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Peroxidação Lipídica em Pacientes Esquistossomóticos Esplenectomizados da Zona da Mata e em Ratos Submetidos à Dieta Básica Regional (DBR)

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Dissertação apresentada para o cumprimento parcial das exigências para obtenção do título de Mestre em Bioquímica pela Universidade Federal de Pernambuco

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RESUMO

O objetivo deste estudo foi investigar o estresse oxidativo em plasma e eritrócitos de pacientes esquistossomóticos submetidos ao tratamento clínico e esplenectomia seguida de reimplante de parte do tecido do baço, bem como, em ratos jovens alimentados com a Dieta Básica Regional (DBR) consumida na área do cultivo de cana do Estado de Pernambuco.

Amostras de sangue foram coletadas de indivíduos saudáveis (n = 18) e de pacientes (n = 18) em jejum de 12 h. A peroxidação lipídica foi avaliada pela determinação de: a) Espécies Reativas do Ácido Tiobarbitúrico (TBARS) no plasma, utilizando a reação com ácido tiobarbitúrico, detectada a 535 nm; b) conjugados dienos extraídos dos eritrócitos com diclorometano/metanol (2:1, v/v) e detectados a 233 nm. Neste estudo foram utilizados ratos de 2, 12, 25 e 60 dias, alimentados com a dieta comercial (LABINA) ou com a DBR durante toda a vida. A susceptibilidade ao estresse oxidativo foi avaliada nos órgãos dos ratos em relação a concentrações crescentes de ferro (Fe^{2+}), haja vista que o Fe^{2+} induz a produção de radicais livres a partir do peróxido de hidrogênio e hidroperóxidos de lipídeo.

Os níveis de conjugados dienos, em eritrócitos de indivíduos esquistossomóticos (584,4 ± 287,65) foram significativamente ($P < 0,05$) aumentados em comparação ao grupo controle (271,72 ± 85,36). Os níveis de TBARS apresentaram 10% de aumento nos pacientes (8,31 ± 3,81), entretanto, este aumento não foi significativo em relação aos controles (7,3 ± 2,35). Foram encontradas correlações positivas entre os níveis de TBARS e conjugados dienos, as quais foram mais significativas para os pacientes ($P < 0,01$, $r = 0,62$) do que para os controles ($P < 0,05$, $r = 0,57$). Esses resultados sugerem maior susceptibilidade dos eritrócitos dos pacientes ao dano oxidativo.

O estresse oxidativo em córtex, fígado e rim de ratos de 2, 12, 25 e 60 dias de idade, alimentados com a DBR, avaliada pela produção de TBARS, apresentou aumento dependente da concentração de Fe^{2+}. No córtex e rim de ratos desnutridos, com 2 dias de idade, houve uma resposta diminuída significantemente ($P < 0,01$) na produção de TBARS, frente a concentrações crescentes de Fe^{2+}, em relação aos controles de mesma idade. Entretanto, com o crescimento do animal desnutrido a produção de TBARS aumentou passando a ser similar aos controles no animal de 12 e 25 dias. Em ratos mais velhos (60 dias de idade), a concentração de TBARS produzida pelo córtex do grupo desnutrido na ausência e presença de Fe^{2+} (2,5 e 5,0 μM), ultrapassou significativamente a do grupo controle, mas no rim os níveis foram significantemente ($P < 0,05$) altos na presença de Fe^{2+}. No fígado, com exceção dos ratos desnutridos de 25 dias que obtiveram valores de produção de TBARS significantemente maiores somente em 10 e 20 μM de Fe^{2+}, todos os animais desnutridos apresentaram níveis de TBARS significantemente ($P < 0,05$) maiores do que os ratos controles, independente da idade e em todas as concentrações de Fe^{2+}.

Os resultados sugerem que: a) nos pacientes as reações de peroxidação lipídica na membrana dos eritrócitos apresentam-se aumentadas sendo, portanto, um forte indicador de estresse oxidativo associado à fisiopatologia da doença; b) experimentalmente a DBR também promove estresse oxidativo indicado pelo aumento na peroxidação lipídica, estimulada pelo Fe^{2+}, em membranas de córtex, rim e fígado.

ABSTRACT

The aim of this study was to investigate the oxidative stress in plasma and erytrhocytes from schistossomiasis mansoni patients subjected to clinical and splenectomy treatment associated with auto-implantation of spleen tissue, as well as, in young rats fed with a Regional Basic Diet (RBD) which has been used in the sugarcane rural area of Pernambuco state.

The blood samples were collected from healthy individuals and from patients after 12 h fasting. The lipid peroxidation was evaluated by measuring: a) plasma Thiobarbituric Acid Reactive Substances (TBARS) by using the reaction with thiobarbituric acid which was read at 535 nm; b) conjugated dienes which were extracted with dichloromethane/methanol (2:1, v/v) and read at 233 nm. In this study it was utilized Wistar rats aged 2-, 12-, 25- and 60-day-old which were fed with commercial or RBD diet during all life. The susceptibility to oxidative stress was determined in organs from rats in the presence of various concentrations of iron (Fe^{2+}), an ion that induces the production of free radical.

It was found significant difference ($P<0.05$) in erythrocytes conjugated dienes level from patients (584.5 ± 287.65) in comparison to the control group (271.72 ± 85.36). On the other hand, TBARS concentration in plasma from patients (8.31 ± 3.83) was increased by 10% in relation to control level (7.3 ± 2.35), but this rise was not significant. Furthermore, a positive correlation between the levels of TBARS and conjugated dienes was found in both groups, patients ($P < 0.01$, $r = 0.62$) and controls ($P < 0.05$, $r = 0.57$). The results sugest that the erythrocyte from patients are more susceptible to oxidative damage.

The oxidative stress in cortex, liver and kidney from rats aged 2-, 12-, 25-, and 60-day olds, fed with DBR, evaluated by TBARS production, show increase-dependent of Fe^{2+} concentration. In cortex and kidney from malnourished rats, with 2-day-old, there was a low significantly ($P < 0.01$) response in TBARS production, in relation to increase in concentratons of Fe^{2+}, in comparison to controls of same age. However, with the growth the malnourished animal the TBARS production increase and it was similar to the controls in 12- and 25-day olds. In old rats (60-day-old), the concentration of TBARS produced by cortex from malnourished group was higher than control in the absence and presence of Fe^{2+} (2.5 e 5.0 μM), but in kidney the levels were significantly ($P < 0.05$) high only in the presence of Fe^{2+}. In the liver, with exception from 25-day-old malnourished rats that had TBARS production significantly ($P < 0.05$) high only in 10 and 20μM of Fe^{2+}, all the malnourished animals show levels of TBARS significantly ($P < 0.05$) higher than the control rats independent of the rat age and Fe^{2+} concentration.

The results suggest that: a) the lipid peroxidation reactions in erythrocytes from patients is increased and this is a strong indicator of oxidative stress associated to physiopathology of the disease. b) under experimental conditions the RBD also promote oxidative stress which is indicated by the increase in the lipid peroxidation stimulated by Fe^{2+}, in membranes of cortex, liver and kidney.

1.0. INTRODUÇÃO

1.1. Radicais livres e peroxidação lipídica

Os radicais livres são espécies portadoras de um ou mais elétrons desemparelhados o que lhes confere uma alta reatividade. O desequilíbrio entre a formação e remoção dos radicais livres no organismo leva a ocorrência de lesões oxidativas em macromoléculas, tais como, proteínas, lipídios, ácidos nucléicos e carboidratos o que pode levar a morte da célula devido a um comprometimento das estruturas celulares (Halliwell & Gutteridge, 1999). Os radicais livres derivados do oxigênio (Espécies Reativas de Oxigênio) são produzidos por várias fontes como, por exemplo: fagocitose (McCormick *et al*., 1996), ciclo das prostaglandinas (Halliwell & Gutteridge, 1999) e pela cadeia respiratória mitocondrial (Abuja, & Albertini, 2001; Kowaltowski, 2000).

O ferro é apontado por muitos como um importante catalisador da peroxidação lipídica (Tadolini & Hakin, 1996; Kappus, 1987; Halliwell, 1996; Emerit *et al*., 2001). O ferro é requerido para o desenvolvimento e crescimento normal da célula. Entretanto, excesso de ferro pode causar danos às estruturas celulares por servir como acelerador da conversão do anion superóxido ($O_2^{\bullet -}$), e peróxido de hidrogênio (H_2O_2) em radical hidroxila ($OH^{\bullet}$) que é um forte oxidante para ácidos graxos poliinsaturados (Emerit *et al*., 2001). Esta reação de formação do radical hidroxila a partir do ferro é chamada de Reação de Fenton e está esquematizada na figura 1. Outro mecanismo proposto para o aumento na peroxidação lipídica pelo ferro é pela estimulação da clivagem do hidroperóxido de lipídeo para alcoxila e $HO^{\bullet}$ (Tadolini & Hakin, 1996). O primeiro propaga a reação de peroxidação lipídica, enquanto o segundo é altamente reativo podendo danificar ainda mais as estruturas celulares.

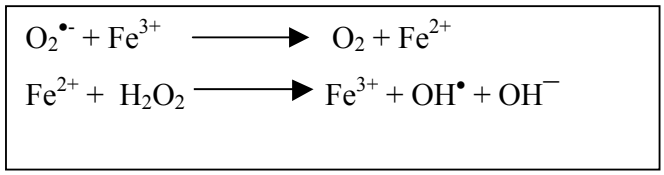

Figura 1 - Reação de Fenton (Emerit *et al*., 2001).

O processo de peroxidação dos lipídeos (Figura 2), começa com a abstração de hidrogênio ($H^{\bullet}$) de um grupo metil ($-CH_2-$) adjacente à dupla ligação do ácido graxo poliinsaturado por ação, por exemplo, das espécies reativas de oxigênio. Esta reação deixa um elétron desemparelhado no carbono, formando um radical lipídico ou radical alila (Girotti, 1998). A seqüência da reação é acompanhada pela mudança de posição, por ressonância, da dupla ligação que dá origem a compostos que contém grupos conjugados dienos (Halliwell & Gutteridge, 1999). Na seqüência de propagação da reação o radical alila, após rearranjo molecular, seguido pela adição do oxigênio, origina o radical peroxila (Abuja & Albertini, 2001). Este, conseqüentemente, pode abstrair um átomo de hidrogênio de um outro ácido graxo poliinsaturado adjacente produzindo hidroperóxido de lipídeo e outro radical alila (Kappus, 1987; Halliwell & Gutteridge, 1999). O radical peroxila pode também formar peróxidos cíclicos que ao final de uma cascata de eventos, ainda não muito bem esclarecida, leva à formação do malondialdeído (MDA) (Halliwell & Gutteridge, 1999). O hidroperóxido de lipídeo na presença de íons férrico (Fe^{2+}) sofre rápida decomposição gerando radical alcoxila and $HO^{\bullet}$, este último é altamente reativo podendo danificar lipídeos, proteínas, DNA e açúcares (Emerit *et al*., 2001). O radical alcoxila favorece a propagação reagindo com um ácido graxo adjacente formando, com isso, um radical lipídico e assim retroalimentando o sistema.

