UNIVERSIDADE DE PASSO FUNDO FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA PROGRAMA DE PÓS-GRADUAÇÃO EM BIOEXPERIMENTAÇÃO

EFEITO DE PROTEÇÃO DE UM COMPOSTO DE SUPLEMENTO (MULTICAFÉ®) EM UM MODELO DE ESTRESSE AGUDO POR CONTENÇÃO

DISSERTAÇÃO DE MESTRADO

Joana Grandó Moretto

Passo Fundo, RS, Brasil
2020

EFEITO DE PROTEÇÃO DE UM COMPOSTO DE SUPLEMENTO (MULTICAFÉ®) EM UM MODELO DE ESTRESSE AGUDO POR CONTENÇÃO

Joana Grandó Moretto

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioexperimentação, Área de Concentração em Bioexperimentação, da Faculdade de Agronomia e Medicina Veterinária da Universidade de Passo Fundo (UPF), como requisito parcial para a obtenção do grau de **Mestre em Bioexperimentação**

Orientador: Prof. Dr. Rômulo Pillon Barcelos

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UNIVERSIDADE DE PASSO FUNDO
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A comissão examinadora, abaixo assinada, aprova a Dissertação de Mestrado

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Elaborada por
Joana Grandó Moretto

Como requisito parcial para a obtenção do grau de
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"Para ter algo que você nunca teve,
é preciso fazer algo que você nunca fez."

(Chico Xavier)

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LISTA DE ABREVIATURAS

AChE - Acetilcolinesterase
ANVISA – Agência Nacional de Vigilância Sanitária
A2A – Receptor de Adenisina
CAT – Catalase
CEUA – Comissão Ética em Uso de Animais
cm – Centímetros
CONCEUA – Comitê Nacional de Controle de Experimentação Animal
EAR/ ARS – Estresse agudo por restrição
ES – Estresse agudo
EROS – Espécies reativas de oxigênio
g – Gramas
h – Horas
ICB – Instituto de Ciências Biológicas
Kg – Quilogramas
MC - MultiCafé®
mcg - Microgramas
mg – Miligrama
min - Minutos
ml – Mililitros
T-SH – Tióies totais
TAC – Capacidade antioxidante total
UFMS – Universidade Federal de Santa Maria
UPF – Universidade de Passo Fundo

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo

EFEITO DE PROTEÇÃO DE UM COMPOSTO DE SUPLEMENTO (MULTICAFÉ®) EM UM MODELO DE ESTRESSE AGUDO POR CONTENÇÃO

Autor: Joana Grandó Moretto
Orientador: Rômulo Pillon Barcelos
Passo Fundo, 26 de Agosto de 2020

O bem-estar de humano e animais é um tema que vem tomando grande importância no mundo, devido às mudanças de hábitos. O primeiro nível de resposta do animal a um ambiente estressante ou aversivo são mudanças nos padrões comportamentais e moleculares. Assim antioxidantes e antiinflamatórios podem estar relacionados aos benefícios para a saúde, a falta de estratégias de tratamento faz com que costumes diários sejam alternativas como solução. Fazendo com que alimentos funcionais movimentem aproximadamente 129 bilhões de dólares. O termo "Estresse" é utilizado para descrever uma variedade de estímulos fisiológicos e/ou psicológicos, que podem ter efeitos diretos ou indiretos na função corporal. Novos estudos surgem com o objetivo de minimizar esses efeitos, como no caso, pesquisas com o MULTICAFÉ® (MC), um suplemento alimentar constituido de cafeína, L-carnitina e zinco, que podem ser obtidos de fontes naturais como semente de guaraná torrada, farinha de arroz e de castanha-do-Brasil. O presente estudo teve por objetivo estudar possíveis efeitos bioquímicos e ansiolíticos deste suplemento em modelos animal submetidos a estresse agudo por restrição de espaço. Avaliamos os potenciais efeitos preventivos do MC no modelo de estresse agudo por restrição (EAR) em camundongos Swuis machos (45 dias). O EAR foi induzido por confinamento individual por duas horas/dia por dez dias. O MC foi administrado por via oral 2,63 mg/dia. Os animais foram submetidos a testes comportamentais de ansiedade e bioquímicos como espécies reativas de oxigênio (ERO) e teor total de tióis não protéicos, lipoperoxidação, capacidade antioxidante total (TAC), atividades das enzimas catalase e acetilcolinesterase. Observamos que a EAR aumentou a produção de EROs, AChE, atividades de CAT no fígado, rim. Por outro lado, o MC foi capaz de atenuar a maioria dessas elevações, diminuindo as espécies reativas, principalmente nos tecidos hepáticos e renais, e restaurando o equilíbrio da atividade antioxidante avaliada pela T-SH no cérebro e nos orgão renais, além disso, o MC apresentou um aumento no TAC no rim. Por fim o MC tem futuro promissor, uma vez que é facilmente produzido através de substâncias naturais e seus efeitos antioxidantes podem ser uma nova alternativa totalmente natural para mudanças em prejuízos a saúde relacionados ao estresse.

