-tretinoin), 9-cisretinoic acid and 9-cis-4-oxo-retinoic acid. Studies examining sebum excretion rates in patients with severe acne have shown that, within 4 weeks, 4-oxo-isotretinoin (30–60 mg/day orally) only produces a 70% mean acne reduction compared to the same dose of oral isotretinoin over 4 weeks. Isotretinoin is also superior to 9-cis-retinoic acid and all-trans-retinoic acid in terms of sebum suppression. Only tretinoin and 4-oxo-tretinoin bind to RAR-γ, which is the receptor thought to be important in retinoid treatment of acne (Layton 2009).

Drugs are absorbed after oral administration as a consequence of a complex array of interactions between the drug, its formulation, and the gastrointestinal (GI) tract. The presence of food within the GI tract impacts significantly on transit profiles, pH, and its solubilization capacity (Charman et al 1997). Absorption of isotretinoin drug is in the intestine and due to the lipophilic nature of isotretinoin, its absorption is increased with concomitant food intake. In the blood, isotretinoin is carried linked to the protein albumin and enters the cell by passive diffusion (Akhtar 2015; Tsukada et al, 2002).

Since the small intestine is the site of drug absorption, some alterations could be found during or after treatment. The recommended dose is about 1mg/kg with a cumulative dosage of about 120-150mg. The treatment is dose cumulative and the duration period is about 2-6 months (Aktar 2015, Leyden 1998, Layton 2009).

The gastrointestinal tract has several important functions besides the absorption and secretion of drugs. This means that the single epithelial layer in the intestine performs the crucial task of regulating the balance between efficient absorption and optimal protection and this affects the permeability of drugs (Lennernäs, 2007). We aimed to investigate the effect of different isotretinoin doses based only on histology and ultrastructure of duodenum, jejunum and ileum mucosa in male Wistar rats.

MATERIAL AND METHODS

Experimental groups

24 Wistar rats (*Rattus norvegicus)* were randomly allocated in the following groups:

Controls:

C: control group with water;

D0: control group with soybean oil (the drug vehicle);

Treatments:

D1: 1mg/kg BM of Isotretinoin for 60 days;

D10: 10mg/kg BM of Isotretinoin for 60 days;

We chose this protocol in order to observe groups with the dose usually indicated for patients' treatment (D1) and a higher (D10) dosage. The rats had free access to rodent feed and water and the animal raising unit had luminosity control with 12 hours of light/dark and temperature control at about 22±1°C. A previous study (Nankervis et al, 1995) demonstrated that soybean oil is an indicated vehicle for isotretinoin. Therefore, the drug was diluted in soybean oil and offered by daily gavage for 60 days. The solutions were prepared every week in the dark and kept in a dark bottle for manipulation.

This experiment followed the established ethical standards in accordance with animal protection laws in Brazil. The research plan was approved by the Ethics Committee for Animal Use, maintained by the State University of Campinas (CEUA/Unicamp/ protocol #2831-1).

Duodenum, jejunum and ileum extraction

After the treatment period of 60 days, the rats were euthanized with a mixture of 10mg/kg of ketamin and 80mg/Kg of xylazine solution. Immediately after, the segments were sectioned and washed in saline solution. The procedures were the usual ones for light microscopy using modified Karnovsky fixative (Karnovsky, 1968) with 4% paraformaldehyde and 2.5% glutaraldehyde solution for 48 hours. The samples were included in paraffin.

Histochemistry

Using 5µm thickness sections, we performed two different histochemical techniques. The first one was the combination of Alcian Blue (AB) pH 2.5 with Periodic Acid Schiff (PAS) (AB+PAS) (Alcian Blue pH 2.5-PAS®, EasyPath) to identify goblet cells according to their mucin type. The second method was Reticulin (Reticulina®, EasyPath) to reveal the reticulin fiber distribution and structure.

Goblet cell evaluation. Using the samples after the histochemical technique combining AB+PAS, pH 2.5, we selected 10 different sites/animal for evaluation. The first step was to determine the mucosal area were the cells would be evaluated. Using the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29), we captured 10 images with 200x magnification. In those images, we determined the mucosa area considering villus and crypts and counted goblet cells in that determined

mucosal area observing the mucin revealed in cytoplasmic granules. The mucin is defined according to the dominant group. Thus, it is possible to estimate the frequency of cells secreting basic (PAS^+, magenta), acidic (AB^+, blue color) and a mucin mixture thereof (AB^+PAS^+, a purple tone).

Light microscopy

After fixation and dehydration, small blocks were embedded in paraffin using the usual procedures. 5µm thick sections were stained with Masson Trichrome Stain (Weigert´s Iron Hematoxilin Set®, Sigma-Aldrich and Masson Trichrome Stain Kit®, Sigma-Aldrich) to show muscle and connective tissue distribution. Other 5µm thick sections were stained with Hematoxilin-Eosin solution for morphometrical analysis. For each section, 30 images were captured with 200x magnification and morphometric data were obtained with the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29).

Villus and crypt morphometry. To analyze the villus, we considered the height of 15 villi/animal. For the Liberkühn crypt evaluation we determined the height of 15 crypts/animal. To determine the relation between villi and crypt height we applied the formula (villi height/crypt height).

Mucosal morphometry. The organ wall thickness (from the villus apex to the base of the muscular layer) was determined for 10 different regions/animal.

Assumed Absorptive Surface. To find the assumed absorptive surface (AS) for duodenum, jejunum and ileum/animal the following formula was used (Hardin et al, 1999):

$AS(\mu m^2)$= villi height (µm) X medium width at 50% of villi heights (µm).

Scanning electron microscopy

In order to show surface structures, we collected other fragments to observe with scanning electron microscopy. The fragments were completely dehydrated (ethanol series of 70 to 100%) after fixation with modified Karnovsky fixative. The next steps were drying in the critical point dryer and gold sputtering.

Statistical analysis

For multiple comparison, we applied the Kruskal-Wallis test followed by Dunn's post test. The tests were carried out considering the significance level of $p < 0.05$. We used Minitab® 16

program (LEAD Technologies, Inc. Charlotte, North Carolina) and the morphometry data are presented as mean ± standard deviation.

RESULTS

This experimental protocol was well tolerated by the animals since it did not cause any obvious signs of health damage. As revealed by morphometry, in the jejunum we have no significant differences in the parameters. In duodenum we found only a narrower duodenum wall in D10 group in relation to C group (Table 1).

