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INTERAÇÕES ENTRE O ESTRESSE REPETIDO E CLOMIPRAMINA: DIFERENTES RESPOSTAS EM RATOS MACHOS E FÊMEAS

TESE DE DOUTORADO

Rodrigo de Souza Balk

Santa Maria, RS, Brasil
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INTERAÇÕES ENTRE O ESTRESSE REPETIDO E CLOMIPRAMINA: DIFERENTES RESPOSTAS EM RATOS MACHOS E FÊMEAS

Rodrigo de Souza Balk

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"Suba o primeiro degrau com fé. Não é necessário que você veja toda a escada. Apenas dê o primeiro passo."

(Martin Luther King)

RESUMO

Tese de Doutorado
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INTERAÇÕES ENTRE O ESTRESSE REPETIDO E CLOMIPRAMINA: DIFERENNTES RESPOSTAS EM RATOS MACHOS E FÊMEAS

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Data e Local da Defesa: Santa Maria, 14 de outubro de 2011.

O estresse pode ser definido como uma variedade de estímulos fisiológicos e psicológicos que podem ter efeitos diretos ou indiretos sobre a função corporal. Neste contexto o estresse repetido ou situações estressantes podem induzir ansiedade e depressão. Diferentes respostas quanto ao estresse repetido e tratamento antidepressivo são observadas em machos e fêmeas. A clomipramina é um antidepressivo tricíclico que inibe a recaptação de serotonina e norepinefrina com ações indiretas sobre o sistema dopaminérgico e eixo límbico-hipotálamo-hipófise-adrenal (LHHA). Seu uso crônico aumenta a habilidade do corpo em combater o estresse, contudo doses elevadas podem potencializar os efeitos colaterais sobre memória, aprendizado e função sensório-motora. Desta forma, este estudo investigou as diferenças sexuais quanto ao estresse repetido por contenção e tratamento crônico com clomipramina sobre parâmetros de estresse oxidativo em córtex cerebral, estriado e hipocampo (**Artigo 1** e **Manuscrito 1**) e comportamento de ratos (**Artigo 2** e **Manuscrito 1**). Machos e fêmeas foram divididos em controle e estresse e subdivididos em tratados ou não com clomipramina durante 40 dias de estresse e 27 dias consecutivos de tratamento com clomipramina (30mg/kg). O modelo de estresse repetido por contenção utilizado neste estudo aumentou o dano oxidativo particularmente em estriado e hipocampo de fêmeas determinando maior sensibilidade ao estresse em comparação aos machos (**Manuscrito 1**). Neste contexto os níveis antioxidantes não-enzimáticos como tióis não-protéicos (NP-SH), enzimáticos tais como a enzima superóxido dismutase (SOD) e catalase (CAT) e Na^+/ K^+-ATPase (**Manuscrito 1**) também sofreram alterações em ambos os sexos. O tratamento antidepressivo com clomipramina potencializou o dano oxidativo causado pelo desequilíbrio entre oxidantes/antioxidantes, particularmente em machos. Em relação às atividades comportamentais (**Manuscrito 1**) fêmeas submetidas à estresse repetido por contenção apresentaram maior ansiedade e menor motivação quando comparado aos machos. Por outro lado, machos apresentaram prejuízo na formação de memória não espacial através das variáveis de memória em curto e longo prazo assim como uma redução na atividade locomotora e exploratória em relação ás fêmeas. Diferenças sexuais foram verificadas também quanto ao tratamento antidepressivo o qual potencializou os déficits sobre a formação da memória não espacial e atividade locomotora e exploratória assim como motivação em machos, mas em contrapartida foi observado um aumento da ansiedade e uma redução quanto ao consumo de alimento doce em fêmeas. Por fim, os resultados deste estudo indicam que fêmeas são sensíveis ao estresse particularmente sobre ansiedade e motivação enquanto machos são sensíveis à formação de memória não espacial assim como atividade locomotora e exploratória. O tratamento antidepressivo com clomipramina na dose utilizada parece ter efeito neuroprotetor em fêmeas, mas citotóxico em machos o que pôde ser observado pelo aumento nos déficits neuroquímicos e comportamentais.

Palavras-chaves: estresse repetido por contenção, clomipramina, estresse oxidativo, comportamento

ABSTRACT

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INTERACTION BETWEEN REPEATED STRESS AND CLOMIPRAMINE: DIFERENCE RESPONSE IN MALE AND FEMALE RATS

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Date and Place of the Defense: Santa Maria, 14th october 2011.

Repeated stress or stressful events has been reported to induce anxiety and depression. Differential responses to repeated stress and antidepressant treatments have been observed in males and females. Clomipramine is a tricyclic antidepressant that inhibits the reuptake of serotonin and norepinephrine by indirect actions on the dopaminergic system and limbic-hypothalamic-pituitary-adrenal (LHHA) axis. Its chronic use increases the body's ability to cope with stress; however, high doses can potentiate its side effects on memory, learning, and sensory motor function. Thus, this study investigated sex-related differential response to repeated restraint stress and chronic administration of clomipramine on parameters of oxidative stress in cerebral cortex, hippocampus and striatum (**Manuscript 1)** and behavior in rats (**Manuscript 1)**. Male and female rats were divided into control and repeated restraint stress, and subdivided into treated or not with clomipramine during 40 days of stress and 27 days of clomipramine treatment (30mg/kg). The model of repeated restraint stress used in this study increased oxidative damage particularly in striatum and hippocampus of females in higher sensitivity to stress compared to males (**Manuscript 1)**. In this context, the levels of non-enzymatic antioxidant such as the levels of non-protein thiol (NP-SH), and enzymatic antioxidants such as the enzyme superoxide dismutase (SOD), catalase (CAT) and Na+/ K+-ATPase (**Manuscript 1)** also changed in both sexes. Antidepressant treatment with clomipramine increased the oxidative damage caused by the imbalance between oxidants/antioxidants, particularly in males. Regarding the behavioral activities (**Manuscript 1**), females subjected to repeated restraint stress had higher anxiety and lower motivation when compared to males. On the other hand, males had impaired non-spatial memory formation through variables such as the short and long term memory as well as a reduction in exploratory and locomotor activity compared to females. Sex differences were also checked as to which antidepressant treatment that potentiate the deficits on the non-spatial memory formation, locomotor and exploratory activities and motivation in males, but in contrast was observed an increase in anxiety and a reduction on the consumption of sweet food in females. Finally, the results of this study indicate that females are particularly sensitive to stress on anxiety and motivation while males on the spatial memory formation as well as locomotor and exploratory acitvities. Antidepressant treatment with clomipramine appears to have neuroprotective effect in females, but cytotoxic in males in what might be observed by the increase in the neurochemical and behavioral deficits.

Keywords: repeated restraint stress, clomipramine, oxidative stress, behavior

LISTA DE FIGURAS

Desenvolvimento

Artigo 1

Artigo 2

Manuscrito 1

LISTA DE TABELAS

Manuscrito 1

LISTA DE ABREVIATURAS

ADN – Ácido Desoxirribonucleico
ADT – Antidepressivo Tricíclico
ATP – Adenosina Trifosfato
CA1 – Corno de Amon 1
CA3 – Corno de Amon 3
CAT – Catalase
DCFH –DA - Diclorofluoresceína-diacetato
EROs – Espécies Reativas de Oxigênio
GC – Glicocorticóides
GPx – Glutationa Peroxidase
H_2O_2 – Peróxido de Hidrogênio
HACT – Hormônio Adrenocorticotrófico
HLC – Hormônio Liberador de Corticotropina
ISRS – Inibidores Seletivos da Recaptação de Serotonina
LHHA – Límbico – Hipotálamo – Hipófise – Adrenal
Na^+/ K^+-ATPase – Sódio/Potássio Adenosina Trifosfato
NP-SH – Tióis não Protéicos
NPV – Núcleo Paraventricular
-$OH^{\bullet}$ – Radical Hidroxil
O_2^- - Radical Superóxido
RG – Receptores de Glicocorticóides
RM – Receptores de Mineralocorticóides
-SH – Grupos Tióis
SNC – Sistema Nervoso Central
SOD – Superóxido Dismutase
TBARS – Espécies Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

1. INTRODUÇÃO

O “Estresse” é um termo utilizado para descrever uma variedade de estímulos, ambos fisiológicos e psicológicos que podem ter efeitos diretos ou indiretos sobre a função corporal (TSIGOS et al., 2002). Assim é de fundamental importância entender como iniciam e terminam as respostas ao estresse a fim de identificar efetivamente quais os objetivos de tratamento das doenças relacionadas ao estresse como exemplo da depressão.

1.1 Neuroendocrinologia do estresse

Os sistemas fisiológicos responsivos ao estresse mais estudados são os que compõem o eixo Límbico-hipotálamo-hipófise-adrenal (LHHA) e o Sistema Neurovegetativo (SNV). O componente simpático de reação ao estresse pode ser visto como a primeira fase desta resposta, e a ativação do eixo LHHA como a segunda. As catecolaminas secretadas pelo SNV induzem cascatas de segundos mensageiros nos tecidos em questão de segundos, enquanto os glicocorticóides (GC) caracterizados como produto final do eixo LHHA, são secretados após uma latência de minutos, com efeitos que podem levar horas para surgir, devido à presença de eventos de transcrição (McEWEN E SAPOLSKY, 1995).

Os GC são hormônios esteróides sintetizados nas glândulas adrenais a partir de ações enzimáticas utilizando o colesterol como precursor. As células adrenocorticais não armazenam grandes quantidades de GC e a secreção destes está associada a uma nova síntese. Desta forma os GC são considerados hormônios hiperglicemiantes e participam das regulações energéticas e da atividade metabólica corpórea, estimulando a ingestão de alimentos e inibindo o armazenamento periférico de energia (STRACK, 1995). A ativação do eixo LHHA e a liberação de GC são adaptativas e essenciais para a sobrevivência imediata do organismo quando em resposta a estímulos agudos, no entanto, elevados níveis de GC durante estímulos crônicos podem produzir lesões neuronais (FONTELLA et al., 2005). A secreção de GC na circulação é mediada principalmente pelo eixo LHHA o qual quando inibido, minimiza a quantidade de tecidos expostos aos GC, reduzindo então os efeitos catabólicos, lipogênicos,

antireprodutivos e imunossupressores destes hormônios (CHARMANDARI et al., 2004) constituindo um sistema de retroalimentação denominado “feedback negativo”.

Frente a um evento estressante, estímulos sensoriais oriundos do tronco cerebral e medula espinhal ou de estruturas prosencefálicas (incluindo recordação de um momento estressante ou a antecipação de um evento de mesma característica), atingem o hipotálamo. Este, por via do sistema simpático, estimula a medula das glândulas adrenais, levando à liberação de catecolaminas (adrenalina e noradrenalina), constituindo uma resposta imediata ao estresse. As catecolaminas apresentam um papel característico no que se refere à mobilização de energia e supressão de outros sistemas desnecessários em situações de alarme, como por exemplo, digestão, crescimento e reprodução (SAPOLSKY, 2000; LEONARD E SONG, 1996) definindo o paradigma da “luta ou fuga” idealizado por Walter Cannon no início do século passado.

Estruturas como o córtex pré-frontal, hipocampo, amígdala e septo originam aferências mono e polissinápticas que convergem para o hipotálamo, agindo como uma espécie de integrador final de resposta ao estresse (DELBENDE et al., 1992).

O eixo LHHA desempenha ações cerebrais e endócrinas, constituindo desta forma o circuito neuroendócrino de resposta ao estresse.

1.2 O modelo de estresse repetido por contenção

O modelo de estresse repetido por contenção, idealizado por Ely et al. (1997), incorpora ambos componentes físicos e psicológicos do estresse sendo utilizado para induzir danos oxidativos e neurotóxicos (ZAFIR et al., 2009), permitindo examinar, em longo prazo, as características da depressão a qual o estresse é um fator contribuinte e predisponente (DE KLOET et al., 2005). O estresse repetido induz dano oxidativo por alterar o equilíbrio entre fatores oxidantes e antioxidantes, particularmente via peroxidação lipídica, deixando o Sistema Nervoso Central (SNC) altamente sensível à lesão (TABAJARA et al., 2003; FONTELLA et al., 2005).

A ativação do eixo LHHA é sexualmente dimórfica, ou seja, fêmeas exibem níveis mais elevados (WOOD & SHORS, 1998) ou similares (CONSOLI et al., 2005) de GC em condições basais e após o estresse por contenção quando comparado aos machos. Assim, o estrogênio dificulta o “feedback negativo” dos glicocorticóides (BURGESS & HANDA,

1992), enquanto a testosterona inibe a atividade do eixo LHHA (SEALE et al., 2004). Neste contexto, o estrogênio pode facilitar a progressão de desordens afetivas induzidas pelo estresse.

1.3 Os antidepressivos tricíclicos (ADTs): clomipramina

Os ADTs têm sido prescritos desde 1950 para tratamento da depressão. As indicações de sua utilização abrangem as desordens alimentares, dolorosas e ansiolíticas (DEMERDASH et al., 2004).

A clomipramina (cloridrato de clomipramina) é um ADT, cujo mecanismo primário de ação se dá pela inibição da recaptação de serotonina e norepinefrina resultando em um aumento destes neurotransmissores na fenda sináptica (FORLENZA et al., 2000; RANG et al., 2004). A injeção repetida de clomipramina provoca estresse crônico leve em modelos de estresse em animais alterando o comportamento (GRIPPO & JOHNSON, 2004). Desta forma, a administração oral é a melhor escolha para o tratamento antidepressivo.

Os efeitos adversos induzidos pela administração de clomipramina, devido ao bloqueio na recaptação de serotonina incluem déficits cognitivos especialmente sobre a memória (BURGOS et al., 2005). Em relação ao mecanismo de toxicidade, *in vitro*, estudos tem evidenciado que a exposição a diferentes classes de ADTs aumentam a produção de espécies reativas de oxigênio (EROs) (DEMERDASH et al., 2004). Desta forma, a interação entre estresse repetido por contenção e o tratamento crônico com clomipramina pode causar vulnerabilidade regional em diferentes estruturas cerebrais. Esta vulnerabilidade pode ser atribuída a diferenças na capacidade antioxidante (DE VASCONCELLOS et al., 2006), metabolismo oxidativo celular e um aumento na formação de radicais livres (GAMARO et al., 2003; GRAUMANN et al., 2002). A espécie e sexo, bem como o tipo, a duração e o grau do estresse podem modificar a resposta induzida pelo estresse e pela clomipramina (MARTI et al., 1994; CONSOLI et al., 2005).

1.4 Justificativa

O estresse pode ser definido como um conjunto de estímulos fisiológicos e psicológicos capazes de alterar a homeostase corporal induzindo à ansiedade e depressão em homens e mulheres. Quanto às conseqüências do estresse crônico sobre os sexos, observa-se que mulheres apresentam prevalência quanto ao desenvolvimento de doenças auto-imunes enquanto homens à doenças infecciosas e cardiovasculares. Quando relacionamos às doenças de natureza psiquiátrica, mulheres apresentam prevalência cerca de duas vezes maior à ansiedade, depressão e síndromes do pânico do que em homens. Desta forma, o conhecimento dos mecanismos biológicos relacionados ao estresse nos diferentes sexos servirá para a prevenção e o tratamento das patologias a ele relacionadas. Entre as classes de antidepressivos mais utilizados para o tratamento de desordens antidepressivas estão os antidepressivos tricíclicos com destaque para a clomipramina que inibe a recaptação de serotonina e norepinefrina com ações indiretas sobre o sistema dopaminérgico e eixo LHHA. O uso crônico aumenta a habilidade corporal em enfrentar os efeitos adversos do estresse, contudo em doses elevadas pode potencializar efeitos colaterais sobre a memória, aprendizado e função sensório-motora. Homens e mulheres podem apresentar diferentes respostas ao tratamento com antidepressivos tricíclicos, contudo, os mecanismos envolvidos nas ações destas substâncias sobre a depressão, bem como sobre a integridade de estruturas cerebrais, não estão claramente estabelecidos. Assim, este estudo investigou as diferentes respostas ao estresse repetido por contenção e tratamento crônico com clomipramina sobre parâmetros comportamentais e de estresse oxidativo em ratos machos e fêmeas.

2. DESENVOLVIMENTO

2.1 Estresse

Em 1936, o cientista e médico húngaro Hans Selye definiu o estresse como "Síndrome da Adaptação Geral", ou seja, resposta adaptativa de um organismo à ação de agentes capazes de ameaçar a homeostase, também chamados "estressores". A partir desta definição, Selye classificou a resposta ao estresse em três estágios: (1) alarme, em que o agente estressor seria notado; (2) resistência, em que o organismo começaria a combater o agente estressor e (3) exaustão, em que o organismo esgotaria toda sua capacidade de resposta ao estresse, advindo desta forma seus efeitos deletérios (KOPIN, 1995). No entanto são poucas as evidências que indicam esta falência referida por Selye. O estresse crônico não é patogênico em função das falhas no organismo, mas sim as próprias defesas tornarem-se patogênicas (SAPOLSKY & STEIN-BEHRENS, 1992)

2.1.1 Os glicocorticóides e o eixo LHHA

Os hormônios desempenham um papel crítico no desenvolvimento e expressão de comportamentos. Um aspecto da influência dos hormônios no comportamento é a potencial contribuição para a fisiopatologia dos transtornos psiquiátricos e para o mecanismo de ação dos psicotrópicos, particularmente na depressão. De todos os eixos endócrinos, o eixo LHHA têm sido o mais amplamente estudado. As anormalidades na função do eixo LHHA têm sido descritas em pessoas que apresentam depressão. Além disso, é bem conhecido o papel fundamental do estresse como precipitante de episódios de transtornos psiquiátricos em indivíduos predispostos (JURUENA et al., 2004). Essas anormalidades parecem estar relacionadas às mudanças na capacidade dos GC circulantes em exercer seu "feedback negativo" na secreção dos hormônios do eixo LHHA por meio da ligação aos receptores de mineralocorticóides (RM) e de glicocorticóides (RG) nos tecidos do eixo LHHA (JURUENA et al., 2004).

O eixo LHHA, ativado pelo estresse, envolve a secreção do hormônio liberador de corticotrofina (HLC) do núcleo paraventricular do hipotálamo (NPV), o qual estimula a liberação do hormônio adrenocorticotrófico (HACT) a partir da glândula hipófise anterior. O HACT, por sua vez, induz a liberação de GC a partir do córtex adrenal. O NPV, portanto, é responsável pela geração de respostas ao estresse. A ativação neuronal no NPV está elevada durante o estresse agudo, mas diminui ou se habitua em resposta à exposição ao estresse repetido (GIROTTI et al., 2006; FENOGLIO et al., 2006). Na condição de estresse repetido, neurônios são ativados em uma sub-região localizada ventralmente no NPV enquanto no estresse agudo a ativação destes neurônios está localizada dorsalmente ao NPV (VIAU et al., 2002). Isto sugere que distintos neurônios no NPV podem existir e ser diferencialmente responsáveis por iniciar as respostas ao estresse e mantê-las em situações repetidas (ZAVALA et al., 2011). Os GC circulantes quando em concentrações elevadas se ligam aos RG os quais estão ativos e servem para mobilização de energia e término do estresse através do feedback negativo em nível de hipotálamo (De KLOET et al., 2005). Além disso, os GC são capazes de se ligar a RG em regiões do cérebro extra-hipotalâmicos que podem posteriormente modificar a atividade do eixo LHHA.

Os estudos realizados em animais mostram que fêmeas apresentam diferentes respostas ao estresse quando comparadas aos machos (ZAVALA et al., 2011). Os níveis circulantes de GC são maiores em fêmeas em condições basais (VIAU et al., 2005). Além disso, os níveis médios diários de GC variam de acordo com estágio do ciclo estral em fêmeas, mostrando níveis equivalentes aos do sexo masculino durante o estro, mas valores maiores durante o proestro, metaestro e diestro (ATKINSON et al., 1997). As fêmeas exibem sensibilização ao estresse repetido, por aumentar os níveis de GC em comparação ao estresse agudo. Desta forma, estudos sugerem que fêmeas liberam maiores níveis de GC e os mantém por um período mais longo em comparação aos machos após estresse repetido por contenção (GALEA et al., 1997).

Esta resposta diferenciada no sexo feminino contribuiu para o entendimento dos mecanismos envolvidos nas diferenças sexuais induzidas pelo estresse repetido.

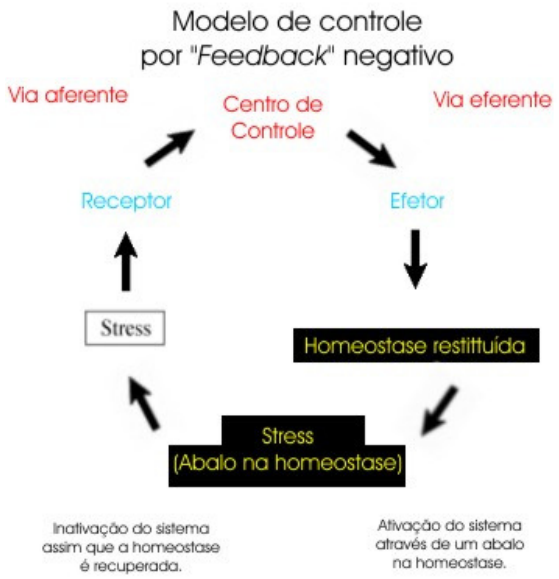

Figura 1. O sistema de retroalimentação " Feedback Negativo" .

2.2 Estresse oxidativo

2.2.1 Os sistemas oxidantes e antioxidantes no cérebro

A "homeostase redox" celular é definida como um equilíbrio entre oxidantes e antioxidantes intracelulares (CASTAGNE et al. 1999). As espécies reativas de oxigênio tais como radical superóxido (O_2^-), radical hidroxil (-$OH^\bullet$) e peróxido de hidrogênio (H_2O_2) são produzidas por processos metabólicos e fisiológicos podendo ocorrer reações oxidativas prejudiciais aos organismos (HALLIWELL, 2006). Os efeitos oxidativos das EROs são controlados por antioxidantes não enzimáticos como o ácido ascórbico e tióis não protéicos (NP-SH) e também por antioxidantes enzimáticos (superóxido dismutase (SOD), que converte os radicais superóxido em H_2O_2, catalase (CAT), responsável pela desintoxicação de H_2O_2 e glutationa peroxidase (GPx), que decompõe os peróxidos, notavelmente aqueles derivados da

oxidação dos fosfolipídios de membrana) (STANGHERLIN et al., 2008; FONTELLA et al., 2005).

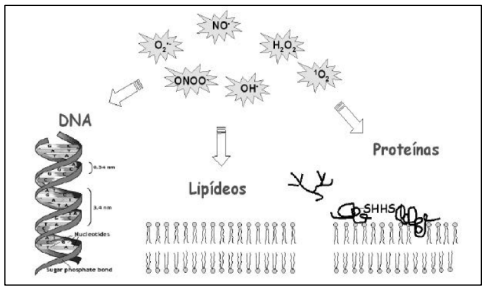

Figura 2. Dano oxidativo às macromoléculas biológicas (TORRES, 2003)

Outra enzima de fundamental importância para a integridade funcional neuronal é a Na^+/ K^+-ATPase responsável pelo transporte ativo de íons sódio e potássio no SNC, mantendo o gradiente iônico necessário para a excitabilidade e regulação do volume celular neuronal. Está presente em alta concentração na membrana celular cerebral, consumindo cerca de 40%–50% do ATP gerado neste tecido. Sua atividade é diminuída nas desordens afetivas como a depressão (GAMARO et al., 2003).

O cérebro é considerado uma estrutura sensível ao dano oxidativo por ser rico em substratos oxidáveis, baixa atividade antioxidante (METODIEWA & KOSKA, 2000) além de apresentar um elevado metabolismo oxidativo e alto índice de ácidos graxos poliinsaturados o que contribui para a reduzida eliminação dos radicais livres (KODAVANTI, 1999; FONTELLA, et al., 2005). O conseqüente aumento nos níveis de radicais livres pode alterar profundamente as funções gliais (FEENEY et al., 2008) e neuronais (FRANTSEVA et al., 2000). Entre as estruturas cerebrais, evidências indicam que o hipocampo e estriado são mais susceptíveis a estresse oxidativo que outras regiões incluindo o córtex cerebral (STANGHERLIN et al., 2008).

Uma das principais conseqüências do estresse oxidativo é a peroxidação lipídica (McBRIDE & KRAEMER, 1999) que imposta às membranas biológicas prejudica a manutenção da homeostase intracelular, uma vez que favorece a entrada e saída indiscriminada de metabólitos e detritos da célula (JOSEPHY, 1997; TIMBRELL, 2000). Alem disso, importantes sistemas biológicos cuja funcionalidade depende da integridade dos grupos tiois não protéicos (NP-SH) podem ser comprometidos (HUSCHENBET et al., 1998; SUN et al., 2001).

Assim, o estresse pode impedir a ação dos sistemas de defesa antioxidante, levando ao dano oxidativo e alterando consideravelmente o equilíbrio entre fatores oxidantes e antioxidantes no cérebro. Com relação às diferenças sexuais, o estrogênio apresenta propriedades antioxidantes capaz de inibir a peroxidação lipídica *in vitro* contribuindo desta forma para seus efeitos neuroprotetores (PREDIGER et al., 2004).