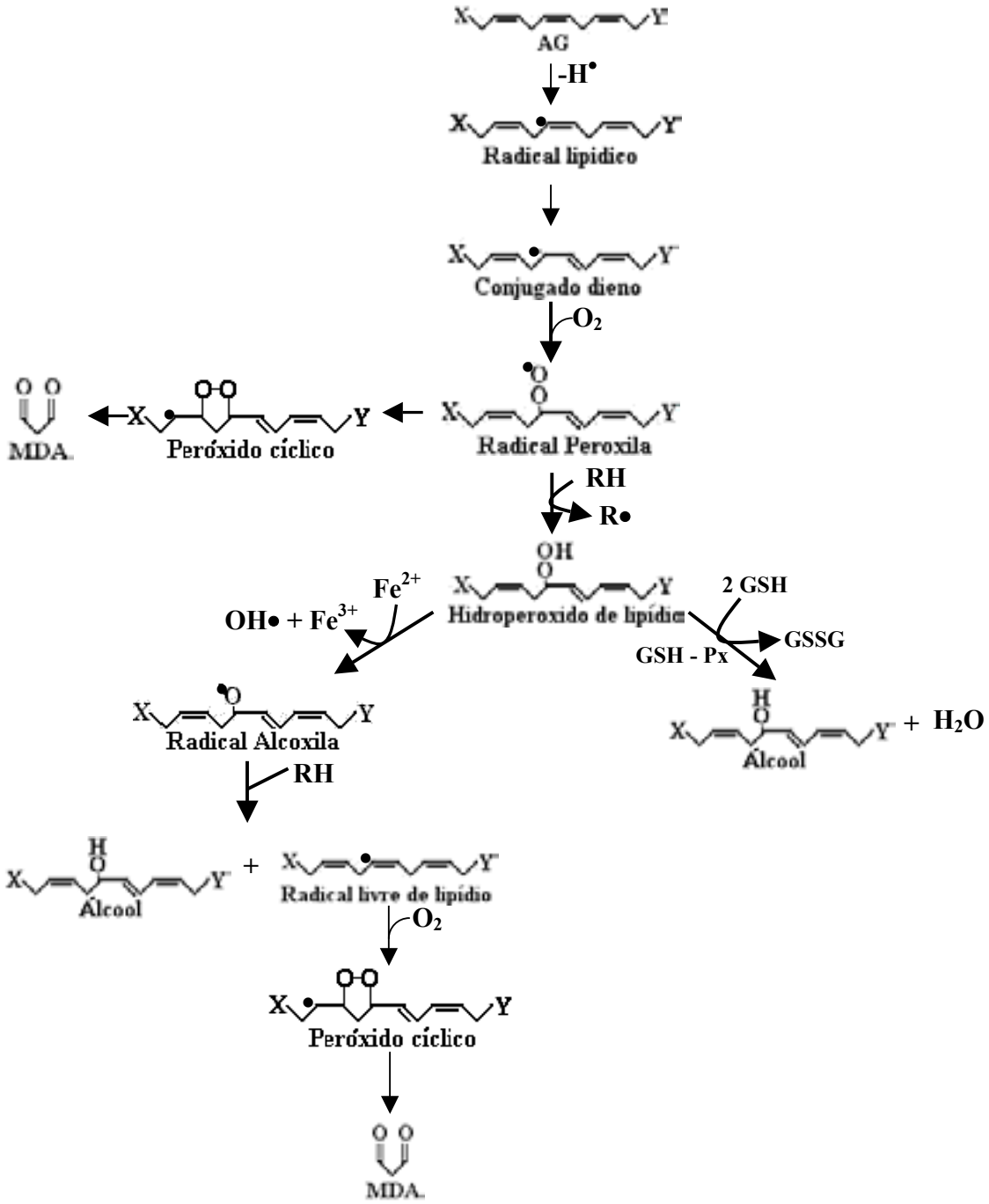

Figura 2 - Esquema das reações de peroxidação lipídica e seus produtos finais. MDA (Malondialdeído), AG (Ácido Graxo), $OH^{\bullet}$(Radical hidroxila), RH (Lipídeo), $R^{\bullet}$ (Radical lipídico), GSH - Px (Glutationa Peroxidase), GSH (Glutationa reduzida), GSSG (Glutationa oxidada).

A peroxidação lipídica, o mais investigado processo induzido por radicais livres (Zwart *et al*., 1999) é, portanto, um processo complexo, onde ácidos graxos poliinsaturados e outros lipídios são oxidados por radicais livres intermediários, levando à reação em cadeia e formando conjugados dienos, hidroperóxidos de lipídeos e MDA entre outros produtos, (Zwart *et al*., *1999* e Buege & Aust, 1978) conforme demonstrado na figura 2. Sobre condições de estresse oxidativo, a propagação da peroxidação lipídica é um processo degenerativo que afeta as membranas celulares e outras estruturas que contém lipídeos (Halliwell & Gutteridge, 1990). O MDA é o mais abundante aldeído produzido durante a peroxidação lipídica, (Esterbauer & Cheeseman, 1990) e sua determinação tem sido, freqüentemente, usada como marcador do estresse oxidativo em doenças e estudos de peroxidação lipídica (Lefèvre *et al*., 1998).

As células eucarióticas são equipadas com uma variedade de moléculas que impedem ou limitam os danos oxidativos sobre as moléculas orgânicas e incluem várias vitaminas como, por exemplo, vitamina C, Vitamina E, vitamina A e β-caroteno (Mccall e Frei, 1999), bem como enzimas antioxidantes (Halliwell & Gutteride, 1999). O desequilíbrio entre a formação e a remoção dos radicais livres no organismo pelos antioxidantes favorece o aparecimento de lesões celulares, com comprometimento de macromoléculas e membranas biológicas, podendo resultar em morte celular (Abdalla, 1996). Em patologias onde há o envolvimento de radicais livres na etiologia da doença como, por exemplo, aterosclerose (Lusis, 2000) e câncer, o aumento das defesas antioxidantes na corrente sangüínea tem sido associada com a diminuição da incidência das mesmas. As vitaminas lipossolúveis antioxidantes e carotenóides têm sido estudados em doenças do fígado e, mais recentemente, em cirrose hepática crônica pelo fato da membrana do fígado ser uma enorme fonte de radicais livres. Rocchi *et al*. (2001) demonstram que existem diferenças significativas nas concentrações de tocoferol, retinol e carotenóides em plasma de pacientes com hepatite crônica o que indica uma verdadeira diminuição nas defesas

antioxidantes. O ácido ascórbico reage diretamente com os radicais livres de oxigênio protegendo as células dos seus efeitos, inibindo com isso a peroxidação lipídica (Halliwell & Gutteridge, 1999). Paradoxalmente, a vitamina C, que é hidrossolúvel, também é associada com efeitos pró-oxidantes por ter a capacidade de reduzir metais que reagem com o O_2 formando iniciadores da peroxidação (Griffiths & Lunec, 2001) .

As enzimas antioxidantes agem na remoção das espécies reativas de oxigênio inibindo o processo de peroxidação lipídica (Figura 3). A superóxido dismutase age acelerando a taxa de dismutação do $O_2^{\bullet-}$ para H_2O_2 (Fridovick, 1986). O H_2O_2 é pouco reativo, entretanto, seu acúmulo em altos níveis pode trazer conseqüências danosas para estruturas celulares (Hyslop *et al*., 1988). Como já foi exposto, muito da toxicidade do $O_2^{\bullet-}$ e do H_2O_2 está relacionada à disponibilidade de ferro no meio e da formação do $OH^{\bullet}$. A glutationa peroxidase é uma enzima que utiliza como cofator o selênio e age na remoção do H_2O_2, acoplada à oxidação da glutationa do meio, formando H_2O e O_2 (Chance *et al*., 1979). A catalase também remove o H_2O_2 convertendo-o para H_2O e O_2. Entretanto, ao contrário da glutathiona peroxidase, essa enzima está localizada nos peroxissomos de células de mamíferos e catalisa a reação em concentrações relativamente altas de H_2O_2, ou seja, tem Km alto (Girotti, 1998).

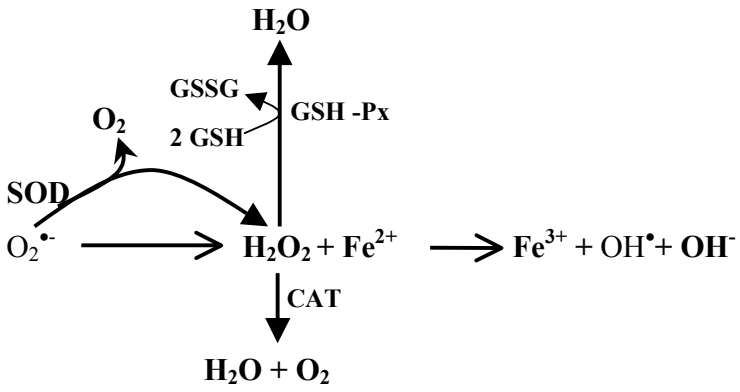

Figura 03: Esquema da formação do radical hidroxila ($OH^{\bullet}$) a partir do peróxido de hidrogênio (H_2O_2). Enzimas antioxidantes podem interromper a reação. SOD (Superóxido dismutase), GSH - PX - Glutationa Peroxidase, CAT (Catalase), GSH (Glutationa reduzida) e GSSG (Glutationa oxidada).

1.2. Esquistossomose mansônica.

A esquistossomose mansônica, doença causada pelo parasita *Schistosoma mansoni*, está entre as mais prevalentes parasitoses, sendo endêmica em 74 países (Chitsulo *et al*., 2000). As infecções causadas por helmintos afetam mais de um quarto da população mundial (Wakelin, 2000). Nos últimos 20 anos, progressos têm sido feitos na tentativa de erradicação da esquistossomose e, bem sucedidos programas de controle têm mudado a distribuição mundial, ainda assim, o número de pessoas infectadas ou com risco de infecção não tem diminuído (Chitsulo *et al*., 2000). Estima-se que 200 milhões de pessoas sejam portadoras da infecção causada pelo *S. mansoni* das quais 120 milhões são sintomáticas e, dentre estes, 20 milhões sofrem severas conseqüências da doença.

O Brasil é considerado um dos maiores focos endêmicos da esquistossomose. Importantes fatores de risco que atestam a intensidade de infecção por *S. mansoni* em comunidades da zona rural do país têm sido identificados. Estes incluem, fatores demográficos, sócio-econômicos e espaciais, bem como, sanitários, ambientais e o risco de contacto com água (Kloos *et al*., 1998;

Lima e Costa *et al*., 1998). Na zona rural do Estado de Pernambuco a esquistossomose é historicamente endêmica (Coutinho *et al*., 1997). A ocupação desordenada de espaços urbanos e a migração de trabalhadores rurais têm determinado a contínua expansão da doença, levando ao estabelecimento de novos focos urbanos e sucessivos relatos de casos agudos da doença (Barbosa *et al*., 1998*;* Barbosa *et al*., 2001). A falta de uma vacina, as falhas na tentativa de erradicar o vetor (molusco) e o recente desenvolvimento de resistência do parasita à drogas esquistossomicidas faz da esquistossomose mansonica um importante problema de saúde pública (Khalife *et al*., 2000).

As três espécies mais importantes de *Schistosoma* que infectam o homem são: *S. mansoni, S. haematobium* e *S. japonicum*. O contato direto com águas contendo a forma infectante do *S. mansoni* leva o hospedeiro humano a contrair a infecção. A forma infectante é denominada cercária que ao penetrar na pele sofre transformação, originando o esquistossômulo este após alguns dias chega ao sistema porta hepático. Quando adultos, o *S. mansoni* migra para as veias mesentéricas iniciando a postura dos ovos, os quais conseguem atravessar a parede intestinal e serem excretados nas fezes. Os ovos que não sofrem este processo caem na circulação e se depositam no fígado, intestino e outros órgãos desenvolvendo reação granulomatosa. A severidade da infecção pelo *S. mansoni* resulta da deposição dos ovos do parasita no fígado onde é associada com uma intensa resposta granulomatosa por parte do hospedeiro humano seguida de fibrose periportal (Rey, 1991). As manifestações clínicas variam desde formas intestinais severas à esquistossomose hepatoesplênica que é associada com fibrose hepática, hipertensão portal, varizes esofagias e esplenomegalia (Bica *et al*., 2000). A morbidade e mortalidade são secundárias à fibrose hepática e, subseqüentes à hipertensão portal (De Jesus *et al*., 2000).

Em indivíduos esquistossomóticos as células de defesa (macrófagos, linfócitos e polimorfonucleares, entre outras) que se posicionam em torno dos ovos do parasita no fígado

formam a lesão típica e elemento anatomopatológico básico do processo crônico da esquistossomose: o granuloma (Rey, 1991). Os eosinófilos, que são os efetores primários no caso de infecções parasitárias, estão presentes nos locais da inflamação granulomatosa e tem habilidade para formar Espécies Reativas de Oxigênio (ROS), mais especificamente, os ânions superóxidos ($O_2^{\bullet -}$) e íons hidroxila ($OH^{\bullet}$), como mostrado por estudos in vitro (McCornick *et al*., 1996). As células inflamatórias liberam peroxidase ativa ao redor dos ovos e, concomitantemente, há a diminuição da capacidade antioxidante do fígado o que leva a geração de peróxidos de lipídeos (Gharib *et al*., 1999). Todos os processos combinados levam ao aumento do estresse oxidativo no fígado, favorecendo, com isso, a morte dos ovos do parasita *in vivo*. Entretanto, o processo oxidativo também leva a reações no hospedeiro que pode ser a estimulação da fibrogênese (Abdallahi *et al*., 2000).