When we consider the thickness of the ileum wall, we found that D10 had higher values in relation to D1. The villi height increased in D1 in relation to D0 after the treatment and the crypt height increased in D10 in relation to the D0 and C groups. With these two results we calculated the assumed absorption surface and we found no difference among the groups. The ratio villus/crypt height showed that in D10 this ratio is greater than in D1, indicating that in D10 the villi are higher than their crypts, in relation to D1 (Table 1).

A decrease in goblet cell frequency was observed in D10 and D1 in relation to C in the duodenum, however no alterations were observed in the jejunum and ileum. In the qualitative observation, we found that the frequency and distribution of goblet cell types along the crypt and villi was as described in the literature. The AB^+ goblet cells were not found and the crypts' basal regions were predominantly occupied by AB^+PAS^+ goblet cells. Their number was reduced in the upper region, towards the lumen. In the villus, goblet cells can be found less frequently and are predominantly of the AB^+PAS^+ type. The PAS^+ goblet cell were found very rarely in the villus and more frequently but still in small numbers in the crypt base (Figure 1). Quantitative analyses showed that the frequency of these three subpopulations of goblet cells were not altered due to the treatment in the duodenum and jejunum. Differences were observed in the ileum where the total number of goblet cells increased in D10 group, in relation to the D1 group. In the ileum we also observed the PAS+ goblet cell frequency diminished in the D10 group in relation to C (Table 1).

The Masson Trichrome stain and Reticulin and scanning electron microscopy showed that, morphologically, the duodenum, jejunum and ileum fragments considered were not altered by the treatment. We found basically the same structure in the treated groups and the control groups, considering villus and crypts, connective tissue distribution, reticulin fiber structure and distribution and the surface characteristics (Figure 1).

DISCUSSION

The intestine is responsible for absorption and due to a rich lymphatic circulation, after absorption and some biotransformation, the substances follow directly to the liver to be metabolized. The small intestine absorptive activity depends on villus distribution, enterocyte and crypt integrity and the motility potential (Bremmer & McCaffery, 2008; Lennernäs, 2007; O'Reilly et al, 2006; Cisneros et al, 2005; Ferguson et al, 2005; Gleeson et al, 1971; Levin, 1969; Stewart et al, 1967; Wilson, 1962). According to Silva et al (2010), healthy intestinal mucosa should provide appropriate morphological and functional characteristics, since the absorption processes are dependent on epithelium integrity.

The small intestine absorptive capacity is initially proportional to the number of villi (Lennernäs, 2007; Pelicano et al, 2003; Gleeson et al, 1971; Levin, 1969;; Stewart et al, 1967; Wilson, 1962). A desirable ratio is shallower crypts than villi (Hayakawa et al, 2014) and this situation was found in all groups (Figure 1). The optimum ratio of villi height to crypts varies; with accepted ratios from 3:1 to 5:1, but this ratio has been found to vary from 2:1, 1.82:1 to 1:1, which has also been accepted as normal (Walker & Talley, 2011). In this study, the average ratio of villus height/crypt height found for the groups followed the trend of about 2.5:1, and is in accordance to the height considered normal in the literature.

Mammals have a rapid turnover of the intestinal epithelium and this is supported by stem cells located at the crypt base. The differentiated Paneth and enteroendocrine cells remain in the crypt while goblet cells and enterocytes are distributed along the crypts and villi (Ritsmaet al, 2014; Walther & Graham, 2014; Yilmaz et al, 2012; Tajbakhsh, 2011).

The experimental protocol, using two different doses, 1mg/kg and 10mg/kg suggests that the effect is dose dependent in the ileum. We observed that the D10 group has greater values in relation to D1 or controls for most parameters considered. This data indicates that the suggested dose used for human treatment, 1mg/Kg, does not lead to significant alterations, since the parameters are similar to the controls. The other dose applied, 10mg/Kg, is not a superdose, because in some cases, patients use even 20mg/Kg per day of the drug. So we chose a higher dose than that usually indicated, but not a toxic one. According to previous authors (Wang 1997) it is well established that vitamin A is essential for normal cell growth, differentiation and maintenance of epithelial tissues. In a previous experiment, the authors observed the stimulating effect of all-trans-retinoic acid (Wang et al, 1997). Using a rat resection model of intestinal adaptation, they observed the effect of retinoic acid stimulating crypt cell proliferation in the adapting remnant of the intestine, verified 6 hours after

surgery. According to the literature, it has been proposed that retinoids act as chemopreventive agents inhibiting cell proliferation and inducing differentiation (Zheng 1997, Wang 1997).

Another cell type was the goblet cell observed with the AB+PAS histochemical technique. In a qualitative observation we found the frequency and distribution of goblet cell types along the crypts and villi was as expected in duodenum, jejunum and ileum (Forster et al, 2014; Yilmaz et al, 2012). The mucin produced and secreted by goblet cells is important for maintaining the microbiota and its composition can be modified due to drug treatment or feed changes. The diet and the use of drugs could have altered the distribution and frequency of these cells and this contributes to the maintenance of bacterial translocation and microbiota (Frankel et al 1995). The change in frequency of that cell type is important because the quality of the mucin secreted also interferes with the absorption capacity of specific groups present in the luminal content. The cells secrete PAS^+ viscous mucus, which contributes to the formation of a laminar flow for lubrification and the processing of particles, allowing food to be transferred from one region to another. It also protects against mechanical injuries caused by the friction of food and protects the mucosa against chemical, physical and biological aggressors, creating an obstacle that modifies the passage of solutes by the epithelium. This mucin contributes by helping to hydrate the bolus (Vieira-Lopes et al 2014). The change in frequency of this cell type was probably in response to the drug, but in no way affected the functioning of the intestinal segment.

It is important to carefully evaluate the parameters involved in the intestinal absorptive capacity. Thus, if its absorptive capacity is changed suddenly, food nutrients and drug absorption will also be affected, as well as the patient's health. The modification found in some segments may not have changed the digestive and absorptive potential. In a similar study, morphometry has been used to show signs of atrophy of the intestinal epithelium height and nucleus diameter in cases of severe malnutrition, or in drug poisoning (Azevedo et al, 2007; Pireset al, 2003). The observations of the samples with scanning electron microscopy showed no differences in surface structure among the groups. All the samples were basically the same as the control group. With the protocol chosen for this study we suggest that no signs of malnutrition or health damage could be clearly attributed to the treatment.