Palavras chaves: Bem-estar animal, Estresse oxidativo, Contenção Mecânica, Cafeína, Antioxidantes.

ABSTRACT

Master's Tesis
Post-Graduation Program in Bioexperimentation – University of Passo Fundo

PROTECTIVE EFFECT OF A SUPPLEMENT (MULTICAFÉ®) COMPOUND IN AN ACUTE RESTRAINT STRESS MODEL

Author**:** Joana Grandó Moretto
Advisor: Rômulo Pillon Barcelos
Passo Fundo, August 26th 2020

The well-being of humans and animals is a topic that has become very important in the world, due to changes in habits. The animal's first level of response to a stressful or aversive environment is changing in behavioral and molecular patterns. So antioxidants and anti-inflammatories can be related to health benefits, the lack of treatment strategies makes daily customs as alternatives as a solution. Making functional foods move approximately $ 129 billion. The term "Stress" is used to describe a variety of physiological and/or psychological stimuli, which can have direct or indirect effects on body function. New studies appear intending to minimize these effects, as in the case, research with the compound MULTICAFÉ®, which is a completely natural food supplement to be obtained from roasted guarana seed powder, rice flour and Brazil nuts. The present study aimed to study possible biochemical and anxiolytic effects of this supplement in animal models subjected to acute stress due to space restriction. We evaluated the potential preventive effects of CM in the acute restriction stress (RAS) model in male Swuis mice (45 days). EAR was induced by individual confinement for two hours/day for ten days. The MC was administered orally 2.63 mg/day. The animals were submitted to behavioral anxiety and biochemical tests such as reactive oxygen species (ROS) and total content of non-protein thiols, lipoperoxidation, total antioxidant capacity (TAC), activities of the enzymes catalase and acetylcholinesterase. We observed that EAR increased the production of EROs, AChE, CAT activities in the liver, kidney. On the other hand, the MC was able to attenuate most of these elevations, decreasing the reactive species, mainly in the liver and kidney tissues, and restoring the balance of the antioxidant activity evaluated by T-SH in the brain and kidney organs, in addition, the MC showed an increase in kidney TAC. Finally, MC has a promising future, since it is easily produced through natural substances and its antioxidant effects can be a new all-natural alternative for changes in stress-related health losses.

Keywords: Animal welfare, Oxidative stress, Mechanical containment, Caffeine, Antioxidants.

1. INTRODUÇÃO

Estresse ambiental e físico afetam continuamente animais e seres humanos, porém, os animais conseguem manter um equilíbrio homeostático por meio de sua força endógena. Mesmo assim exposições intensas e extremas a fatores estressores acabam por terem efeitos marcantes no organismo e também gerando distúrbios comportamentais (1).