2.3 Estresse e neurotoxicidade

2.3.1 Glicocorticóides e o status oxidativo cerebral

O modelo de estresse repetido por contenção promove o aumento na liberação de GC interferindo diretamente sobre o "feedback negativo" do eixo LHHA nos diferentes sexos, conforme descrição anterior. Os elevados níveis de GC liberados pelas adrenais em resposta a estressores físicos e psicológicos podem exacerbar a geração de EROs causando danos neuronais através do estresse oxidativo (MCINTOSH & SAPOLSKY, 1996; PREDIGER et al., 2004). Assim, o estresse por restrição é seguido por aumento na produção de EROs, reduzida atividade antioxidante, gerando então o dano oxidativo (LIU et al., 1994) que tem sido proposto como um dos mecanismos pelos quais os GC aumentam a vulnerabilidade de diferentes regiões cerebrais, como hipocampo, à insultos metabólicos (McINTOSH & SAPOLSKY,. 1996). O cérebro consome grande aporte de oxigênio, apresenta conteúdo abundante de lipídeos e insuficiência de antioxidantes comparado a outros tecidos (HALLIWELL & GUTTERIDGE, 1985). O ânion superóxido, o radical hidroxila e o peróxido de hidrogênio promovem a peroxidação lipídica especialmente nas membranas (KOVÁCS et al., 1996) e danificam as proteínas e o ácido desoxirribonucléico (ADN) celular (COCHRANE, 1991).

Uma falência neuronal via deficiência de ATP, bem como a geração de EROs pode também acarretar em uma inibição da síntese protéica e conseqüente diminuição da atividade (SANDERS et al., 1970) e funcionalidade da enzima Na+/K+-ATPase induzida pelo estresse (CREMA et al., 2010). Como conseqüência, a sinalização de neurotransmissores e atividade neuronal bem como o comportamento animal podem ter suas funções comprometidas (JAMME et al., 1995). Três isoformas da subunidade α da Na+/K+-ATPase são encontradas

no cérebro, mas variam quanto à expressão e tipo celular além de modular a atividade locomotora, ansiedade , aprendizado espacial e memória (MOSELEY et al., 2007). A isoforma α 1 é encontrada em muitos tipos celulares no SNC, a isoforma α 2 predomina em células gliais e a isoforma α 3 é somente expressa em neurônios(MOSELEYet al., 2003). A deficiência na atividade da Na+/K+-ATPase quanto a isoforma alfa pode estar diretamente relacionada com aumento nos níveis de ansiedade .

As alterações oxidativas são reconhecidas como responsáveis pela fisiopatologia de desordens psiquiátricas induzidas pelo estresse (NG et al., 2008). Desta forma, o aumento no estresse oxidativo em reposta ao estresse pode ser evidenciado pela associação entre a deficiência no sistema de defesa antioxidante e aumento nos níveis de peroxidação lipídica (BILICI et al., 2001).

2.4 Estresse e comportamento

2.4.1 Hipocampo

O hipocampo apresenta a maior densidade de receptores de glicocorticóides (RG) no cérebro e está envolvido na regulação do eixo LHHA e nas respostas comportamentais relacionadas ao estresse (BOWMAN et al., 2002). No hipocampo existem estruturas importantes para o processo de aprendizagem, como os campos CA1 e CA3, no corno de Amon e o giro denteado (BEAR et al., 2002). Os neurônios hipocampais são altamente sensíveis ao estresse em resposta aos GC em níveis elevados. Três semanas de estresse por restrição causam uma retração dendrítica de neurônios CA3 no hipocampo de ratos. Contudo quatro semanas potencializam os danos dentríticos, podendo levar até a morte neuronal se o estresse prolongar-se para meses ou anos. (CONRAD et al., 2003). Os estudos farmacológicos indicam que a formação hipocampal é requerida para a aquisição de experiências prévias de ordem temporal e detalhes contextuais (BALDERAS et al., 2008). Assim o hipocampo mostra ser de grande importância para a aquisição, consolidação e evocação da memória (IZQUIERDO & MEDINA, 1995; RUBIN et al., 2000).

O desempenho em tarefas de memória parece estar dependente de um circuito neuronal envolvendo córtex pré-frontal, hipocampo, tálamo e estriado. Contudo, uma

desconexão entre hipocampo e córtex pré-frontal é sugerida alterar este desempenho (BURGOS et al., 2005). Assim, o estresse pode causar profundas alterações quanto à memória de curto e longo prazo (ELIZALDE et al., 2008). A influência do estresse repetido sobre a memória é complexa. Semanas de estresse repetido parecem dificultar o desempenho sobre aprendizado e memória?

2.4.2 Estriado

O estriado é considerado de fundamental importância para o aprendizado e comportamento decisório. Neurônios estriatais diminuem ou aumentam sua atividade de acordo com a progressão do aprendizado (TREMBLAY et al., 1998; BRASTED & WISE, 2004). A atividade destes neurônios pode ser modulada pela quantidade, probabilidade e presença esperada de recompensa assim como a magnitude de atenção e memória necessária para executar as tarefas propostas (KAWAGOE et al., 1998; SHIDARA et al., 1998; CROMWELL & SHULTZ, 2003). Estudos sobre os mecanismos sinápticos no estriado têm mostrado que a potencialização em longo prazo (PLP) que pode ser considerado um modelo celular de consolidação de memória (CLARKE et al., 2010), ou a depressão em longo prazo (DLP) pode ocorrer em sinapses corticoestriatais dependendo da combinação de inputs corticais, output estriatais e inputs dopaminérgicos D1 e D2 (REYNOLDS & WICKENS, 2002).

O sistema dopaminérgico e seus locais de projeção (estriado e córtex pré frontal), desempenham ação crucial na modulação de comportamentos motivados, emoção e funções cognitivas superiores relacionadas com o processamento de recompensa. As funções de recompensa incluem a capacidade de prever situações potencialmente gratificantes, servindo como uma vantagem evolutiva para o enfrentamento de ambientes imprevisíveis. A integridade do sistema dopaminérgico é importante para o processamento eficiente da informação de recompensa (DREHER et al., 2009). Desta forma, quando o animal é submetido a situações de estresse, a habilidade diminuída para associar a recompensa com o lugar onde a mesmo foi colocado, pode ser um sinal de prejuízo no sistema dopaminérgico com dificuldades de aprendizado assim como pode interferir no consumo de alimentos palatáveis doces (PAPP et al., 1991).

2.4.3 Interações estruturais e influência do sexo sobre o comportamento

As conexões diretas entre hipocampo e estriado (principalmente o núcleo accumbens) assim como estriado e hipocampo via córtex pré frontal e entorrinal (ROSSATO et al., 2006) tem sido estudadas recentemente quanto ao seu papel colaborativo e cooperativo nos sistemas de memória. Os esteróides gonadais apresentam importantes efeitos sobre a estrutura e funcionamento do cérebro adulto. Contudo é necessário o conhecimento de como estes esteróides, principalmente o estrogênio, influenciam o aprendizado e memória em ratos adultos. Por exemplo, existem divergências com relação aos efeitos do estrogênio sobre o aprendizado e memória no que se refere às dificuldades e melhoras no desempenho de diferentes tarefas em fêmeas. Isto pode estar relacionado a uma variedade de fatores como hormônios, sexo e tipo de tarefa realizada (KOROL & KOLO, 2002). Estudos relatam diferenças entre os sexos quanto ao comportamento em tarefas de memória não-espacial onde machos apresentam maiores dificuldades quando comparados às fêmeas na tarefa de reconhecimento de objetos (BOWMAN et al., 2001). Quando exposto ao mesmo estressor, que dificulta o desempenho em tarefas cognitivas, fêmeas apresentam melhor desempenho que machos (LUINE et al., 2007). Desta forma, as flutuações dos níveis de estrogênio podem influenciar a aquisição de aprendizado e memória, com diferentes desempenhos (TROPP e MARKUS, 2001) dependendo das demandas específicas para cada tarefa (KOROL et al., 2002).

2.5 Antidepressivos tricíclicos

Os ADTs representam uma classe de fármacos prescritos ao longo do tempo para o tratamento da depressão crônica, fases depressivas dos distúrbios bipolares e dor neuropática. Outras indicações estão associadas com ansiedade e distúrbios alimentares (DEMERDASH et al. 2004). O mecanismo pelo qual os ADTs exercem seus efeitos antidepressivos não está claramente estabelecido. Contudo, a capacidade em inibir a recaptação de neurotransmissores é geralmente aceito como seu modo de ação primária (POTTER et al., 1998) permitindo um aumento nas concentrações de serotonina, norepinefrina e dopamina na fenda sináptica o que resulta no aumento da velocidade de transmissão sináptica (DALMIZRAK et al., 2011).

Os ADTs são absorvidos pelo intestino delgado e metabolizados no fígado. Sua marcante característica lipofílica permite que facilmente cruzem a barreira cérebro-sangue (BARANCZYK-KUZM et al., 2004). Estudos realizados *in vitro*, evidenciam que a exposição a diferentes ADTs aumenta a produção de EROs (DEMERDASH et al., 2004) assim como podem inibir a atividade da pois alteram a estrutura organizacional da mambrana lipídica induzindo déficits cognitivos (PEDRAZZA et al., 2007). O sucesso quanto ao tratamento se deve á normalização na função do eixo LHHA em pacientes deprimidos, o que reflete à normalização dos níveis de CRH e GC (ELAKOVIC et al., 2009)

2.5.1 Clomipramina

A clomipramina, uma amina terciária, é classificada como um ADT com meia vida de aproximadamente 12 a 24 horas em humanos e 4 horas em ratos, sendo utilizada para o tratamento de transtornos obssessivo compulsivo, síndrome do pânico e depressão em humanos (WEIGMANN et al., 2000) inibindo principalmente a recaptação de serotonina e norepinefrina no SNC (PETERS et al., 1990; TRIMBLE et al., 1990). A sua aplicação pode ser observada na prática clínica e em modelos de estresse em animais (LIU et al., 2008). A biotransformação da clomipramina ocorre no fígado através da desmetilação em seu principal metabólito N-desmetilclomipramina. Desta forma estão presentes dois princípios ativos in vivo: clomipramina e N- desmetilclomipramina. Esta apresenta meia-vida cerca de duas vezes maior possibilitando que seu uso crônico aumente a concentração em relação à clomipramina. Diferenças no metabolismo hepático de antidepressivos sugerem diferentes impactos dos esteróides gonadais em ambos os sexos (DANNAN et al., 1986). Em doses relativamente baixas, as concentrações de clomipramina e N- desmetilclomipramina são maiores no SNC do que em plasma (WEIGMANN et al., 2000). Entre as estruturas cerebrais, o córtex cerebral apresenta maiores concentrações de clomipramina e N- desmetilclomipramina quando comparado ao estriado e hipocampo (AITCHISON, et al., 2009).

Estas mesmas doses reduzem os déficits comportamentais e disfunções colinérgicas induzidas por estresse em ratos (D'AQUILA et al., 2000) enquanto doses elevadas são tóxicas em modelos experimentais (CALEGARI et al., 2007). Contudo, a espécie e sexos dos animais, quanto o tipo, duração severidade do estresse podem modificar as respostas induzidas pelo estresse e clomipramina (CONSOLI et al., 2005).

2.5.2 Clomipramina: efeitos endócrinos e comportamentais

Uma vez que o desequilíbrio oxidante/antioxidante é determinante para a depressão induzida pelo estresse, espera-se que o tratamento crônico com antidepressivos normalize as funções neuroquímicas juntamente com os parâmetros comportamentais. Estudos indicam que o processo de neurogênese encontra-se reduzido no hipocampo o que pode induzir à depressão, assim o tratamento antidepressivo proporciona aumento na proliferação neuronal e consequentemente na neurogênese hipocampal influenciando a atividade comportamental (LIU et al., 2008). Contudo alguns questionamentos podem se fazer necessários para as ações da clomipramina: 1º) há influência sobre o sistema endócrino e cognitivo? 2º) a dose além dos níveis terapêuticos influencia a neuroquímica e conseqüentemente o comportamento? 3º) diferenças sexuais e hormonais influenciam as respostas cognitivas ? O comprometimento no desempenho de tarefas cognitivas é comum em pacientes com depressão (ELLIOTT et al. 1998). Um antidepressivo pode ter um efeito deletério sobre a cognição, que é agravado com um sintoma cognitivo residual causado, por exemplo, por estresse, contrariando eventuais benefícios de recuperação nas funções cognitivas. Na verdade, muitos antidepressivos apresentam propriedades farmacológicas que podem explicar por que tal disfunção pode ser um resultado esperado. Processos de memória são influenciados por inúmeros sistemas de neurotransmissão como colinérgicos, serotoninérgicos e dopaminérgicos os quais são afetados pela maioria dos agentes antidepressivos (GORENSTEIN, et al. 2006).

Contudo a administração crônica de clomipramina pode ter efeitos adversos como dificuldades de aprendizado e memória (BURGOS et al., 2005) assim como um aumento no consumo de alimento doce que são caracterizados como sintomas iniciais da depressão e ansiedade (ELY et al., 1997). Determinados autores defendem que as dificuldades no desempenho principalmente no que se refere à memória na terapia com ADTs, não se deve à sedação, pois doses terapêuticas podem causar estes déficits. Os mesmos autores sugerem estudos com a utilização de doses maiores para análise de potencialização dos prejuízos envolvendo cognição e memória (CARVALHO et al., 2002). Doses consideradas terapêuticas (10–20 mg/kg/dia) aumentam a função e expressão dos RG em células neuronais (LAI et al., 2003). Contudo, a função reduzida dos RG *in vitro* por antidepressivos tem sido referida, o que pode aumentar a produção de EROs, pois os antidepressivos controlam a função dos RG *in vitro* por regular a concentração intracelular de GC (PARIANTE et al., 2001). Na presença de GC, os quais não são substratos para transportadores esteróides, a ativação reduzida e

down-regulation dos RG induzida por antidepressivos ocasionam uma função reduzida dos RG (CARVALHO et al., 2008). Assim, o desequilíbrio entre GC e RG poderia causar a produção de EROs.

A depressão é duas vezes mais comum em mulheres que em homens o que sugere diferenças na eficácia do tratamento antidepressivo (WOHLFARTH et al., 2004). Estudos recentes indicam resultados conflitantes quanto as ações terapêuticas de ADTs em ambos os sexos. As mulheres parecem responder melhor a ISRS enquanto homens a ADTs (KORNSTEIN et al., 2000). Contudo, esta resposta é questionada por alguns autores (STEINER et al., 1990; LEWIS – HALL et al., 1997). A vantagem da ação terapêutica de ADTs em mulheres também é defendida por outros autores. Em contrapartida, dados da literatura referem que não há diferenças sexuais em resposta ao tratamento com ADTs (LEWIS – HALL et al., 1997).

As diferenças sexuais no metabolismo, entrada no SNC e distribuição da clomipramina e seu metabólito ativo (KOKRAS et al., 2009) assim como os hormônios sexuais podem resultar em diferente captação e retenção da clomipramina, contribuindo desta forma para as diferenças entre as ações antidepressivas e efeitos sobre os GC e RG (ELAKOVIC et al., 2009).

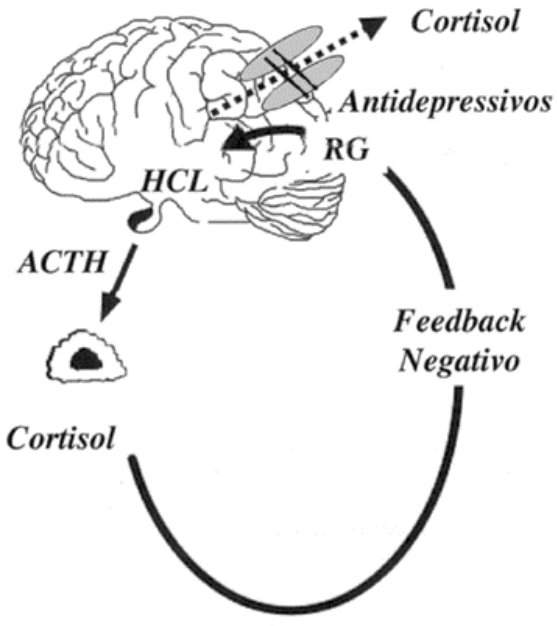

Figura 3. Ação dos antidepressivos sobre os receptores de glicocorticóides (RG) (JURUENA, 2004)

3. OBJETIVOS

3.1 Objetivo Geral

O presente estudo teve como objetivo investigar os efeitos da interação de uma dose elevada de clomipramina com estresse repetido por contenção sobre possíveis alterações comportamentais e na homeostase redox em cérebro de ratos machos e fêmeas.

3.2 Objetivos Específicos

Capítulo I

- Investigar os efeitos induzidos pelo estresse repetido por contenção sobre a homeostase redox através das análises de DCFH-DA e TBARS assim como dos principais sistemas de defesa antioxidantes enzimáticos (CAT e SOD) e não enzimáticos (NP-SH) em córtex cerebral, hipocampo e estriado de ratos machos;
- Verificar a atividade enzimática da Na^+/ K^+-ATPase em córtex cerebral, hipocampo e estriado de ratos machos submetidos a estresse repetido por contenção;
- Verificar se a dose administrada de clomipramina recupera a homeostase redox em córtex cerebral, hipocampo e estriado de ratos machos estressados repetidamente;
- Verificar se a ingestão crônica de clomipramina na dose utilizada protege contra possível redução na atividade enzimática da Na^+/K^+-ATPase em córtex cerebral, hipocampo e estriado induzido por estresse repetido por contenção em ratos machos;

Capítulo II

- Analisar a relação existente quanto a parâmetros comportamentais e a atividade enzimática da Na^+/ K^+-ATPase em córtex cerebral, hipocampo e estriado de ratos machos submetidos a estresse repetido por contenção;
- Analisar se a interação entre estresse repetido por contenção e clomipramina interferem nos parâmetros comportamentais e na atividade enzimática da Na^+/K^+-ATPase em córtex cerebral, hipocampo e estriado de ratos machos;
- Avaliar os efeitos do estresse repetido por contenção e clomipramina sobre a memória não espacial, motivação, comportamento alimentar, ansiedade e atividade exploratória em ratos machos;

Capítulo III

- Investigar as interações do estresse repetido por contenção e clomipramina quanto aos efeitos sobre a homeostase redox através das análises de DCFH-DA e TBARS assim como dos principais sistemas de defesa antioxidantes enzimáticos (CAT e SOD) e não enzimáticos (NP-SH) em córtex cerebral, hipocampo e estriado de ratos machos e fêmeas;
- Verificar a atividade enzimática da Na^+/ K^+-ATPase em córtex cerebral, hipocampo e estriado de ratos machos submetidos a estresse repetido por contenção em ratos machos e fêmeas;
- Verificar se a clomipramina recupera a homeostase redox em córtex cerebral, hipocampo e estriado de ratos machos e fêmeas estressados repetidamente;
- Verificar se a ingestão crônica de clomipramina protege contra a possível redução na atividade enzimática da Na^+/K^+-ATPase em córtex cerebral, hipocampo e estriado induzido por estresse repetido por contenção em ratos machos e fêmeas;
- Avaliar os efeitos da interação entre estresse repetido por contenção e clomipramina sobre a memória não espacial, motivação, comportamento alimentar, ansiedade e atividade exploratória em ratos machos e fêmeas;

4. ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese serão apresentados sob a forma de dois artigos científicos e um manuscrito, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão dos Resultados e Referências Bibliográficas, encontram-se no artigo científico e no manuscrito.

4.1 Artigo Científico 1:

4.1.1 Tratamento com clomipramina e estresse repetido por contenção alteram parâmetros de estresse oxidativo em cérebro de ratos

Clomipramine Treatment and Repeated Restraint Stress Alter Parameters of Oxidative Stress in Brain Regions of Male Rats

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ORIGINAL PAPER

Clomipramine Treatment and Repeated Restraint Stress Alter Parameters of Oxidative Stress in Brain Regions of Male Rats

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Abstract This study aimed to compare the effects of repeated restraint stress alone and the combination with clomipramine treatment on parameters of oxidative stress in cerebral cortex, striatum and hippocampus of male rats. Animals were divided into control and repeated restraint stress, and subdivided into treated or not with clomipramine. After 40 days of stress and 27 days of clomipramine treatment with 30 mg/kg, the repeated restraint stress alone reduced levels of Na^+, K^+-ATPase in all tissues studied. The combination of repeated restraint stress and clomipramine increased the lipid peroxidation, free radicals and CAT activity as well as decreased levels of NP-SH in the tissues studied. However, Na^+, K^+-ATPase level decreased in striatum and cerebral cortex and the SOD activity increased in hippocampus and striatum. Results indicated that clomipramine may have deleterious effects on the central nervous system especially when associated with repeated restraint stress and chronically administered in non therapeutic levels.

Keywords Antioxidant enzymes · Repeated restraint stress · Clomipramine · Free radicals · Oxidative stress

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Introduction

The repeated or chronic stress can alter neuronal functions by complex neuro-hormonal changes and consequently contribute to the development of diverse disorders in the central nervous system (CNS), such as clinical manifestations of depression. The main physiological structures involved in response to stress are the limbic-hypothalamic-pituitary-adrenal axis (LHPA) and the autonomic system. The secretion of catecholamines (epinephrine and norepinephrine) by autonomic system is considered an immediate response to stress and central to the mobilization of energy [1, 2] and release of glucocorticoids (GC) [3]. In the LHPA axis, catecholamines are secreted after a latency of minutes, with effects that may take hours to appear [3]. Thus, the activation of the LHPA axis and the release of glucocorticoids are fundamental for the adaptive response and immediate survival of the organism in reaction to acute stimuli. However, studies have suggested that high levels of glucocorticoids in the brain may produce neuronal injury by increasing oxidative stress, particularly via lipid peroxidation [4]. These events are associated with the overproduction of reactive oxygen species (ROS) [5] and deficits in the antioxidant defense systems. In addition, changes of the LHPA hormones have been described in depressed patients [6] and in stress-related models of depression. In line with this, it has been demonstrated that antidepressant not only reduces the depressive-like behavior, but also counteracts stress-induced hormonal changes in rats [7].

Tricyclic antidepressants (TCAs) have been prescribed along time for the treatment of depression. Moreover, the indications for the use of TCAs are associated with anxiety, eating disorders and chronic pain syndromes [8]. The mechanism by which TCAs exert their antidepressant

effects is not clearly established until now. However, the capacity of TCAs in blocking the reuptake of centrally active neurotransmitters is generally accepted as their primary mode of action [9]. Indeed, TCAs, as well as serotonergic receptors, can also connect to several other receptors, such as histamine (H1E H2), adrenergic (alpha-1 and alpha-2), GABA-A, and muscarinic receptors [9, 10].

Clomipramine belongs to a class of TCAs and is widely used for the treatment of depression and obsessive compulsive disorder mainly by inhibiting the reuptake of serotonin and norepinephrine in the brain [11, 12]. It has been shown that the chronic administration of clomipramine, at doses relatively low, evokes benefits on depression [13, 14] and reduces the behavioral deficits and cholinergic dysfunction induced by stress in rats [15]. However, the species and sex of animals as well as type, duration and severity of stress can modify the response induced by stress and clomipramine [16]. Literature data have also reported that high doses ($\geq$30 mg/kg) of clomipramine are toxic to experimental animals [17]. The adverse effects induced by clomipramine administration, due to the blockade of serotonin reuptake, include difficulties in learning and memory as well as an increase in the consumption of sweet foods, which are characterized as initial symptoms of depression and anxiety, [18]. Regarding the mechanism of toxicity, in vitro studies have evidenced that the exposure to different classes of TCAS drugs increases the production of ROS [8].

In this context, the objective of this study was to evaluate the effects of the repeated restraint stress treatment in combination with clomipramine in order to verify whether the association between stress condition and use of TCAs could affect the antioxidant defense and ROS production in central nervous system in different brain structures such as hippocampus, striatum and cerebral cortex of adult rats.

Experimental Procedures

Chemicals

Clomipramine, Thiobarbituric acid (TBA), 2′-7′-dichlorofluorescein (DCF), trichloroacetic acid (TCA), *p*-dimethylaminobenzaldehyde, reduced glutathione (GSH), 5.5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Animals

Forty adult male Wistar rats (250–300 g) from our own breeding colony were used. The animals were placed in groups of five animals in boxes made of Plexiglas measuring 42 × 34 × 16 cm, with the floor covered with sawdust. They were kept in a room with light–dark cycle of 12 h with the lights on between 0700 and 1900 hours and temperature (20–25°C) controlled receiving water and food ad libitum.

Animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of Santa Maria, Brazil.

Experimental Groups

Animals were divided into two groups: control group, only manipulated for the necessary maintenance of their cages in good health, and stress group, which received a treatment of repeated restraint stress for a 40-day period. On day 14 of this experimental period, each initial group was subdivided into two different groups:

1) control group: control (C) and control plus clomipramine group (CC)
2) stress group: stress (S) and stress plus clomipramine group (SC)

Repeated Restraint Stress Model

Stress condition was performed according to the model of repeated restraint stress as described by Ely et al. [18]. Animals were immobilized in plastic tubes measuring 25 × 7 cm of diameter that were adjusted to the size of the animal. The animal behavior was carried out 1 h per day, 5 days a week, for 40 days in the morning between 0800 and 1000 hours. The control group was just handled and thus not subject to repeated stress situation.

Clomipramine Treatment

The animals of CC and SC groups received clomipramine (30 mg/kg) for 27 consecutive days, according to Calegari [17], in the drinking water after 14 days of the start of the trial period. The untreated rats with the drug (C and S groups) received regular water. Clomipramine was placed in dark bottles due to its photosensitivity. The water consumption by animals was analyzed daily in order to adapt the dose to be administered. Oral administration was chosen because it is the most common manner of using antidepressants in patients with psychiatric disorders described by Lucassen et al. [19].

Tissue Preparation

At the end of the treatment period, rats were killed by decapitation. The brain was removed and structures such as

hippocampus, striatum and cerebral cortex were quickly dissected, placed on ice, and immediately homogenized in cold 50 mM Tris–HCl pH 7.4. Homogenates were centrifuged at 4,000×*g* for 10 min to yield the low-speed supernatant fractions that were used for different biochemical assays in all trials.