Histochemistry showed a tissue with the expected characteristics that are well described in medical literature (Cachay et al, 2014; Hajková et al, 2014; Kovalčinovà et al, 2014; Kumar et al, 2014; Otte et al, 2014; Kierszenbaum & Tres, 2011) (Figure 1). We found an integrative epithelium, followed by a thin connective tissue layer with lymphatic and blood vessels. Below these were the

mucosa muscularies layer, followed by connective tissue and close to the intestine wall, the muscular layer. Reticulin fibers were found throughout the structure, providing support for the tissue, and comparing treated groups with control groups, their frequency and structure were not different.

CONCLUSIONS

The duodenum and jejunum are less susceptible to alterations due to isotretinoin treatment. The changes were found in the ileum, where goblet cell frequency was noted to increase during intestinal tissue development. These results confirm the safety of the treatment and no signs of consistent and lasting changes were found in the small intestine with 1mg/kg and 10mg/kg of isotretinoin in male Wistar rats.

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Table 1: Body weight, stereologic and morphometric analysis of the duodenum, jejunum and ileum mucosa after 60 days of treatment with isotretinoin in male young Wistar rats.

Groups	**C- control with water**	**D0- control with soybean oil**	**D1-1mg/Kg BM of isotretinoin**	**D10- 10mg/kg BM of isotretinoin**
Initial body weight (g)	192, 82±13,96	193,5±10,17	193,46±15,24	192,78±3,8
Final body weight (g)	468,43±40,15	459,44±53,34	466,68±22,54	474,25±41,73
Duodenum				
Duodenum Wall Thickness(µm)	955,47±529,22^{a}	733,07±118^{ab}	776,44±163,35^{ab}	621,65±121,44^{b}
Mucosal área (mm²)	2,93±0,12^{a}	2,85±0,22^{ab}	2,69±0,06^{b}	2,37±0,34^{b}
Absorption surface (mm²)	0,043±0,011	0,046±0,01	0,041±0,077	0,037±0,058
Villus Height *(µm)*	419,61±98,46	442,19±85,19	390,05±75,17	403,48±83,82
Crypt height (µm)	223,40±139,74	169,32±28,33	160,65±16,92	152,02±16,52
Villus height:Crypt height ratio	2,19±0,79	2,63±0,42	2,44±0,47	2,63±0,35
Goblet cells- Units/mm²	559,80±134,54^{ab}	657,83±5,45^{ab}	528,09±146,42^{b}	686,13±118,86^{a}
Goblet cells- PAS^{+}/mm² (%)	97,30±1,17^{a}	98,96±0,27^{b}	98,82±0,25^{ab}	98,78±0,89^{ab}
Goblet cells- PAS^{+}AB^{+}/mm² (%)	2,70±1,17^{a}	1,04±0,27^{b}	1,18±0,25^{ab}	1,22±0,89^{ab}
Jejunum				
Jejunum Wall Thickness(µm)	734.69±40.26	717.88±82.68	746.95±39.32	744.47±19.43
Mucosal área (mm²)	3.12±0.31	2.97±0.28	2.93±0.25	2.90±0.38
Absorptionsurface (mm²)	0.49±0.07	0.46±0.12	0.44±0.05	0.44±0.07
Villus Height *(µm)*	468.00±21.22	442.38±80.42	446.42±30.35	433.69±33.81
Crypt height (µm)	176.73±18.24	180.95±29.98	175.01±9.46	166.29±19.44
Villus height:Crypt height ratio	2.67±0.25	2.53±0.72	2.55±0.18	2.63±0.26
Goblet cells- Units/mm²	62.46±13.02^{a}	76.01±1.60^{b}	71.57±9.53^{ab}	78.35±10.90^{b}
Goblet cells- PAS^{+}/mm²	99.18±0.46	99.54±0.20	99.15±0.52	99.48±0.64
Goblet cells-AS^{+}AB^{+}/mm²	0.81±0.46	0.46±0.20	0.85±0.52	0.52±0.64

Mean ± standard deviation. Averages in the same row followed by different letters differ by Kruskal-Wallis followed by Dunn post test at a 5% significance level.

Continuation of Table 1: Body weight, stereologic and morphometric analysis of the duodenum, jejunum and ileum mucosa after 60 days of treatment with isotretinoin in male young Wistar rats.

Groups	**C- control with water**	**D0- control with soybean oil**	**D1-1mg/Kg BM of isotretinoin**	**D10- 10mg/kg BM of isotretinoin**
Ileum				
Ileum Wall	580,05±68,33^{ab}	618,25±76,57^{ab}	538,08±91,73^{a}	632,18±59,87^{b}
Mucosal área (mm²)	0,25±0,01	0,24±0,06	0,26±0,04	0,27±0,02
Absorptionsurface	0,30±0,04	0,30±0,06	0,37±0,05	0,33±0,13
Villus Height *(μm)*	333,89±7,51^{ab}	313,65±30,98^{a}	365,06±22,55^{b}	327,68±78,27^{ab}
Crypt height (μm)	145,47±19,39^{a}	137,61±19,87^{a}	161,40±18,27^{ab}	180,68±17,63^{b}
Villus height:Crypt	2,33±0,27^{a}	2,30±0,28^{ab}	2,28±0,24^{ab}	1,81±0,40^{b}
Goblet cells- Units/mm²	236,58±19,35	234,52±61,06	227,02±64,63	224,45±37,26
Goblet cells-	4,20±1,86^{a}	1,73±0,96^{bc}	3,32±1,01^{ac}	0,66±0,26^{b}
Goblet cells-	98,2±0,76^{a}	99,2±0,45^{bc}	98,51±0,32^{ac}	99,71±0,09^{b}

Mean ± standard deviation. Averages in the same row followed by different letters differ by Kruskal-Wallis followed by Dunn post test at a 5% significance level.