O primeiro nível de resposta do animal a um ambiente estressante ou aversivo são mudanças nos padrões comportamentais. No meio científico o bem-estar animal é um tema que vem tomando grande importância, um importante indicador do seu bem-estar são os padrões comportamentais (2). Baseando-se nestas ideias devemos considerar eventos estressantes como importantes fatores de risco no desenvolvimento de distúrbios neuropsiquiátricos que incluem distúrbios afetivos, alimentares. Alterações significativas nos sistemas cognitivo, neurotransmissor e neuroendócrino de humanos e modelos animais, podem estar atribuídas a eventos estressantes/traumáticos (3).

Há interesse na formulação tanto em modelos animais quanto em ensaios clínicos, de um suplemento alimentar constituido de cafeína, L-carnitina e zinco, que podem ser obtidos de fontes naturais como semente de guaraná torrada, farinha de arroz e de castanha-do-Brasil, foi concebido. Estes três compostos químicos são considerados seguros para consumo, possuindo propriedades capazes de serem extraídas por processos de extração aquosa a quente, a frio, em um pH ácido, e são comercialmente nacionais, tornando-se assim viáveis logisticamente e economicamente se utilizadas como matéria prima para o suplemento Multicafé® em questão (4).

Por isso, nosso objetivo neste trabalho foi testar o produto Multicafé®, o qual é formulado com Guaraná , Castanha do Brasil e Farina de Arroz e verificar se o mesmo é capaz de afetar os mecanismos bioquímicos e comportamentais do estresse físico em animais.

Esta dissertação está estruturada da seguinte forma, uma breve revisão sobre estresse, radicais livres e os componentes do suplemento. Logo em seguida como Capítulo 1, o artigo como forma de apresentação de resultados da pesquisa, este que foi submetido a revista Journal of Ethonopharmacology. E na sequência a conclusão e considerações finais.

2. REVISÃO BIBLIOGRÁFICA

2.1 ESTRESSE OXIDATIVO

O termo “Estresse” é utilizado para descrever uma variedade de estímulos fisiológicos e/ou psicológicos, que podem ter efeitos diretos ou indiretos na função corporal (5). O estresse repetido provoca alterações neuroquímicas e morfológicas que afetam negativamente o funcionamento do cérebro. Assim, o estresse repetido é um gatilho ou um fator de risco para distúrbios neuropsiquiátricos, ou seja, depressão, tanto em humanos quanto em modelos animais (6). Em 1963 o estresse foi definido pelo cientista e médico Hans Selye como uma síndrome, a “Síndrome da Adaptação Geral” sendo, a resposta adaptativa de um organismo à ação de agentes capazes de ameaçar a homeostase. Selye ainda classificou o estresse em três estágios: 1. Alarme, momento que o agente estressor é notado; 2. Resistência, quando o organismo inicia combater o agente estressor e 3. Exaustão, momento que o organismo esgota toda sua capacidade de reposta, sofrendo assim efeitos deletérios exaustão (7). Contrapondo a ideia de Selye, indica-se que o estresse crônico é patogênico, não em função das falhas do organismo, mas sim, pelas próprias defesas se tornarem patogênicas (8). Hans Selye apontou ainda *″o* estresse é um conceito científico que recebeu a benção mista de ser muito conhecido e pouco compreendido ″, e continuou salientando que ainda não existe uma definição perfeita de estresse que seja comumente vista como a resposta do cérebro a uma demanda e / ou desafio (conhecidos como estressores). Estes agentes estressores podem ser reais ou percebidos. E não dependem somente do sujeito, mas também da dinâmica temporal distinta, variando sua intensidade (9).

O estresse oxidativo é um processo que vem da decorrência de um desiquilíbrio entre compostos oxidantes e antioxidantes (figura 1), em relação a uma excessiva de radicais livres (figura 2) ou prejuízo da remoção dos mesmos (10).

Figura 1: Processo de desiquilíbrio do qual o estresse oxidativo decorre.