Oxidized Diclorofluoresceine (DCFH-DA) Levels Determination

2′-7′-Dichlorofluorescein diacetate (DCFH-DA) levels were determined as an index of the peroxide production by the cellular components. This experimental method of analysis is based on the deacetylation of the probe DCFH-DA, and its subsequent oxidation by reactive species to DCFH-DA, a highly fluorescent compound [20]. The supernatant fraction of hippocampus, striatum and cerebral cortex was added to a medium containing Tris–HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (Excitation at 488 nm and Emission at 525 nm, and both slit widths used were at 1.5 nm). DCFH-DA levels were determined using a standard curve of DCF and results were corrected by the protein content.

Thiobarbituric Acid Reactive Substance (TBARS) Level Determination

Lipid peroxidation was estimated by measuring TBARS and expressed in terms of malondialdehyde (MDA) content, according to the method of Ohkawa et al. [21]. In this method, MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Briefly, the supernatant fraction of brain structures was incubated at 100°C for 60 min in acid medium containing 8.1% sodium dodecyl sulfate, 0.5 ml of acetic acid buffer (500 mM, pH 3.4) and 0.6% TBA. TBARS levels were measured at 532 nm and the absorbance was compared with the standard curve using malondialdehyde.

Non Protein Thiol (NP-SH) Level Determination

NP-SH levels of hippocampus, striatum and cerebral cortex samples were determined according to the method proposed by Ellman with some modifications [22]. Samples were precipitated with TCA (10%) and subsequently centrifuged at 4,000×*g* for 10 min. After the centrifugation, the supernatant fraction (200 µl) was added to a reaction medium containing K^+-phosphate (0.5 M and pH = 7.4) and DTNB (0.5 mM). NP-SH levels were measured spectrophotometrically at 412 nm. Results were calculated in relation to a standard curve constructed with reduced glutathione (GSH) and also corrected by the protein content.

Enzyme Assays

Catalase (CAT) Activity

The measurement of the CAT activity was determined according to the method proposed by Aebi [23]. The supernatant fraction of hippocampus, striatum and cerebral cortex (40 µl) was added to a medium containing K^+-phosphate buffer (50 mM; pH 7.4) and H_2O_2 (10 mM). The kinetic analysis of catalase was started after H_2O_2 addition and the rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s. One unit of the enzyme was considered as the amount of enzyme which decomposes 1 µmol H_2O_2/min at pH 7.

Superoxide Dismutase (SOD) Activity

The SOD enzyme activity was determined in hippocampus, striatum and cerebral cortex according to the method proposed by Misra and Fridovich [24]. This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. Briefly, the supernatant fraction (20–60 µl) was added to a medium containing glycine buffer (50 mM; pH 10.5) and adrenaline (1 mM). The kinetic analysis of SOD was started after adrenaline addition, and the color reaction was measured at 480 nm.

Sodium Potassium (Na^+, K^+-ATPase) Activity

The cerebral Na^+, K^+-ATPase activity was determined according to the method proposed by Musbeck et al. [25] with some modifications. Briefly, the supernatant fraction of brain (20 µl) was added to a reaction medium containing NaCl (125 mM), $MgCl_2$ (3.0 mM), KCl (20 mM), and Tris–HCl buffer (50 mM and pH 7.4), with or without ouabaine (5 µM). The method for ATPase activity measurement was based on the determination of the inorganic phosphate (*Pi*) released to the reaction medium by the hydrolysis of the ATP according to the method proposed by Atkinson et al. [26]. The reaction was initiated with the addition of the substrate ATP (1.5 mM) to the reaction medium, and was finished by the addition of the color reagent (1 ml) containing ammonium molibdate (2%) triton-100X(5%) and H2SO4 1,8 M(10%) after 15 min of incubation at 37°C. The formed molibdate-*Pi* complexes were measured spectrophotometrically at 405 nm. The values were calculated in relation to a standard curve constructed with *Pi* at known concentrations and also corrected by the protein content.

Protein Determination

The protein content was determined according to Lowry et al. [27] using bovine serum albumin (BSA) as standard.

Statistical Analysis

Data were analyzed by two-way ANOVA followed by Duncan's multiple range test (SPSS version 10.0 for Windows 98—SPSS Inc, Chicago, Illinois, USA). Differences between groups were considered significant when $P < 0.05$. Results are expressed as mean (standard error of the mean (SEM).

Results

Oxidized Diclorofluoresceine (DCFH-DA) Levels

Statistical analysis demonstrated that the control group submitted to clomipramine treatment had a significant increase in DCFH-DA production in the hippocampus [two way ANOVA, $F(1, 19) = 27.81$, $P < 0.05$; Fig. 1b] and striatum [two way ANOVA, $F(1, 19) = 37.34$, $P < 0.05$; Fig. 1c], when compared to the untreated group (Fig. 1). This effect of chronic treatment with clomipramine was not observed in the cerebral cortex structure. However, we found that the combination of repeated restraint stress and clomipramine showed a significant increase compared to the respective group treated with clomipramine [two way ANOVA, $F(1,19)] = 8.37$, $P < 0.05$; Fig. 1a]. Two-way ANOVA shows that the association between chronic clomipramine treatment and stress condition also caused significant elevation of DCF levels in hippocampus, striatum and cerebral cortex compared to the control group [$F(1,19) = 8.37$, $P < 0.05$]. On the other hand, no alteration in the DCF production of brain structures was observed in the groups that received only repeated restraint stress.

Thiobarbituric Acid Reactive Substance (TBARS) Levels

Figure 2 shows the effects of chronic treatment with clomipramine and repeated restraint stress on lipid peroxidation in cerebral cortex (Fig. 2a), hippocampus (Fig. 2b) and striatum (Fig. 2c). Chronic clomipramine treatment caused *per se* an increase of the lipid peroxidation in striatum [two way ANOVA, $F(1, 19) = 70.68$, $P < 0.05$] and cerebral cortex [two way ANOVA, $F(1, 19) = 69.77$, $P < 0.05$]. Indeed, two-way ANOVA revealed that clomipramine treatment associated with stress condition caused a further increase in the levels of TBARS in all brain structures analyzed [$F(1, 19) = 10.76$, $P < 0.05$], when compared to the respective control group with clomipramine. Moreover, the repeated restraint stress alone changed TBARS levels only in striatum [$F(1, 19) = 40.62$, $P < 0.05$], when compared to values found in the control group.

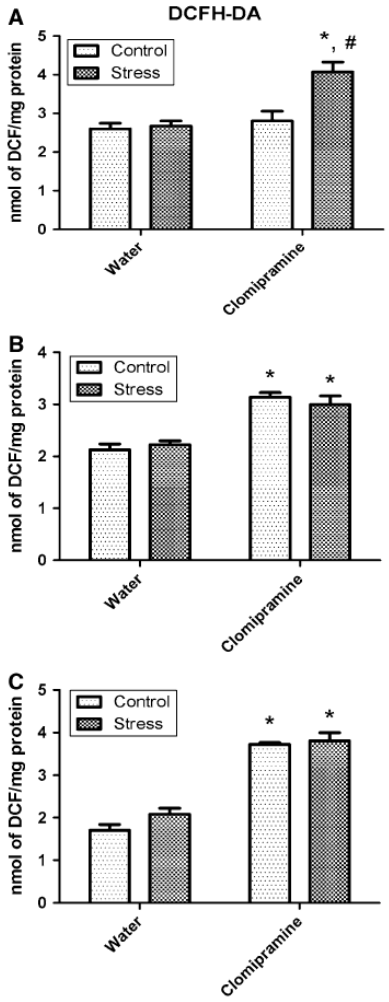

Fig. 1 Effects of repeated restraint stress and chronic treatment with clomipramine on free radical production (DCF–DA) in cerebral cortex (**a**), hippocampus (**b**) and striatum (**c**) of male rats. Data are expressed as means ± SEM, *N* of five animals/group. Data of reactive species (ROS) levels are presented as nmol of DCF/mg protein. * Significant effect of treatment with clomipramine when compared to control group (Two-way ANOVA, $P < 0.05$). # Significant effect of stress in relation to the respective control group (Two-way ANOVA, $P < 0.05$)

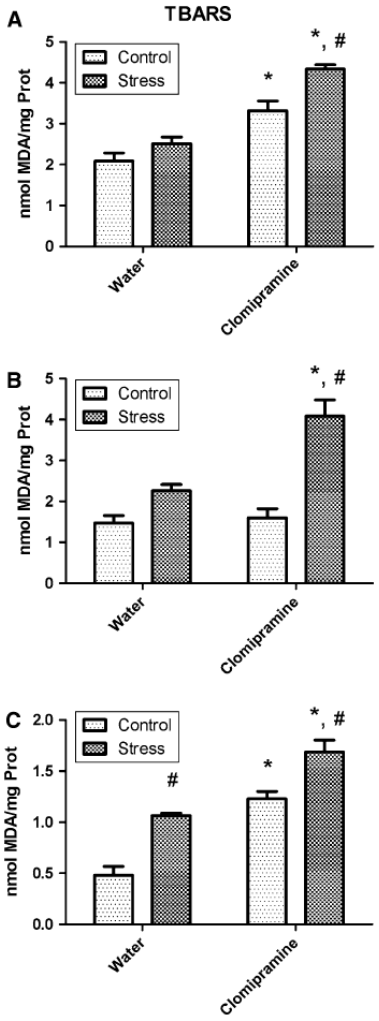

Fig. 2 Effects of repeated restraint stress and chronic treatment with clomipramine on lipid peroxidation (TBARS) in cerebral cortex (**a**), hippocampus (**b**) and striatum (**c**) of male rats. Data are expressed as means ± SEM, *N* of five animals/group. TBARS levels are expressed as nmol of MDA/mg protein. * Significant effect of treatment with clomipramine when compared to control group (Two-way ANOVA, $P < 0.05$). # Significant effect of stress in relation to the respective control group (Two-way ANOVA, $P < 0.05$)

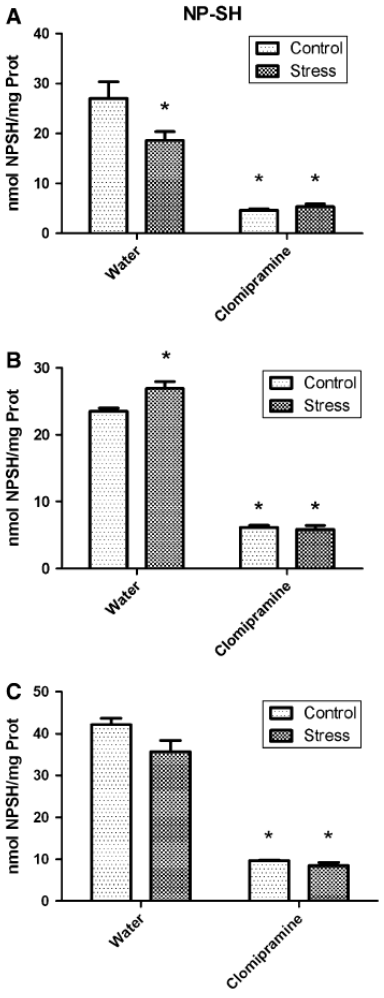

Fig. 3 Effects of repeated restraint stress and chronic treatment with clomipramine on NP-SH levels in cerebral cortex (**a**), hippocampus (**b**) and striatum (**c**) of male rats. Data are expressed as means ± SEM, *N* of five animals/group. * Significantly different from the control group (Two-way ANOVA, $P < 0.05$)

Non Protein Thiol (NP-SH) Levels

The results of Fig. 3 indicate that clomipramine treatment significantly reduced the levels of non protein thiol (NP-SH) in cerebral cortex [two way ANOVA, $F(1,19) = 5.68$, $P < 0.05$; Fig. 3a], hippocampus [two way ANOVA, $F(1,19) = 5.45$, $P < 0.05$; Fig. 3b] and striatum [two way ANOVA, $F(1,19) = 46.98$, $P < 0.05$; Fig. 3c] when compared to the control group. Two-way ANOVA shows that the association between chronic clomipramine treatment and stress condition also caused a significant reduction of NP-SH levels in hippocampus [$F(1, 19) = 7.70$, $P < 0.05$], striatum [$F(1, 19) = 5.67$, $P < 0.05$] and cerebral cortex [$F(1, 19) = 5.68$, $P < 0.05$] compared to the control group. The group subjected only to repeated restraint stress showed that there was a reduction in the levels of NP-SH in the cerebral cortex [$F(1, 19) = 15.43$,

$P < 0.05$] and an increase in the hippocampus [$F(1, 19) = 5.45$, $P < 0.05$] compared to the control group.

Enzyme Activities

CAT Activity

Statistical analysis of two way ANOVA showed that the CAT activity was significantly increased in the groups submitted to clomipramine and clomipramine plus stress treatment in cerebral cortex [$F(1, 19) = 59.23$, $P < 0.05$; Fig. 4a], hippocampus [$F(1, 19) = 35.87$, $P < 0.05$; Fig. 4b] and striatum [$F(1, 19) = 30.62$, $P < 0.05$; Fig. 4c] when compared to the control group (Fig. 4). There was a reduction of the enzymatic activity in the hippocampus [two way ANOVA, $F(1, 19) = 20.90$, $P < 0.05$] in the group undergoing repeated restraint stress and clomipramine compared to the respective control group with clomipramine. However, the enzyme activity showed an increase when the stress was applied alone in striatum [$F(1, 19) = 35.32$, $P < 0.05$] when compared to the control group. There was no significant difference in cerebral cortex.

SOD Activity

Figure 5 illustrates the activity of SOD in samples of cerebral cortex (Fig. 5a), hippocampus (Fig. 5b) and striatum (Fig. 5c) of rats subjected to repeated restraint stress and chronic treatment with clomipramine. Data show that clomipramine treatment caused a significant increase in the SOD activity of striatum and hippocampus when compared to the control group [two way ANOVA, $F(1, 19) = 8.48$, $P < 0.05$]. Likewise, an enhanced SOD activity was verified in the group submitted to both chronic clomipramine treatment and stress condition in striatum and hippocampus [two way ANOVA, $F(1, 19) = 6.61$, $P < 0.05$]. No significant difference was observed in cortical SOD activity between groups. In striatum there was a reduction of the SOD activity [two-way ANOVA, $F(1, 19) = 52.30$, $P < 0.05$] in the group subjected to the repeated stress of restraint when compared to the control group.

Na^+, K^+-ATPase

The data of Fig. 6 show that clomipramine treatment caused *per se* a significant reduction in the Na^+, K^+-ATPase activity in cerebral cortex [two way ANOVA, $F(1,19) = 9.23$, $P < 0.05$; Fig. 6a] and striatum [two way ANOVA, $F(1,19) = 19.09$, $P < 0.05$; Fig. 6c] compared to the control group. Two-way ANOVA revealed that the inhibition of the enzyme was also significant in the animals submitted to chronic clomipramine treatment plus stress condition [$F(1, 19) = 5.41$, $P < 0.05$] in striatum and cerebral cortex [$F(1,19) = 8.89$, $P < 0.05$]. Unlike, repeated restraint stress promoted a significant decrease in the Na^+, K^+-ATPase activity in cerebral cortex [$F(1,19) = 27.88$, $P < 0.05$], hippocampus [$F(1,19) = 23.33$, $P < 0.05$] and striatum [$F(1,19) = 15.51$, $P < 0.05$] when compared to the control group.

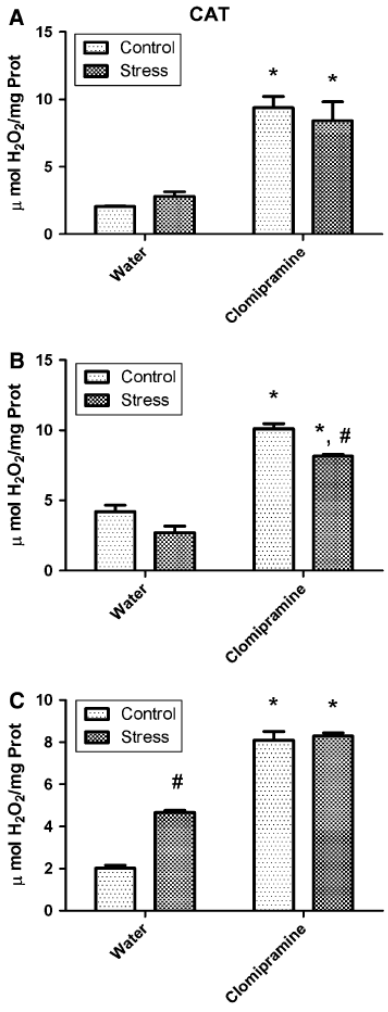

Fig. 4 Effects of repeated restraint stress and chronic treatment with clomipramine on the activity of catalase in cerebral cortex (**a**), hippocampus (**b**) and striatum (**c**) of male rats. Data are expressed as means ± SEM, *N* of five animals/group. * Significant effect of chronic treatment with clomipramine when compared to control group (Two-way ANOVA, $P < 0.05$). # Significant effect of stress in relation to the respective control group (Two-way ANOVA, $P < 0.05$)

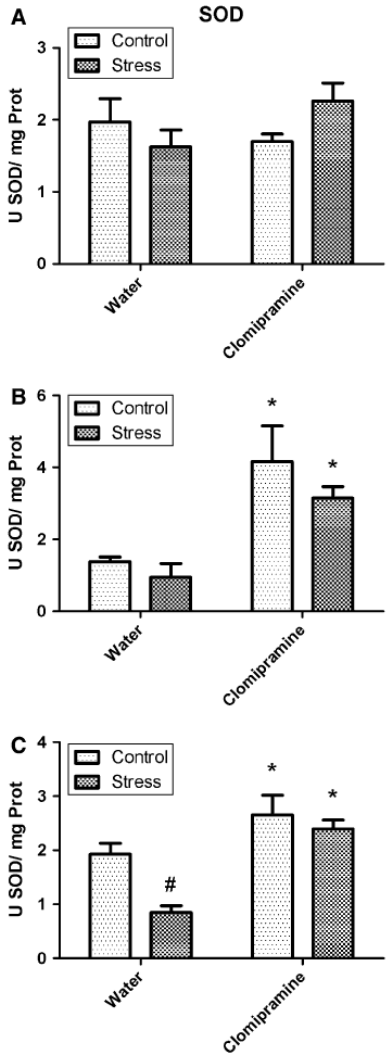

Fig. 5 Effects of repeated restraint stress and chronic treatment with clomipramine on the antioxidant activity of superoxide dismutase (SOD) of cerebral cortex (**a**), hippocampus (**b**) and striatum (**c**) of male rats. Data are expressed as means ± SEM, *N* of five animals/group. * Significant effect of chronic treatment with clomipramine when compared to control group (Two-way ANOVA, $P < 0.05$). # Significant effect of stress in relation to the respective control group (Two-way ANOVA, $P < 0.05$)

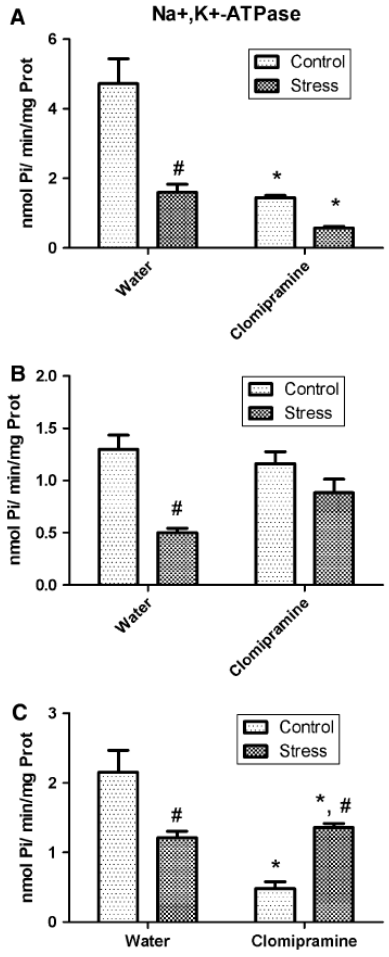

Fig. 6 Effects of repeated restraint stress and chronic treatment with clomipramine on the activity of the enzyme Na^+K^+ATPase cerebral cortex (**a**), hippocampus (**b**) and striatum (**c**) of male rats. Clomipramine was administered with (30 mg/kg/day) for 27 consecutive days, alone or associated with 40 days of repeated restraint stress for 1 h/day. Untreated groups are represented by repeated restraint stress or not. Data are expressed as means ± SEM, *N* of five animals/group. * Significant effect of chronic treatment with clomipramine when compared to control group (Two-way ANOVA, $P < 0.05$). # Significant effect of stress in relation to the respective control group (Two-way ANOVA, $P < 0.05$)

Discussion

This study was conducted to investigate the levels of oxidative stress and the action of the main antioxidant defense systems in the brain of male rats subjected to chronic treatment with clomipramine and repeated restraint stress. The dose and duration of the administration of antidepressant associated with repeated stress model in this study suggests neurochemical changes opposed to beneficial effects of antidepressants under stress conditions. Interestingly, we found that the treatment with clomipramine associated with repeated restraint stress resulted in changes in the antioxidant defense system, lipid peroxidation and

production of free radicals in cerebral cortex, hippocampus and striatum.

The increase in the lipid peroxidation observed in the striatum of the stressed group was not identical to the production of free radicals analyzed by the DCFH–DA test. During the application of repeated restraint stress, there is an increased production of free radicals. However, just 24 h after the last session of stress, the levels of DCFH–DA may be reduced suggesting that free radicals could dissipate after the end of the stressor stimulus in this brain region [28]. Stress provides a deficit in the signaling of glucocorticoid receptors (GR) in the brain [29] by increasing the levels of circulating glucocorticoids and therefore the production of ROS in sensitive regions to the axis LHPA [30]. We suggest that repeated restraint stress and the treatment with clomipramine cause a regional vulnerability [4]. Clomipramine is distributed in the cerebral cortex and in a lesser part in striatum and hippocampus during the treatment of psychiatric disorders [31]. The vulnerability can be attributed to differences in the antioxidant capacity [30], cellular oxidative metabolism and production of compounds that can lead to an increase in the formation of free radicals [32, 33].

Clomipramine is the most potent antidepressant tricycilc and has two clinically significant serotonergic effects efficacy in obsessive compulsive disorder (other TCAs do not have) and adverse effects at high doses [34]. Therapeutic doses (10–20 mg/kg/dia) determine an increase GR function and expression in neuronal cell cultures [35]. However, reduced GR function in vitro by antidepressants has also been described [36], which may suggest increased production of ROS because antidepressants control GR function in vitro by regulating the intracellular concentration of glucocorticoids [37]. In the presence of glucocorticoids, which are not substrates for steroid transporters, the antidepressant-induced GR activation and downregulation lead to a reduced GR function [38]. We suggest that according to the dose administered here, it could occur an imbalance between GR and glucocorticoids causing ROS production.

On the other hand, the exposure of rats to different antidepressant drugs produces long-lasting alterations in cortical beta-adrenoreceptors and in 5-HT2 receptors. Furthermore, the exposure to monoamine uptake blockers results in more permanent changes in monoaminergic function in various brain regions, which demonstrates a dysfunction of serotonergic and noradrenergic neurotransmission [39]. The blockade of histamine receptors, cholinergic and alpha-adrenergic receptors may cause poor tolerability and risk of toxicity. However, the precise mechanisms involved in the unexpected potentiation of DCFH–DA and TBARS in groups receiving clomipramine and repeated restraint stress are still unknown.

It is important to consider that the increase in ROS generation does not necessarily indicate that there is oxidative damage to cellular macromolecules, since they can be detoxified by antioxidant defense systems [40]. The imbalance between the production of free radicals and antioxidant defense system is the key to understand the oxidative stress.

The levels of NP-SH reduced in the group subjected to repeated stress of restraint plus clomipramine in all structures analyzed indicating a failure in the antioxidant defense system of GSH, which is not in accordance with other studies [41]. A decrease in cerebral glutathione was found in recent studies using a model of depression induced by stress in rats [42]. In conditions of cytotoxicity a depletion of GSH levels may occur mainly in the hippocampus [43, 44]. A similar result was observed in the stress plus clomipramine group but only in the hippocampus and striatum. The chronic administration of antidepressants at therapeutic doses recover GSH levels in animals subjected to stress, which indicates the beneficial effects of treatment with restraint stress (4 h/day) followed by 10 mg/kg/day, for 21 days [41]. However, in our study a reduction of glutathione levels through the analysis of the NP-SH which may suggest an oxidative stress. According to our data, the loss of GSH may occur due to an event, such as an oxidative mechanism caused by the use of antidepressants.

CAT and SOD are the most important antioxidant enzymes in response to antidepressant treatments [41]. We observed that repeated restraint stress reduced the activity of SOD but enhanced CAT in striatum, suggesting that increased lipid peroxidation in this study associated with reduced activity of these enzymes explains the effects of repeated restraint stress in this structure. The impairment of antioxidant enzyme defenses has been reported as a possible component of GC-mediated neuroendangerment [45, 46]. Thus, the alterations induced in the present study may be induced by different aspects of the stress response, and may not necessarily involve GC. On the other hand, when we associate the stress of repeated restraint with clompramine we found an increased activity of CAT and SOD. Although, the precise mechanisms involved in the unexpected potentiation of CAT and SOD activity in rats receiving both clomipramine and stress treatments, as observed in this study, are still unknown, it has been reported that antidepressants increase the levels of corticoesterone suggesting that a further activation of the HPA axis in chronically stressed rats may be induced by these drugs possibly causing this interaction [30]. In essence, the simultaneous and synchronized elevation of CAT and SOD levels in combination with reduced levels of NP-SH by the treatment with clomipramine suggests the reduced resistance of chronically stressed rats compared to animals exposed to stress, but did not receive any treatment.