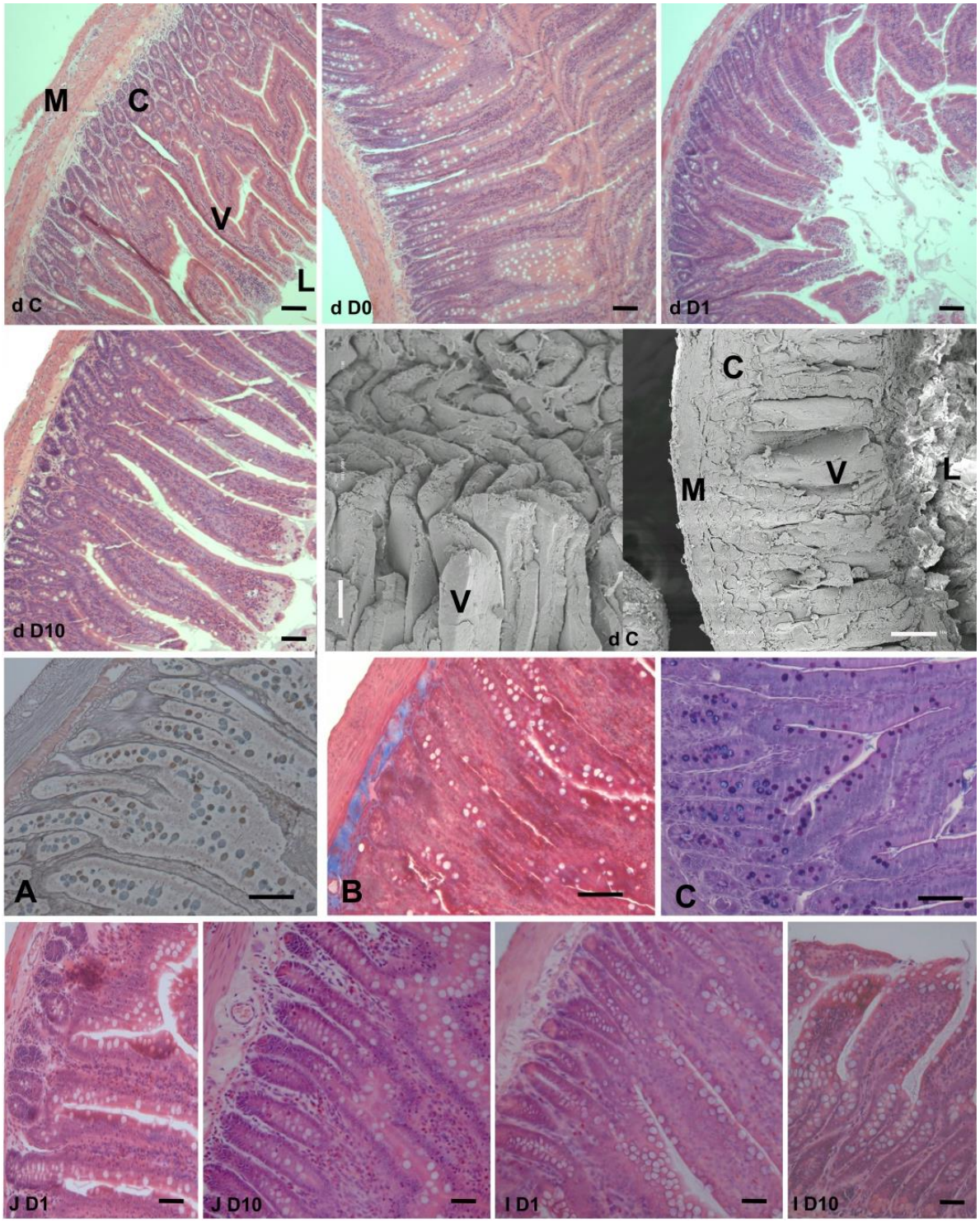

Figure 1: Light microscopy images of duodenum, jejunum and ileum mucosal. d: duodenum; J: jejunum; I: ileum; C: control group, D0: control group with soybean oil, D1: 1mg/kg of isotretinoin, D10: 10mg/kg of isotretinoin. These images showed the general structure for the groups. In the first image, the small intestine structures are indicated. C: crypt, M: muscle layer, V: villus, L: lumen. A: Duodenum of the control group using the histochemical technique for reticulin fibers that are stained brown. B: Duodenum of the control group with Masson´s Trichrome histochemistry. In blue is the connective tissue and in red the muscle layer and general epithelium, C: Duodenum of the control group applying AB+PAS histochemical technique. The pink regions are PAS+ goblet cells and the purple ones are AB+PAS+ goblet cells. Since the general structure seems to be unaltered, we choose these images to illustrate the three small intestine regions. The two scanning electron microscopy images were obtained for the duodenum fragments and represent the patterns found also for the jejunum and ileum. Bar: 100 µm.

Capítulo 4:

Effect of isotretinoin treatment on the small intestine mucosa structure of young male Wistar rats after 60 and 90 days: A morphological study.

Abstract

Isotretinoin is the substance used in cases of severe acne and acne resistant to other treatments. This skin disease affects patients of all ages, depending on various factors and can hinder social life, especially in adolescents. The drug acts suppressing sebaceous gland activity and creating an inhospitable environment for *Propionibacterium acne*. During treatment, the symptoms improved but some side effects occurred, such as mucosal dryness, and these should disappear after terminating the treatment. Drugs and food nutrients are absorbed in the small intestine and the integrity of this organ is important for correct nutrition and patient treatment. We intended to verify the small intestine structure, after the treatment with 5g/kg Isotretinoin, and after a period without the drug, which could be considered a rest period after the treatment. 24 young male Wistar rats were separated randomly into 4 groups (n=6): *C: control with water; D0: control with soybean oil;* D5a: 5mg/kg of Isotretinoin; D5b: 5mg/kg of Isotretinoin for the 60 days of treatment and another 30 days of rest period. The drug was diluted in soybean oil and offered daily by gavage for 60 days. After treatment, the animals were euthanized and duodenum, jejunum and ileum were collected for analysis with light and scanning electron microscopies. The treatment stimulated tissue proliferation in the jejunum and ileum, but had no significant effect in the duodenum. The results also showed a modification in goblet cells frequency due the treatment, in the duodenum and ileum. Another conclusion reached after this treatment is that some modifications disappear during the rest period. The observations are in agreement with the literature that associates retinoids with tissue proliferation.

Key-words: small intestine, drugs, Wistar rats, acne

INTRODUCTION

Acne is a skin disorder that can impact on social relations, with skin scars often leading to low self-esteem, mainly in adolescents. Oral isotretinoin (13-cis-retinoic acid) is a synthetic vitamin A analogue that was approved by the Food and Drug Administration in 1982 for the treatment of severe cystic acne. This substance is currently the most effective acne treatment available, with reported long-term remission rates as high as 89%. This treatment is specially indicated in cases of resistant disease, unresponsive to other therapies (1-5).