O tratamento cirúrgico para pacientes esquistossomóticos é indicado quando se instala um quadro de hepatoesplenomegalia descompensada que induz à hemorragias nas varizes esofágicas associadas à hipertensão portal (Petroianu & Antunes, 1998). O tratamento cirúrgico mais freqüente quando este quadro se instala é a esplenectomia com ligadura da veia gástrica esquerda e esclerose das varizes esofágicas. A inclusão, a este tratamento, do autotransplante de parte do tecido do baço, a qual é uma nova técnica empregada no Hospital das clínicas da UFPE pela equipe do Professor Dr. Carlos Teixeira Brandt no intuito de prevenir a septicemia originada pela proliferação de microorganismos patógenos, tem se mostrado muito eficaz na redução da hipertensão portal, além de manter a função hepática de reserva. Fatores como aumento dos níveis de protrombina plasmática, melhora do fluxo sangüíneo hepático e função de reserva (Brandt *et al*., 1997), aumentos significantes de peso do corpo e discretos aumentos da densidade mineral óssea são indicadores de melhora do estado geral dos pacientes após esplenectomia (Brandt *et al*., 1998). No entanto, a avaliação do metabolismo desses indivíduos através do

estresse oxidativo pode auxiliar o entendimento da fisiopatologia da doença. Mais especificamente no fígado (McCornick *et al.*, 1996; Gharib *et al.,* 1999; Abdallahi *et al.*, 2000) faz-se necessário o aprofundamento do esclarecimento da importância do estresse oxidativo sobre a fisiopatologia da doença. Neste aspecto o presente trabalho avaliou o estresse oxidativo, pela quantificação de indicadores da peroxidação lipídica, no sangue de indivíduos tratados com cirurgia no Hospital das Clínicas - UFPE, realizada pelo Dr. Carlos Teixeira Brandt.

1.3. Desnutrição e Dieta Básica Regional

A desnutrição constitui um problema universal de saúde pública e resulta primariamente da pobreza, das más condições ambientais e da marginalização social em que vivem certas populações de áreas urbanas periféricas e rurais (Carrazza, 1994). Em países em desenvolvimento, inclusive no Brasil (Saraiva *et al*.,1992), a desnutrição calórico-protéica materna e em crianças é um grande problema de saúde pública (Pissaia *et al*., 1980; Olubodum, 1992) sendo responsável por uma alta taxa de mortalidade pós-natal e infantil (Wharton *et al*., 1991).

Estudos epidemiológicos têm mostrado que a deficiência protéico-calórica materna é um importante problema de saúde pública nestes países (Olobodun *et al*., 1992). Segundo Desai e Hales (1996) a desnutrição materna e fetal provoca alterações adaptativas da relação entre os índices de desenvolvimento que são indicativas de doenças futuras. Isto é confirmado por estudos que têm mostrado que a desnutrição pré- ou pós-natal, pode programar modificações estruturais e funcionais básicas por toda a vida podendo, com isso, afetar o crescimento, comportamento, aprendizagem, a taxa de lipídeos sangüíneos, a pressão arterial e provocar obesidade, além de alguns processos patológicos como, por exemplo, diabetes, hipertensão arterial, arteriosclerose, entre outras (Lucas, 1998).

A nutrição fetal depende dos nutrientes fornecidos, via placenta, e também de quantidades extra de proteínas e gorduras armazenadas pela mãe no início da gestação. Em fêmeas má nutridas e com baixo ganho de peso durante a gestação, há redução na reserva de nutrientes estocados e se a desnutrição for severa prejudicará o crescimento e o desenvolvimento fetal (Pessoa, 1997). Neste contexto, o estudo conduzido por Johnson e Dunhan (1988) demonstra que a ingestão de apenas 6% de proteínas durante a gestação retarda significantemente o crescimento fetal. Os órgãos se apresentam com pesos reduzidos quando a desnutrição é mantida durante a amamentação (Garofano *et al*., 1998). O fígado, rim e pâncreas de ratos submetidos à restrição alimentar durante a vida intra-uterina têm seu desenvolvimento afetado. A deficiência em proteínas compromete a nefrogênese com redução do número de néfrons na prole, seja durante a gestação (Merlet-Benichou *et al*., 1994; Langley-Evans, 1999), ou quando a deficiência protéica é mantida durante o aleitamento (Paixão *et al*., 2001) o que evidência o caráter irreversível das alterações produzidas durante a formação dos órgãos ou organogênese (Langley-Evans *et al*., 1994). Em lâminas histológicas de fígado de ratos alimentados com dieta pobre em proteína foi evidenciado a presença de infiltração de ácidos graxos no fígado(Rana *et al*., 1996).

Foi evidenciado que durante o período de crescimento rápido, que normalmente ocorre durante o período de aleitamento, a pobre oferta nutricional diminui a capacidade orgânica de utilizar combustíveis endógenos (Boxwell *et al*., 1995). Segundo Nashimith e Morgan (1976) é o período de aleitamento a fase crítica ou vulnerável do desenvolvimento do rato. Portanto, o estado nutricional materno durante esse período é de suma importância. Os efeitos da má nutrição durante as primeiras semanas de vida pós-natal, quando o animal está em um período de crescimento e desenvolvimento rápidos, são bem aparentes (Girard *et al*., 1992).

A desnutrição fetal acarreta prejuízos ao desenvolvimento e também favorece o aparecimento de precoces e severas doenças crônicas na vida adulta, danificando

permanentemente a expressão do potencial genético do feto e de crianças, com outros fatores, como, por exemplo, os efeitos adversos do álcool, fumo entre outras drogas podendo ser adicionados (Scrinshaw *et al*., 1997).

A Dieta Básica Regional (DBR) é uma dieta multideficiente e foi preparada de acordo com a dieta consumida por populações que vivem na região rural de cultivo de cana de açucar, no Estado de Pernambuco, Região nordeste do Brasil. Essa dieta foi desenvolvida pelo Departamento de Nutrição da UFPE baseada em inquéritos realizados na Zona da Mata de Pernambuco, e vem sendo considerada por muitos como um útil modelo experimental para estudos em desnutrição humana, cujos efeitos assemelham-se aos efeitos nutricionais observados em comunidades carentes do Nordeste (Teodósio *et al*., 1990). A DBR é deficiente não somente em proteínas, lipídeos, vitaminas como também em sódio e outros minerais (Monteiro *et al*., 1997; Teodósio *et al*., 1990). Estudos demonstram que em ratos submetidos à esta dieta durante o período fetal há diminuição do peso corpóreo no nascimento (Teodósio *et al*., 1990; Paixão *et al*., 2001) e quando é mantida durante a lactação provoca uma perda de massa corpórea ainda mais significante (Paixão *et al*., 2001).

Alta produção de ROS e baixas defesas antioxidantes têm sido apontadas como importantes fatores na fisiopatologia do kwashiorkor (Sive *et al*., 1993; Golden & Ramadath, 1987). Tem sido relatado que sob dieta deficiente em proteína a atividade da glutationa peroxidase é drasticamente diminuída enquanto a superóxido dismutase no fígado se mantem constante (Pélissier *et al*., 1993) ou foi diminuída (Rana *et al*., 1996) . Este quadro favorece o acúmulo de $O_2^{\bullet-}$ e H_2O_2, (figura 3) estes podem reagir para formar $OH^{\bullet}$ que inicia a cascata de peroxidação lipídica (figura 2) resultando no dano tecidual (Halliwell *et al*., 1991; Emerit *et al*., 2001; Halliwell & Gutteridge, 1999). O H_2O_2 é pouco reativo, entretanto, as estruturas celulares podem sofrer conseqüências se ocorrer acúmulo em altos níveis (Hyslop *et al*., 1988). A

sindrome do kwashiorkor tem sido associada a várias alterações nas defesas antioxidantes e podem ser listadas como: 1) Baixas concentrações de vitamina E, selênio, carotenóides e glutationa; 2) Baixa relação NADPH/ $NADP^+$; 3) Baixa atividade de glutationa peroxidase (para revisão ver Golden & Ramadath, 1987). O déficit nos níveis de glutationa e na atividade da glutationa peroxidase tem sido relatado serem maiores em kwashiorkor do que no marasmo (Sive, *et al*., 1993).

A DBR é, também, quantitativamente deficiente em aminoácidos essenciais e não essenciais, como por exemplo, o aminoácido metionina que está presente em baixas concentrações (Teodósio *et al*., 1990) e é utilizado pelas células hepáticas para a síntese de glutationa (Reed & Orrenius, 1997) que age protegendo as células dos efeitos danosos do estresse oxidativo (Reed, 1990). Foi demonstrado que em ratos alimentados com dietas deficientes em metionina o metabolismo do selênio está alterado e, conseqüentemente, menos selênio estaria disponível para a síntese da enzima glutationa peroxidase (Sunde *et al*., 1981). A deficiência de vitaminas na DBR é outro fator que poderia induzir a um quadro de estresse oxidativo, em decorrência de baixas quantidades de vitaminas antioxidantes como, por exemplo, vitamina E e C. Neste aspecto, tem sido reduzido nível de vitaminas antioxidantes (Ashour *et al.,* 1999) e as defesas antioxidantes totais (Fechner *et al.,* 2001) no sangue de crianças com desnutrição protéico-calórica.

O desenvolvimento de modelo experimental de má nutrição por DBR tem possibilitado um melhor conhecimento de muitos aspectos biológicos da nutrição da população nordestina (Coutinho *et al.,* 1992 a, b). A existência de relatos na literatura que associam o estresse oxidativo à fisiopatologia da má nutrição (Ashour *et al.,* 1999; Sive *et al.,*1993; Fechner *et al.,* 2001) torna importante o desenvolvimento de trabalhos que investiguem as conseqüências da DBR sobre a peroxidação lipídica.

2.0. OBJETIVOS

2.1. Geral

Avaliar o estresse oxidativo em plasma e eritrócitos de pacientes esquistossomóticos submetidos ao tratamento clínico e esplenectomia, bem como, em ratos jovens alimentados com a Dieta Básica Regional (DBR) consumida na área do cultivo de cana de açucar no litoral do Estado de Pernambuco.

2.2. Específicos

1. Avaliar a peroxidação lipídica pela investigação dos níveis de TBARS e conjugados dienos em plasma e eritrócitos, respectivamente, de humanos infectados com *S. mansoni* após tratamento clínico e esplenectomia com reimplante de parte do tecido do baço.

2. Correlacionar os indicadores da peroxidação (MDA e conjugados dienos) em pacientes, bem como, em indivíduos saudáveis.

3. Avaliar o efeito da desnutrição induzida pela DBR sobre o peso corporal e dos órgãos de ratos.

4. Investigar o estresse oxidativo em ratos jovens submetidos à DBR durante a gravidez e lactação, pela determinação dos níveis de TBARS produzidos em córtex, fígado e rim frente a concentrações crescentes de ferro (Fe^{2+}) durante o crescimento e desenvolvimento.

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4.0. TRABALHOS A SEREM SUBMETIDOS PARA PUBLICAÇÃO.

4.1. Trabalho submetido ao Brazilian Journal of Medical and Biological Research. Aceito com correção em andamento.

Increased lipid peroxidation in schistosomiasis mansoni patients as indicated by conjugated dienes level but not by malondialdehyde level.

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Running title: Lipid peroxidation in patients with schistosomiasis mansoni.

Key words: Lipid peroxidation; Conjugated dienes; Malondialdehyde; Schistosomiasis mansoni.

Abstract

Schistosoma mansoni causes liver disease by inducing granulomatous inflammation and favour the formation of certain types of reactive oxygen species such as superoxide ions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) which may induce lipid peroxidation. The aim of this study was to evaluate the lipid peroxidation in hepatosplenic schistosomiasis mansoni patients by measuring the conjugated dienes and malondialdehyde (MDA) levels. The patients (n = 18) were subjected to treatment with oxaminiquine and underwent splenectomy, ligature of the left gastric vein and auto-implantation of spleen tissue. Age-matched control group (n = 18) consisted of healthy individuals. The erythrocyte conjugated dienes were extracted with dichloromethane/methanol and the absorbance was determined at 233 nm. Plasma malondialdehyde level was determined by reaction with thiobarbituric acid and the chromogen was spectrophotometrically read at 535 nm. MDA concentration in plasma from patients was increased by 10% only. However, a significant difference ($p<0.05$) was found in erythrocyte conjugated dienes level. Furthermore, the levels of MDA were significantly ($P < 0.05$) and positively correlated with conjugated dienes in both groups control and patient. The high level of conjugated dienes in erythrocyte suggested that patients infected by *Schistosoma mansoni* have increased lipid peroxidation in cell membrane, which was not evident using MDA, the most common product marker. Therefore, in schistosomiasis patients it remains difficult to ascertain whether abnormal lipid peroxidation is a consequence of the disease process with free fatty acids being oxidized to conjugated dienes, but perhapes having the process stopped by the redox capacity of antioxidants agents. In conclusion, our data show that patients who underwent splenectomy, ligature of the left gastric vein and auto-implantation of spleen tissue present abnormal lipid peroxidation, and the end product of lipid peroxidation may be responsible for cell membrane dysfunction.