Lipid peroxidation of membrane modifies the release of neurotransmitters and absorption, ion channel activity, and the function of ATPases [47]. Since Na^+, K^+-ATPase is crucial for maintaining ionic gradients in neurons [48], it is acceptable that a reduction in the activity of this enzyme may affect neural activity and memory storage. We found a decrease in the activity of Na^+, K^+-ATPase in the group subjected to repeated stress by the restraint that agrees with other studies [32]. In this context, different studies have demonstrated the effect of antidepressant drugs on the activity of Na^+, K^+-ATPase [49, 50]. Tricyclic antidepressants inhibit the activity of the enzyme Na^+, K^+-ATPase in the rat brain and there is evidence that tricyclic drugs alter the structural organization of the lipid membranes [50]. We observed that the stress associated with treatment with clomipramine and control plus clomipramine reduces the activity of Na^+, K^+-ATPase in specific brain regions related to cognitive function.

In conclusion, the present findings suggest that the treatment with clomipramine alters the antioxidant defense system and the production of free radicals mainly in the hippocampus and striatum when therapeutic doses are surpassed. Clomipramine is one of the most effective TCAs known and is regarded as the drug of choice for treatment of severely depressed patients. However, when it is administered chronically at doses above the therapeutic range, it may cause biochemical imbalances in the antioxidant defense system when combined with repeated restraint stress. Thus the treatment used here was not able to prevent lipid peroxidation induced by repeated restraint stress leading to imbalance with enzymatic activity of SOD, CAT, Na^+, K^+-ATPase and a decrease in NP-SH levels. Further studies are needed to understand the molecular mechanisms involved between treatment with clomipramine and repeated restraint stress as observed in this study.

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4.2 Artigo Científico 2:

4.2.1 Efeitos do estresse repetido por contenção e clomipramina sobre a atividade da Na+/K+-ATPase e comportamento em ratos

Effect of repeated restraint stress and clomipramine on Na+/K+-ATPase activity and behavior in rats

Rodrigo de Souza Balk , Michele Hinerasky da Silva, Jessika Cristina Bridi, Nelson Rodrigues Carvalho, Rafael de Lima Portella , Fernando Dobrachinski, Guilherme Pires Amaral, Rômulo Barcellos , Glaecir Roseni Mundstock Dias , João Teixeira Batista da Rocha, Nilda Berenice Vargas Barbosa , Felix Alexandre Antunes Soares

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Effect of repeated restraint stress and clomipramine on Na^+/K^+-ATPase activity and behavior in rats

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ABSTRACT

Activation of the limbic-hypothalamic-pituitary-adrenal axis (LHPA) and the release of glucocorticoids are fundamental for the adaptive response and immediate survival of an organism in reaction to acute stimuli. However, high levels of glucocorticoids in the brain may produce neuronal injury and a decrease of Na^+/K^+-ATPase activity, with effects on neurotransmitter signaling, neural activity, as well as the whole animal behavior. Clomipramine is a tricyclic antidepressant that inhibits the reuptake of serotonin and norepinephrine by indirect actions on the dopaminergic system and LHPA axis. Its chronic use increases the body's ability to cope with stress; however, high doses can potentiate its side effects on memory, learning, and sensory motor function. The purpose of the present study was to compare the effect of repeated restraint stress and clomipramine treatment on Na^+/K^+-ATPase activity and on the behavior of male rats. Changes in the behavioral response were evaluated by measuring the memory, learning, anxiety, and exploratory responses. Our results showed that exposure to repeated restraint stress reduced levels of Na^+/K^+-ATPase in brain structures and changed short and long-term memory, learning, and exploratory response when compared to the control group. Exposure to clomipramine treatment increased anxiety levels and reduced Na^+/K^+-ATPase activity in the cerebral cortex as well as short term memory, learning, and exploratory response. In conclusion, the present results provide additional evidence concerning how repeated restraint stress and clomipramine chronically administered at higher dose levels affect the neural activity and behavior of male rats.

1. Introduction

Na^+/K^+-ATPase is the enzyme responsible for the active transport of sodium and potassium ions in the nervous system, thus maintaining the ionic gradient necessary for neuronal excitability and regulation of neuronal cell volume. Its activity is decreased in patients with bipolar affective disorder and other psychiatric disorders such as depression (Zanatta et al., 2001). As a consequence, a decrease of Na^+/K^+-ATPase activity directly affects neurotransmitter signaling and neural activity, as well as the whole animal behavior (Jamme et al., 1995). Chronic stress models alter the functionality of this enzyme (Crema et al., 2010). Three isoforms of the Na^+/K^+-ATPase are found in the brain, but vary by cell type and level of expression, and differentially modulate locomotor activity, anxiety-like behavior, spatial learning, and memory (Moseley et al., 2007). The α1 isoform is found in many central nervous system (CNS) cell types; the α2 isoform is predominantly expressed in glia cells, and the α3 isoform is only expressed in neurons (Moseley et al., 2003). Recent studies have shown a significant correlation between chronic treatment with clomipramine and repeated restraint stress on Na^+/K^+-ATPase activity in the brain regions of male rats (Balk et al., 2010).

Activation of the LHPA axis and the release of glucocorticoids are fundamental for the adaptive response and immediate survival of an organism in reaction to acute stimuli. However, high levels of glucocorticoids in the brain may produce neuronal injury (Fontella et al., 2005). A substantial amount of literature has focused on anatomical, behavioral, and neuroendocrine changes associated with exposure to chronic stress.

Tricyclic antidepressants (TCAs) have been prescribed for a long time for the treatment of depression. Moreover, indications for their use are associated with anxiety, eating disorders, and chronic

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DN-1493; No. of Pages 8
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pain syndromes (Demerdash and Mohamadin, 2004). The mechanism by which TCAs exert their antidepressant effects is not clearly established to date. However, the capacity for blocking the re-uptake of centrally active neurotransmitters is generally accepted as their primary mode of action (Potter et al., 1998).

Clomipramine is a tricyclic antidepressant widely used for the treatment of depression and obsessive compulsive disorder, mainly by inhibiting the re-uptake of serotonin and norepinephrine in the brain (Peters et al., 1990; Trimble, 1990). These neurotransmitters can influence the neuroplasticity in the brain and both are involved in mediating the therapeutic effects of most currently available antidepressants (Delgado, 2004). It has been shown that the chronic administration of clomipramine at relatively low doses reduces depression (Bhagya et al., 2008; Srikumar et al., 2006) as well as the behavioral deficits and cholinergic dysfunction induced by stress in rats (D'Aquila et al., 2000). However, the species and sex of animals, as well as type, duration, and severity of stress can modify the response induced by stress and clomipramine (Consoli et al., 2005). Literature data have also reported that high doses (30 mg/kg) of clomipramine are toxic to experimental animals (Calegari et al., 2007). The adverse effects induced by clomipramine administration due to the blockade of serotonin reuptake include difficulties in learning and memory (Burgos et al., 2005). Recent studies have revealed that endogenous serotonin and norepinephrine can modulate cognitive processes, particularly learning and memory. However, at present, the mechanisms, locations, and durations of serotonergic and noradrenergic system involvement remain unclear (Millan et al., 2000). TCAs inhibit the activity of the enzyme Na^+/K^+-ATPase in the rat brain and there is evidence that tricyclic drugs alter the structural organization of the lipid membranes (Pedrazza et al., 2007), causing cognitive deficits.

In this context, the objective of this study was to evaluate the effects of repeated restraint stress in combination with a high concentration of clomipramine to verify whether the association between a stress condition and the use of TCA could affect the Na^+/K^+-ATPase activity in different brain structures, and produce changes in the behavior of adult male rats.

2. Experimental procedures

2.1. Animals

Forty adult male Wistar rats (250–300 g in weight and 60 days old) from our own breeding colony were used. The animals were placed in groups of five animals in cages made of Plexiglas measuring 42 cm × 34 cm × 16 cm, with the floor covered with sawdust. They were kept in a room with light-dark cycle of 12 h with the lights on between 7:00 and 19:00 h and temperature (20–25 °C) controlled receiving water and food *ad libitum*. Rats were first habituated in the room where the behavioral tasks were performed by at least 30 min. When the group was exposed to more than one subsequent behavioral task, there was a period of three days of resting between tasks.

The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources (number of protocol: 23081.007146/2010-17) of the Federal University of Santa Maria, Brazil.

2.2. Experimental groups

The animals were divided into two groups: control group, only manipulated for the necessary maintenance of their cages in good health, and stress group, which received a treatment of repeated restraint stress for a forty-day period. On day 14 of this experimental period, each initial group was subdivided into 4 different groups: (1) control, (2) stress, (3) clomipramine, and (4) stress + clomipramine.

2.3. Repeated restraint stress model

Stress condition was performed according to the model of repeated restraint stress as described by Ely et al. (1997). The animals were immobilized in plastic tubes measuring 25 cm × 7 cm of diameter adjusted to the size of the animal. The animal behavior was carried out 1 h per day, five days a week, for forty days in the morning between 8:00 and 10:00. The control group was just handled and thus not subject to any repeated stress situation. The repeated restraint stress was applied at least one hour after exposure to behavioral tasks.

2.4. Clomipramine treatment

The animals received clomipramine (30 mg/kg) for 27 consecutive days previously described by Calegari et al. (2007) in the drinking water from the fourteenth day of the trial period after the stress-induced behavioral alterations had been established. This dose was chosen because it provides, when administered chronically, possible toxic effects on the body in rodents according to Calegari et al. (2007). The repeated restraint stress continued during the whole treatment period. The rats untreated with the drug received regular water. Clomipramine was placed in dark bottles due to its photosensitivity and the water consumption by animals was analyzed daily in order to adapt the dose to be administered. Oral administration was chosen because it is the most common use of antidepressants in patients with psychiatric disorders according to Lucassen et al. (2004).

2.5. Tissue preparation

At the end of the treatment period, rats were killed by decapitation. The brain was removed and the structures like hippocampus, striatum and cerebral cortex were quickly dissected, placed on ice, and immediately homogenized in cold 50 mM Tris–HCl pH 7.4. The homogenates were centrifuged at 4000 × *g* for 10 min to yield the low-speed supernatant fractions that were used for different biochemical assays in all trials.

2.6. Sodium potassium (Na^+/K^+-ATPase) activity

The Na^+/K^+-ATPase activity was estimated by the method of Muszbek (1977). The enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of hippocampal and striatal aliquots. Before, the slices were incubated with Meth (0.05, 0.1, 0.5 and 1 μM) at different times (5 or 15 min). Then, the reaction mixture containing 95 mM NaCl, 15 mM KCl, 1.0 mM ATP (disodium salt), 38 mM Tris–HCl buffer (pH 7.4) was added to aliquot of homogenized slices (50 μg of protein) in a final volume of 0.3 mL. After a 5-min pre-incubation at 37 °C in the presence of 0.1 mM ouabain to specifically inhibit Na^+/K^+-ATPase, the reaction was initiated by addition of ATP and terminated after 15 min of incubation by addition of 1 mL of color reagent (Ammonium Molybdate 2%, Triton X 5% solubilized in H_2SO_4 1.8 M). The released inorganic phosphate was measured spectrophotometrically at λ = 405 nm. Na^+/K^+-ATPase activity was calculated from the difference between amounts of inorganic phosphate found after incubation in the absence and presence of 1.5 M ouabain.

2.7. Behavioral analysis

Aiming to verify whether chronic treatment with clomipramine could or not potentiate the effects caused by repeated restraint stress on cognitive function in animals we performed four behavioral tasks. The Video-Track® system, used for recording the animal behavior, was composed of a camera connected to a computer.

2.7.1. Object recognition task

The object recognition task was performed according to Stangherlin et al. (2008). Novel object recognition is a type of non-aversive and non-spatial memory. Rodents naturally tend to approach and explore novel objects, which are assumed to have no natural significance to the animal and which have never been paired with a reinforcing stimulus. They also show an innate preference for novel over familiar objects. Rodents readily approach objects and investigate them physically by touching and sniffing the objects, rearing upon and trying to manipulate them with their forepaws. This behavior can be easily quantified and utilized to study simple recognition memory as well as more complex spatial-, temporal- and episodic-like memory in rodents. The standard object recognition task measures the spontaneous behavior. The novelty-preference paradigm does not require lengthy training and does not induce high levels of arousal and stress (Stangherlin et al., 2008). The behavioral task was performed in a 45 cm × 45 cm open field surrounded by 30 cm height walls made of brown plywood. The behavioral task was conducted in a moderately lighted room (30 lx). All animals were given a habituation session where they were left to freely explore the open field for 5 min. No object was placed in the box during the habituation trial. Subsequently, four objects were used: A1, A2, B and C. The "A" objects were two identical triangles, the "B" object was a ball and the "C" object was a rectangle. All objects were made of plastic material, with 10 cm × 10 cm (length × height). Each object had the pattern of color, as follows: blue, red and yellow. Twenty-four hours after habituation, training was conducted by placing each individual rat for 5 min into the field, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners, 10 cm from the walls. In a short-term memory (STM) test given 1.5 h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. All objects presented similar textures, colors, and sizes, but distinctive shapes. The percentage of the total exploration time that the animal spent investigating the novel object was

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DN-1493; No. of Pages 8
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the measure of recognition memory. Between trials the objects were washed with 10% ethanol solution. In a long-term memory (LTM) test given 24 h after training, the same rat explored the field for 5 min in the presence of a familiar object A and a novel object C. Recognition memory was evaluated as for the STM test. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Data are expressed as the mean ± SEM percentage time exploring any of the objects (training) or the novel objects. Exploratory preference in: Training = (A2/(A1 + A2)) × 100; STM = (B/(A1 + B)) × 100; LTM = (C/(A1 + C)) × 100.

2.7.2. Open field task

The open field task is a simple assessment used to determine general activity levels, gross locomotor activity and exploration habits in rats. A 50-cm high, 40 cm × 60 cm open field made of brown plywood was used. The floor was subdivided with white lines into 12 equal 13.3 cm × 15.0 cm rectangles. The behavioral task was conducted in a moderately lighted room (30 lx). The animals were gently placed facing the left corner and allowed to explore the arena for 3 min. The number of rearings and line crossings was counted as a parameter of motor activity. The rat is placed in the center of the open field arena and allowed to freely move around for 5 min while being tracked by an automated tracking system. At the conclusion of each trial the surface of the arena is cleaned with 90% ethanol (Walsh and Cummins, 1976).

2.7.3. Elevated plus maze task

Elevated plus maze task is based on the natural aversion of rats for open spaces and uses an elevated plus-maze (60 cm from the floor) consisting of two open arms (50 cm long × 10 cm wide) and two enclosed arms of equal length and width (50 cm × 10 cm with 40 cm high walls) forming a square cross with a 10 cm square center piece. The behavioral task was conducted in a moderately lighted room (30 lx as measured at the center of the maze). This task is considered sensitive to the anxiety state of the animal based on the principle that exposure to an elevated and open arm maze leads to an approach conflict that is stronger than that evoked by exposure to an enclosed arm maze. On the day of the experiment, the test initiated after a period of 5 min in the behavioral testing room. Animals were placed individually in the center of the maze, in the junction between open and closed arms, facing one of the open arms, and performance was scored for 5 min. The maze was cleaned thoroughly with warm water between trials. The following parameters were measured: percent time spent in open and closed parts of the maze as well as frequencies of open and closed entries (an arm entry was considered when the rat stepped with all four paws into it) (Conde et al., 1999).

2.7.4. Conditioned place preference task

Conditioned place preference (environmental place conditioning) is a commonly used technique to evaluate preferences for environmental stimuli associated with a positive or negative reward. Moreover, it evaluates the interest in a different environment used as an indicator of integrity of dopaminergic pathways (Figlewicz et al., 2001). The task was performed for eight days. Before beginning the task, the animals were subjected to dietary restriction receiving 90% of their daily diet for 24 h in order to increase the interest for the new food. The animals were placed in a rectangular box divided into two compartments: one white wall illuminated moderately with 30 lx and a black wall, separated by a partition high. On day one, the time that the rats remained in the light compartment and number of crossings made between the two compartments were analyzed for 15 min. No food was available in the apparatus on day one of exposure. From day two to seven, the division between the two compartments was lowered, the compartments were separated and twenty units of fresh food, donut-shaped, 1 cm in diameter (of Kellogg's Froot Loops) were placed daily in the light compartment. The animal remained in the room light or dark for half an hour, alternately; one day the animal stayed in the light compartment and next day in the dark compartment. On the test day (day eight), the sweet food was removed, a mean of sweet food intake between day two and day seven was performed, and the procedure repeated as the day one. We compared the time spent in the light compartment and the number of crossings between the day eight and day one presented by Δ.

2.8. Protein determination

The protein content was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.9. Statistical analysis

Data were analyzed by one or two-way ANOVA followed by Duncan's multiple range test when *F* test was significant (Statistical Packcage for the Social Science version 10.0 for Windows 98 – SPSS Inc., Chicago, IL, USA). Differences between groups were considered significant when $p < 0.05$. Results are expressed as mean (standard error of the mean (SEM)).

3. Results

3.1. Enzyme activities

3.1.1. Na^+/K^+-ATPase

In Fig. 1, one way ANOVA shows that clomipramine treatment caused *per se* a significant reduction in the Na^+/K^+-ATPase activity in cerebral cortex [$F(1, 19) = 9.23$, $p < 0.05$, followed by Duncan's multiple range test] and striatum [$F(1, 19) = 19.09$, $p < 0.05$, followed by Duncan's multiple range test] compared to the control group. The inhibition of the enzyme was also significant in the stress + clomipramine group [$F(1, 19) = 5.41$, $p < 0.05$, followed by Duncan's multiple range test] in striatum and cerebral cortex [$F(1, 19) = 8.89$, $p < 0.05$, followed by Duncan's multiple range test]. On the other hand, repeated restraint stress promoted a significant decrease in the Na^+, K^+-ATPase activity in cerebral cortex [$F(1, 19) = 27.88$, $p < 0.05$, followed by Duncan's multiple range test], hippocampus [$F(1, 19) = 23.33$, $p < 0.05$, followed by Duncan's multiple range test] and striatum [$F(1, 19) = 15.51$, $p < 0.05$, followed by Duncan's multiple range test] when compared to the control group.

3.2. Behavioral analysis

3.2.1. Object recognition task

Fig. 2 shows the effects of chronic treatment with clomipramine and repeated restraint stress on training, short term memory and long term memory. Chronic clomipramine treatment caused *per se* a decrease of exploratory preference in STM [one way ANOVA, $F(1, 29) = 30.61$, $p < 0.05$, followed by Duncan's multiple range

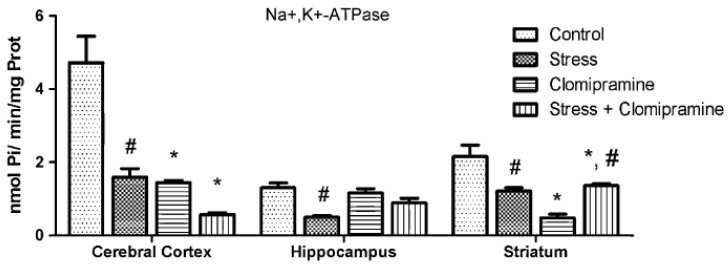

Fig. 1. Effects of repeated restraint stress and chronic treatment with clomipramine on the activity of the enzyme Na^+K^+ATPase in cerebral cortex, hippocampus and striatum of male rats. Clomipramine was administered (30 mg/kg/day) for 27 consecutive days, alone or associated with 40 days of repeated restraint stress for 1 h/day. Untreated groups are represented by repeated restraint stress or not. Data are expressed as mean ± SEM with *N* of five animals/group. *Significant effect of treatment with clomipramine on the control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). #Significant effect of the stressor when compared to the control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$).

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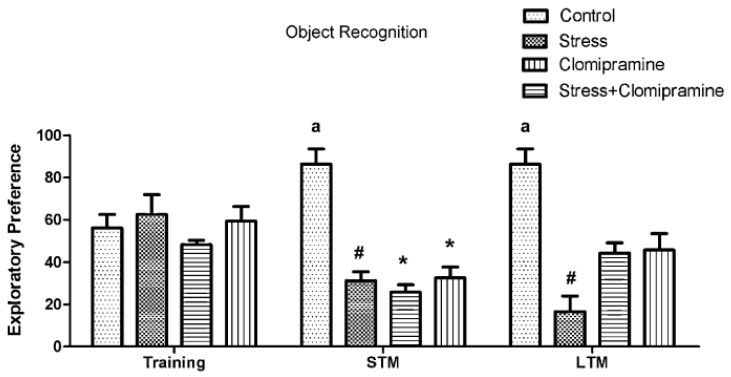

Fig. 2. Effects of repeated restraint stress and chronic treatment with clomipramine on object recognition task in male rats during Training, Short Term Memory (STM) and Long Term Memory (LTM). The percentage of the total exploration time that the animal spent investigating the novel object was the measure of recognition memory. Data are expressed as mean ± SEM, *N* of ten animals/group. [a]Significantly different from control training group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). *Significant effect of treatment with clomipramine when compared to the control group during training (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). #Significant effect of stress in relation to the respective control group during training (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$).

test] and when associated with repeated restraint stress condition caused a further decrease in STM [one way ANOVA, $F(1, 29) = 20.47$, $p < 0.05$, followed by Duncan's multiple range test] when compared to the respective control training group. Moreover, the repeated restraint stress alone changed STM [$F(1, 29) = 20.48$, $p < 0.05$, followed by Duncan's multiple range test] and LTM [$F(1, 29) = 11.94$, $p < 0.05$, followed by Duncan's multiple range test], when compared to values found in the training group. The control groups showed an increase in the exploratory performance in STM and LTM [$F(1, 29) = 21.50$, $p < 0.05$, followed by Duncan's multiple range test] when compared to the control group trained.

3.2.2. Open field task

Statistical analysis of one way ANOVA showed that there was an increase of the crossings [$F(1, 31) = 6.11$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 3A] in the stress + clomipramine group compared to the respective clomipramine group. However, the crossings showed a decrease in the stress + clomipramine group or only clomipramine when compared to the control group [$F(1, 31) = 15.59$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 3A]. The rearings were significantly decreased in the stress [$F(1, 31) = 7.51$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 3B], clomipramine and stress + clomipramine groups [$F(1, 31) = 27.87$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 3B], when compared to the control group.

3.2.3. Plus maze task

Fig. 4 indicates that clomipramine treatment significantly reduced the number of entries in closed arms [one way ANOVA, $F(1, 36) = 9.56$, $p < 0.05$, followed by Duncan's multiple range test Fig. 4B] and open arms [one way ANOVA, $F(1, 36) = 11.94$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 4D] when compared to the control group. The association between chronic clomipramine treatment and stress condition also caused a significant reduction of time spent in open arms [$F(1, 36) = 7.13$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 4C], entries in closed arms [$F(1, 36) = 11.03$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 4B] and open arms [$F(1, 36) = 12.24$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 4D], compared to the control group. There was no significant difference in the stressed group.

3.2.4. Conditioned place preference task

Fig. 5 illustrates the conditioned place preference (CPP) task on the difference between time in the light compartment and crossings in the days eight and one and mean intake of sweet food between day seven and day two in male rats. One way ANOVA show that clomipramine treatment alone or in combination with stress caused a significant decrease in crossings [$F(1, 36) = 21.82$, $p < 0.05$, followed by Duncan's multiple range test, Fig. 5A] and in

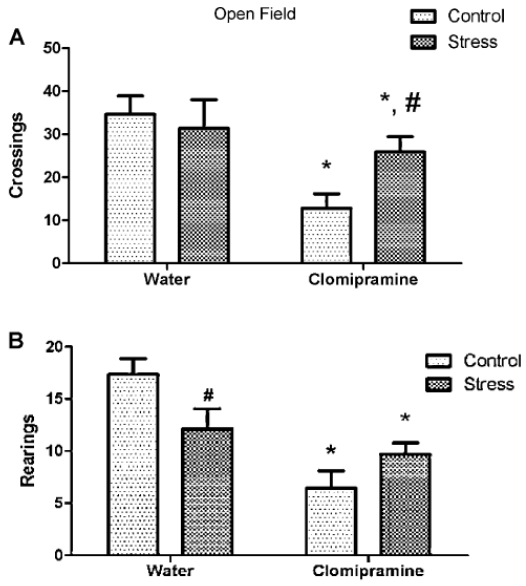

Fig. 3. Effects of repeated restraint stress and chronic treatment with clomipramine on number of crossings (A) and rearings (B) of male rats in open field exposure. Data are expressed as mean 1 SEM, *N* often animals/group. *Significant effect of treatment with clomipramine when compared to control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). #Significant effect of stress in relation to the respective control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$).