The treatment usually initiates at a daily dose of 0.5mg/kg (or higher) and can be increased to 1.0mg/kg. A low dose of isotretinoin, such as 0.15-0.40mg/kg, has been reported to be effective with a low incidence of severe side effects. Aiming at a total dose of 120–150mg/kg per treatment, it may last for 3–7 months depending on the daily doses used (4,6,7). About 25% of patients treated with isotretinoin have elevated triglyceride plasma levels, which in some cases may be associated with the onset of acute pancreatitis. Isotretinoin can also cause a slight decrease in plasma HDL cholesterol and increased LDL and VLDL cholesterol. Changes in serum triglycerides and cholesterol are reversible upon treatment interruption. Less frequent adverse reactions that are reversible include vomiting, gastrointestinal bleeding, appendicitis, gut inflammation, oesophagitis, anorexia, weight loss and ulcerative colitis (7-12). Unspecific symptoms have also been observed, including nausea, diarrhea and abdominal pain. There is also evidence that the drug may worsen the symptoms of inflammatory diseases. However, it has been administered successfully to patients with Crohn's disease and ulcerative colitis without discomfort to the patients (10).

In the intestine, ingested food is converted into a small particle nutrient solution (13,14). Isotretinoin absorption occurs, then, in the intestine, where the esterified chylomicron with a retinol group is absorbed by the lymphatic system. Due to the lipophylic nature of isotretinoin, its absorption is increased with concomitant intake of food. In the blood, isotretinoin is linked to the protein, albumin, and enters the cell by passive diffusion (15). Although it is widely used and many side effects have been described, information about the direct effect in the healthy small intestine is not available. We considered that duodenum is the main digestion site and jejunum and ileum seem to be the absorption sites of drugs and nutrients. So, the aim of this study was to investigate in young male Wistar rats the structure of the duodenum, jejunum and ileum after treatment with isotretinoin and after a rest period with no exposure to this component.

MATERIAL AND METHODS

Experimental groups

24 male Wistar rats (*Rattus norvegicus) were randomly allocated in the following groups:*

C: control group with water;

D0: control group with soybean oil, the vehicle where we dissolved the substance;

D5a: 5mg/kg BW of Isotretinoin for 60 days;

D5b: 5mg/kg BW of Isotretinoin for the 60 days of treatment and another 30 days drug free.

The four groups received the treatment for 60 days but the D5b group remained another 30 days without the medicine in order to elucidate any alterations right after the treatment. The rats had free access to rodent food and water and the animal house had luminosity control with 12 hours of light/dark and temperature control about 22±1°C. We diluted the drug in soybean oil (16), offered by daily gavage for 60 days. The solutions were prepared every week in the dark and kept in a dark bottle for manipulation.

The small intestine collection

After the treatment the groups C, D0 and D5a were euthanized with a mixture of 10mg/kg of ketamin and 80mg/Kg of xylazine solution and the duodenum, jejunum and ileum were immediately sectioned and washed in saline solution. The preparations followed the usual procedure for light microscopy using Karnovsky's modified fixative (17) which includes 4% paraformaldehyde and 2.5% glutaraldehyde solution for 48 hours. After 30 days, this same procedure was repeated for group D5b.

Light microscopy

In order to analyze the treatment effect, we chose the morphometric tool linked to stereological evaluation. After fixation, the fragments were embedded in paraffin using the usual dehydration protocol in alcohol solutions. Sections with 5µm thickness and 40µm of interval were stained with Hematoxilin-Eosin solution.

Villus and Crypt Morphometry. All data were obtained with the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29). To analyze the villus we considered the height of 15 villi. For the Liberkühn crypt evaluation we determined the height of 15 crypts. To determine the relation between villi and crypt height, we applied the formula (villi height/crypt height).

Mucosal Morphometry. We determined the thickness of the organ wall of 10 different regions.

Absorptive Surface. To find the assumed absorptive surface (AS) for the duodenum we used the following formula (19):

AS(µm²)= villi height (µm) X medium width at 50% of villi height (µm).

Histochemistry

In the 5µm thickness section we performed the combination of Alcian Blue (AB) pH 2.5 with Periodic Acid Schiff (PAS) (AB+PAS) (Alcian Blue pH 2.5-PAS®, EasyPath) to stain the goblet cells according to their mucin type. The second technique was Reticulin (Reticulina®, EasyPath) to reveal reticulin fiber distribution and structure. The third one was the Masson Trichrome Stain (Weigert´s Iron Hematoxilin Set®, Sigma-Aldrich and Masson Trichrome Stain Kit®, Sigma-Aldrich) to show muscle and connective tissue distribution.

Goblet Cell Evaluation. The first step was to determine the area occupied by villi and crypts. In this area we selected ten different fields of the same samples using the histochemical technique combining AB+PAS pH 2.5. Using the software Image Pro Plus® (Media Cybernetics, version 4.5.0.29), we counted the goblet cells considering the type of mucin revealed in the cytoplasm. The different mucins were revealed according to their dominant group. Thus, it is possible to estimate the frequency of cells secreting basic (PAS^+, magenta color), acidic (AB^+, blue color) and a mixture thereof (AB^+PAS^+, purple color).

Scanning electron microscopy

In order to show surface structure we collected fragments to observe with scanning electron microscopy. The fragments were completely dehydrated (ascending ethanol series of 70 to 100%), after fixation with a modified Karnovsky fixative. The next steps were critical point drying, followed by gold sputtering.

Ethical permissions

This experiment followed the established ethical standards in accordance to the animal protection laws of Brazil. The research procedure was approved by the Ethics Committee of Animal Use of the State University of Campinas (CEUA/Unicamp/ protocol #2831-1).

Statistical analysis

Considering the sample size, we applied the non-parametric Kruskal-Wallis statistic test followed by Dunn's post test. Data are presented as mean± standard deviation. The tests considered

P<0.05 statistically significant and were performed with Minitab® 16 program (LEAD Technologies, Inc. Charlotte, North Carolina).

RESULTS

The treatment did not cause obvious signs of damage to the rat's health. Gavage was performed without difficulty and, as shown in Table 1, all groups had body mass gain during the treatment.

The morphometric data indicates that the duodenum parameters did not alter with the treatment. The stereology of goblet cells showed that the effects were dosage-dependent and that the rest or recovery period was sufficient, since their parameters were like that of the controls. We found diminished jejunum wall thickness in the D5a group in relation to the control D0. Crypt evaluation showed that their height decreased in the recovery group D5b in relation to control DO. Considering the villus data, the treatment did not affect its height but it could have affected the assumed absorption surface because these measurements were smaller in the D5b group in relation to D5a. Stereology and morphometry of the cell types indicated that goblet cell frequency increased in group D5b in relation to C while the frequency of subtypes showed no statistical difference among the groups (Table 1 and Figure 1).