Schistosomiasis mansoni is an endemic disease in northeastern Brazil, especially in the rural area of Pernambuco. Transmission of *Schistosoma mansoni* infection by people, who moves from one place to another, has extended the disease to new areas. Recently, several cases of acute schistosomiasis mansoni have been confirmed in Pernambuco, for example in Itamaracá island which is a vacation city (1). Frequently, schistosomiasis patients with the most serious form of the disease develop periportal fibrosis, portal hypertension and hepatosplenomegaly. In this case, the classical surgery treatment for the severe hepatosplenic schistosomiasis is splenectomy, associated with obliterative suture of the esophageal varices and ligature of left gastric vein and, in the past decade, autoimplantation of spleen tissue was added to the surgical process, and that has increased the survival-time of the patients after splenectomy (2). Amongst the tropical diseases the *schistosomiasis mansoni* is the second major worldwide cause of morbidity and mortality (3). The disease persists mainly in developing countries, and has significant economic and public health consequences (1). The morbidity depends on genetics and enviromental factors, as well as on the intensity of infection. These three factors may influence the granulomatous inflamation of the liver and posterior fibrosis around eggs. Eosinophil cells associated with Schistosome-induced granuloma have the ability to form oxygen free radicals such as superoxide and hydroxyl radical (3), and to release active eosinophil peroxidase around the eggs granuloma (4). The effect of free radical production in schistosomiasis mansoni is still unknown, although lipid peroxidation induced by free radicals is one of the most extensively investigated processes where polyunsaturated fatty acids and other lipids are oxidized by intermediate free radicals. This process forms conjugated dienes, malondialdehyde (MDA) and lipid hydroperoxides, among other products (5). MDA is the most abundant aldehyde produced during lipid peroxidation, and its determination has been largely used as a marker product in studies involving the oxidative stress and lipid peroxidation in diseases (6). Oxidative stress is

thought to play a major role in pathogenesis of several diseases such as Alzheimers disease. Other pathological conditions, such as accelerated aging and atherosclerosis, may be related to the hyper-production of free radicals (7). In liver disease the free radical has been implicated in the inflamation process, and a significant increase in lipid peroxidation has been found (8). The lipid peroxidation process starts with the abstration of $H^{\bullet}$ from a $-CH_2-$ group of the polyunsaturated fatty acids where the carbon radical is usually stabilized by a molecular rearrangement forming the conjugated dienes which are compounds containing two double bonds separated by a single bond. These compounds react with O_2 forming peroxyl radical that can react with $H^{\bullet}$ atom from another lipid producing lipid hydroperoxide or forming cyclic peroxide, and several products are formed, among these, the MDA (9).

Under experimental condition, the antioxidant capacity of a liver damaged by *S. mansoni* is reduced has been reported, and this results in the generation of lipid peroxide (10). The aim of this study was to evaluate the degree of lipid peroxidation in schistosomiasis mansoni patients subjected to a new surgery process of splenectomy (2). In this study it was examined the plasma from some schistosomiasis patients for markers of increased oxidative stress. So, the patients were evaluated on the basis of its conjugated dienes and of thiobarbituric acid reactive substances (TBARS).

Thiobarbituric acid, malondialdehyde and methanol were obtained from Sigma (St. Louis, USA). Dichloromethane and trichloroacetic acid were obtained from Vetec (Rio de Janeiro, Brazil). All solvent and chemicals were of analytical grade.

Patients with hepatosplenic schistosomiasis mansoni (n = 18), from both sexes, were recruited from the general pediatric service. They were outpatients of the Clinical Hospital of the Federal University of Pernambuco – UFPE, Recife, PE, Brazil, and had been subjected to treatment with oxaminiquine (an antischistosome drug) followed by splenectomy, ligature of the left gastric vein and auto-implantation of spleen tissue. A group of age- and sex-matched

healthy subjects were also included in the study. All the healthy volunteers and patients were young subjects aged between 11 and 20 years. None of the women in either group were pregnant or using oral contraceptive preparations at the time of the study, and also, patients with renal, cardiac, hepatitis, or other parasites/microbial associated disease were excluded of this study. Informed consent for the study to be performed was obtained from the parents or persons responsible. This study was approved by the Ethics Committee of the Clinical Hospital - UFPE (Process number 193/99-CEP/CCS).

Blood samples were collected, in the early morning, into ice-cold tubes containing EDTA (1mg/ml), and they were centrifuged at 3,000 x *g*, for 15 minutes, at 4 °C, for erythrocyte and plasma isolation. Considering that the determination performed in fresh samples has the advantage of lack of storage since it is known that prolonged freezing at –20 °C may increase lipid peroxidation, the samples were immediately analysed. All the experiments were done in duplicate samples.

The level of thiobarbituric acid reactive substance (TBARS) in fresh plasma was measured by the method of Buege and Aust (5). Brifly, plasma sample was added into a reaction mixture made with 15% (w/v) trichloroacetic acid and 0.375% (w/v) thiobarbituric acid. The sample was heated at 100 °C, for 15 min, then cooled at room temperature and centrifuged at 3,000 x g for 5 min. Spectrophotometric absorption was measured at 535 nm against a reaction mixture lacking plasma but subjected to the whole procedure, and malondialdehyde level was calculated by using a standard curve prepared with malondialdehyde bis (diethyl acetal) as the MDA source. Similar to recent works, TBARS value was stated as nmol of MDA per liter of plasma (11).

Conjugated dienes in well-washed erythrocytes (duplicate samples) were extracted with dichloromethane/methanol (2:1, v/v) according to the method of Buege and Aust (5). 0.05 M potassium chloride were added to the solution, and after overnight at 4 °C the upper phase

was discarded and the concentration of conjugated dienes in the low phase was obtained by UV detection at 233 nm against dichloromethane.

The Body Mass Index (BMI) was calculated as the body weight (kg) per height^2 (m^2). Results were analyzed by unpaired student`s "t" test, and the differences were considered to be significant when the probability was $P<0.05$. Linear regression analysis was used for testing correlations between variables. Since no sex-related difference was observed in the correlation analysis, data from both sexes were used as a single group.

Most studies exclude patients considered to be malnourished, and this is often decided on the basis of body mass index scores or plasma proteins and haemoglobin levels. In this study it was used BMI and dietary history to exclude patients who were supposed to be malnourished. All the patients included in this study were living at home with their mother or a carer who was responsible for the preparation of the food, and no significant changes in their dietary habits was evident as a result of the disease. Also, the BMI was similar for the patients and control groups, since no significant difference was found in the BMI of patient (19.3 ± 4.8 kg/m^2) in comparison with the control (21.7 ± 2.1 kg/m^2) group.

Analysis of the parameter that indicate lipid peroxidation revealed that MDA concentration in plasma of schistosomiasis mansoni patients was increased by 10 % (Figure 1A), but the difference was not significant. However, a significant increase ($P = 0.0001$) was found in conjugated dienes extracted from erythrocyte of patients. As show in Figure 1B, the level of conjugated dienes of erythrocyte from schistosomiasis patients increased by 53,5 %, in comparison to the control group. Correlation analysis between erythrocyte conjugated dienes and MDA levels in the groups of patient and control is shown in Figure 2. The two indicators of lipid peroxidation, circulating MDA and erythrocyte conjugated dienes, were significantly correlated in both groups, control ($P = 0.012$) and patient ($P = 0.006$). A comparative study with several different methods used for measuring the lipid peroxidation in

liver samples have shown that although simples both methods used in this work to evaluate MDA and conjugated dienes have comparable reability (12). Also, it is important to consider that the practical and simple measurement of MDA by TBARS test is still largely used, and its concentration is commonly stated as nmol MDA per liter plasma (13), although the assay is not specific for the MDA since others compounds including sugars, amino acids and billirubin may cross-react with thiobarbituric acid, it has been taken into consideration instead using the more complex HPLC analysis.

The clinical manifestations of schistosomiasis range from mild to severe intestinal and hepatosplenic forms. The infection caused by the parasite *S. mansoni* can induce granulomatous inflammation of the liver and lead to the formation of certain types of reactive oxygen species, such as superoxide ions ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) (3) responsible for lipid peroxidation. Such events may be responsible, at least in part, for the pathology associated with schistosomiasis. In this study, evidence of the lipid peroxidation in circulating erythrocyte cells from schistosomiasis mansoni patients was found by the presence of significant high concentration of conjugated dienes, a product of lipid peroxidation which measure the initial phase of lipid peroxidation. Neverthless, the other circulating product evaluated by TBARS test, which measure MDA in the degradation phase of lipid peroxidation, was only slightly rose in plasma of these patients. The apparent lipid peroxidation increase in erythrocyte of patients seems to be rather a consequence of the disease than the clinical treatment with oxaminiquine. Previous studies (14) showed that in a group of *Schistosoma mansoni*-infected mice treated with praziquantel (a drug used for schistosomiasis mansoni treatment) there was not significant difference in plasma MDA level. The patients of the present study do not have granulomas inflammation, which could explain the increase in lipid peroxidation detected by the increased concentration of conjugated dienes, but they still have moderate liver disease. Previous study has demonstrated that in

patients with moderate liver disease caused by alcoholism the plasma MDA level is normal, although lipid peroxidation could be detected by measuring other indicator of oxidative stress (15). Experimental evidence of oxidative damage has been reported in Alzheimer's disease brains. However, even in Alzheimer's disease the use of markers in the peripheral circulation to show oxidative stress is also less convincing or controversial.

It is noteworthy that the increased peroxidation events do not reflect exclusively the production of MDA by eosinophyl, but also by other cell elements such as platelets, erythrocytes (3) and leukocytes (4). Erythrocytes constitute a well established model for the study of the cytotoxic damage to membranes by chemical/physical free radicals promoters. As demonstrated in the present study, schistosomiasis mansoni may induce chemical erythrocyte membrane alterations through an oxidative stress pathway. Superoxide anion, singlet oxygen and H_2O_2 concur in the lipid oxidation of erythrocyte membranes. It has been reported that the formation of the intermediate product (conjugated diene) proceed that of ultimate aldehydic products (TBARS), and takes place when the main endogenous lipophilic antioxidant vitamin E is significantly consumed. There are evidences that vitamin E interfere with the production of free radicals in some cells (16). Under experimental schistosomiasis mansoni, supplementation of vitamin E lowered the activity of liver enzymes involved in the antioxidant mechanisms, such as catalase and glutatione peroxidase (17). In the present study, alterations at the membrane levels detected as increased erythrocyte conjugated diens concentration may indicate that the lipid peroxidation is facilitated in the patients, in spite of the redox capacity of the GSH/GSSG as well as measurement of antioxidants such as vitamin E and C levels has yet to be established. On the other hand, the protective effect in MDA formation may be due to redox effect of GSH / protein thiol preservation. It has been shown that in aged people the blood antioxidant defenses are significantly reduced, and in subjects suffering from unstable hemoglobin disorders or with low levels of glucose-6-phosphate

dehydrogenase: G6PD-deficient red blood cells are more susceptible to oxidative attack than normal erythrocytes, predisposing subjects to drug- or infection-mediated hemolytic crisis (18). Furthermore, experimental studies have shown that granulomas that appear in schistosomiasis liver triggers the production of reactive oxigen species and leads to the alteration of antioxidants defences (4). Thus, it is important to further investigate whether the lipid peroxidation measured as the rise in conjugated dienes level of erythrocyte membranes from schistosomiasis mansoni patients is associated with the reduction of antioxidants defences. Therefore, this study examined the plasma from some schistosomiasis patients for markers of increased oxidative stress, but it still remains important to ascertain whether any changes in lipid peroxidation marker is a consequence of the disease process with ascorbate or other antioxidant nutrient being consumed during scavenging of free radicals.

The present finding of a positive correlation between serum MDA and conjugated diene from erythrocyte cell may support the utility of using a simple procedure to identify subjects with high-risk of increasing the severity of lipid peroxidation in schistosomiasis. The correlation between the MDA and conjugated dienes demonstrated in this study is in agreement with the ROS reactions that lead to the lipid peroxidation process. The high correlation coefficient observed in our study may be due to the fact that the polyunsaturated fatty acids are susceptible to hydrogen abstration by free radical attack what results in the molecular rearrangement of a double bond forming conjugated dienes (19) which are able to produce MDA or lipid hydroperoxide (9). Thus, the conjugated dienes are intermediates of the MDA and this could explain the positive and significant correlation between both. In addition, the lipid peroxidation could be potentially influenced by nutrition and diet. In fact, conjugated dienes from patients proved to be best correlated with the MDA than conjugated dienes from controls.