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R.d.S. Balk et al. / Int. J. Devl Neuroscience xxx (2011) xxx–xxx 5

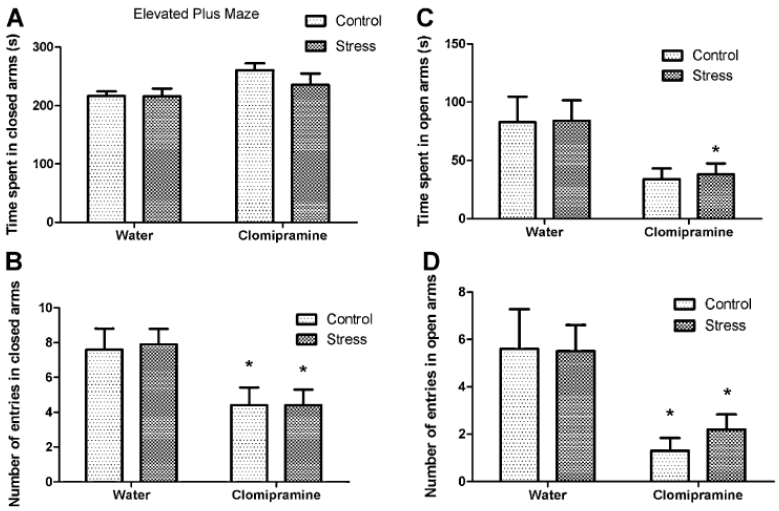

Fig. 4. Effect of repeated restraint stress and chronic treatment with clomipramine on time spent in closed arms (A), number de entries in closed arms (B), time spent in open arms (C) and number of entries in open arms (D) of male rats in elevated plus maze test. Data express in mean ± SEM, *N* of ten animals/group. *Significantly different from the control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$).

the time in the light compartment [F (1, 36) = 18.28, $p < 0.05$, followed by Duncan's multiple range test, Fig. 5B] when compared to the control group. The repeated restraint stress decreased the time in the light compartment when compared to the control group [F (1, 36) = 4.75, $p < 0.05$, followed by Duncan's multiple range test, Fig. 5B]. An increase in the intake of sweet food was verified in the stress + clomipramine group [F (1, 19) = 3.81, $p < 0.05$, followed by Duncan's multiple range test, Fig. 5C] while a decrease was observed in the stressed group [F (1, 19) = 6.60, $p < 0.05$, followed by Duncan's multiple range test, Fig. 5C].

4. Discussion

In our study, we analyzed the effects of chronic treatment with clomipramine and repeated restraint stress on the activity of Na^+/K^+-ATPase and its relationship regarding the behavior of male rats, which have been used as animal models of depression.

We have applied models of anxiety-like and depression-like behavior and memory assessment. The main findings in the present investigation indicated a correlation between repeated restraint stress and administration of 30 mg/kg of clomipramine, evidenced by a reduction in the Na^+/K^+-ATPase activity, which considerably influenced performance in the proposed activities for behavioral assessment. Taken together, these results strongly implicate in behavioral deficits caused in stressful situations and with high doses of clomipramine.

The repeated restraint stress model employed in the present study incorporated both physical and psychological components of stress, and is widely used to induce oxidative and neurotoxic damage (Zafir et al., 2009), allowing researchers to examine the long-term features of depression, for which stress is a predisposing and contributing factor (De Kloet et al., 2005).

Clomipramine is a reference drug for the treatment of emotional disorders such as obsessive–compulsive disorder and panic disorder, and has been widely used for the treatment of psychiatric disorders (Calegari et al., 2007).

Because Na^+/K^+-ATPase is crucial for maintaining ionic gradients in neurons (Xiong and Stringer, 2000), it is accepted that a reduction in the activity of this enzyme may affect neural activity, and consequently, memory storage (Stangherlin et al., 2008). Ouabain, previously known as a cardiotonic steroid capable of inhibiting Na^+/K^+-ATPase, has recently been identified as an endogenous compound produced by the adrenals and hypothalamus. Ouabain is found circulating in the plasma and is also released under stress conditions (Crema et al., 2010). Na^+/K^+-ATPase inhibition by ouabain is known to decrease norepinephrine, dopamine, and serotonin uptake, and increase acetylcholine release (Moseley et al., 2007). Hamid et al. (2009), showed that brain areas that upregulated sodium pump isoforms (frontal cortex and basal ganglia) maintained normal pump activity, while the hippocampus did not upregulate any isoforms, and experienced a decrease in pump activity. The alpha 3 isoform is neuron-specific. Additionally, glucocorticoids have been shown to be positive modulators of the Na^+ K^+-ATPase alpha 3 subunit mRNA in some regions of the brain, and this isoform has been reported to be altered in animal models related to changes in behavior. On the other hand, other subunits of this enzyme are present in the brain and may be involved in the reduction observed in the enzyme activity (Crema et al., 2010).

Central catecholaminergic and serotonergic activities may be modulated by exposure to stress situations. The systems mediating 5-HT regulatory mechanisms for Na^+/K^+-ATPase activity have been shown to depend on the brain area and on the particular physiological conditions.

Depression, particularly stress-associated cases, may result from the atrophy of pyramidal neurons in the hippocampus (Gamaro et al., 2003). This atrophy, with a reduction in the number of synapses, may be involved in several repeated stress effects, including the reduced Na^+/K^+-ATPase activity (Yamamoto and Quinton, 2007). The reduction of Na^+/K^+-ATPase activity in the cerebral cortex, hippocampus, and striatum could also be due to the direct influence of high concentrations of stress hormones. In contrast, it is unknown whether stress produces damage to the

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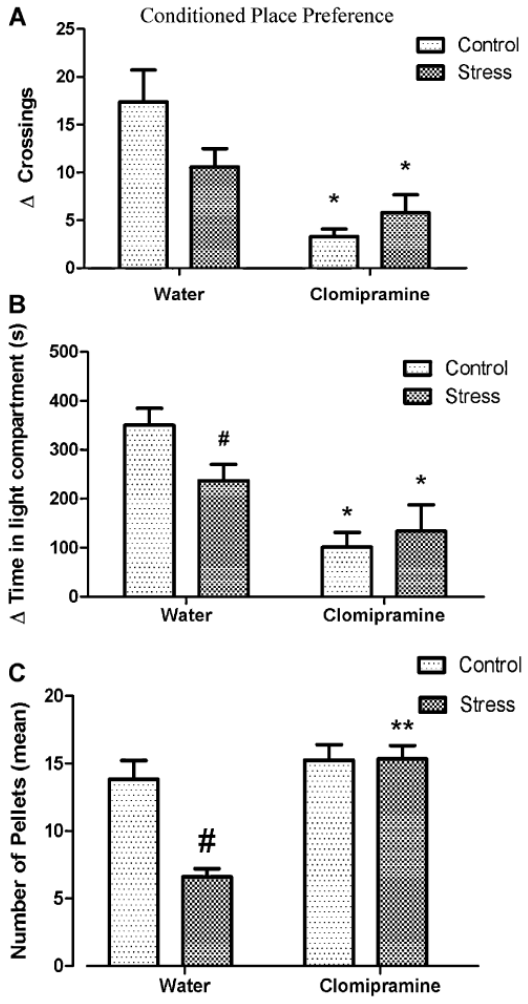

Fig. 5. Effects of repeated restraint stress and chronic treatment with clomipramine on the conditioned place preference task. Data are expressed as the difference between number of crossings (A) and time in light compartment in the day eight and day one (B) without the presence of sweet food and mean intake of sweet food from the seven day and two day (C) of male rats. Data are expressed as means ± SEM, *N* of ten animals/group. *Significant effect of treatment with clomipramine when compared to control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). #Significant effect of repeated restraint stress in relation to respective control group (One-way ANOVA, followed by Duncan's multiple range test, $p < 0.05$).

striatum, despite evidence that stress can also increase glutamate release in this area (Moghaddam, 1993). According to Balk et al. (2010), TCAs inhibit the activity of Na^+/K^+-ATPase in the rat brain, and especially in the cerebral cortex, by decreasing ATP and ADP hydrolysis in synaptosomes of rats (Barcellos et al., 1998).

This could be explained according to studies conducted by Moseley et al. (2007), which showed that deficiency of the Na^+/K^+-ATPase alpha 2 isoform results in increased anxiety-related behavior and reduced locomotor activity, and deficiency of the Na^+, K^+-ATPase 3 alpha isoform results in spatial learning and memory deficits.

Considering that the maintenance of Na^+/K^+-ATPase activity is essential for brain function and the reduction of this activity is associated with excitotoxicity and neuronal damage, we suggest that the reduction of Na^+/K^+-ATPase activity induced by repeated restraint stress and chronic treatment with clomipramine may be deleterious and, that the alpha isoform can be associated with increased anxiety behavior.

The hippocampus is essential for memory processing during the object recognition task (Clark et al., 2000) and pharmacological studies indicate that hippocampal formation is required for acquisition and storage of the contextual details and temporal order of previous experiences (Balderas et al., 2008). The performance in the recognition memory task appears to be dependent on interconnected neural circuits involving the pre-frontal cortex, hippocampus, thalamus, and ventral striatum. Moreover, a disconnection between the hippocampus and the pre-frontal cortex has been shown to disrupt this performance (Burgos et al., 2005), while clomipramine has been shown to impair memory performance in rats (Naudon et al., 2007). Chronic stress also has a dramatic influence on short-term and long-term episodic memory performance (Elizalde et al., 2008). Evidence indicates that poor memory is common in patients suffering from major depression and that memory performance can significantly improve after chronic treatment with antidepressants (Burt et al., 1995). However, antidepressants, which alleviate depression symptoms, can also have a negative impact on cognitive functions (Danion, 1993).

In line with this concept, and based on our findings, the clear decrease in short-term and long-term memory following repeated restraint stress application could be associated with a respective decrease in Na^+/K^+-ATPase activity resulting from high glucocorticoid levels, especially in the cerebral cortex and hippocampus. Additionally, clomipramine treatment could produce changes in the structural organization of the lipid membranes and affect the connections between the cerebral cortex, hippocampus, and striatum.

Concerning emotional behavior, there is some evidence suggesting that repeated restraint stress produces an increase in anxiety behavior, and the "open field" has been suggested as a good model for the normal anxiety that animals experience when confronted with a stressful or threatening situation (Bravin et al., 2005). In addition, differences in brain excitability due to changes in Na^+/K^+-ATPase activity have been suggested to produce changes in behavior in the open field model (Alves et al., 2005). Repeated restraint stress significantly reduced the number of rearings in the open field task as well as the number of crossings (not significantly), according to studies by Liu et al. (2008). Clomipramine treatment improves behavioral activities at doses of 5 mg/kg (Liu et al., 2008). These findings oppose our results showing a reduction in the frequency of rearing and crossing in the open field, indicating a probable anxiogenic effect of this drug, possibly due to a direct relationship with dosage (Bravin et al., 2005).

The elevated plus-maze includes 2 additional anxiety-provoking environmental parameters (height and a totally open area); therefore, levels of anxiety might be more easily detectable in this test. When exposed to an elevated plus maze, rats tend to avoid the open arms and prefer to stay in the enclosed arms. This way, drugs that elicit a decrease of the time spent in the open arms are considered as anxiogenic (Pellow et al., 1985). This is consistent with our study, which demonstrates that chronic treatment with clomipramine could increase the levels of anxiety.

The striatum may be an important brain area in decision-making. Many striatal neurons decrease or increase their activity as learning progresses (Tremblay et al., 1998; Brasted and Wise, 2004). Importantly, the activity of these neurons has been found to be modulated by the expected presence, amount, probability of reward, magnitude of attention, and memory required to execute

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the task (Kawagoe et al., 1998; Shidara et al., 1998; Cromwell and Schultz, 2003). Indeed, studies on the synaptic mechanisms in the striatum have shown that long-term potentiation (LTP) or long-term depression (LTD) can occur in the corticostriatal synapses, depending on the combination of cortical inputs, striatal outputs, and D1 and D2 dopaminergic inputs (Reynolds and Wickens, 2002). The place conditioning data could also be explained as a decreased ability, following stress, to associate the reward with the environment in which it was presented (Papp et al., 1991). The most important environmental events that may lead to reinstatement are re-exposure to the drug itself, presentation of drug-associated stimuli or cues, and exposure to a stressful event (Do Couto et al., 2006).

We suggest that the reduction of time in the light compartment and crossings could be associated with a reduction in Na^+/K^+-ATPase activity occurring during dysregulation of the dopaminergic system in the striatum, because there is further evidence of limbic–striatal interactions underlying reward-related processes. The reduction in Na^+/K^+-ATPase activity could change this interaction (Everitt et al., 1991).

The behavioral passivity displayed by restraint stressed animals is an analogue of decreased motivation, which is a behavioral correlate of depressive symptoms in humans (Porsolt, 1979). Control animals typically exhibited a high preference for sweet food (Plaznik et al., 1989), while this preference was markedly reduced following exposure to restraint stress, in agreement with earlier reports (Zafir et al., 2009). The repeated restraint stress induced a significant decrease in sweet food ingestion, an effect that was reversed by the chronic treatment with a tricyclic antidepressant, in accordance with other studies (Clark et al., 2000).

The precise mechanisms involved in the reduction of Na^+/K^+-ATPase activity and deficits in behavioral tasks in rats receiving both clomipramine and stress treatments, as observed in this study, are still unknown. Doses of 10–20 mg/kg/day demonstrated increased glucocorticoid receptor (GR) function and expression in neuronal cell cultures (Lai et al., 2003). However, reduced GR function *in vitro* by antidepressants has also been described (Pariante et al., 1997), which may suggest deficits in behavioral tasks because antidepressants control GR function *in vitro* by regulating the intracellular concentration of glucocorticoids (Pariante et al., 2001). In the presence of glucocorticoids, the antidepressant-induced GR activation and downregulation lead to a reduced GR function (Carvalho et al., 2008). We suggest that according to the dose administered here, an imbalance between GR and glucocorticoids could occur.

In summary, clomipramine is one of the most effective TCAs known and is considered the drug of choice for treatment of severely depressed patients. However, when it is chronically administered at higher doses, it may produce behavioral changes induced by a decreased activity of Na^+/K^+-ATPase. These results contribute to other studies conducted in our laboratory, which indicated that the dose of clomipramine used can lead to oxidative stress in brain structures, and the reduced activity of Na^+/K^+-ATPase results in an increase in cognitive deficit. Further studies are needed to understand the molecular mechanisms involved regarding treatment with clomipramine and repeated restraint stress as observed in this study.

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4.3 Manuscrito 1:

4.3.1 Diferentes respostas do estresse repetido por contenção e administração crônica de clomipramina relacionadas ao sexo sobre parâmetros de estresse oxidativo e comportamento em ratos

Sex-related differential response to repeated restraint stress and chronic administration of clomipramine on parameters of oxidative stress and behavior in rats.

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Sex-related differential response to repeated restraint stress and chronic administration of clomipramine on parameters of oxidative stress and behavior in rats.

Running title: Clomipramine alters brain oxidative parameters and behavior

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ABSTRACT

Repeated stress or stressful events has been reported to induce anxiety and depression. Differential responses to repeated stress and antidepressant treatments have been observed in males and females. Clomipramine is a tricyclic antidepressant that inhibits the reuptake of serotonin and norepinephrine by indirect actions on the dopaminergic system and limbic-hypothalamic-pituitary-adrenal (LHPA) axis. Its chronic use increases the body's ability to cope with stress; however, high doses can potentiate its side effects on memory, learning, and sensory motor function. Thus, this study investigated sex-related differential response to repeated restraint stress and chronic administration of clomipramine on parameters of oxidative stress in cerebral cortex, hippocampus and striatum and behavior in rats. Male and female rats were divided into control and repeated restraint stress, and subdivided into treated or not with clomipramine during 40 days of stress and 27 days of clomipramine treatment (30mg/kg). Our results showed that in female rats, the repeated restraint stress induced an increase of the free radicals production in all brain structures and lipid peroxidation in striatum and modified CAT and NP-SH activities. No significant difference was observed in SOD and Na+/ K+-ATPase activity. On the other hand, in male, NP-SH decrease only in cerebral cortex, SOD activity in striatum, Na +/ K+-ATPase in all brain structures while CAT activity increased in striatum. The negative effects of stress are enhanced with the clomipramine, particularly in male rats with an increase in lipid peroxidation in all brain structures, free radicals production in cerebral cortex and enzymatic activity of CAT modified in the hippocampus. Interestingly these effects in females were observed only in the cerebral cortex that increased production of free radicals and decreased NP-SH activity as well as an decrease in activity of CAT. Changes in the behavioral response were evaluated by measuring the memory, learning, anxiety, and exploratory responses. Repeated restraint stress decrease short and long term memory only in male rats. Motivation was the main behavioral change in female rats and the anxiety in male rats submitted to stress model used in this study. Clomipramine combined with stress, increased levels of anxiety and reduced long-term memory in both sexes. However, in other tasks performed, clomipramine showed a protective activity in females but not males. In conclusion, the present results provide additional evidence concerning how repeated restraint stress and clomipramine chronically administered at higher dose levels affect the neural activity and behavior of both sexes.

Keywords: sexes, repeated restraint stress, clomipramine, oxidative stress, behavior.

1. INTRODUCTION:

Repeated stress or stressful events can lead to the development of a number of medical and psychopathological disorders, including anxiety and depression (Kamarack and Jennings, 1991; Kessler, 1997; Lancman et al., 1993). The nature of the stress response and its impact on health depends on the type of stressor as well as the sensitivity of the organism to the stress (Chadda and Devaud, 2005). Activation of the limbic-hypothalamic-pituitary-adrenal (LHPA) axis and the release of glucocorticoids (GC) are fundamental for the adaptive response and immediate survival of an organism in reaction to acute stimuli. However, high levels of GC in the brain may produce neuronal injury (Fontella et al., 2005). Immobilization stress is followed by an increase in free radical levels, especially in lipid peroxidation, in plasma and many structures of brain, such as cerebral cortex, cerebellum, hippocampus, and midbrain changing the balance between oxidative and antioxidant factors in brain leading to oxidative damage (Tabajara et al., 2003).

A substantial amount of literature has focused on anatomical, behavioral, and neuroendocrine changes associated with exposure to chronic stress. Differential responses to stress have been observed in males and females, and sex-specific outcomes have been noted in terms of stress-related disease incidence, but the mechanisms through which these outcomes are mediated remain unclear (Zavala et al., 2011).

Estrogens are known to have antioxidant properties and they can inhibit lipid peroxidation in vitro, which might contribute directly to their neuroprotective effect (Prediger et al., 2004) and has been demonstrated to impair glucocorticoid negative feedback (Burgess and Handa, 1992), whereas testosterone inhibits LHPA axis activity (Seale et al., 2004). Acting this way, estrogen might facilitate the onset and progression of stress induced affective disorders. Significant effects of gender on oxidant levels were also found in humans: men present greater values of hydrogen peroxide production in plasma when compared to women (Lacy et al., 2000). Apart from gender-related differences in the prevalence of depression, the differences in treatment response to antidepressants are also observed (Elaković et al., 2009).

Tricyclic antidepressants (TCAs) have been prescribed for a long time for the treatment of depression. Moreover, indications for their use are associated with anxiety, eating disorders, and chronic pain syndromes (Demerdash and Mohamadin, 2004). The mechanism by which TCAs exert their antidepressant effects is not clearly established to date. However, the capacity for blocking the re-uptake of centrally active neurotransmitters is generally accepted as their primary mode of action (Potter et al., 1998). There is still conflicting

evidence on whether current antidepressant treatments exert a sex-dependent action (Kokras et al., 2009). Studies reported that women may have a better outcome when treated with selective serotonin re-uptake inhibitors (SSRIs) instead of TCAs (Kornstein, et al., 2000). On the other hand, studies have reported negative results (Steiner et al., 1990) and (Lewis –Hall et al., 1997). Indeed, the efficacy of antidepressant drugs in experimental model of depression may be deeply influenced by application of different kinds of stressor prior to behavioral testing (Consoli et al., 2005).

Clomipramine is a TCA widely used for the treatment of depression and obsessive compulsive disorder, mainly by inhibiting the re-uptake of serotonin and norepinephrine in the brain (Peters et al., 1990; Trimble, 1990), increase their concentrations in the synaptic cleft which will result in the speed up of synaptic transmission (Dalmizrak et al.,2010). These neurotransmitters can influence the neuroplasticity in the brain and both are involved in mediating the therapeutic effects of most currently available antidepressants (Delgado, 2004). TCAs are well absorbed from small intestine and are lipophilic so they can easily cross the blood–brain barrier.

It has been shown that the chronic administration of clomipramine at relatively low doses reduces depression (Bhagya et al., 2008; Srikumar et al., 2006) as well as the behavioral deficits and cholinergic dysfunction induced by stress in rats (D'Aquila et al., 2000). However, the species and sex of animals, as well as type, duration, and severity of stress can modify the response induced by stress and clomipramine (Consoli et al., 2005). Literature data have also reported that high doses (30 mg/kg) of clomipramine are toxic to experimental animals (Calegari et al., 2007). The adverse effects induced by clomipramine administration due to the blockade of serotonin reuptake include difficulties in learning and memory (Burgos et al., 2005). Recent studies have revealed that endogenous serotonin and norepinephrine can modulate cognitive processes, particularly learning and memory. However, at present, the mechanisms, locations, and durations of serotonergic and noradrenergic system involvement remain unclear (Millan et al., 2000).

In this context, the objective of this study was to evaluate the effects of the repeated restraint stress treatment in combination with clomipramine in order to verify whether the association between stress condition and use of TCAs could affect the antioxidant defense and ROS production in CNS in different brain structures such as cerebral cortex, hippocampus and striatum as well as behavior of male and female adult rats.

2. RESULTS

2.1 Parameters of Oxidative Stress

In male rats (Table 1A), one way ANOVA demonstrated that the clomipramine treatment *per se* had a significant increase in DCFH-DA production in the hippocampus [F (1, 19) = 27.81, p<0.05] and striatum [F (1, 19) = 37.34, p<0.05], and lipid peroxidation in cerebral cortex [F (1, 19) = 69.77, p<0.05] and striatum [F (1, 19) = 70.68, p<0. 05] when compared to the untreated group . However, reduced the levels of non protein thiol (NP–SH) in cerebral cortex [F (1,19) = 5.68, p <0.05], hippocampus [F (1,19) = 5.45, p <0.05] and striatum [F (1,19) = 46.98, p <0.05] when compared to the control group. The combination of repeated restraint stress and clomipramine showed a significant stress effect in DCFH-DA when compared to the respective group treated with clomipramine [F (1,19)] = 8.37, p<0.05] in cerebral cortex and TBARS in all brain structures analyzed [F (1, 19) = 10.76, p< 0.05] .This combination also caused significant elevation of DCFH-DA levels in cerebral cortex, hippocampus and striatum [F(1,19) = 8.37 , p<0.05] and a reduction of NP-SH levels in cerebral cortex [F (1, 19) = 5.68 p<0.05], hippocampus [F (1, 19) = 7.70, p <0.05] and striatum [F (1, 19) = 5.67, p <0.05] compared to the control group. The group subjected only to repeated restraint stress showed that there was a reduction in the levels of NP-SH in the cerebral cortex [F (1, 19) = 15.43, p <0.05] and a increase on TBARS levels only in striatum [F (1, 19) = 40.62, p< 0.05], when compared to values found in the control group. On the other hand, no alteration in the DCFH-DA production of brain structures was observed.

The clomipramine treatment in combination or not with repeated restraint stress caused a significant increase in the CAT activity in cerebral cortex [F (1, 19) = 59.23, p<0.05], hippocampus [F (1, 19) = 35.87, p< 0.05] and striatum [F (1, 19) = 30.62, p< 0.05] as well as the SOD activity of hippocampus and striatum submitted to clomipramine [F (1, 19) = 8.48, p <0.05] and clomipramine + stress [F (1, 19) = 35.32, p <0.05] when compared to the control group . Clomipramine treatment caused per se a significant reduction in the Na^+/ K^+-ATPase activity in cerebral cortex [F(1,19) = 9.23, P<0.05] and striatum [F(1,19) = 19.09, P<0.05] compared to the control group. CAT [F(1,19) = 35.32, < P0.05] and SOD [F(1, 19) = 52.30, P<0.05] activities showed an increase in striatum and a reduction in Na^+/ K^+-ATPase activity [F (1, 19) = 5.41, p <0.05] in cerebral cortex [F(1,19) = 27.88, P<0.05], hippocampus [F(1,19)

= 23.33, P<0.05] and striatum [F(1,19) = 15. 51, P<0.05] when the stress was applied alone . No significant difference was observed in cortical CAT and SOD activity between groups.

In female rats (Table 1B) the analysis revelated that antidepressant treatment increased the DCFH-DA levels [F (1, 17) = 48.47, p< 0.05] and decreased the NP-SH [F (1, 17) = 37.89, p< 0.05] when combined with the repeated restraint stress in cerebral cortex. This combination also increased significantly the lipid peroxidation in hippocampus and striatum [F (1, 17) = 6.44, p< 0.05] compared to control group. The clomipramine increased CAT activity in hippocampus and striatum [F (1, 17) = 43.54, p< 0.05] Repeated restraint stress increased DCFH-DA levels in cerebral cortex [F (1,17) =17.32, p<0.05], hippocampus [F (1, 17) = 7.87, p< 0.05] and striatum [F (1, 17) = 4.94, p< 0.05], TBARS levels in striatum [F (1, 17) = 7.16, p< 0.05] with a reduction in the levels of NP-SH in cerebral cortex [F (1, 17) = 17.41, p< 0.05] and hippocampus [F (1, 17) = 6.25, p< 0.05]. The CAT had its enzymatic activity increased in hippocampus [F (1, 17) = 32.82, p< 0.05] . However there was a reduction in its activity in cerebral cortex [F (1, 17) = 6.44, p< 0.05] and striatum [F (1, 17) = 17.35, p< 0.05]. No significant difference was observed in brain structures on SOD and Na^+/ K^+-ATPase activity between groups.