Fonte: https://ri.ufs.br/bitstream/riufs/4930/1/PATRICIA_MORGANA_FERREIRA_SANTOS.pdf

O estado de desequilíbrio celular entre as defesas antioxidantes e a produção de espécies reativas de oxigênio é caracterizado como estresse oxidativo, onde o aumento dos níveis dessas espécies reativas de oxigênio (EROS) são danosos ao organismo(11). Pode-se definir também como um desequilíbrio entre antioxidantes endógenos e espécies oxidativas, em um sistema biológico pode ser causador de excesso de espécies reativas de oxigênio ou diminuição de antioxidantes ou ambos. Este desequilíbrio está associado a modificação de proteínas, oxidação lipídica e quebra de ácido nucleicos, o que prejudica as funções celulares(12).

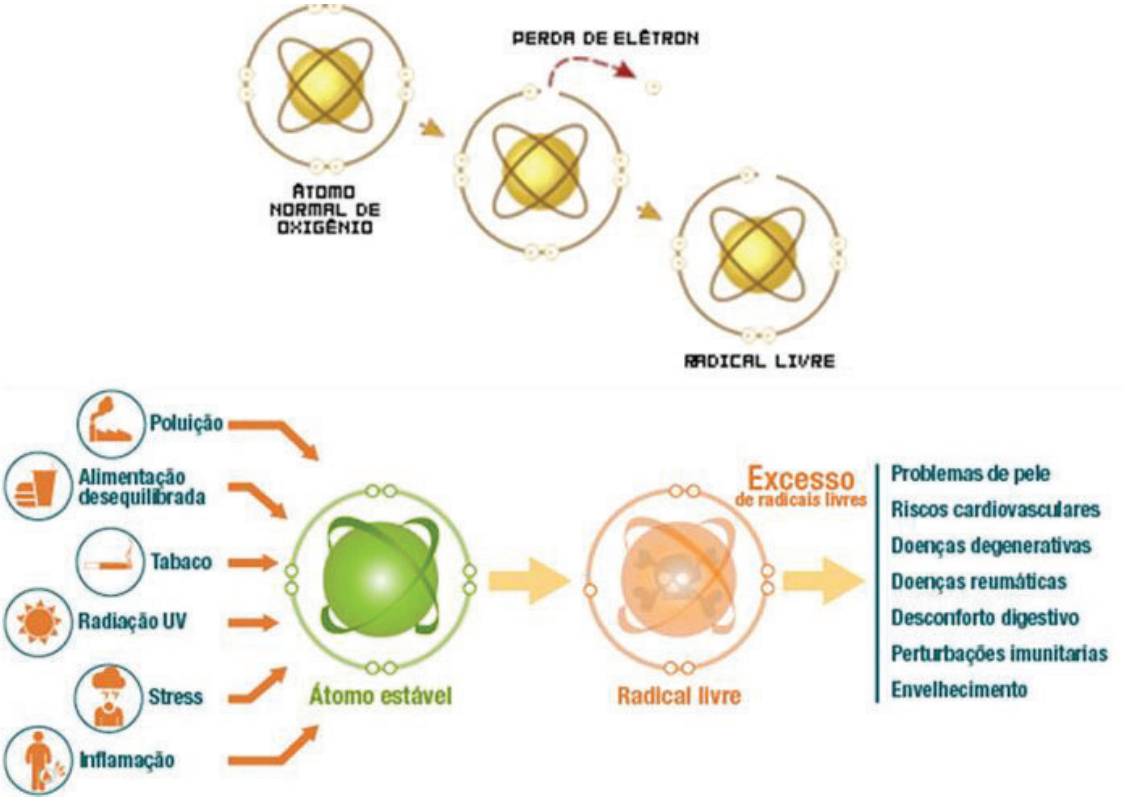

Figura 2: Ilustrações de radicais livres, e exemplos de situações que podem levar a produção destes.