Reactive oxygen species such as superoxide anions, hydrogen peroxide and hydroxyl radicals are generated by platelets and can damage the plasma membranes by triggering polyunsaturated fatty acid peroxidation, polysaccharide depolymerization and protein cross-linking and fragmentation, membrane receptors and enzyme inactivation (Salvemini et al, 1993). Previous studies have reported activation of phospholipases A_2 and C by ROS (21). Study from Silva et al (16) reported a significante reduction in LCAT activity in plasma of schistosomiasis patients subjected to spleen surgery, even though the plasma total cholesterol/phospholipid ratio was unaltered (22). LCAT catalize the transfer of an unsaturated fatty acid of the lecithin to free cholesterol forming cholesteryl ester and lysolecithin. Although a clear correlation between LCAT activity and lipid peroxidation is yet to be demonstrated, it is reasonable to suppose that in vivo production of ROS by eosinophyls, erythrocytes and other cell elements may affect the substrate of LCAT and decrease its enzymatic activity.

This study suggests that the high level of conjugated dienes affect the composition of the cell membrane, and is an indicator that the lipid peroxidation is an important reaction in patients infected by *Schistosoma mansoni*. Measurement of MDA by TBARS is feasible but may be inappropriate for the diagnosis of the stage of lipid peroxidation in schistosomiasis. Nevertheless, this study represents the first step to evaluate the degree of lipid peroxidation process in patients with schistosomiasis mansoni who underwent splenectomy, ligature of the left gastric vein and auto-implantation of spleen tissue. We concluded that lipid peroxidation in patients with schistosomiasis mansoni is enhanced even after a long-term survival after splenectomy (around 6 years). However, a low sensitivity due to the procedures used to detect MDA and conjugated diene levels in the samples studied, and the low number of schistosomiasis patients can not be excluded.

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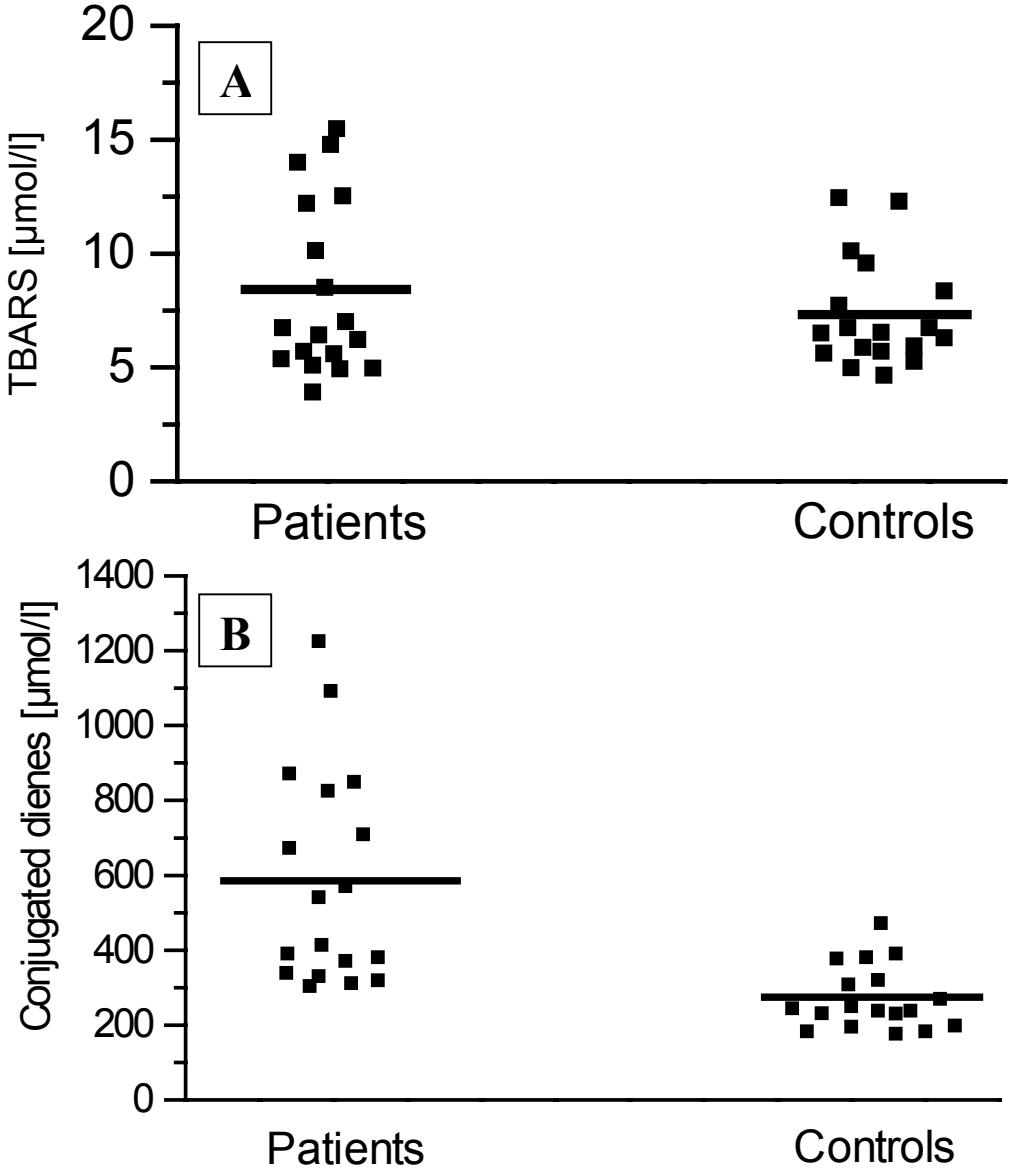

Figure 1 - Concentration of lipid peroxide products in plasma and erythrocyte of hepatosplenic schistosomiasis mansoni patients. Malondialdehyde (A) and erythrocyte conjugated diene levels (B) from 18 schistosomiasis mansoni patients, which were subjected to splenectomy associated with obliterative suture of the esophageal varices, ligature of the left gastric vein and auto-implantation of spleen tissue. Scatter plot showing mean value (horizontal bar). In comparison to the 18 controls, a statistical difference ($p < 0.0001$) was found only in patients by using the unpaired Student "t" test B.

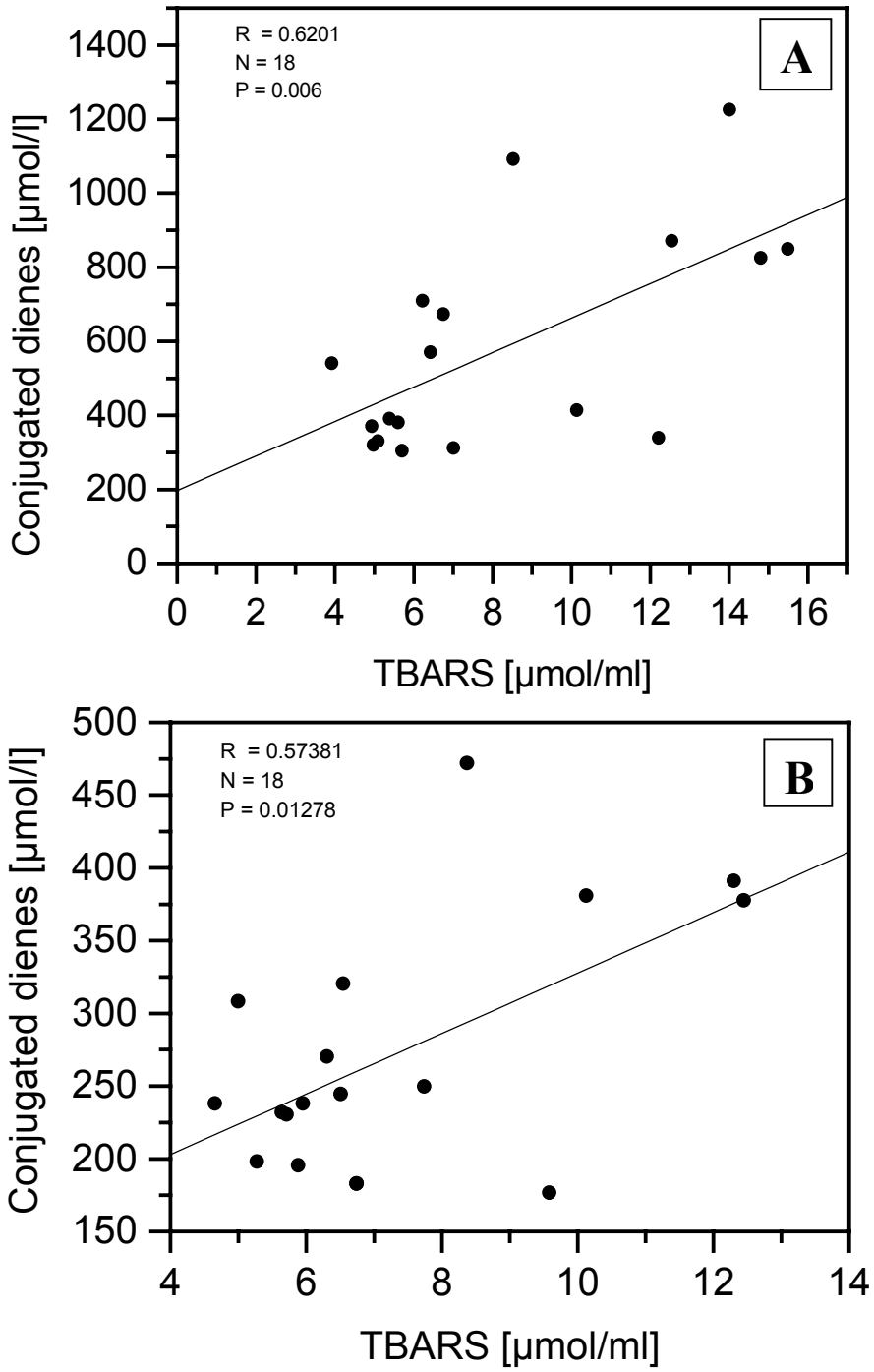

Figure 2 - Correlation between plasma MDA and erythrocyte conjugated diene levels. A, patients with schistosomiasis mansoni, B, Control group. Significant correlation is show as a P value.

4.2. Trabalho a ser submetido para publicação no periódico British Journal of Nutrition

LIPID PEROXIDATION IN ORGANS FROM MALNOURISHED RATS FED WITH A MULTIDEFICIENT DIET.

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Shortened version of the paper´s title: Lipid Peroxidation in malnourished rats

Abstract

Nutrition studies have shown that in Northeast Brazil the Regional Basic Diet (RBD) is deficient in protein, lipids and vitamins. Reactive Oxygen Species has been implicated in the pathogenesis of many conditions including protein-energy malnutrition. The purpose of the present study was to evaluate the lipid peroxidation in membranes of cerebral cortex, liver and kidney from malnourished Wistar rats in presence of Fe^{2+}. The Thiobarbituric Acid Reactive Substances (TBARS) produced by rats cerebral cortex, liver and kidney, in the presence of Fe^{2+} (2.5, 5.0, 10, 20 and 30 μM). The malnourished rats were fed with RBD during all life. The malnourished rats showed significant ($P < 0.05$) lower body weight than the control rats. The production of TBARS in cortex and kidney from malnourished young rats was low in 2-day-old, similar in 12- and 25-day-old, but in older animals (60-day-old) the production of TBARS was significantly ($P < 0.05$) higher in malnourished group than in control. On the other hand, in the liver from undernourished group in all ages the TBARS production was significantly ($P < 0,05$) higher than that from control group. The results sugest that: a) according to the age the organs from malnourished rats have abnormal and different respose to lipid peroxidation stimulated by Fe^{2+}; b) the organ more susceptible to oxidative stress in malnourished rats is the liver; c) During growth and development control rats improve the lipid peroxidation while it is not observed for malnourished rats**.**

Introduction

Epidemiological studies have implicated that severe malnutrition represents one of the most severe socioeconomic and healthy problems in underdeveloped countries. Maternal malnutrition is known to be the most significant factor for high infant and postnatal mortality rates in these countries (Wharton, 1991). Reduced nutrition has been shown to have acute and chronic effects on anatomy, physiology and longevitity (Boxwell *et al*. 1995). Thus, glomerular hypertrophy, metabolism, blood lipids, diabetes, blood pressure, obesity and atherosclerosis may be programed by pre- or postnatal nutrition manipulation (Lucas, 1998).

The envelopment of Reactive Oxygen Species (ROS) in pathophysiologic changes observed in kwashiorkor, has attracted interest over the last years. Golden & Ramdath (1987) postulates that exist a close relation between an imbalance in ROS generation and the antioxidant capacity and its contribution in the pathophysiology of kwashiorkor. Occurs also an redution of the antioxidant defence system and increase oxidative stress in marasmic children showing, consequently, the importance of the envolviment these parametres in the pathophysiology of marasmus (Tatli *et al*. 2000).