Two way ANOVA showed that there was a significant interaction between stress exposure and clomipramine treatments in cerebral cortex (DCFH – DA [F (1, 30) = 4.46; TBARS [F (1, 30) = 5.45; NP-SH [F (1, 30) = 13.57; p<0.05 in all cases), hippocampus (TBARS [F (1, 30) = 6.05; CAT [F (1, 30) = 4.27; p<0.05 in all cases) and striatum (TBARS [F (1, 30) = 5.27; CAT [F (1, 30) = 9.77; Na^+/ K^+-ATPase [F (1, 30) = 6.87; p<0.05 in all cases). We observed a sex difference on repeated restraint stress in cerebral cortex (two way ANOVA, TBARS [F (1, 30) = 10.87; Na^+/ K^+-ATPase [F (1, 30) = 15.41; NP-SH [F (1, 30) = 6.56; CAT [F (1, 30) = 12.17; p<0.05 in all cases) and hippocampus (two way ANOVA, TBARS [F (1, 30) = 13.90; DCFH-DA [F (1,30)= 6.97; NP-SH [F (1, 30) = 11.68; CAT [F (1, 30) = 50.34; p<0.05 in all cases) as well as sex difference on the clomipramine treatment in cerebral cortex (two way ANOVA, TBARS [F (1, 30) = 5.75; Na^+/ K^+-ATPase [F (1, 30) = 17.40; NP-SH [F (1, 30) = 119.56; CAT [F (1, 30) = 8.94; p<0.05 in all cases),hippocampus (TBARS [F (1, 30) = 8.57; NP-SH [F (1, 30) = 220.68; CAT [F (1, 30) = 12.87; Na^+/ K^+-ATPase [F (1, 30) = 47.23; p<0.05 in all cases.) and striatum (TBARS [F (1, 30) = 4.77; DCFH-DA [F (1, 30) = 52.41; NP-SH [F (1, 30) = 134.31; CAT [F (1, 30) = 8.94; Na^+/ K^+-ATPase [F (1, 30) = 47.23; p<0.05 in all cases)

2.2 Behavioral analysis

2.2.1 Object recognition task

Figure 1 shows the effects of chronic treatment with clomipramine and repeated restraint stress on training, short term memory and long term memory. In male rats (Figure 1B), chronic clomipramine treatment caused *per se* a decrease of exploratory preference in STM [one way ANOVA, F (1, 29) = 30.61, p<0. 05] and when associated with repeated restraint stress condition caused a further decrease in STM [one way ANOVA, F (1, 29) = 20.47, p<0. 05] when compared to the respective control training group. Moreover, the repeated restraint stress alone changed STM [F (1, 29) = 20.48, p< 0.05] and LTM [F (1, 29) = 11.94, p< 0.05], when compared to values found in the training group. The control groups showed an increase in the exploratory performance in STM and LTM [F (1, 29) = 21.50, p< 0.05] when compared to the control group trained.

In female rats (Figure 1A), chronic clomipramine treatment when associated with repeated restraint stress condition induced an increase in STM [one way ANOVA, F (1, 24) = .8.56, p<0. 05] but decreased LTM [one way ANOVA, F (1, 24) = 13.89, p<0. 05] when compared to the respective control training group.

Two way ANOVA showed sex difference only in STM [F (1, 55) = 12.47, p <0.05] and there were interactions between repeated restraint stress and clomipramine treatments in STM [F (1, 55) = 7.94, p <0.05] and LTM [F (1, 55) = 13.65, p <0.05].

2.2.2 Open field task

In male rats, statistical analysis of one way ANOVA showed that there was an increase of the crossings [F (1, 31) = 6.11, p<0.05, Figure 2A] in the stress + clomipramine group compared to the respective clomipramine group. However, the crossings showed a decrease in the stress + clomipramine group or only clomipramine when compared to the control group [F (1, 31) = 15.59, p <0.05, Figure 2A]. The rearings were significantly decreased in the stress [F (1, 31) = 7.51, p<0.05, Figure 2B], clomipramine and stress + clomipramine groups [F (1, 31) = 27.87, p<0.05, Figure 2B], when compared to the control group.

In female rats the results showed a decrease in crossings of the stress + clomipramine group or only clomipramine [F (1, 34) = 20.65, p <0.05, Figure 2A] and rearings [F (1, 31) = 7.93, p <0.05, Figure 2A] only in the stress + clomipramine group when compared to the control group.

Two way ANOVA showed sex difference on crossings [two way ANOVA, $F(1, 70) = 20.41$, $p < 0.05$] and rearings [two way ANOVA, $F(1, 70) = 25.94$, $p < 0.05$] but there were no interactions between treatments.

2.2.3 Plus maze task

Figure 3 indicates that, in male rats, clomipramine treatment significantly reduced the number of entries in closed arms [one way ANOVA, $F(1,36) = 9.56$, $p < 0.05$, Figure 3B] and open arms [one way ANOVA, $F(1,36) = 11.94$, Figure 3D] when compared to the control group. The association between chronic clomipramine treatment and stress condition also caused a significant reduction of time spent in open arms [$F(1, 36) = 7.13$, $p < 0.05$, Figure 3C], entries in closed arms [$F(1, 36) = 11.03$, $p < 0.05$, Figure 3B] and open arms [$F(1, 36) = 12.24$, $p < 0.05$, Figure 3D], compared to the control group. There was no significant difference in the stressed group.

In female rats the association between chronic clomipramine treatment and stress condition caused a significant reduction of time spent in open arms [$F(1, 39) = 10.94$, $p < 0.05$, Figure 3C] and showed an increase in the time spent in closed arms [$F(1, 39) = 21.96$, $p < 0.05$, Figure 3C].

Two way ANOVA showed sex difference of time spent in open arms [$F(1, 75) = 20.56$, $p < 0.05$], number of entries in open arms [$F(1, 75) = 42.51$, $p < 0.05$] and time spent in closed arms [$F(1, 75) = 6.48$, $p < 0.05$]. There were no interactions between treatments.

2.2.4 Conditioned place preference task

Figure 4 illustrates the conditioned place preference task (CPP) on the difference between time in the light compartment and crossings in the days eight and one and mean intake of sweet food between day seven and day two in male and female rats. In male rats one way ANOVA show that clomipramine treatment alone or in combination with stress caused a significant decrease in crossings [$F(1, 36) = 21.82$, $p < 0.05$, Figure 4A] and in the time in the light compartment [$F(1, 36) = 18.28$, $p < 0.05$, Figure 4B] when compared to the control group. The repeated restraint stress decreased the time in the light compartment when compared to the control group [$F(1, 36) = 4.75$, $p < 0.05$, Figure 4B]. An increase in the intake of sweet food was verified in the stress + clomipramine group [$F(1, 19) = 3.81$, p

<0.05, Figure 4C] while a decrease was observed in the stressed group [F (1, 19) = 6.60, p <0.05, Figure 4C].

In female rats the repeated restraint stress decreased the time in the light compartment [F (1, 38) = 22.21, p <0.05, Figure 4B] when compared to the control group. This effect was reversed by treatment with clomipramine on the time in the light compartment [F (1, 38) = 8.39, p <0.05, Figure 4B] and crossings [F (1, 38) = 9.29, p <0.05, Figure 4B]. A decrease in the intake of sweet food was verified in the stress + clomipramine group [F (1, 38) = 8.77, p <0.05, Figure 4C], clomipramine [F (1, 38) = 18.01, p <0.05, Figure 4C] and in the stressed group [F (1, 38) = 5.17, p <0.05, Figure 4C].

A significant interaction between repeated restraint stress exposure and clomipramine treatment was observed on the time in light compartment [two way ANOVA, F (1, 74) = 15.42, p <0.05] , crossings [two way ANOVA, F (1, 74) = 22.04, p <0.05] and intake of sweet food [two way ANOVA, F (1, 74) = 7.69, p <0.05]. On the other hand, two way ANOVA showed sex difference on crossings [two way ANOVA, F (1, 74) = 15.42, p <0.05] and intake of sweet food [two way ANOVA, F (1, 74) = 19.75, p <0.05].

3. DISCUSSION

In our study, we analyzed the sex differences of chronic treatment with clomipramine in a repeated restraint stress model on the levels of free radicals, lipid peroxidation and the action of the main antioxidant defense systems in the brain structures and their relationship regarding the behavior of male and female rats, which have been used as animal models of depression. The dose and duration of the administration of antidepressant associated with repeated restraint stress model in this study suggests neurochemical changes opposed to beneficial effects of antidepressants under stress conditions in male and female rats. The repeated restraint stress model employed in the present study incorporated both physical and psychological components of stress, and is widely used to induce oxidative and neurotoxic damage (Zafir et al., 2009).

The brain is considered as a sensitive organ prone to oxidative damage because it has a high rate of oxidative metabolism, low levels of protective enzymes to eliminate free radicals (Kodavanti 1999) that are particularly good substrates for peroxidation reactions. The oxidative effects of ROS are controlled by non-enzymatic antioxidants (NP-SH) and also by enzymatic antioxidants (SOD and CAT). The imbalance between the production of free

radicals and antioxidant defense system is the key to understand the oxidative stress (Stangherlin et al., 2009).

Animal studies have also found that females can display differential responses to stress compared to males (Zavala et al., 2011). It has been shown previously that circulating GC levels are higher in females than males under resting conditions (Viau et al., 2005). Furthermore, average daily GC levels fluctuate according to estrus cycle stage in females, showing equivalent levels to males during estrus but higher values during proestrus, metestrus and diestrus (Atkinson et al., 1997). These findings support a previous report, which found that female rats released more GC and for a longer period compared to males following repeated restraint stress (Galea et al., 1997). This enhanced GC response in females contributed to mechanisms underlying the repeated stress-induced sex differences (Chadda and Devaud, 2005).

Our results showed that female rats submitted to repeated restraint stress induced formation of ROS in cerebral cortex, hippocampus and striatum while lipid peroxidation levels increased only in striatum when compared to male rats. The non-enzymatic antioxidant activity as NP-SH decreased in hippocampus and cerebral cortex of female rats and only in cerebral cortex of male rats. However, the enzymatic antioxidant activitiy as CAT increased its activity in hippocampus and striatum of female rats when compared to male rats. No significant difference was observed in Na^+/ K^+-ATPase activity in female rats while a reduction of its activity was observed in all brain structures in male rats. Antidepressant treatment under conditions of stress showed clearly oxidative stress in all brain structures of male rats. Interestingly these effects were not observed in female rats.

These results are in accordance to numerous publications, which have provided evidence that the hippocampus and striatum are more susceptible to oxidative stress than other regions including the cerebral cortex (Stangherlin et al., 2009) and suggest that repeated restraint stress, induced an increase of lipid peroxidation and free radicals production in hippocampus and striatum than cerebral cortex of female rats when compared to male rats. On the other hand, the negative effects of stress are enhanced with the dose of clomipramine, particularly in male rats.

Stress provides a deficit in the signaling of glucocorticoid receptors (GR) in the brain (Juruena et al., 2004) by increasing the levels of circulating GC and therefore the production of ROS in sensitive regions to the axis LHPA. Therapeutic doses (10-20mg/kg/dia) determine an increase GR function and expression in neuronal cell cultures (Lai et al., 2003). However, reduced GR function *in vitro* by antidepressants has also been described (Pariante et al.,

1997), which may suggest increased production of ROS because antidepressants control GR function *in vitro* by regulating the intracellular concentration of GC (Pariante et al., 2001). In the presence of GC, which are not substrates for steroid transporters, the antidepressant-induced GR activation and downregulation lead to a reduced GR function (Carvalho et al., 2008).

Research has suggested some theoretical reasons for suspecting that male-female differences in antidepressant response exist.

First, female gonadal hormones may play a permissive or inhibitory role in antidepressant activity, enhancing response to SSRIs or inhibiting response to TCA (Kornstein et al., 2000). Second, sex differences in the metabolism, brain penetration and distribution of clomipramine and its active metabolites (Kokras, et al., 2009) may result in different uptake and retention of the drug, and consequently may contribute to herein observed gender-related differences in the effects of GC on GR (Elaković et al., 2009). Third, a relative difference between right and left brain function may be related to depression and antidepressant response. Therefore, apparent sex differences in cognitive styles that may reflect differences in right and left brain function or less well-lateralized function in female might also contribute to difference in antidepressant response (Quitkin et al., 2002) In contrast, the results of a data synthesis of nine studies suggested no gender difference in response to tricyclics (Wohlfarth et al., 2004).

We have applied models of anxiety-like and depression-like behavior and memory assessment. The hippocampus is essential for memory processing during the object recognition task (Clark et al.,2000) and pharmacological studies indicate that hippocampal formation is required for acquisition and storage of the contextual details and temporal order of previous experiences (Balderas et al., 2008). The performance in the recognition memory task appears to be dependent on interconnected neural circuits involving the pre-frontal cortex, hippocampus, thalamus, and ventral striatum. Moreover, a disconnection between the hippocampus and the pre-frontal cortex has been shown to disrupt this performance (Burgos et al., 2005), while clomipramine has been shown to impair memory performance in rats. (Naudon et al., 2007). Chronic stress also has a dramatic influence on short-term and long-term episodic memory performance (Elizalde et al., 2008). Evidence indicates that poor memory is common in patients suffering from major depression and that memory performance can significantly improve after chronic treatment with antidepressants (Burt et al., 1995). However, antidepressants, which alleviate depression symptoms, can also have a negative impact on cognitive functions (Danion, 1993).

It has been shown that behavioral sex differences exist in non-spatial tasks in response to chronic stress: males are impaired while females are not impaired in the object recognition task (Luine et al., 2001) which is in agreement with our results.

Females have a higher level of activation of GR-expressing cells than males after both acute and repeated stress exposure, suggesting that they may be able to respond more rapidly to hormonal signals designed to terminate the response to stress (Zavala et al., 2011). Estrogen results in female resistance to stress-induced impairments by exerting either a direct protective effect on the hippocampus or by modifying the LHPA cascade in females influencing GC release or GR density (Galea et al., 1997).

In line with this concept, and based on our findings, the clear decrease in short-term and long-term memory following repeated restraint stress application in male but not female rats could be associated with a these studies. Additionally, clomipramine treatment could produce changes in the structural organization of the lipid membranes and affect the connections between the cerebral cortex, hippocampus, and striatum in both sexes.

The striatum receives inputs from the hippocampus, and may be an important brain area in decision-making and involved in the learning of cognitive and motor activities (Gubert et al., 2011). Many striatal neurons decrease or increase their activity as learning progresses (Tremblay et al., 1998; Brasted and Wise, 2004). Importantly, the activity of these neurons has been found to be modulated by the expected presence, amount, probability of reward, magnitude of attention, and memory required to execute the task (Kawagoe et al., 1998; Shidara et al., 1998; Cromwell and Schultz, 2003). Indeed, studies on the synaptic mechanisms in the striatum have shown that long-term potentiation (LTP) or long-term depression (LTD) can occur in the corticostriatal synapses, depending on the combination of cortical inputs, striatal outputs, and D1 and D2 dopaminergic inputs (Reynolds and Wickens, 2002). The place conditioning data could also be explained as a decreased ability, following stress, to associate the reward with the environment in which it was presented (Papp et al., 1991). The most important environmental events that may lead to reinstatement are re-exposure to the drug itself, presentation of drug-associated stimuli or cues, and exposure to a stressful event (Do Couto et al., 2006).

The reduction of time in the light compartment and crossings in both sexes could be associated with a dysregulation of the dopaminergic system in the striatum due to decreased striatal dopaminergic activity (Bekris et al., 2005), because there is further evidence of limbic-striatal interactions underlying reward-related processes. The behavioral passivity displayed by restraint stressed animals is an analogue of decreased motivation, which is a behavioral

correlate of depressive symptoms in humans (Porsolt, 1979). Thus, we suggest that striatum could be the brain structure most sensitive to hormonal changes and motivation is the main behavioral change in female rats submitted to stress model used in this study. Control animals typically exhibited a high preference for sweet food (Plaznik et al., 1989; Dalla et al., 2005), while this preference was markedly reduced following exposure to restraint stress in agreement with earlier reports (Bekris et al., 2005; Zafir et al., 2009), an effect that was reversed in male rats by the chronic treatment with a tricyclic antidepressant, in accordance with other studies (Clark et al., 2000) but not in female rats.

Concerning emotional behavior, there is some evidence suggesting that repeated restraint stress produces an increase in anxiety behavior, and the "open field" has been suggested as a good model for the normal anxiety that animals experience when confronted with a stressful or threatening situation (Bravin et al., 2005).

Repeated restraint stress significantly reduced the number of rearings in the open field task as well as the number of crossings (not significantly), according to studies by Liu (2008) in male rats because males displayed decreased moving behavior during six weeks and females seem to decline earlier (Dalla et al., 2005) resulting in enhanced anxiety in males (Fernandes et al., 1999). However, it is not clear whether direct activational influences of organisational steroid effects resulting in sexual dimorphism in the brain are responsible for the observed sex differences in stress responses. Clomipramine treatment improves behavioral activities at doses of 5 mg/kg (Liu et al., 2008). These findings oppose our results showing a reduction in the frequency of rearing and crossing in the open field, indicating a probable anxiogenic effect of this drug, possibly due to a direct relationship with dosage (Bravin et al., 2005) in both sexes.

The elevated plus-maze includes two additional anxiety-provoking environmental parameters (height and a totally open area); therefore, levels of anxiety might be more easily detectable in this test. When exposed to an elevated plus maze, rats tend to avoid the open arms and prefer to stay in the enclosed arms. This way, drugs that elicit a decrease of the time spent in the open arms are considered as anxiogenic (Pellow et al., 1985). This is consistent with our study, which demonstrates that chronic treatment with clomipramine could increase the levels of anxiety in male and female submitted to repeated restraint stress.

In line with the well-established notion that TCAs are effective in both men and women, clomipramine treatment com dose de 10mg/kg, effectively reversed relevant behavioural indices in both sexes (Kokras et al., 2009) but with the dose of 30mg/kg found opposite effects of clomipramine particulary in male rats.

These results demonstrate that duration and intensity of stressful stimuli may deeply affect the behavioral response of rats and influence clomipramine effect in this behavioral model depending on gender-based variables, probably of the hormonal type. Plasma GC levels correlate with the behavioral response to clomipramine treatment suggesting that reactivity of LHPA axis to stress may be involved in the antidepressant effect of this drug. (Consoli et al., 2005). So, we believe that the central question of our study was appropriately answered since our findings clearly revealed sex difference on oxidative stress and behavioral parameters of rats exposed to repeated restraint stress and clomipramine treatments. In conclusion, our results contribute to improve the knowledge regarding to the tricyclic antidepressants, especially clomipramine that showed greater sensitivity in males particularly in this stress model used in our study.

4. EXPERIMENTAL PROCEDURES

4.1 Chemicals

Clomipramine hydrochloride, Thiobarbituric acid (TBA), 2'-7'-dichlorofluorescein (DCF), trichloroacetic acid (TCA), p-dimethylaminobenzaldehyde, reduced glutathione (GSH), 5.5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

4.2 Animals

Forty adult male and female rats (250-300g in weight and 60 days old) from our own breeding colony were used. The animals were placed in groups of five animals in cages made of Plexiglas measuring 42X34X16cm, with the floor covered with sawdust. They were kept in a room with light-dark cycle of 12 h with the lights on between 7:00 and 19:00 h and temperature (20°C - 25°C) controlled receiving water and food *ad libitum*. Rats were first habituated in the room where the behavioral tasks were performed by at least 30 min. When the group was exposed to more than one subsequent behavioral task, there was a period of three days of resting between tasks.

The animals were maintained and used in accordance with the guidelines of the Committee on Care and Use of Experimental Animal Resources (number of protocol: 23081.007146/2010-17) of the Federal University of Santa Maria, Brazil.

4.3 Experimental Groups

The animals were divided into two groups: control group, only manipulated for the necessary maintenance of their cages in good health, and stress group, which received a treatment of repeated restraint stress for a forty-day period. On day 14 of this experimental period, each initial group was subdivided into 4 different groups: 1) control, 2) stress, 3) clomipramine, and 4) stress + clomipramine.

4.4 Repeated Restraint Stress Model

Stress condition was performed according to the model of repeated restraint stress as described by Ely et al. (1997). The animals were immobilized in plastic tubes measuring 25 x 7 cm of diameter adjusted to the size of the animal. The animal behavior was carried out 1 h per day, five days a week, for forty days in the morning between 8:00 and 10:00. The control group was just handled and thus not subject to any repeated stress situation. The repeated restraint stress was applied at least one hour after exposure to behavioral tasks.

4.5 Clomipramine Treatment

The animals received clomipramine (30 mg/kg) for 27 consecutive days previously described by Calegari et al. (2007) in the drinking water from the fourteenth day of the trial period after the stress-induced behavioral alterations had been established. This dose was chosen because it provides, when administered chronically, possible toxic effects on the body in rodents according to Calegari et al. (2007). The repeated restraint stress continued during the whole treatment period. The rats untreated with the drug received regular water. Clomipramine was placed in dark bottles due to its photosensitivity and the water consumption by animals was analyzed daily in order to adapt the dose to be administered. Oral administration was chosen because it is the most common use of antidepressants in patients with psychiatric disorders according to Lucassen et al. (2004).

4.6 Tissue Preparation

At the end of the treatment period, rats were killed by decapitation. The brain was removed and structures such as cerebral cortex, hippocampus and striatum were quickly dissecated, placed on ice, and immediately homogenized in cold 50 mM Tris-HCl pH 7.4. Homogenates were centrifuged at 4,000 x g for 10 min to yield the low-speed supernatant fractions that were used for different biochemical assays in all trials.

4.7 Oxidized diclorofluoresceine (DCFH-DA) levels determination

2'-7'-dichlorofluorescein diacetate (DCFH-DA) levels were determined as an index of the peroxide production by the cellular components. This experimental method of analysis is based on the deacetylation of the probe DCFH-DA, and its subsequent oxidation by reactive species to DCFH-DA, a highly fluorescent compound (Halliwell and Gutteridge, 2007). The supernatant fraction of hippocampus, striatum and cerebral cortex was added to a medium containing Tris-HCl buffer (10 mM; pH 7.4) and DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (Excitation at 488 nm and Emission at 525 nm, and both slit widths used were at 1.5 nm). DCFH-DA levels were determined using a standard curve of DCF and results were corrected by the protein content.

4.8 Thiobarbituric acid reactive substance (TBARS) level determination

Lipid peroxidation was estimated by measuring TBARS and expressed in terms of malondialdehyde (MDA) content, according to the method of Ohkawa et al. (1979). In this method, MDA, an end product of fatty acid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex. Briefly, the supernatant fraction of brain structures was incubated at 100ºC for 60 min in acid medium containing 8.1% sodium dodecyl sulfate, 0.5 mL of acetic acid buffer (500 mM, pH 3.4) and 0.6% TBA. TBARS levels were measured at 532 nm and the absorbance was compared with the standard curve using malondialdehyde.

4.9 Non protein thiol (NP-SH) level determination

NP–SH levels of cerebral cortex, hippocampus and striatum samples were determined according to the method proposed by Ellman (1952) with some modifications. Samples were precipitated with TCA (10%) and subsequently centrifuged at 4,000 x g for 10 min. After the centrifugation, the supernatant fraction (200µL) was added to a reaction medium containing K^+-phosphate (0.5M and pH = 7.4) and DTNB (0.5mM). NP–SH levels were measured spectrophotometrically at 412 nm. Results were calculated in relation to a standard curve constructed with reduced glutathione (GSH) and also corrected by the protein content.

4.10 Enzyme Assays

4.10.1 Catalase (CAT) activity

The measurement of the CAT activity was determined according to the method proposed by Aebi (1984). The supernatant fraction of cerebral cortex, hippocampus and striatum (40 µL) was added to a medium containing K^+-phosphate buffer (50 mM; pH 7.4) and H_2O_2 (10mM). The kinetic analysis of catalase was started after H_2O_2 addition and the rate of H_2O_2 decomposition was measured spectrophotometrically at 240nm during 120s. One unit of the enzyme was considered as the amount of enzyme which decomposes 1 µmol H_2O_2/min at pH 7.

4.10.2 Superoxide dismutase (SOD) activity

The SOD enzyme activity was determined in cerebral cortex, hippocampus and striatum according to the method proposed by Misra and Fridovich (1972). This method is based on the capacity of SOD in inhibiting autoxidation of adrenaline to adrenochrome. Briefly, the supernatant fraction (20-60µL) was added to a medium containing glycine buffer (50 mM; pH 10.5) and adrenaline (1mM). The kinetic analysis of SOD was started after adrenaline addition, and the color reaction was measured at 480 nm.

4.10.3 Sodium potassium (Na+/ K+-ATPase) activity

The Na+/K+-ATPase activity was estimated by the method of Muszbek (1977). The enzyme activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP during the incubation of hippocampal and striatal aliquots. Before, the

slices were incubated with Meth (0.05, 0.1, 0.5 and 1 μM) at different times (5 or 15 min). Then, the reaction mixture containing 95 mM NaCl, 15 mM KCl, 1.0 mM ATP (disodium salt), 38 mM Tris –HCl buffer (pH 7.4) was added to aliquot of homogenized slices (50 μg of protein) in a final volume of 0.3 mL. After a 5-min pre-incubation at 37 °C in the presence of 0.1 mM ouabain to specifically inhibit Na+/K+-ATPase, the reaction was initiated by addition of ATP and terminated after 15 min of incubation by addition of 1 mL of color reagent (Ammonium Molybdate 2%, Triton X 5% solubilized in H2SO4 1.8 M). The released inorganic phosphate was measured spectrophotometrically at λ=405 nm. Na+/K+-ATPase activity was calculated from the difference between amounts of inorganic phosphate found after incubation in the absence and presence of 1.5 M ouabain.

4.11 Behavioral analysis

Aiming to verify whether chronic treatment with clomipramine could or not potentiate the effects caused by repeated restraint stress on cognitive function in animals we performed four behavioral tasks. The Video-Track® system, used for recording the animal behavior, was composed of a camera connected to a computer.