Observing all data obtained for the ileum segment we found indicative of tissue recover and others indicating direct effect of the treatment. The crypt height was higher in D5a group in relation to controls groups. In the other side, villus height was higher in D5b group in relation to control, indicating that even after the end of the treatment, the drug, since have dose dependent effect, was still active until its complete elimination, interfering in this parameter. For the goblet cells, we also found some indication of recovery in the D5b group after treatment and a drug-free period. We found no AB+ goblet cells. The frequency of PAS+ goblet cell was smaller in D5a group in relation to both controls, and the frequency of AB+PAS+ goblet cells was higher in the D5a group in relation to both controls. Since we found no difference in the D5b group in relation to D5a or control groups, we can assume that the alterations observed were recovered. The mucosa area considered in this analysis was higher in D5b group in relation to C. The total number of goblet cells was smaller in the D5a group than in D5b and higher in relation to the C group.

Goblet cells were observed throughout the mucosa, more concentrated at the base of the crypt and villus, diminishing in direction of the apex. Cells secreting acidic mucins (AB^+) were not found and secretory cells with basic mucins (PAS^+) were found more frequently in the crypts, reducing in

number as the villi are reached, where they occurred rarely or not at all. Regarding secreting Goblet cells with mucin composed of a mixture of basic and acidic mucin ($AB^{+}PAS^{+}$), these were infrequent in the crypts and increased to predominate in the villi. This pattern is repeated for all groups, an indication that the proposed treatment did not change the basic distribution of secretory cell types.

Reticulin immunohistochemistry is based on silver impregnation of collagen fibers. The fibers were found throughout the connective tissue, forming a framework. This distribution was not altered in the different groups (Figure 1). In the samples stained with Masson's Trichrome technique, the connective tissue appeared in blue. All groups showed the same distribution pattern of connective and muscle tissue labeling.

Scanning electron microscopy performed for small samples of the different groups showed the expected morphology, already described in the literature. We found villi extending into the lumen and intact absorptive epithelium with microvilli on the surface. The crypt below the villi was observed and connective tissue was found just below this tissue. The muscle layer was found sealing the wall of the intestinal segment. This pattern was found in all groups.

DISCUSSION

Many publications can be found concerning the role of isotretinoin in cancer and effects on the nervous system of patients (19-22), but its direct effects on the structure of the gastrointestinal tract is still little discussed. The exposure to isotretinoin did not cause any obvious signs of damage to the rat’s health although there was a trend to reduced food and water intake. In a previous study (21) the authors observed a reduction in food intake, linked to consumption of isotretinoin. In our study, since the consumption of food and water was reduced in both groups receiving the drug and the control groups, this reduction cannot be directly linked to the consumption of the drug, but it does follow a trend already suggested in the literature (20,21).

Intestinal mucosa should provide appropriate morphological and functional characteristics, since the absorption processes are dependent on epithelium integrity. Numerous infectious or noninfectious agents can damage the intestinal mucosa and compromise the digestive processes. Inflammatory bowel disease has been attributed to the use of some substances, among them, isotretinoin (23-25). Inflammatory diseases could be related to treatment with the substance, but the symptoms disappeared at the end of the medication period (24).

The gastrointestinal tract has a primarily mechanical function. The overall rating of each histological section of the small intestine regions stained with hematoxylin-eosin revealed the general

morphology corresponding to that already described in the literature (14, 26, 27). The absorptive capacity of the gut is initially proportional to the number of villi present (28). A desirable ratio describes crypts that are shallower when compared to the villi and this situation was found in all groups. The optimum ratio of villus height:crypt height varies, and the accepted ratios are 3:1 to 5:1, but ratios of 2:1, 1.82:1 and 1:1 have also been found and are accepted as normal (29). In this study, the average found for the groups followed the trend of approximately 2:1, in agreement with descriptions proposed in the literature.

These results are also related to this drug's potential to interfere with cell proliferation. The crypt is the stem cell zone, located in the epithelium base. Increased epithelium proliferation potential was observed in the ileum and jejunum. Crypts are predominantly made up of goblet cells, Paneth cells in the base, enteroendocrine cells along the structure and some enterocytes. In the villi are found predominantly the enterocyte and some goblet cells. We found a normalize of goblet cells frequency after the treatment interruption, and this results are in accordance with the alteration found in D5a in duodenum and jejunum.

Mucus is a viscoelastic, gel-like and the primary function of mucus is to protect the surface mucosal cells from acids and peptidases. In addition, it serves as a lubricant for the passage of solids and as a barrier to antigens, bacteria and viruses. Mucins are likely to be the first molecules that invading pathogens interact with at the cell surface and thus, can limit binding to other glycoproteins and neutralize the pathogen (30-33). The diet and the use of drugs can alter the distribution and frequency of goblet cells and it is related to the maintenance of bacterial translocation and microbiota (34-37). The modifications concerning goblet cells were concentrated in the duodenum and ileum and the results are so interesting considering the typical goblet cell type in both regions. In duodenum the tendency of increase in PAS+ cell during the treatment and a decrease of the same cell after the treatment interruption indicates a potential of bolus hydration, linked to this type of mucin (34-37). In ileum, the oppose situation was observed, with the decrease of PAS+ cell during the treatment and an increase after it. In ileum we have an increase in PAS+AB+ goblet cell. This results indicates a potential of microbiota maintained for PAS+AB+ goblet cell secretion (34-37). The difference found in these proportion are related to the treatment, considering the result after the interruption of it, but this alteration is not enough to cause loss to small intestine function. Considering the three segments duodenum, jejunum and ileum we observed that jejunum and ileum are the two region more sensitive to the treatment with isotretinoin in relation to duodenum and that ileum is the most affected. The activity potential seems to be not modified but in jejunum we observed a increased thickness of the

wall during the treatment and the reduce of it after the interruption while in ileum we observed a tendency of continue increasing the thickness along the time.

The evaluation of the reticulin fibers and connective tissue distribution showed the distribution following the expected structure, as described in the literature (14,27), with fibers throughout the submucosa, and particularly in the connective tissue surrounding the villi and crypts.

CONCLUSIONS

We hypothesized that ileum and jejunum are the most sensitive regions for retinoid treatment, since these are the main absorption sites for these substances. Goblet cells types frequency have altered in jejunum and ileum indicative of treatment effect. In general, no signs of damage to the small intestine were found with this protocol.