The Regional Basic Diet used here was derived from a diet available in the sugarcane cultivation in rural area along the cost of the state of Pernambuco, Brazil (Teodósio *et al*. 1990). In this region has been reported pronounced incidence of different stages of malnutrition specially among the children.

The release of free radicals is a physiological process found in all living tissues and more recently it has been implicated in cell signaling (Thannickal & Fanburg, 2000). Under normal conditions oxidative reactions are instantly neutralized by antioxidant system, however, when cellular production of ROS overhelms its antioxidant capacity occur damage in lipids, proteins and DNA (Halliwell & Gutteridge 1999). The imbalance between ROS generation and antioxidant status can be accelerates by free iron (Fe^{2+}) that, from H_2O_2 by fenton reaction, forms hydroxyl radical ($OH^{\bullet}$). The $OH^{\bullet}$ is highly reactive and capable of iniciating the lipid peroxidation. The lipid hydroperoxide, an intermediary product formed in lipid peroxidation, can be cleaved by Fe^{2+}, in an addicional reaction, forming again $OH^{\bullet}$ (Emerit *et al*. 2001). To confirm the correlation between oxidative stress and malnutrition we have investigated the Thiobarbituric Acid Reactive Substances (TBARS) production in response to iron, which has been frequently used by estimulate lipid peroxidation in vitro (Driver *et al*. 2000), in cerebral cortex, liver and kidney from developing malnourished rats.

Materials and methods

Chemicals

Stock solutions of Fe^{2+} [purchased from Merck as ferrous ammonium sulfate; $Fe(NH_4)_2(SO_4)_2$] were prepared in distilled water at 10 times the final concentration. Thiobarbituric acid (TBA), methanol and trichloacetic acid (TCA) were purchased from Merck. EGTA, Tris and sucrose were obteined form Sigma Chemical Company (St. Louis, MO, USA). All chemicals were of analytical grade and used without further purification.

Diets

The diet used to induce malnutrition during pregnancy and lactation in rats was developed from a Regional Basic Diet (RBD) consumed in the rural area of sugar-cane cultivation in rural area from Pernambuco State northeast - Brazil. Its ingredients composition (g/100 g) is beans (*Phaseolus vulgaris*; 18.34), manioc flour (*Manihot esculenta*; 64.81), dried and salted meat (3.74), and sweet potato (*Ipomaea batatas*;12.76). The diet was prepared as follows: all ingredients (except manioc flour) were cooked, dehydrated for 24-60 h (according to the type of ingredient) at 60 ºC and pulverized. Each component was mixed with manioc flour by humidifying. Meat fat (0.35%) was then added, and the mixture was shaped into balls which were dehydrated for 24 h at 60 ºC. Standard pellet chow (Purina do Brasil Ltd., São Paulo, SP, Brazil) was used to feed control rats. Table 1 shows the centesimal composition of the RBD, which was determined according to the Laboratory of Experimentation and Analysis of Food (LEAAL), Nutrition Department of Universidade Federal de Pernambuco, Recife, Brazil.

Animals and tissue preparation

Male and female Wistar rats with 2-day-old (n = 6), 12-day-old (n = 9), 25-day-old (n = 6), and 60-day-old (n = 9), were kept under conditions of constant temperature (23 ± 2 ºC) with a 12 hours light/dark cycle (12-h/12-h), with free access to food and water. Throughout lactation (postnatal day 2-24) each litter and its dam were housed separately in a plastic cage with wire mesh cover and wood shavings for bedding. Control dams were fed ad libitum with standard diet (Purina do Brasil Ltd., São Paulo, SP, Brazil) while restricted dams were fed with RBD diet (RBD group). The malnourished rats were fed with RBD during all life. Prior

to sacrificed, rats were anesthetized with ether. Subsequently, the liver, cerebral cortex and kidney were removed and its wet weight was obteined. Each tissue was first homogenized with a teflon homogenizer (100 mg of tissue/ml buffer) in ice-cold buffer with the following composition 10 mM tris-HCl and 1 mM EGTA, pH 7.4 containing sucrose 20% and allowed to warm to room temperature for 5 min. The homogenates were centrifuged 12,000 g for 30 minutes at 4º C . The pellet was ressuspended in 50 mM de Tris-HCl buffer, pH 7.4 and stored at -20º C for analysis of the oxidation with Fe^{2+} and protein content.

Thiobarbituric acid reactive substances (TBARS)

TBARS were estimated in brain, liver and kidney mambranes using the method of Buege and Aust, 1978. Briefly, membranes suspension was diluted in PBS buffer to a concentration of 20 mg tissue/ml, and a 0.45-ml aliquot was transfered to a glass test tube and allowed to warm to room temperature for 5 min. After, 50 µl of Fe^{2+} solution (2.5, 5.0, 10, 20 e 30 µM final concentration) was then added and the misture incubated at 4 ºC for a further 20 min. The reaction was stopped by the addition of 1.0 ml of TBARS reagent (15% (w/v) TCA, 0.38% (w/v) TBA) and tubes were heated at 95 ºC for 15 min. After cooling, the tubes were centrifuged for 5 min at 3,000 g and the supernatant read on a spectrophotometer at 535 nm. MDA is an important byproduct of lipid peroxidation that reacts with thiobarbituric acid. Results were expressed as nmol MDA formed/mg protein. Protein concentration was determined by the Lowry method using BSA as a standard.

Analitical analysis

All numerical data are expressed as mean ± standard error (SE). Data on Fe^{2+} stimulating lipid peroxidation were analyzed using analysis of variance (One-way ANOVA) followed by Newman-Keuls multiple comparison test. Changes in body and organ weight were analyzed by unpaired student`s "t" test. A $P < 0.05$ was considered statistically significant.

Results

In all ages the rats from malnourished group had significantly ($P < 0.01$) lower body weight than rats from the control groups (Table 2). Table 3 shows data on cerebral cortex, liver and kidney absolutes and relatives weight. Compared with control group the rats submitted to muldeficient diet exhibited significant ($P < 0.01$) decreases in liver absolute

weight in all ages and cerebral cortex and kidney absolute weights in 12-day-old, 25-day-old, and 60-day-old.

In order to study the effect of the oxidative stress stimulated by Fe^{2+} in increasing concentrations in cortex, liver and kidney of the malnourished rats in development we used TBARS assay according to Buege and Aust, 1978. The concentration-dependent effects of 30μM Fe^{2+} (maximal concentration in this study) in the cortex, liver and kidney in malnourished (RBD) and control rats are shown in Table 4. Analyzing each group separately we observed that the concentration of TBARS produced in cortex in the control group decreased significantly with increase of age. On the other side, in RBD group occured the contrary, there was a significant age-dependent increase in the levels of TBARS produced in cortex. The liver of 2-day-old rats controls and RBD had higher TBARS prodution compared to all other ages. After the birth (2-day-old) the kidney of control rats has a major suscetibility to oxidation, showing the higher TBARS production in relation to all other ages, while the maximum TBARS generation, stimulated by Fe^{2+}, in RBD group occured in 60-day-old.

The fig. 1 (A-D) shown the effects of Fe^{2+} on TBARS production in cortex of RBD and control rats during its development. There was a concentration-dependent increase, in MDA production, in response to Fe^{2+}. The 2-day-old RBD rats shown lower TBARS production in this context. The cortex of the 2-day-old rats, fed with Regional Basic Diet (RBD group), showed a significant ($P < 0.01$) decrease in the levels of TBARS produced in comparison to the age-matched control rats (Fig. 1A). On the other hand, the production of TBARS in control and RBD rats with 12-day-old and 25-day-old was similar (Fig. 1B and Fig. 1C, respectively). The figure 1D show that the production of TBARS in cortex from 60-day-old rats was significantly higher in undernourished group than in control in the absence and presence of Fe^{2+} (2.5 and 5.0 μM).

The effect of exposing the liver membranes from RBD and control rats to in vitro oxidative stress is shown in Fig. 2 (A-D). The concentration of TBARS was significantly increased in liver from 2-day-old (Fig. 2A), 12-day-old (Fig. 2B) and 60-day-old (Fig. 2C) malnourished rats in relation to control rats. In 25-day-old rats the production of TBARS was significantly higher in undernourished group than in control only in 10 and 20 μM. The increase in the TBARS production (in response to increasing concentration of Fe^{2+}) was substatially lower in liver from control rats with 2-, 12- and 60-day-old than in malnourished rats.

The impact of oxidative stress stimulated by Fe^{2+} in kidney strutures from RBD and control rats is shown in Fig. 3 (A-D). There was a concentration-dependent increase, in

TBARS production, in response to Fe^{2+}. The 2-day-old RBD rats showed the lower TBARS production in this context. In comparison to age-matched control rats, exists a statistically significant decrease ($P < 0.01$) in TBARS produced levels in kidney of 2-day-old malnourished rats (Fig. 3A). However, in 60-day-old the TBARS produced levels is higher in cortex of RBD than in controls rats (Fig. 1D). On the other hand, the production of TBARS in control rats with 12-day-old and 25-day-old compared to malnourished rats at the same age were similar.

Discussion

In recent years, ROS has been implicated in the pathogenesis of many conditions including protein-energy malnutrition (Golden & Ramadath, 1987: Sive *et al*. 1993). In this study we examined the suscetibility of different organs from malnourished rats to in vitro oxidative stress. Compared with the control diet, the Regional Basic Diet is not only deficient in proteins, but also in lipids, vitamins and some minerals (Table 1). The rats fed with the Regional Basic Diet showed low body and liver weights in all ages, and low cerebral cortex and kidney weights in 12-day-old, 25-day-old, and 60-day-old. Considerable retardation of the rate of growth in pups it was observed by Boxwell *et al*. 1995 caused by nutritional deprivation of lactating dams. Using as reference 2-day-old rats to represent the weight after the birth, the rats fed with multideficient diet has born with low body wheight, which is conformity with prenatal malnutrition. Undernutrition retards growth in all differents parts of the body. Thus, the current finding confirms the negatives effects of malnutrition in the development and growth of the body and organs of rats.

The envelopment of oxidative stress in the severe malnutrition, firstly postulated by Golden & Ramdath (1987), has awaked interesting of some scientists, over the last year, by clearing of the evidence of the role of Reactive Oxygen Species (ROS) in the pathophysiology of Kwashiorkor (an edematous form of undernutrition) (Becker *et al*. 1995; Golden, 1998). In the present study, tissue (cerebral cortex, liver and kidney) oxidative damage in relation to development of malnourished young rats fed with a multideficient diet has been studied by TBARS generation in response to increase of Fe^{2+} concentration. The Fe^{2+} can damage tissue by catalyzing the conversion of H_2O_2 and lipid hydroperoxyde to free radical species that attack cellular membranes, proteins and DNA (Gutteridge *et al*. 1982). The concentration of Fe^{2+} used is similar to those used in previous studies to examine lipid peroxidation (Andorn *et al*. 1996) and in vitro ROS production (Driver *et al*. 2000).

Tissue oxidative damage to malnourished and control rats, in this study, was analyzed by estimating the lipid peroxidation with increasing of Fe^{2+} concentration. Age-related changes in TBARS production in controls and malnourished rats in the maximal concentration of Fe^{2+} (30μM) used in this work are shown in table 4. The concentration of TBARS in cerebral cortex, liver and kidney in the control and liver from malnourished group decreased significantly with the age, while in the cortex and kidney from malnourished group there was a age-dependent increase in the levels of TBARS produced (Table 4). These result confirms the idea proposed by Muller *et al*. (1987) that normally newborns are particularly susceptible to ROS and in this work the newborn rats (2-day-old) show higher levels of TBARS in all organs than adult age and notable similar changes were also observed in serum levels of MDA in newborn infants compared with adults (McCarthy *et al*. 1984). There have been relatively few studies examining vulnerability of the developing rat cortex and liver to oxidative stress, in kidney and in organs from malnourished young rats fed with RBD this is non-existent. The measures of lipid peroxidation has been shown not change (Gupta *et al*. 1995) or to decrease (Sahoo & Chainy, 1997) during the first postnatal month in brain of control rats, as well as the levels of antioxidants enzymes has been reported to increase after first month postnatal (Scarpa *et al*. 1987; Schreiber *et al*. 1995). Gupta *et al*. 1995 showed that superoxide dismutase, catalase and glutathione peroxidase, enzymes that act to protect against ROS, are found at low levels in postnatal period and increase with age. The different profile in the lipid peroxidation stimulated by Fe^{2+}, during the development of the malnourished rats, can be related with loss of the capacity of synthesis of antioxidants enzymes, that protect against ROS, in cerebral cortex. This may be because the Regional Basic Diet is quantitatively deficient in essencial and non-essencial aminoacids (Teodósio *et al*. 1990) once that the lack of one essencial aminoacid could limit the synthesis of this antioxidant enzymatic machinery. In this study rats were fed with multideficient diet, during all life which affected the development of the body and organs (Table 2 and 3).