Fonte: https://www.nutergia.pt/pt/nutergia-conselheiro/dossiers-bem-estar/antioxidantes.php

2.2 ANTIOXIDANTES

Ocorre produção de EROS em todos tipos de células, podendo assim afetar várias funções celulares como a angiogênese, apoptose e proliferação celular. Dessa forma, as defesas antioxidantes endógenas que irão neutralizar os danos causados pelo estresse oxidativo (figura 3) (13). Pode -se usar como definição de antioxidante, qualquer substância que atrasa, previne ou remove um processo oxidativo (14). Salienta-se também que essa neutralização é feita por antioxidantes enzimáticos (como a glutationa peroxidase - GSHpx e a Catalase – CAT) e antioxidantes não enzimáticos (como glutationa reduzida – GSH, vitaminas A e E) (15).

Mecanismos antioxidantes e anti-inflamatórios podem estar relacionados aos benefícios para a saúde através de dietas à base de alimentos vegetais. Assim aumentam as pesquisas na identificação de alimentos e extratos de alimentos que sejam capazes de modular eficientemente o estresse oxidativo e inflamatório que possam trazer benefícios a saúde (16).

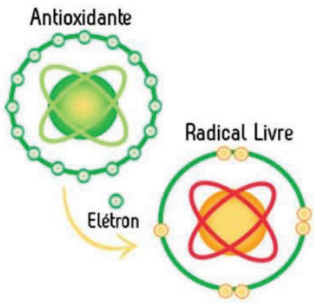

Figura 3: Antioxidante doa o elétron ao radical neutralizando sua atividade nociva

Fonte: https://www.camim.com.br/dicas-de-como-combater-os-radicais-livres/

2.3 MULTICAFÉ®

Atualmente por falta de estratégias de tratamento para condições de estresse, busca-se alternativas em costumes diários como solução. A cafeína é considerada a droga psicoativa mais comum utilizada (6). Além disso considera-se que no mercado mundial em 2015, alimentos funcionais movimentaram em torno de 129 bilhões de dólares americanos e com aumento ainda ativo (17).

Alimentos funcionais são assim considerados em relação aos papeis metabólicos ou fisiológicos que uma molécula desempenha no organismo, significando que esses alimentos auxiliam no equilíbrio e nos hábitos de uma vida saudável, não tendo assim intuito de tratamento, prevenção ou cura de doenças (18).

A farinha de arroz, é fonte de L-Carnitina, que tem um papel crucial no metabolismo energético das células e a qual pesquisas concluem que possui propriedades antioxidantes e pode proteger as células de espécies reativas de oxigênio tóxicas em alguns distúrbios (19). O selênio encontrado na Castanha do Brasil, é um elemento que sua importância para o organismo é contraditória depende de sua dosagem, tendo assim então uma recomendação de 34 mcg/dia. Mas é sabido que ele atua como antioxidante e anticoagulante (20). Enquanto isso o guaraná produz uma semente rica em alcalóides e polifenóis com propriedades antimicrobianas, antioxidantes e anti-inflamatórias, onde a cafeína é o principal composto (21).

Atualmente estudos consistentes ressaltam que a cafeína contém propriedades benéficas a saúde humana, apresentando um alto potencial para ser utilizado como suplemento energético, antioxidante e antiinflamatório, e também sendo relacionada na alteração do estado redox celular. Consideranto a possibilidade do desenvolvimento de uma bebida funcional contendo o Guaraná, também contribuindo com bioativo a cafeina (4,22,23). Podendo ser encontrada em muitos produtos e bebidas e até mesmo em medicamentos, acredita-se que na atualidade é o psicoestimulante mais frequentemente ingerido, fazendo parte dos consumos diários em diversos países (24,25). Seu consumo pode causar estado de alerta, e tem interferência no humor e bem estar no comportamento, pois tem ação na liberação de catecolaminas (25).