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Table 1: Body weight, stereologic and morphometric analysis of the duodenum, jejunum and ileum mucosal after 60 days of treatment with isotretinoin and a recovey period in Wistar male young rats.

Groups	**C- control with water**	**D0- control with soybean oil**	**D5a-1mg/Kg of isotretinoin**	**D5b- 5mg/kg of isotretinoin and a recovered period**
Initial body weight (g)	192, 82±13,96	193,5±10,17	193.46±15.24	192.78±3.8
Final body weight (g)	468,43±40,15	459,44±53,34	466.68±22.54	474.25±41.73
Duodenum				
Duodenum Wall Thickness(µm)	955,47±529,22	733,07±118	761,52±168,47	679,24±122,47
Mucosal área (mm²)	2,93±0,12	2,85±0,22	2,83±0,38	2,97±0,10
Assumed Absorption surface (mm²)	0,043±0,011	0,046±0,01	0,05±0,0063	0,039±0,005
Villus Height *(µm)*	419,61±98,46	442,19±85,19	449,80±70,16	388,97±59,29
Crypt height (µm)	223,40±139,74	169,32±28,33	162,96±34,75	148,44±20,04
Villus height:Crypt height ratio	2,19±0,79	2,63±0,42	2,79±0,34	2,64±0,37
Goblet cells- Units/mm²	559,80±134,54^{ab}	657,83±5,45^{a}	426,48±62,44^{b}	663,99±163,81^{a}
Goblet cells- PAS^{+}/mm² (%)	97,30±1,17^{a}	98,96±0,27^{ab}	99,39±0,35^{b}	99,10±0,24^{ab}
Goblet cells- PAS^{+}AB^{+}/mm² (%)	2,70±1,17^{a}	1,04±0,27^{ab}	0,61±0,35^{b}	0,90±0,24^{ab}
Jejunum				
Jejunum Wall Thickness(µm)	734.69±40.26^{ab}	717.88±82.68^{b}	803.28±71.50^{ab}	760.91±56.81^{a}
Mucosal área (mm²)	3.12±0.31	2.97±0.28	2.87±0.26	2.89±0.36
Assumed Absorption surface (mm²)	0.49±0.07^{ab}	0.46±0.12^{ab}	0.55±0.063^{b}	0.45±0.09^{a}
Villus Height *(µm)*	468.00±21.22	442.38±80.42	502.61±28.14	475.49±46.85
Crypt height (µm)	176.73±18.24^{ab}	180.95±29.98^{a}	176.98±13.91^{ab}	155.29±9.38^{b}
Villus height:Crypt height ratio	2.67±0.25	2.53±0.72	2.85±0.25	3.08±0.40
Goblet cells- Units/mm²	62.46±13.02^{a}	76.01±1.60^{ab}	70.35±10.09^{ab}	81.74±6.51^{b}
Goblet cells- PAS^{+}/mm² (%)	99.18±0.46	99.54±0.20	99.66±0.32	99.27±0.23
Goblet cells- PAS^{+}AB^{+}/mm²(%) (%)	0.81±0.46	0.46±0.20	0.34±0.33	0.73±0.23
Ileum				
Ileum Wall Thickness(µm)	580,05±68,33^{b}	618,25±76,57^{ab}	583,54±42,53^{a}	585,65±41,75^{a}
Mucosal área (mm²)	0,25±0,01^{a}	0,24±0,06^{ab}	0,28±0,02^{ab}	0,29±0,03^{b}
Assumed Absorption surface (mm²)	0,30±0,04	0,30±0,06	0,32±0,06	0,27±0,03
Villus Height *(µm)*	333,89±7,51^{ab}	313,65±30,98^{a}	308,31±27,95^{ab}	296,88±25,04^{b}
Crypt height (µm)	145,47±19,39^{a}	137,61±19,87^{a}	171,69±11,26^{b}	156,31±12,41^{ab}
Villus height:Crypt height ratio	2,33±0,27^{a}	2,30±0,28^{ab}	1,80±0,11^{b}	1,91±0,18^{b}
Goblet cells- Units/mm²	236,58±19,35^{a}	234,52±61,06^{a}	192,8±21,80^{b}	245,4±9,35^{a}
Goblet cells- PAS^{+}/mm² (%)	4,20±1,86^{a}	1,73±0,96^{a}	0,60±0,39^{b}	1,41±0,19^{ab}
Goblet cells- PAS^{+}AB^{+}/mm²(%) (%)	98,2±0,76^{a}	99,2±0,45^{a}	99,7±0,18^{b}	99,42±0,07^{ab}

Mean ± standard deviation. Averages in the same row followed by different letter differ by the Kruskal-Wallis test followed by Dunn post test at a 5% significance level.