These present data add to the above data showing that this higher susceptility of newborn to oxidative stress also occur in the liver from malnourished rats fed with RBD and that the liver of these rats is particularly more susceptible than cortex and kidney to damage by oxidative stress in this age in malnourished rats (Table 4). The liver of malnourished rats showed an improve, during development, in the levels of TBARS produced, nevertheless, this was not sufficient to decrease the very high susceptibility to oxidative stress. On the other hand, control rats showed low levels of TBARS production in liver and there was a significant decrease in levels of TBARS in 12-day-old and 60-day-old compared with 2-day-

old. This results may be also related with compromisement, during the development of the malnourished rats, of activity liver antioxidant enzymes, since that antioxidative enzymes of tissue are considered to be a primary defense that prevents biological macromolecules from oxidative damage (Halliwell & Gutteridge, 1999). In the previous study conduced by Rana *et al*. 1996 rats fed with 5% protein showed decreased activity in liver antioxidants enzymes (superoxide dismutase, glutathione peroxidase and catalase) with concomitant increased of lipid peroxidation. Of the same form the Regional Basic Diet used in this study provide 8% protein and is deficient in methionine (Monteiro *et al*. 2001) it has been proposed this aminoacid to play an important role in antioxidant defense mechanism by reacting with oxidant (Livine *et* al. 1999) and would favour the imbalance between prooxidants and antioxidants in liver of these rats fed with Regional Basic Diet.

It is know that physiological and biochemical events in the brain is modified in several levels by developmental protein malnutrition (Resnick *et al*. 1979). Our data show that the lipid peroxidation in cortex from 2-day-old rats fed with Regional Basic Diet was significantly decrease in comparison to age-matched control rats, however, in 12-day-old this decrease was not so apparent. The effects of the reduced nutrition are more evidents when the animal is experiencing a period of rapid development (sucling period ,post-natal day 1 to 21) of the brain and peripheral tissues (Boxwell *et al*. 1995). The elevated level of TBARS in 2-day-old control rats in comparison to malnourished rats may be related to rapid development and concentration of polyunsturated fatty acid of the brain. Free radical formation is linked to normal cellular processes including cell metabolism, mitochondrial respiration, lipoxigenase, and cicloxigenase activity (Coyle & Puttfarcken, 1993). Neuronal tissue is particularly susceptible to oxidative damage due to high concentration of polyunsaturated fatty acids, which are susceptible to oxygen radicals attack (Rice-evans & Burdon, 1993). On the other side, undernutrition reduces the biosynthesis of the main sphingolipids during the period of brain growth spurt (Rotta *et al*. 1999). Maybe the low levels of TBARS on cerebral cortex from 2-day-old malnourished rats would be explained by mobilization of antioxidants aparatus from the mother and this fact is able to compensate the consequences of multideficient diet in the fetus. In 12- and 25-day-old the levels of TBARS is similar to control. On the other hand, in 60-day-old the malnourished rats showed higher levels of TBARS in relation to control group. In brain of control rats the levels of antioxidants enzymes has been reported to increase after first month postnatal (Scarpa *et al*. 1987; Schreiber *et al*. 1995). The increased level of TBARS in 60-day-old malnourished animals in comparison to control may be linked to low capacity of synthesis of antioxidants enzymes, that protect

against ROS, in cerebral cortex. This may be explained by fact of the Regional Basic Diet to be poor in protein and also quantitatively deficient in essencial and non-essencial aminoacids (Teodósio *et al*. 1990).

The liver of malnourished rats was highly susceptible to oxidative injury, stimulated by Fe^{2+}, in all ages in comparison to control. In relation to control, in 2-day-old, 12-day-old and 25-day-old the deleterious effect of protein deficiency appeared to more marked in the liver than in the cortex and kidney. The diet employed in this study was prepared according to diet consumed by some populations in coastal Pernambuco, northeast Brazil, and produce in rats a type of malnutrition similar to that prevalent among children from that region (Teodósio *et al*. 1990). Reduced levels of plasma antioxidants vitamins has been shown previosly in the children with protein-energy malnutrition living in Cairo, Egypt (Ashour *et al*. 1999). In rats fed with diets low in protein occured a high oxidative stress, and hepatic injury by fatty infiltration was also discribed (Rana *et al*. 1996). In this study the diet employed to produce malnutrition is deficient in protein, lipids as well as vitamins (Table 2). In this aspect, the high levels of TBARS in liver of malnourished rats may be related with low levels of antioxidants vitamins and, possibly, with the high infiltration of fatty acids. On the other side, in the liver of rats fed with deficienty diet in protein the activity of the glutathione peroxidase was reduced (Pellisier *et al*. 1993). It was described by Sunde et al. (1981) that metabolism of selenium might be altered by suboptimal level of dietary methionine, consequently, reducing the glutathione peroxidase biosynthesis. This enzyme remove H_2O_2, using selenium as cofator, by coupling its redution to H_2O with oxidation of reduced glutathione (Halliwell & Gutteridge, 1999). Methionine is readily taken up by the hepatocytes for the direct sinthesis of glutathione (Reed & Orrenius, 1997). and was reported to be present at the lowest level in Regional Basic Diet, (Teodósio *et al*. 1990).This parametres taken together may result in high acumulate of H_2O_2 or lipid hydroperoxide in hepatic cells which may to form hydroxil radical in presence of Fe^{2+} increasing the tissue damage.

The Regional Basic Diet is deficient in several nutrientes and some like, for instance, sodium cloride. This deficiency as showed by Paixão *et al*. (2003) change the kidney hemodinamic by renal vasodilatation. Kidney show deficiency in the number of nephron and glomerular hypertrophy when rats were fed with a multideficient diet during pregnancy and lactation (Paixão *et al*. 2001). In this study we show the high lipid peroxidation in rats with 60-day-old as another factor that indicates damage to kidney in young rats. These different profile of response to oxidative injury may be linked to fact that the muldeficient diet used in this study to be poor in vitamins (Table 1) so in this point important role, for intance of the

vitamin E, in defense against oxidative injury are impaired (Abuja & Albertini, 2001) . Paradoxically, the malnourished rats were fed with Regional Basic Diet the levels of TBARS produced was lower (2-day-old) or similar (12-, 25-day-old) than to the control rats. This may be explained by fact that is the suckling period of this animals and some antioxidant may to be available from the mother due to the stress related to diet poor in protein. The high levels of TBARS in 60-day-old could to be linked with the fact that rats fed with multideficient diet (providing 8% protein) during pregnancy and lactation has shown reduced number of nephron in the adult age (Paixão *et al*. 2001). Previous studies have shown that component renal antioxidant is important to its normal functioning, for instance, that dietary supplementation with the antioxidant vitamin E slowed the rate of progression of renal deterioration (Fryer, 1997) and ameliorated the glomerulosclerosis occurring in the nephrectomy remnant kidney model in the rat (Hahn *et al*. 1999). Another possible factor to increase the susceptibility of the kidney membranes of malnourished rats to in vitro oxidative stress would be the deficit in developing of the enzymatic antioxidant apparatus with the age. It is known that glutathione is used by glutathione peroxidase to scavenger H_2O_2 in several organs including kidney (Halliwell & Gutteridge, 1999), in the malnourished rats the synthesis of glutathione peroxidase may be jeopardized by deficiency of the multideficient diet in methionine (Sunde *et al*. 1981). However, more researches in this point in relation to rats fed with Regional Basic Diet will be necessaries.

Conclusions

The results sugested that: a) according to the age the organs from malnourished rats have abnormal and different respose to lipid peroxidation stimulated by Fe^{2+}; b) the organ more susceptible to oxidative stress in malnourished rats is the liver; c) the growth and development improve the lipid peroxidation better in control than in malnourished rats.

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Table 1. Composition (g/100g) of the multideficient diet and standard diet.

	Standard diet[1]	Multideficient diet[2]
Protein	23	8,87
Carbohydrates	41	77,7
Ether extract	2.5	1.12
Fibers	9	7
Minerals	8	3,96
Vitamin supplement	Yes	No
Sodium	0.37	0.17
Calcium	1.8	0.25
Phosphorus	0.8	0.08
Moisture	13	0
Kcal/100 g	278	356

[1] As indicated by the manufacturer (Ralston-Purina)
[2] According to the Laboratory of Experimentation and Analysis of Food (LEAAL), Nutrition department/UFPE, Recife, Brazil.

Table 2. Body weight of malnourished (RBD) and control rats.

	Body weight, g	
Ages	Control	RBD
2 (n = 6)	8.24 ± 0.26	5.17 ± 0.10*
12 (n = 9)	33.98 ± 1.11	11.75 ± 0.35*
25 (n = 6)	56.5 ± 1.11	20.5 ± 0.67*
60 (n = 9)	251.25 ± 12.6	53.33 ± 1.27*

Values are expressed as means ± SE. Differences between groups were analyzed by unpaired student`s "t" test. Values marked with asterisks differ significantly from the corresponding control values (* $P < 0.01$).

Table 3. Organ weight of malnourished (RBD) and control rats.

Age (days)	Tissue/organ					
	Cortex		Liver		Kidneys	
	Absolute weight, g					
	Control	RBD	Control	RBD	Control	RBD
2	0.13 ± 0.005	0.14 ± 0.01	0.34 ± 0.02	0.24 ± 0.01	0.07 ± 0.004	0.06 ± 0.001
12	0.77 ± 0.01	0.55 ± 0.04	1.22 ± 0.03	0.36 ± 0.02	0.37 ± 0.02	0.14± 0.008
25	0.92 ± 0.03	0.79 ± 0.01	2.96 ± 0.22	1.34 ± 0.11	0.83 ± 0.02	0.28 ± 0.009
60	1.29 ± 0.09	0.92 ± 0.01	12.07 ± 0.5	2.4 ± 0.06	2.06 ± 0.08	0.47 ± 0.01
	Relative weight (x 100)					
2	1.63 ± 0.08	2.7 ± 0.22*	4.19 ± 0.24	4.65 ± 0.23	0.92 ± 0.05	1.22 ± 0.03*
12	2.24 ± 0.09	4.8 ± 0.35*	3.62 ± 0.12	2.98 ± 0.19**	1.07 ± 0.05	1.18 ± 0.07
25	1.61± 0.07	3.83 ± 0.12*	5.25 ± 0.41	6.52 ± 0.34**	1.46 ± 0.03	1.39 ± 0.04
60	0.49 ± 0.05	1.74 ± 0.06*	4.83 ± 0.15	4.54 ± 0.15	0.81± 0.01	0.89± 0.02**

Values are expressed as means ± SE for 2-day-old (n = 6), 12-day-old (n = 9), 25-day-old (n = 6) and 60-day-old (n = 6). Relative weight refers to organ weight divided by body weight. Differences between groups were analyzed by unpaired student`s "t" test. Values marked with asterisks differ significantly from the corresponding control values (* $P < 0.01$; ** $P < 0.05$).

Table 4. Age related changes in TBARS production stimulated by Fe^{2+} in cortex, liver and kidney of developing malnourished rats (RBD) and controls.

Age (days)	Tissue/organ					
	Cortex		Liver		Kidney	
	Control	RBD	Control	RBD	Control	RBD
2 (n = 6)	37.34 ± 1.71	14.16 ± 1.54	16.16 ± 2.9	53.01 ± 2.22	19.54 ± 1.9	6.43 ± 1.77
12 (n = 9)	30.37 ± 1.68	27.01 ± 2.34*	6.1 ± 0.36*	20.15 ± 0.27*	6.44 ± 0.31*	5.88 ± 0.67
25 (n = 6)	23.37± 0.57*	20.66 ± 2.7	12.16 ± 2.3	15.34 ± 1.9*	12.46 ± 2.1*†	8.76 ± 1.89
60 (n = 9)	25.97 ± 5.31*	33.7 ± 3.04**‡	6.0 ± 2.67*	19.0 ± 2.4*	8.21 ± 1.58*	15.9 ± 2.1*†‡

TBARS production was determined in the presence of 30 μM of Fe^{2+} for 20 minutes at 4 ºC. Results are expressed as nmol of TBARS produced/mg protein and are mean ± SE. Differences between groups were analyzed one-way ANOVA, followed by Newman-Keuls multiple comparison test. The significance levels of differences between parameters within each group are shown as follow: * $P < 0.01$ in relation 2-day -old; ** $P < 0.05$ in relation 2-day-old; $^{\dagger}P < 0.01$ in relation 12-day-old; $^{\ddagger}P < 0.01$ in relation 25-day-old.