Segundo Kaster et al (2015), em condições estressantes o consumo da cafeína aumenta, traçando assim uma correlação inversa com a incidência de depressão e suicido. O grupo de pesquisa e o autor mencionam também que em doses não tóxicas, os alvos da

cafeína serão principalmente os receptores de adenosina no cérebro, tendo como mecanismo de ação o aumento da ação do sistema nervoso simpático e bloqueando estes receptores. Barcelos et al., (2020) acrescenta ainda que os efeitos deste bioativo são dose dependentes, e que propriedades químicas e físicas, pH e rota de administração podem vir a influenciar sua absorção. Seu mecanismo de ação esta relacionada aos receptores de adenosina, mais especificamente no A2A, podendo se tornar um regulador inflamatório em várias condições patofisiológicas (25).

Estudos vem apontando que o estresse oxidativo e a inflamação estão ligados a diversas doenças crônicas não transmissíveis. Assim como, evidenciando que a cafeína possuem efeito neuroprotetor através da diminuição da oxidação e da sua capacidade de modulação inflamatória (25).

3. CAPITULO 1

PROTECTIVE EFFECT OF A SUPPLEMENT (MULTICAFÉ®) IN AN ACUTE RESTRAINT STRESS MODEL

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ABSTRACT

Ethnopharmacological relevance

Consistent studies emphasize that coffee contains properties beneficial to health, with high potential for use as an energy supplement, antioxidant and anti-inflammatory. The object of the study is a supplement based on caffeine, L-carnitine and zinc, which can be obtained from natural sources such as roasted guarana seed *(Paullinia cupana)*, rice flour *(Oryza sativa)* and Brazil nut *(Bertholletia excelsa)*. With this evaluation, we seek to establish the beneficial and adverse effects, seeking a better understanding of the action of the supplement, through the analysis of oxidative stress and the evaluation of the modulations of the behavioral parameters of mice subjected to mechanical stress by containment.

Aim of the study

We aimed to study possible biochemical and anxiolytic effects of this supplement in animal models subjected to acute stress through space restriction.

Materials and methods

We evaluate the potential preventive effects of a supplement name MULTICAFÉ® (MC) on acute stress restriction (ARS) model in male Swiss mice (45 days old). ARS was induced by individual confinement for two hours/day for ten days. MC was administered orally 2.63 mg/day. Animals were submitted to anxiety behavioral and biochemical tests as reactive oxygen species (ROS) and total non-protein thiols content, lipoperoxidation, total antioxidant capacity (TAC), catalase, and acetylcholinesterase enzyme activities.