4.11.1 Object recognition task

The object recognition task was performed according to Stangherlin et al. (2008). Novel object recognition is a type of non-aversive and non-spatial memory. Rodents naturally tend to approach and explore novel objects, which are assumed to have no natural significance to the animal and which have never been paired with a reinforcing stimulus. They also show an innate preference for novel over familiar objects. Rodents readily approach objects and investigate them physically by touching and sniffing the objects, rearing upon and trying to manipulate them with their forepaws. This behavior can be easily quantified and utilized to study simple recognition memory as well as more complex spatial-, temporal- and episodic-like memory in rodents. The standard object recognition task measures the spontaneous behavior. The novelty-preference paradigm does not require lengthy training and does not induce high levels of arousal and stress (Stangherlin et al., 2008).The behavioral task was performed in a 45×45 cm open field surrounded by 30 cm height walls made of brown plywood. The behavioral task was conducted in a moderately lighted room (30 lx). All

animals were given a habituation session where they were left to freely explore the open field for 5 min. No object was placed in the box during the habituation trial. Subsequently, four objects were used: A1, A2, B and C. The “A” objects were two identical triangles, the “B” object was a ball and the “C” object was a rectangle. All objects were made of plastic material, with 10 cm×10 cm (length×height). Each object had the pattern of color, as follows: blue, red and yellow. Twenty-four hours after habituation, training was conducted by placing each individual rat for 5 min into the field, in which two identical objects (objects A1 and A2) were positioned in two adjacent corners, 10 cm from the walls. In a short-term memory (STM) test given 1.5 h after training, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. All objects presented similar textures, colors, and sizes, but distinctive shapes. The percentage of the total exploration time that the animal spent investigating the novel object was the measure of recognition memory. Between trials the objects were washed with 10% ethanol solution. In a long-term memory (LTM) test given 24 h after training, the same rat explored the field for 5 min in the presence of a familiar object A and a novel object C. Recognition memory was evaluated as for the STM test. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Data are expressed as the mean±SE percentage time exploring any of the objects (training) or the novel objects. Exploratory preference in: Training = (A2 / (A1+A2))×100; STM=(B/(A1+B))×100; LTM=(C/ (A1+C))×100.

4.11.2 Open field task

The open field task is a simple assessment used to determine general activity levels, gross locomotor activity and exploration habits in rats. A 50-cm high, 40X60 cm open field made of brown plywood was used. The floor was subdivided with white lines into 12 equal 13.3X15.0 cm rectangles. The behavioral task was conducted in a moderately lighted room (30 lx).The animals were gently placed facing the left corner and allowed to explore the arena for 3 min. The number of rearings and line crossings was counted as a parameter of motor activity. The rat is placed in the center of the open field arena and allowed to freely move around for 5 min while being tracked by an automated tracking system. At the conclusion of each trial the surface of the arena is cleaned with 90% ethanol (Walsh and Cummins, 1976).

4.11.3 Elevated plus maze task

Elevated plus maze task is based on the natural aversion of rats for open spaces and uses an elevated plus-maze (60 cm from the floor) consisting of two open arms (50 cm long X10 cm wide) and two enclosed arms of equal length and width (50X10 cm with 40 cm high walls) forming a square cross with a 10 cm square center piece. The behavioral task was conducted in a moderately lighted room (30 lx as measured at the center of the maze). This task is considered sensitive to the anxiety state of the animal based on the principle that exposure to an elevated and open arm maze leads to an approach conflict that is stronger than that evoked by exposure to an enclosed arm maze. On the day of the experiment, the test initiated after a period of 5 min in the behavioral testing room. Animals were placed individually in the center of the maze, in the junction between open and closed arms, facing one of the open arms, and performance was scored for 5 min. The maze was cleaned thoroughly with warm water between trials. The following parameters were measured: percent time spent in open and closed parts of the maze as well as frequencies of open and closed entries (an arm entry was considered when the rat stepped with all four paws into it) (Conde et al., 1999).

4.11.4 Conditioned place preference task

Conditioned place preference (environmental place conditioning) is a commonly used technique to evaluate preferences for environmental stimuli associated with a positive or negative reward. Moreover, it evaluates the interest in a different environment used as an indicator of integrity of dopaminergic pathways (Figlewicz et al., 2001). The task was performed for eight days. Before beginning the task, the animals were subjected to dietary restriction receiving 90% of their daily diet for 24 h in order to increase the interest for the new food. The animals were placed in a rectangular box divided into two compartments: one white wall illuminated moderately with 30lx and a black wall, separated by a partition high. On day one, the time that the rats remained in the light compartment and number of crossings made between the two compartments were analyzed for 15 minutes. No food was available in the apparatus on day one of exposure. From day two to seven, the division between the two compartments was lowered, the compartments were separated and twenty units of fresh food, donut-shaped, 1 cm in diameter (of Kellogg's Froot Loops) were placed daily in the light compartment. The animal remained in the room light or dark for half an hour, alternately; one day the animal stayed in the light compartment and next day in the dark compartment. On the test day (day eight), the sweet food was removed, a mean of sweet food intake between day

two and day seven was performed, and the procedure repeated as the day one. We compared the time spent in the light compartment and the number of crossings between the day eight and day one presented by Δ.

4.12 Protein determination

The protein content was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

4.13 Statistical analysis

Data were analyzed by one or two-way ANOVA followed by Duncan's multiple range test when *F* test was significant(Statistical Packcage for the Social Science version 10.0 for Windows 98 - SPSS Inc, Chicago, Illinois, USA). Differences between groups were considered significant when $p < 0.05$. Results are expressed as mean (standard error of the mean (SEM).

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FIGURE LEGENDS

Figure 1. Effects of repeated restraint stress and chronic treatment with clomipramine on object recognition task during Training, Short Term Memory (STM) and Long Term Memory (LTM). Exploratory preference in female (A) e male rats (B). The percentage of the total exploration time that the animal spent investigating the novel object was the measure of recognition memory. Data are expressed as mean ± SEM, N of ten animals/ group. a Significantly different from control training group (One-way ANOVA,followed by Duncan's multiple range test, $p < 0.05$). * Significant effect of treatment with clomipramine when compared to the control group during training (One-way ANOVA,followed by Duncan's multiple range test, $p < 0.05$). # Significant effect of stress in relation to the respective control group during training (One-way ANOVA,followed by Duncan's multiple range test , $p < 0.05$). a Significant interaction between stress and clomipramine treatments (two way ANOVA <0,05) b Significant sex difference of clomipramine in relation to the respective group in female rats (two -way ANOVA,$p<0.05$).

Figure 2. Effects of repeated restraint stress and chronic treatment with clomipramine in open field exposure. Performance on number of crossings (A) and rearings (B) of male and female rats. Data are expressed as mean ± SEM, N of ten animals/group. * Significant effect of treatment with clomipramine when compared to control group(One -way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). # Significant effect of stress in relation to the respective control group (One -way ANOVA,followed by Duncan's multiple range test, $p < 0.05$). b Significant sex difference of clomipramine in relation to the respective group in female rats (two -way ANOVA, $p<0.05$). c Significant sex difference of stress in relation to the respective group in female rats (two -way ANOVA, $p<0.05$).

Figure 3. Effect of repeated restraint stress and chronic treatment with clomipramine in elevated plus maze test. Time spent in closed arms (A), number de entries in closed arms (B), time spent in open arms (C) and number of entries in open arms (D) in male and female rats. Data express in mean ± SEM, N of ten animals/group. * Significantly different from the control group (One-way ANOVA, followed by Duncan's multiple range test, $p< 0.05$). # Significant effect of stress in relation to the respective control group (One -way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). b Significant sex difference of clomipramine in relation to the respective group in female rats (two -way ANOVA, $p<0.05$). c Significant sex difference of stress in relation to the respective group in male rats (two -way ANOVA, $p<0.05$).

Figure 4. Effects of repeated restraint stress and chronic treatment with clomipramine on the conditioned place preference task. Data are expressed as the difference between number of crossings (A) and time in light compartment in the day eight and day one(B) without the presence of sweet food and mean intake of sweet food from the seven day and two day (C) of male and female rats. Data are expressed as means + SEM, N of ten animals/group. * Significant effect of treatment with clomipramine when compared to control group (One -way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). # Significant effect of repeated restraint stress in relation to respective control group (One -way ANOVA, followed by Duncan's multiple range test <0.05). ** Significant effect of treatment with clomipramine when compared to control group (One -way ANOVA, followed by Duncan's multiple range test, $p < 0.05$). a Significant interaction between stress and clomipramine treatments (two way ANOVA <0, 05) b Significant sex difference of clomipramine in relation to the respective group in female rats (two -way ANOVA, $p<0.05$). c Significant sex

difference of stress in relation to the respective group in male rats (two -way ANOVA,$p<0.05$).

TABLE LEGEND

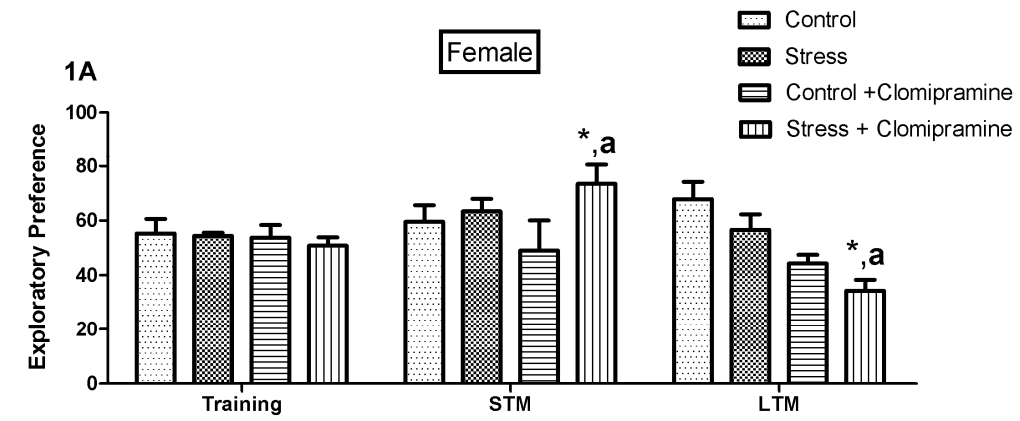
Table 1. Effects of repeated restraint stress and clomipramine treatment on the ROS formation, lipid peroxidation and antioxidant activity in cerebral cortex, hippocampus and striatum of male (1A) and female (1B) rats. Data are expressed as mean ± SEM with N of five animals/group. # Significant effect of the stressor when compared to the control group (One - way ANOVA, followed by Duncan's multiple range test, $p <0.05$). * Significant effect of clomipramine treatment when compared to the control group (One - way ANOVA, followed by Duncan's multiple range test, $p <0.05$). a Significant interaction between stress and clomipramine treatments (two way ANOVA, $p<0.05$). b Significant sex difference of the clomipramine treatment (two way ANOVA, $p<0.05$). c Significant sex difference of the repeated restraint stress (two way ANOVA, $p<0.05$).

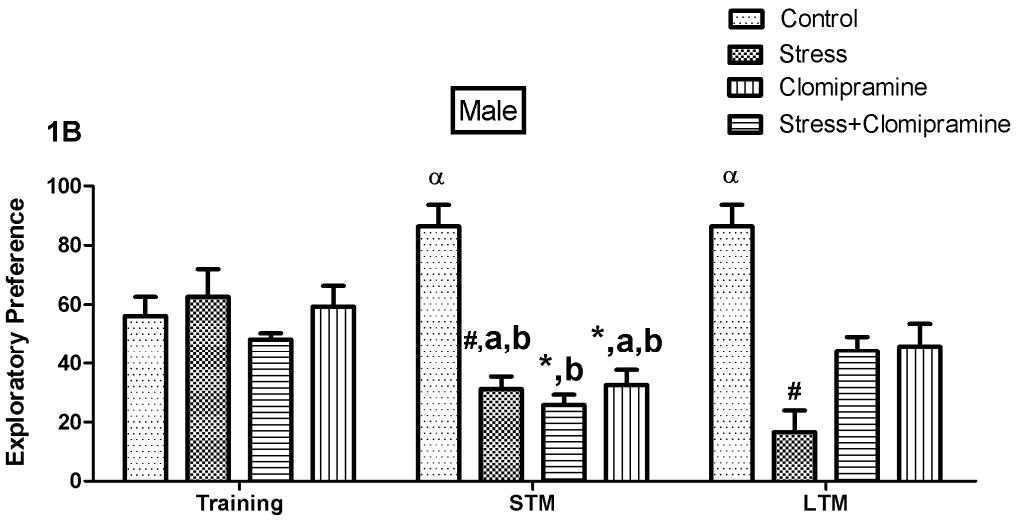
1A Male Rats

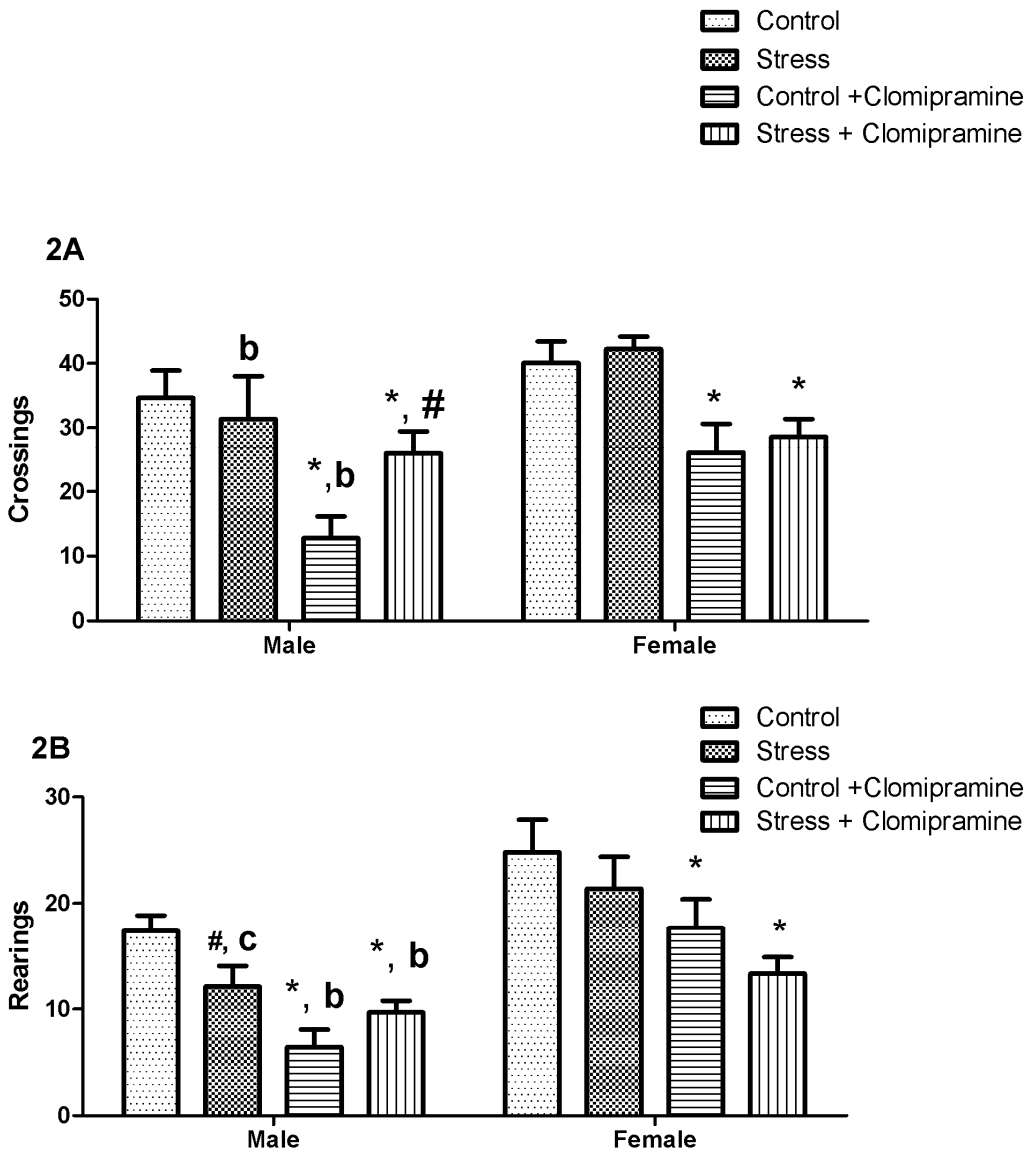
	Control		Stress	
	Water	Clomipramine	Water	Clomipramine
Cerebral Cortex				
DCFH-DA	2.60 ± 0.14	2.80 ± 0.25	2.67 ± 0.13	4.07 ± 0.25*, #, a
TBARS	2.09 ± 0.19	3.32 ± 0.23*,b	2.50 ± 0.16c	4.34 ± 0.11*, #,a,b
NP-SH	27.03 ± 3.33	4.65 ± 0.26*,b	18.63 ± 1.76#	5.34 ± 0.54*,a,b
CAT	2.05 ± 0.37	9.38 ± 0.83*	2.81 ± 0.34	8.42 ± 1.42*,b
SOD	1.97 ± 0.32	1.70 ± 0.10	1.62 ± 0.23	2.26 ± 0.25
Na+/ K+-ATPase	4.72 ± 0.72	1.44 ± 0.19*,b	1.60 ± 0.23#,c	0.57 ± 0.04*,b
Hippocampus				
DCFH-DA	2.12 ± 0.11	3.13 ± 0.58*	2.22 ± 0.40	2.99 ± 0.16*
TBARS	1.47 ± 0.17	1.59 ± 0.22	2.25 ± 0.15 c	4.08 ± 0.39*,#,a,b
NP-SH	23.51 ± 0.48	6.13 ± 0.31*, b	26.90 ± 1.03	5.84 ± 0.59*,b
CAT	4.20 ± 0.46	10.11 ± 0.35*,b	2.70 ± 0.46	8.15 ± 0.13*,#,a,b
SOD	1.37 ± 0.12	4.16 ± 0.99*	0.95 ± 0.38	3.15 ± 0.31*
Na+/ K+-ATPase	1.20 ± 0.11	1.16 ± 0.11b	0.49 ± 0.04#	0.88 ± 0.13 b
Striatum				
DCFH-DA	1.70 ± 0.14	3.72 ± 0.15*,b	2.08 ± 0.13	3.81 ± 0.19*,b
TBARS	0.48 ± 0.06	1.23 ± 0.17*	1.06 ± 0.19#	1.69 ± 0.11*,#, a
NP-SH	42.14 ± 1.54	9.69 ± 0.14*	35.65 ± 2.70	8.45 ± 0.78*,b
CAT	2.02 ± 0.13	8.09 ± 0.41*,b	4.66 ± 0.10#	8.29 ± 0.15*,b
SOD	1.93 ± 0.20	2.65 ± 0.36*	0.85 ± 0.12#	2.40 ± 0.15*
Na+/ K+-ATPase	2.15 ± 0.31	0.48 ± 0.10*,b	1.21 ± 0.05#	1.36 ± 0.14*,#,a

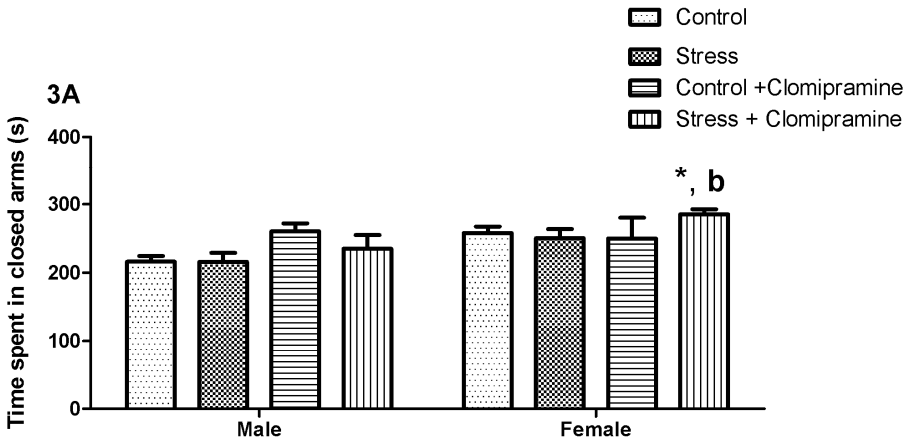
1B Female Rats

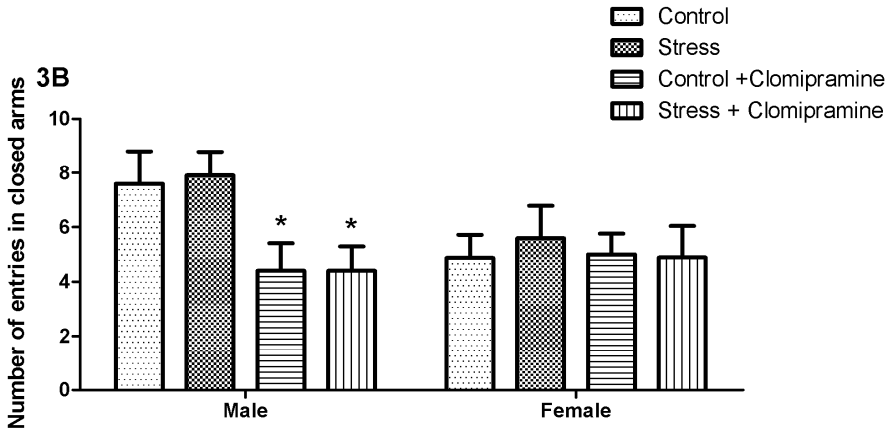
	Control		Stress	
	Water	Clomipramine	Water	Clomipramine
Cerebral Cortex				
DCFH-DA	2.35 ± 0.16	3.69 ± 0.11*, b	2.86 ± 0.22#	4.14 ± 0.13*
TBARS	1.35 ± 0.24	2.11 ± 0.40	1.04 ± 0.09	1.65 ± 0.21
NP-SH	6.55 ± 1.17	16.12 ± 2.08	2.31 ± 0.31#,c	8.95 ± 0.44#
CAT	17.69 ± 1.97	9.65 ± 1.95	10.00 ± 1.15#,c	16.79 ± 3.98
SOD	1.83 ± 0.40	1.78 ± 0.21	1.73 ± 0.21	2.13 ± 0.28
Na+/ K+-ATPase	1.90 ± 0.25	1.77 ± 0.11	1.74 ± 0.20	1.28 ± 0.14
Hippocampus				
DCFH-DA	2.62 ± 0.33	3.13 ± 0.15	3.81 ± 0.21#,c	3.36 ± 0.22
TBARS	1.29 ± 0.16	2.26 ± 0.37*	1.23 ± 0.19	2.37 ± 0.34*
NP-SH	8.97 ± 2.01	10.10 ± 0.70	3.59 ± 0.73#,c	9.38 ± 0.65
CAT	9.09 ± 0.89	7.60 ± 0.59	15.79 ± 2.37#,c	18.23 ± 0.59#
SOD	3.17 ± 0.40	4.92 ± 0.86	3.55 ± 0.30	4.42 ± 0.89
Na+/ K+-ATPase	2.91 ± 0.67	4.17 ± 0.94	4.00 ± 0.38	3.81 ± 1.14
Striatum				
DCFH-DA	2.62 ± 0.33	3.00 ± 0.24	3.82 ± 0.21#	3.36 ± 0.22
TBARS	0.72 ± 0.14	1.39 ± 0.16*	1.62 ± 0.29#	2.37 ± 0.34*,a,b
NP-SH	9.34 ± 1.50	8.49 ± 0.31	9.73 ± 0.96	9.38 ± 0.65
CAT	13.17 ± 2.15	13.26 ± 1.50	5.95 ± 0.59#	21.25 ± 1.43*,#,a
SOD	1.89 ± 0.14	2.57 ± 0.33	2.49 ± 0.19	4.42 ± 0.89
Na+/ K+-ATPase	1.03 ± 0.23	1.44 ± 0.81	0.75 ± 0.32	0.77 ± 0.04

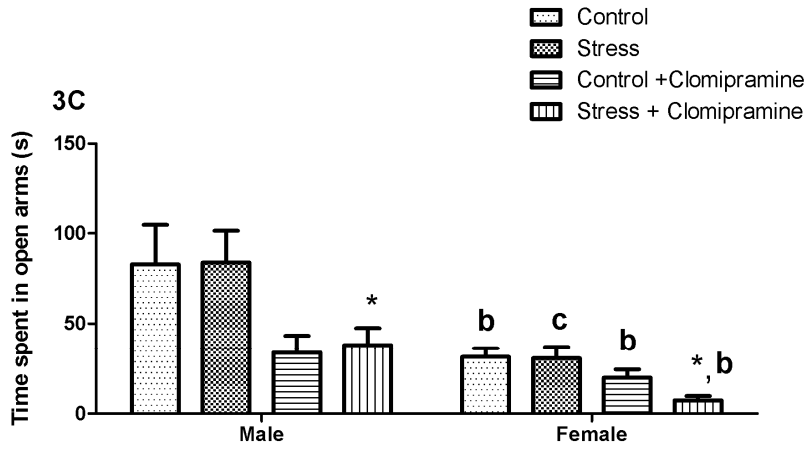

1A
Female
Control
Stress
Control +Clomipramine
Stress + Clomipramine
Exploratory Preference
100
80
60
40
20
0
*,a
*,a
Training
STM
LTM

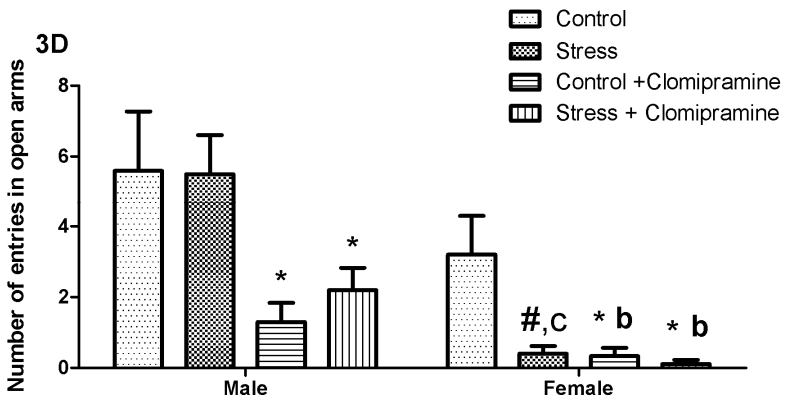

1B
Male
Control
Stress
Clomipramine
Stress+Clomipramine
Exploratory Preference
100
80
60
40
20
0
α
α
#,a,b
*,b
*,a,b
#
Training
STM
LTM