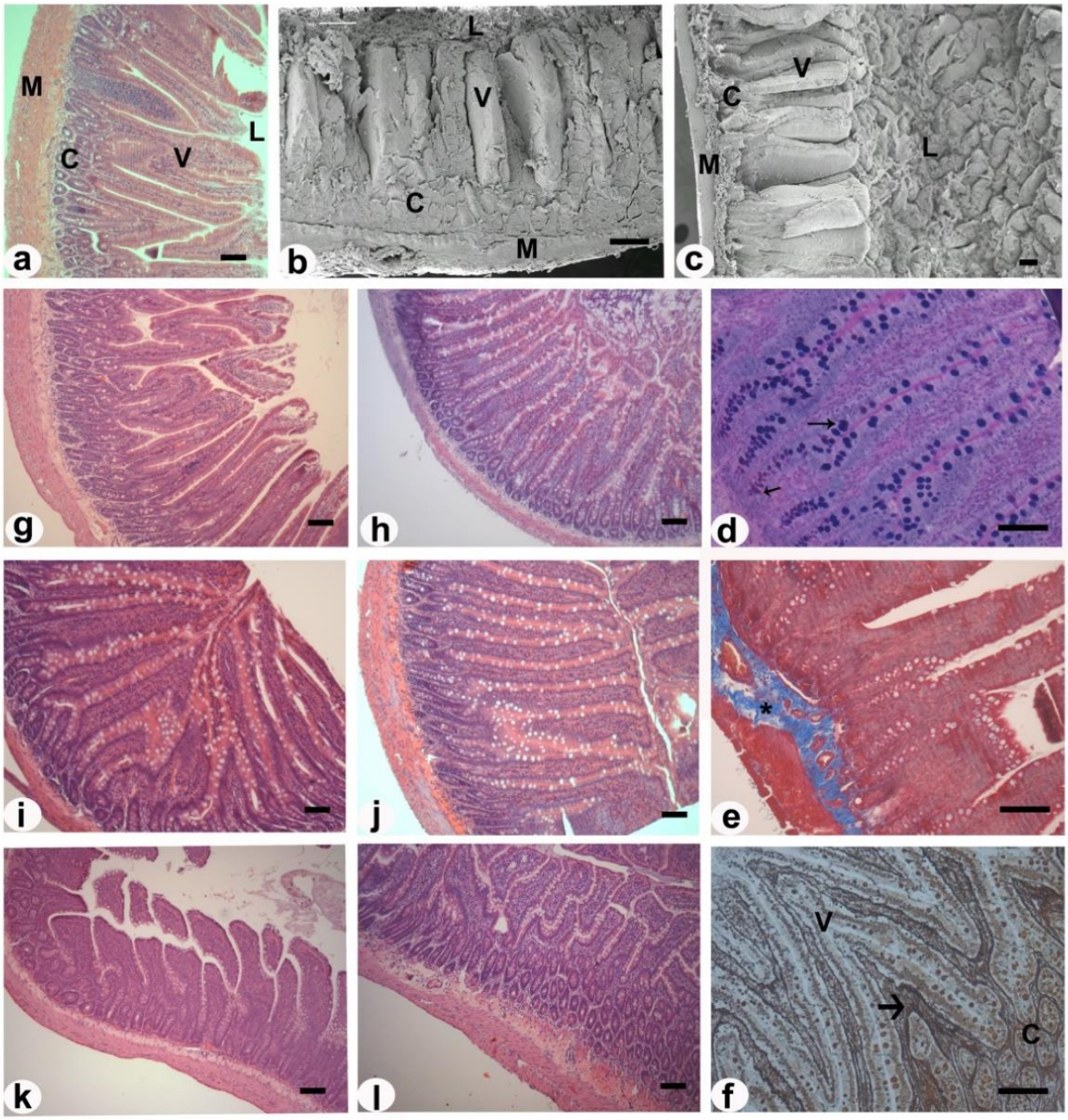

Figure 1: Light microscopy and scanning electron microscopy images of duodenum, jejunum and ileum mucosa. a-f: images of duodenum in control group (Group C). Image *a,* shows the expected structure in light microscopy. V: villus; C: crypt; M: muscle; L: lumen. This image was chosen as a model to show the structure of the two regions, jejunum and ileum. Images *b* and *c*: duodenum in control group obtained using scanning electron microscopy. V: villus; C: crypt; M: muscle; L: lumen. We choose this image as a model to show the structure that can be applied to the other two regions, jejunum and ileum. Image *d*: using the histochemical technique of Alcian Blue pH 2.5 combined with PAS. In purple (large arrow), the goblet cells secreting neutral mucin and in pink (small arrow) the goblet cells secreting basic mucin. *e*: Masson´s Trichrome technique. The connective tissue is stained in blue (*) and the normal structure of epithelium and muscle in red. *f*: results after Reticulin technique. The arrow indicates reticulin fibers, seen throughout the connective tissue, providing support for the organ. *g:* duodenum in group D5a; *h*: duodenum in group D5b; *i*: jejunum in group D5a; *j*: jejunum in group D5b; *k*: ileum in group D5a; *l*: ileum in group D5b. Images *a, g-l*: Hematoxilin-Eosin staining; Bar: 100u.

7- CONCLUSÕES GERAIS

Com o protocolo adotado não foi observado alteração no consumo de água e ração, como no ganho de peso dos animais;

Foi possível confirmar a segurança deste tratamento tendo em vista a pouca variação de estrutura e ultraestrutura verificada para o intestino delgado e fígado de ratos Wistar machos;

O uso do medicamento alterou o perfil lipídico sérico e de enzimas indicativas de danos hepáticos. Essas alterações não foram percebidas na dose de 1mg/kg, que é a dose usualmente utilizada no tratamento da acne, mas foram percebidas com 10mg/kg;

O tempo de readaptação sem medicamento foi adequado para reverter a maioria das alterações. Entretanto, o efeito do medicamento continua a ser observado mesmo após 30 dias do término de seu uso contínuo, demonstrado por algumas alterações ainda dentro deste período;

O fígado aumenta a sua atividade metabólica sob o efeito desta droga, como evidenciado pela modificação da ultraestrutura deste tecido com aumento de área de reticulo endoplasmático rugoso e mitocôndrias que ocorre de forma dose dependente;

As porções do jejuno e do íleo são mais susceptíveis a isotretinoína, demonstrado pela alteração da frequência de células caliciformes e, portanto, a modificação do padrão de mucinas no lúmen. Tal modificação pode levar a uma alteração do potencial de manter microbiota ou hidratação do bolo alimentar;

Estes resultados abrem portas para novos ensaios com outras drogas pois o uso seguro foi novamente confirmado, mas não deixa de demonstrar a reação do organismo ao uso de retinóides por via oral. Seria interessante a utilização de marcadores para desenvolvimento de alergias ou intolerâncias pois apesar de não ter sido encontrado sinais de inflamação, seria interessante investigar se ocorre uma predisposição a intolerâncias advindas de tratamento com protocolo similar.

ANEXO I

Comprovante de aprovação do projeto ao Comitê de Ética de Uso de Animais em Experimentação.

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"Avaliação bioquímica do soro e da histomorfometria do trato gastrointestinal e rins de ratos juvenis tratados com ácido 13-cis-retinóico"** (protocolo nº **2831-1**), sob a responsabilidade de **Profa. Dra. Mary Anne Heidi Dolder / Bruna Fontana Thomazini**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **05 de setembro de 2012**.

Campinas, 05 de setembro de 2012.

Profa. Dra. Ana Maria A. Guaraldo
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ANEXO II

Declaração de não infringência da lei de direitos autorais.

Profa. Dra. Rachel Meneguello
Presidente
Comissão Central de Pós-Graduação
Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"SEGURANÇA DO TRATAMENTO COM ISOTRETINOÍNA MENSURADO PELOS PARÂMETROS DE ESTRESSE OXIDATIVO, ESTRUTURA E ULTRAESTRUTURA DO FÍGADO E INTESTINO DELGADO DE RATOS WISTAR MACHOS."**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 16 de junho de 2016

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