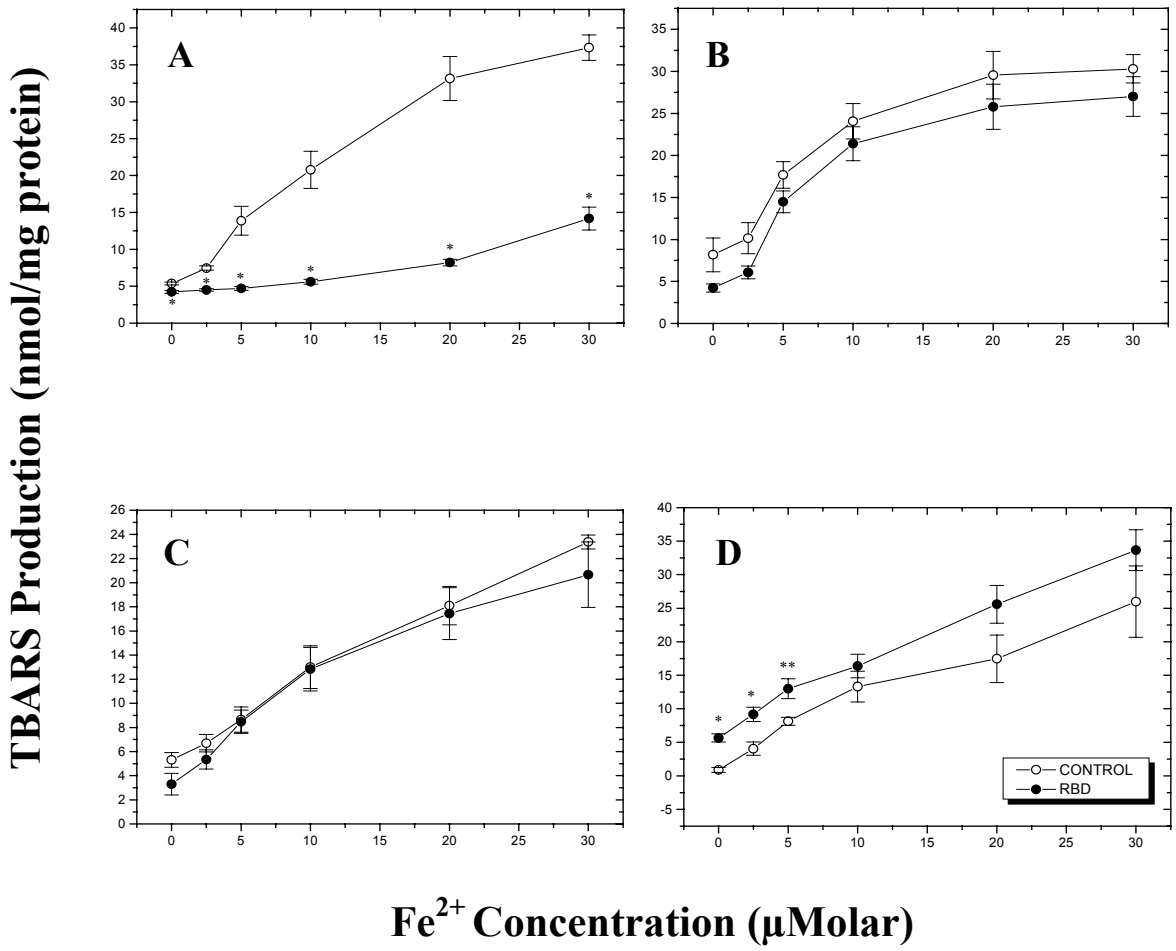

Fig. 1. The effect of exposing cortex membranes from 2-day-old (A, n = 6), 12-day-old (B, n = 9), 25-day-old (C, n = 6) and 60-day-old (D, n = 9) rats submitted to a muldeficient diet (RBD) and age-matched control rats, to in vitro oxidative stress using iron (Fe^{2+}) in increasing concentrations for 20 minutes at 4 ºC. Data are the mean ± SE and expressed as nmol of MDA produced / mg protein. * Significantly different from control ($P < 0.01$), ** Significantly different from control ($P < 0.05$).

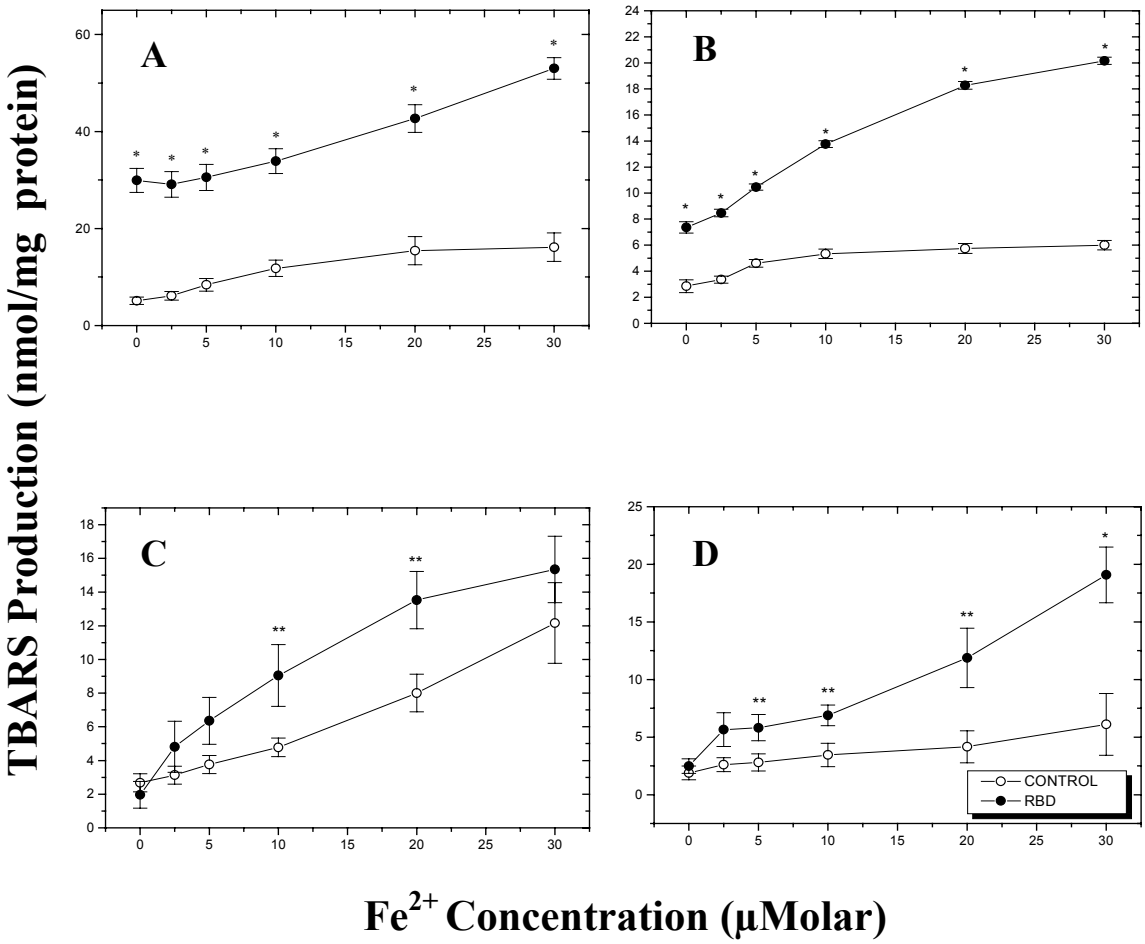

Fig. 2. The effect of exposing liver membranes from 2-day-old (A, n = 6), 12-day-old (B, n = 9), 25-day-old (C, n = 6) and 60-day-old (D, n = 9) rats submitted to a muldeficient diet (RBD) and age matched controls rats, to in vitro oxidative stress using iron (Fe^{2+}) in increasing concentrations for 20 minutes at 4 ºC. Data are the mean ± SE and expressed as nmol of MDA produced / mg protein. * Significantly different from control ($P < 0.01$), ** Significantly different from control ($P < 0.05$).

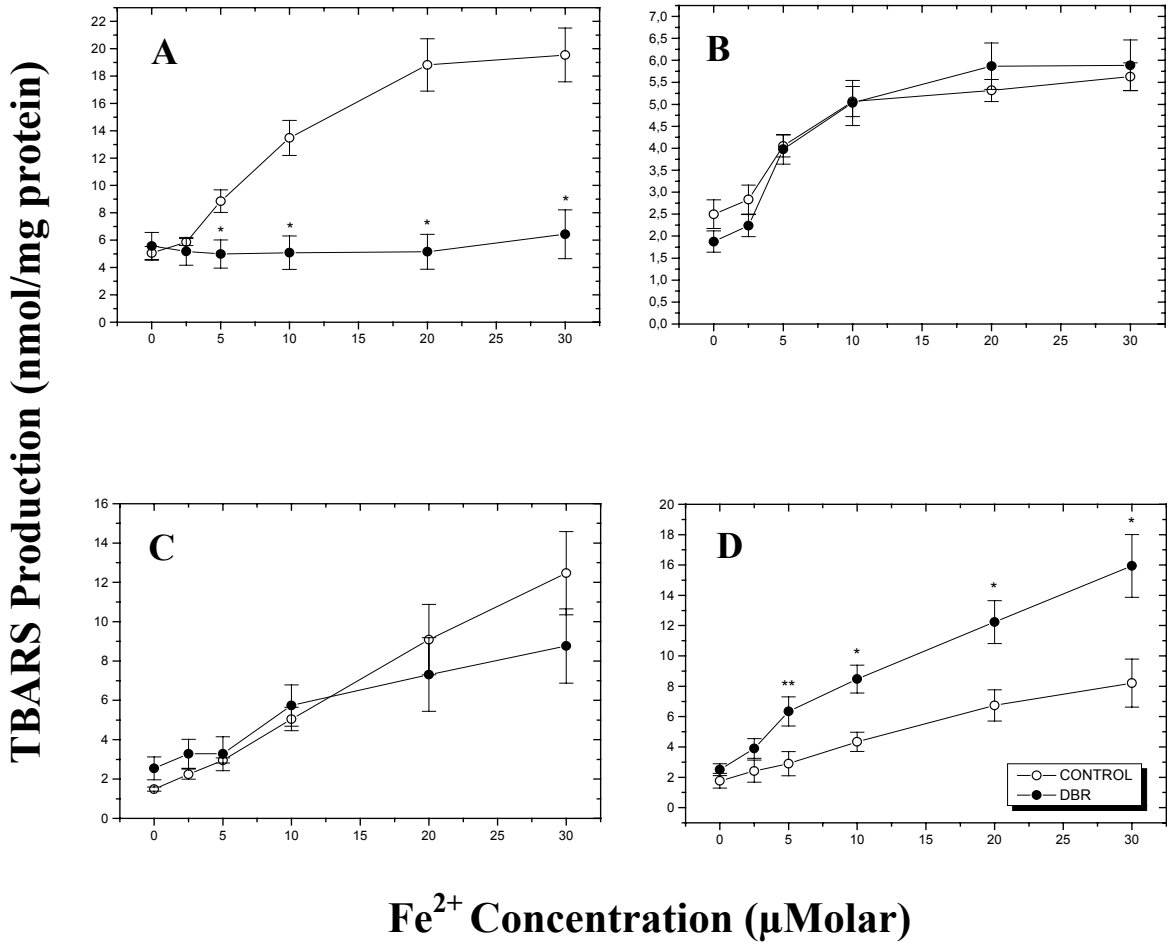

Fig. 3. The effect of exposing kidney membranes from 2-day-old (A, n = 6), 12-day-old (B, n = 9), 25-day-old (C, n = 6) and 60-day-old (D, n = 9) rats submitted to a muldeficient diet (RBD) and age matched controls rats, to in vitro oxidative stress using iron (Fe^{2+}) in increasing concentrations for 20 minutes at 4 ºC. Data are the mean ± SE and expressed as nmol of MDA produced / mg protein. * Significantly different from control ($P < 0.01$), ** Significantly different from control ($P < 0.05$).

5.0. Conclusão

- Os resultados sugerem que: a) nos pacientes as reações de peroxidação lipídica na membrana dos eritrócitos apresentam-se aumentadas sendo, portanto, um forte indicador de estresse oxidativo associado à fisiopatologia da doença; b) experimentalmente a DBR também promove estresse oxidativo indicado pelo aumento na peroxidação lipídica, estimulada pelo Fe^{2+}, em membranas de córtex, rim e fígado.