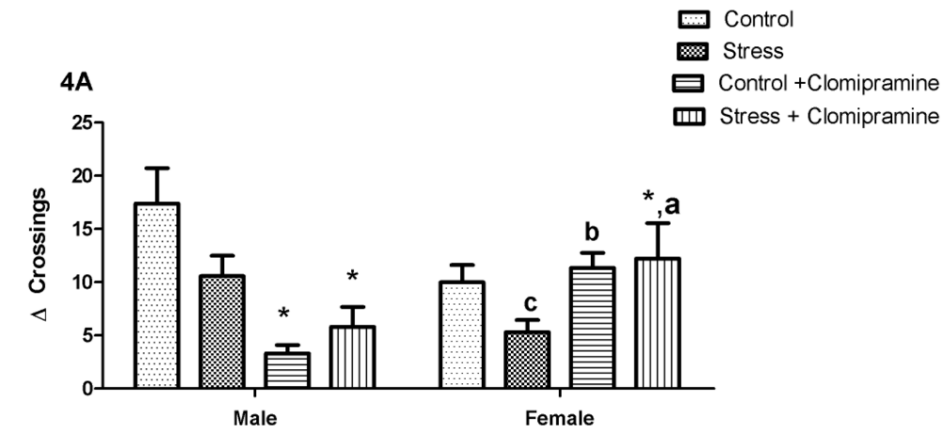

Control
Stress
Control +Clomipramine
Stress + Clomipramine
2A
Crossings
50
40
30
20
10
0
b
*,b
*, #
*
*
Male
Female
2B
Rearings
30
20
10
0
#, c
*, b
*, b
*
*
Male
Female

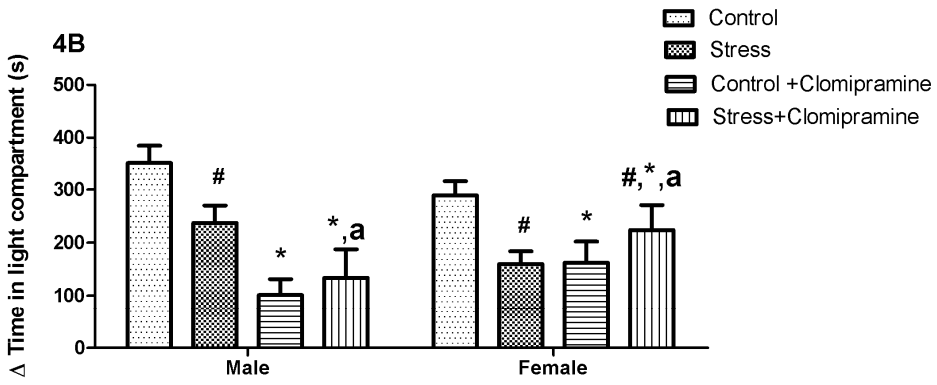

3A
Control
Stress
Control +Clomipramine
Stress + Clomipramine
Time spent in closed arms (s)
400
300
200
100
0
*, b
Male
Female

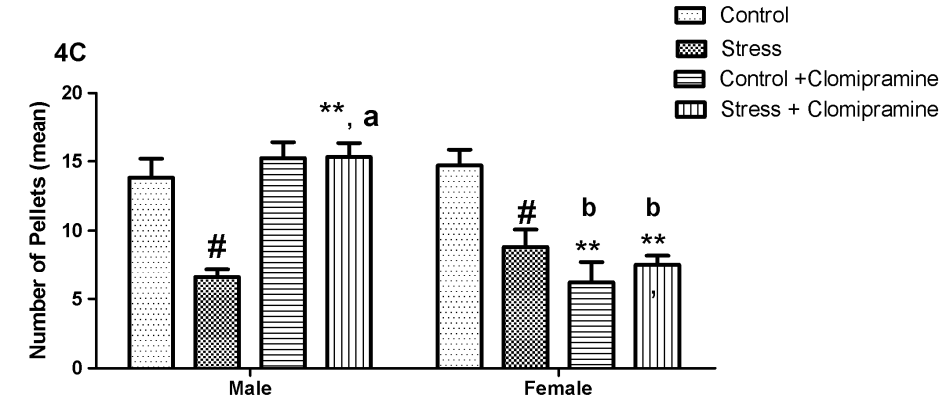

3B
Control
Stress
Control +Clomipramine
Stress + Clomipramine
Number of entries in closed arms
10
8
6
4
2
0
*
*
Male
Female


3C
Control
Stress
Control +Clomipramine
Stress + Clomipramine
Time spent in open arms (s)
150
100
50
0
*
b
c
b
*, b
Male
Female


3D
Number of entries in open arms
8
6
4
2
0
Male
Female
Control
Stress
Control +Clomipramine
Stress + Clomipramine
*
*
#,c
* b
* b


4A
Control
Stress
Control +Clomipramine
Stress + Clomipramine
Δ Crossings
25
20
15
10
5
0
*
*
c
b
*,a
Male
Female


4B
Control
Stress
Control +Clomipramine
Stress+Clomipramine
Δ Time in light compartment (s)
500
400
300
200
100
0
#
*
*,a
#
*
#,*,a
Male
Female


4C
Control
Stress
Control +Clomipramine
Stress + Clomipramine
Number of Pellets (mean)
20
15
10
5
0
#
**, a
#
b
**
b
**
Male
Female

5. DISCUSSÃO

O estresse repetido pode ser visto como um modelo importante na indução de desordens psiquiátricas como a depressão. A deficiência nos sistemas de defesa antioxidantes associado com aumento da peroxidação lipídica e formação específica de EROs podem induzir ao dano oxidativo e conseqüentemente causar vulnerabilidade cerebral com diferentes respostas em machos e fêmeas. Por outro lado, a clomipramina enquanto auxilia na redução dos sintomas oriundos do estresse, pode apresentar efeitos colaterias sobre as funções cognitivas. A ação dos antidepressivos tricíclicos em ambos os sexos ainda precisa ser melhor entendida.

Desta forma este estudo propôs investigar em um primeiro momento os efeitos da clomipramina sobre os parâmetros de estresse oxidativo em cérebro de ratos machos submetidos a estresse repetido por contenção (**Artigo 1**), a possível relação na atividade da Na^+/ K^+-ATPase em cérebro sobre as alterações comportamentais induzidas por estresse repetido e clomipramina em ratos machos (**Artigo 2**) e se as respostas ao dano oxidativo em cérebro e disfunções comportamentais apresentam respostas diferentes em machos e fêmeas submetidos ao estresse repetido e tratamento com clomipramina (**Manuscrito 1**).

Observamos diferenças significativas quanto aos níveis de DCFH-DA e TBARS quando comparamos machos e fêmeas. Machos submetidos a estresse não apresentaram modificação nos níveis de DCFH-DA nos tecidos cerebrais estudados, enquanto os níveis de TBARS aumentaram somente em estriado (Figuras 1 e 2 – **Artigo 1** e Tabela 1A – **Manuscrito 1**). Por outro lado, fêmeas parecem apresentar maior sensibilidade a DCFH-DA em todas as estruturas analisadas enquanto TBARS somente em estriado (Tabela 1B – **Manuscrito 1**). Neste contexto, durante o tratamento antidepressivo, machos apresentaram níveis elevados de DCFH-DA em estriado e hipocampo enquanto os níveis de TBARS foram maiores em córtex cerebral e estriado. Contudo, quando associamos a clomipramina ao estresse, somente córtex cerebral apresentou aumento nos níveis de DCFH-DA enquanto os níveis de TBARS aumentaram todas as estruturas analisadas (Figuras 1 e 2 – **Artigo 1** e Tabela 1A – **Manuscrito 1**). Em contrapartida, em fêmeas não foi observado este aumento em DCFH-DA e TBARS em todas as estruturas estudadas (Tabela 1B – **Manuscrito 1**).

Os resultados indicam que os efeitos impostos pelo estresse sobre os padrões de formação específica de EROs e peroxidação lipídica, são aumentados em fêmeas

particularmente em estriado e hipocampo. Neste contexto, os resultados estão de acordo a diferentes estudos que demonstraram sensibilidade ao dano oxidativo em estriado e hipocampo (HOMI et al., 2002; STANGHERLIN et al. 2008), influência dos níveis de GC sobre a peroxidação lipídica (FONTELLA et al., 2005) e de estrogênio sobre os GC (PREDIGER et al., 2004) que os mantém elevados por um período prolongado demonstrando maior sensibilização em fêmeas (GALEA et al., 1997) e déficits neuronais. Particularmente, a formação específica de EROs pode ocorrer durante a terapia antidepressiva com ADTs (DEMERDASH et al., 2004), contudo, verificamos uma redução de EROs em fêmeas. Isto vai contra estudos que enfatizam possível melhora terapêutica com IRSS em fêmeas e a ADTs em machos. Poucos estudos revelam um aumento na formação de EROs e peroxidação lipídica após tratamento antidepressivo quanto ao sexo em ratos. Desta forma, as diferenças sexuais quanto ao modelo de estresse repetido (mais agressivo em fêmeas), metabolismo, distribuição, captação e retenção da clomipramina contribuem para um desequilíbrio entre GC e RG determinando vulnerabilidade regional quanto à funcionalidade neuronal e assim potencializando estes efeitos. Os estudos laboratorias realizados com ambos os sexos evidenciam que fêmeas são mais sensíveis ao estresse que machos (CHADDA & DEVAUD, 2005), sendo assim o tratamento antidepressivo reduziria possíveis déficits causados pelo estresse.

Os efeitos oxidativos das EROs são controlados por antioxidantes não enzimáticos (–SH) e enzimáticos (CAT e SOD) utilizados neste estudo. Assim, uma alteração estrutural e funcional destas enzimas assim como a redução em suas atividades contribuem para condições associadas à excessiva geração de EROs (MEISTER, 1983; MEIJER, 1991; SIES, 1997; VERTUANI et al., 2004). Os grupos –SH não protéicos são encontrados especialmente na estrutura do tri-peptídeo de função antioxidante não-enzimático glutationa (GSH). A diminuição na GSH cerebral foi encontrada em estudos com modelos de depressão induzidos por estresse (PAL et al., 1994) caracterizando efeitos de citotoxicidade principalmente em hipocampo (GILBERTI et al., 2000; SAIJA et al., 1994) o que foi evidenciado somente em fêmeas.

A quantidade de NP-SH encontra-se reduzida em córtex cerebral e hipocampo de fêmeas, o que não foi observado em machos submetidos a estresse (Tabela 1A e 1B – **Manuscrito 1**). Diferenças sexuais são encontradas também quanto à atividade da Na^+ /K^+ ATPase que se encontra reduzida em todas estruturas cerebrais analisadas em machos submetidos e estresse, fato não observado em fêmeas (Tabela 1 A e 1B – **Manuscrito 1**). A atividade destas enzimas dependem em parte da integridade dos grupos –SH para que tenham

sua funcionalidade não prejudicada (ZHENG et al., 2003). A terapia antidepressiva com ADTs em doses terapêuticas recupera os níveis de GSH em animais indicando seus efeitos benéficos na depressão induzida pelo estresse (ZAFIR et al., 2009). Contudo, a dose utilizada determinou dano oxidativo em todas as estruturas analisadas independente do estresse em machos. Por outro lado, este efeito foi observado somente em córtex cerebral de fêmeas. Destacamos também a reduzida atividade da Na^+ /K^+ ATPase em córtex cerebral e estriado quando da combinação de clomipramina com estresse ou aplicada sozinha em machos (Figura 6 – **Artigo 1** e Tabela 1A – **Manuscrito 1**). Os ADTS inibem a atividade desta enzima por alterar a organização estrutural das membranas lipídicas (PEDRAZZA et al., 2007). Assim é possível sugerir que córtex cerebral e estriado são as estruturas mais sensíveis à clomipramina interferindo para a excitabilidade e regulação do volume celular neuronal referente à dose aplicada quanto à atividade da Na^+ /K^+ ATPase.

Os sistemas de defesa antioxidante enzimáticos como CAT e SOD, por sua vez, também foram alterados em decorrência do estresse em ambos os sexos. Observamos que fêmeas apresentaram redução significativa na atividade da enzima CAT em córtex cerebral e estriado enquanto a SOD não apresentou alteração significativa em fêmeas (Tabela 1B – **Manuscrito 1**). As atividades da CAT e SOD estiveram alteradas somente em estriado de machos (Figuras 4 e 5 – **Artigo 1** e Tabela 1A – **Manuscrito 1**). O tratamento antidepressivo aumentou a atividade da CAT em todas as estruturas cerebrais quando em combinação com estresse ou aplicado sozinho em machos, enquanto a atividade da SOD apresentou atividade reduzida em estriado e hipocampo de machos quando comparado às fêmeas (Tabela 1 A e 1B – **Manuscrito 1**).

Sugere-se que este resultado esteja relacionado a uma resposta compensatória dos tecidos, especialmente estriado e hipocampo lesados na tentativa de neutralizar uma maior formação de H_2O_2 condicionada ao estresse e tratamento antidepressivo. Os resultados obtidos a partir da análise das atividades enzimáticas dependentes da integridade dos grupos NP–SH e dos sistemas de defesa antioxidantes enzimáticos e não enzimáticos corroboram indiretamente a excessiva geração de EROs subseqüente ao estresse e tratamento antidepressivo em ambos os sexos. É importante considerar que o aumento na formação específica de EROs, não necessariamente indica que há dano oxidativo nas macromoléculas celulares, uma vez que elas podem ser destoxificadas por sistemas de defesa antioxidante (ORHAM et al., 2006)

Os resultados até o momento descritos permitem fazer algumas reflexões acerca do tema proposto particularmente quanto ao estresse oxidativo: 1º) fêmeas são mais susceptíveis

ao modelo de estresse utilizado sugerindo maior propensão à depressão. 2º) estriado e hipocampo parecem ser as estruturas cerebrais mais atingidas por danos oxidativos quanto ao aumento nos níveis de peroxidação lipídica e formação de EROs assim como nas atividades enzimáticas de CAT e SOD em fêmeas. 3º) o tratamento antidepressivo sugere menor desequilíbrio na homeostase redox em fêmeas e maior em machos com relevância na dose utilizada. 4º) a excitabilidade neuronal parece estar comprometida principalmente em machos, o que é revertido pelo tratamento antidepressivo em estriado e hipocampo de ambos os sexos.

Outro importante fenômeno investigado na tentativa de elucidar os mecanismos envolvidos paralelamente ao dano oxidativo foi a análise comportamental e suas diferenças quanto ao sexo. Nossos resultados iniciais mostram que o estresse pode influenciar a memória não-espacial em ambos os sexos. Na tarefa de reconhecimento de objetos, machos apresentaram prejuízo quanto à memória de curto e longo prazo (Figura 2 – **Artigo 2** e Figura 1B - **Manuscrito 1**), o que não foi observado em fêmeas (Figura 1A – **Manuscrito 1**). Estes resultados estão de acordo com estudos demonstrando prejuízo quanto à memória de curto e longo prazo (ELIZALDE et al., 2008) em machos, mas não em fêmeas (BOWMAN et al., 2009). As fêmeas são mais sensíveis ao estresse, o que poderia potencializar as disfunções quanto à memória. Assim, sugerimos que o modelo de tarefa realizada associado às flutuações ou efeito neuroprotetor dos níveis de estrogênio podem ter influenciado o desempenho nesta tarefa e conseqüentemente sobre a formação de memória (TROPP E MARKUS, 2001). Com referência ao tratamento antidepressivo, os prejuízos quanto à memória de curto prazo foi marcante em machos (Figura 2 – **Artigo 2** e Figura 1B- **Manuscrito 1**) enquanto a memória a longo prazo foi alterada em fêmeas (Figura 1A- **Manuscrito 1**) quando associado ao estresse repetido por contenção. Neste contexto, os resultados vão ao encontro de outros estudos determinando efeitos adversos da administração crônica de clomipramina sobre a memória (BURGOS et al., 2005). As diferenças sexuais de acordo com inúmeros estudos permanecem ainda inconclusivas, mas direcionam para uma melhora terapêutica em fêmeas.

A atividade exploratória e locomotora em ambos os sexos foi utilizada através da tarefa de campo aberto. Verificamos diferenças sexuais sobre a atividade locomotora quanto ao número de cruzamentos (Figura 2A – **Manuscrito 1**) e elevações (Figura 2B – **Manuscrito 1**) sendo ambos menores em machos em comparação às fêmeas em estresse de acordo a outros estudos (CHADDA & DEVAUD, 2005). Durante o tratamento com antidepressivo, verificamos que machos e fêmeas apresentaram redução na atividade locomotora evidenciado pelo número de cruzamentos e elevações, contudo esta redução foi significativamente acentuada em machos (Figuras 3A e 3B - **Artigo 2** e Figuras 2A e 2B

respectivamente – **Manuscrito 1**). A clomipramina melhora as atividades locomotoras com doses menores equivalentes 5 mg/kg (LIU et al., 2008) mas verificamos que a dose utilizada em nosso estudo induziu à um efeito ansiogênico, possivelmente relacionado com a dosagem (BRAVIN et al., 2005).

As diferenças sexuais quanto ao número de cruzamentos e o tempo de permanência nos braços abertos que são consideradas variáveis importantes na análise de ansiedade em animais foram analisadas na tarefa de labirinto em cruz elevado. Este é um modelo de ansiedade mais fidedigno em relação ao campo aberto. Os resultados obtidos evidenciam que fêmeas apresentam maior nível de ansiedade quando comparados aos machos (Figuras 3C e 3D – **Manuscrito 1**). Estes resultados são contrários a maioria dos estudos que apontam geralmente, maior efeito de ansiedade em machos (MITRA et al., 2005; LUINE et al., 2007; BOWMAN et al., 2009) embora dados de literatura apontem maior suscetibilidade ao estresse em fêmeas (BOURKE et al., 2011) as quais são afetadas pela ansiedade com elevado índice de desordens afetivas (MYIN-GERMEYS et al., 2004; SONNE et al., 2003). Recentemente, um estudo realizado por Huynh (2011) verificou que o estresse crônico diminui a tempo e entrada nos braços abertos no labirinto em cruz elevado em fêmeas o que vai ao encontro de nossos resultados.

Este fato pode ter ocorrido devido a maioria destes estudos utilizarem 1-3 semanas de estresse por restrição o que poderia não apresentar as mesmas alterações sobre os sistemas oxidantes e antioxidantes em nosso protocolo de 6 semanas e também pela variação hormonal que pode alterar os níveis de GC e RG contribuindo para a presença de ansiedade. Neste contexto, após o tratamento com clomipramina, verificamos que ambos os sexos apresentam alteração nos níveis de ansiedade, mas particularmente este efeito foi maior em fêmeas (Figuras 3C e 3D – **Manuscrito 1**) determinando um possível efeito ansiogênico deste antidepressivo (BRAVIN et al., 2005).

Contudo, a ansiedade maior em fêmeas sob estresse parece não ter efeitos impactantes sobre a memória já que não houve prejuízos quanto à memória nestes animais sugerindo diferenças importantes quanto ao sexo.

A motivação por um novo ambiente determinado pela tarefa condicionada de lugar, mostrou que machos e fêmeas permanecem menos tempo em um novo ambiente (claro) (Figura 4B – **Manuscrito 1**) assim como apresentam redução no número de cruzamentos entre os ambientes claro e escuro (Figura 4A – **Manuscrito 1**) quando submetidos ao estresse. Contudo, esta redução parece ser mais evidenciada em fêmeas quando comparamos aos machos. Ambos apresentam também uma redução quanto ao consumo de alimento doce o que

parece ser mais evidenciado em machos em relação ás fêmeas (Figura 4C – **Manuscrito 1**). Neste contexto o tratamento antidepressivo também apresenta impacto negativo na realização desta tarefa principalmente em machos quando analisamos estas duas varáveis. Por outro lado, o consumo de alimento doce (recompensa) apresenta efeitos opostos. Machos aumentam enquanto fêmeas reduzem o consumo de alimento doce (Figura 4C – **Manuscrito 1**).

Este comportamento pode ter ocorrido por uma desregulação do sistema dopaminérgico no estriado, pois há evidências significativas que interações límbico-estriatais estejam relacionadas com o processo de recompensa. A passividade observada nesta tarefa pelo estresse é comparada a uma motivação reduzida que é característica de sintomas depressivos em humanos (PORSOLT, 1979). Quanto ao consumo de alimento doce, nossos resultados vão ao encontro de outros estudos que demonstraram aumento da preferência doce em animais controles (PLAZNIK et al., 1989) seguido por uma redução quando em exposição ao estresse por restrição (ZAFIR et al., 2009). A reversão destes sintomas é observada pelo tratamento crônico com clomipramina (CLARK et al., 2000), contudo não observamos esta melhora em fêmeas.

Em conclusão, os resultados apresentados nesta tese contribuem para aprimorar o conhecimento sobre os antidepressivos tricíclicos, especialmente a clomipramina a qual em uma dose elevada mostrou induzir maior sensibilidade quanto aos parâmetros de estresse oxidativo e comportamentais em machos a partir do modelo de estresse utilizado.

6. CONCLUSÕES

De acordo com os objetivos apresentados nesta tese podemos concluir que:

Capítulo I

- No presente estudo, o modelo de estresse repetido por contenção alterou a homeostase redox evidenciada pelo aumento nos níveis de TBARS e desequilíbrio quanto as atividades antioxidantes de SOD e CAT em estriado de ratos machos;
- A Na^+/ K^+-ATPase teve sua atividade reduzida em todos os tecidos estudados em ratos machos repetidamente estressados;
- O tratamento com clomipramina não foi apto a reverter o dano oxidativo causado pelo estresse. Isto pôde ser verificado pelo aumento nos níveis de DCFH-DA e TBARS assim como nas atividades enzimáticas de SOD e CAT e redução na atividade de NP-SH mais evidenciado em estriado e hipocampo de ratos machos;
- A clomipramina somente reduziu a atividade da Na^+/ K^+-ATPase em córtex cerebral de ratos machos repetidamente estressados.

Capítulo II

- Os resultados deste estudo demonstraram uma relação entre a atividade reduzida da Na^+/ K^+-ATPase e comportamento em ratos machos submetidos à estresse repetido por contenção. A atividade reduzida da Na^+/ K^+-ATPase em hipocampo, estriado e córtex cerebral pode estar relacionada respectivamente ao prejuízo na formação de memória a curto e longo prazo além de motivação e atividade exploratória reduzidas. A análise de comportamento alimentar demonstrou redução no consumo de alimento doce neste grupo;

- A clomipramina reduziu a atividade da Na^+/ K^+-ATPase em córtex cerebral, contudo verificamos aumento da ansiedade, redução na formação da memória a curto prazo, na motivação e atividade exploratória em ratos machos. O aumento no consumo de alimento doce foi marcante neste grupo.

Capítulo III

- O modelo de estresse repetido por contenção induziu maior sensibilidade ao dano oxidativo em fêmeas determinado pelo desequilíbrio oxidante/antioxidante principalmente em estriado e hipocampo;
- O modelo de estresse repetido por contenção reduziu a atividade enzimática da Na^+ / K^+-ATPase em todas as estruturas cerebrais analisadas em machos o que não observamos em fêmeas;
- O modelo de estresse repetido por contenção limitou a formação da memória não espacial e atividade exploratória em ratos machos enquanto uma redução no comportamento motivado e aumento nos níveis de ansiedade foram mais evidenciados em fêmeas;
- O comportamento alimentar evidenciado pelo consumo de alimento doce foi reduzido em ambos os sexos submetidos a estresse repetido por contenção;
- O tratamento antidepressivo com clomipramina na dose utilizada não recuperou o equilíbrio oxidante/antioxidante potencializando o dano oxidativo em ratos machos repetidamente estressados;
- O tratamento antidepressivo com clomipramina induziu atividade reduzida a Na^+ / K^+-ATPase em córtex cerebral e hipocampo de ratos machos quando comparamos às fêmeas;
- O tratamento antidepressivo com clomipramina limitou a formação da memória não espacial a curto prazo em machos e a longo prazo em fêmeas, limitou a atividade exploratória em ratos machos, aumentou a ansiedade em fêmeas enquanto uma redução no comportamento motivado foi evidenciado em ambos os sexos paticularmente em machose aumento nos níveis de ansiedade foram mais evidenciados em fêmeas;

- O comportamento alimentar evidenciado pelo consumo de alimento doce apresentou maior atividade em machos submetidos ao tratamento com clomipramina.

7. PERSPECTIVAS

A partir dos resultados apresentados nesta tese, poderíamos realizar estudos com os seguintes objetivos:

- Investigar os efeitos induzidos pelo estresse repetido por contenção e clomipramina sobre a viabilidade celular mitocondrial em cérebro de ratos machos e fêmeas;
- Investigar os efeitos induzidos pelo estresse repetido por contenção e clomipramina através de análises histológicas em cérebro de ratos machos e fêmeas;
- Investigar os efeitos induzidos pelo estresse repetido por contenção e clomipramina sobre a homeostase redox através da análise de DCFH-DA e TBARS assim como dos principais sistemas de defesa antioxidantes enzimáticos (CAT e SOD) e não enzimáticos (NP-SH) em fígado e rim de ratos machos e fêmeas;
- Avaliar os efeitos benéficos do treinamento físico moderado sobre alterações dos parâmetros de estresse oxidativo e viabilidade celular mitocondrial em cérebro assim como análises comportamentais em machos e fêmeas induzidos por estresse repetido por contenção e clomipramina.

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