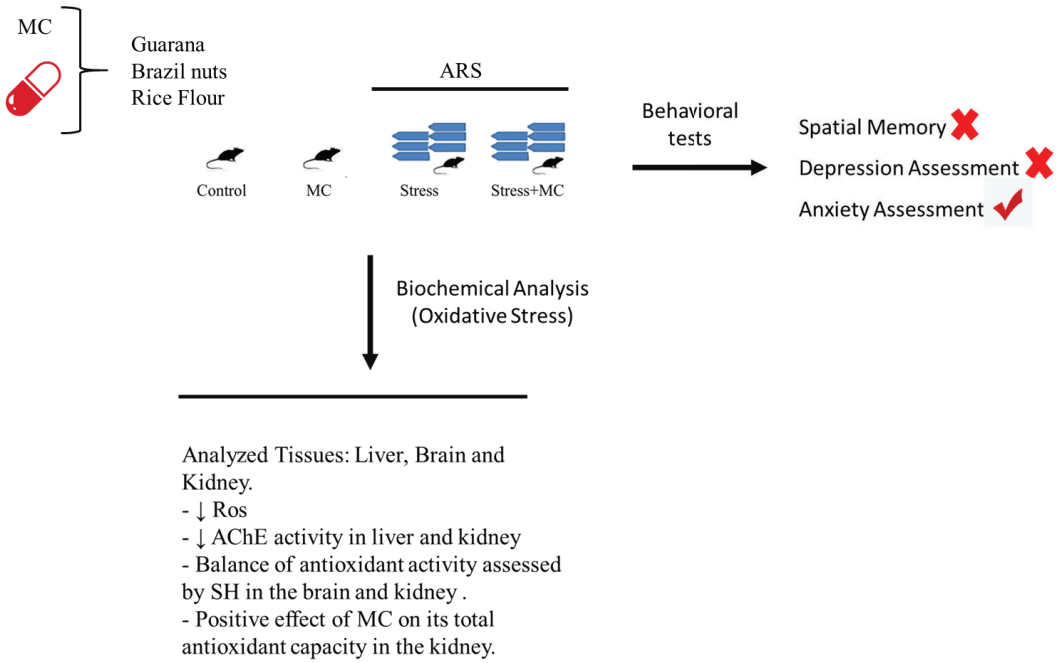
Results

We observed that ARS increased ROS production, AChE, CAT activities in the liver, kidney. On the other way, MC was able to blunt most of these elevations by decreasing the reactive species, mainly in liver and kidney tissues, and by restoring the balance of antioxidant activity assessed by SH in the brain and kidney organs. And MC had a increase in the TAC in the kidney.

Conclusions

MC has promising future since it is easily produced through natural substances and its antioxidant effects may be a new all-natural alternative for changes in stress-related diseases.

Graphical abstract



Keywords: Animal welfare, Oxidative stress, Mechanical containment, Caffeine, Antioxidants;

1. Introduction

Environmental and physical stress affected animals and humans, which can maintain the homeostatic balance through their endogenous strength until moderate levels of stress. However, intense and extreme exposures to stressors end up having significant effects on the body and also generating behavioral disorders (Toyoda, 2017).

Based on these ideas, we should consider stressful events as significant risk factors in the development of neuropsychiatric disorders that include affective, eating disorders, and illegal substances. Substantial changes in the cognitive, neurotransmitter, and neuroendocrine systems of humans and animal models, can be attributed to stressful/traumatic events (Cadet, 2016).

Stress is defined as a "general adaptation syndrome," an organism's adaptive response against agents capable of threatening homeostasis (Kopin, 1995). Psychological stress is a scientific concept that received the mixed blessing of being very well known and poorly understood, commonly known as the brain's response to an adverse situation and challenge (known as stressors). These stressors can be real or perceived. And they do not depend only on the subject, but also on the distinct temporal dynamics, varying their intensity and on the environment (Sousa, 2016).

In the same line, oxidative stress is a process that results from an imbalance between oxidizing and antioxidant compounds, about an excess of free radicals or loss of their removal (Green et al., 2004). This imbalance or increased levels of these reactive oxygen species (ROS) is harmful to the organism (Seddon et al., 2007). It can also be defined as an imbalance between endogenous antioxidants and oxidative species, leading to protein modification, lipid peroxidation, and nucleic acid breakdown, which impairs cellular functions (He et al., 2018).

Currently, due to the lack of treatment strategies for stress conditions, alternatives are sought in daily customs as a solution. Caffeine is considered the most common psychoactive drug used worldwide (Kaster et al., 2015). There are also reports of the growing interest in functional foods, related to the metabolic or physiological roles that a molecule plays on the body, helping to maintain the balance and habits of a healthy life (Grosso et al., 2017). So, we came with a supplement studied in this research - Multicafe® (MC), developed based on caffeine, zinc and L-carnitine compounds from Guarana *(Paullinia cupana)*, Castanha do Pará *(Bertholletia excelsa)* and Rice Farina *(Oryza sativa)*, claiming antioxidant properties, antifatigue and aid to the immune system. These ingredients are considered safe for consumption and can be easily extracted by hot and cold aqueous extraction processes, as they are marketed nationally, making them logistically and economically viable if used as a raw material for the product in question (Hedström et al. , 2016).

Therefore, the objective of this study was to test the MC supplement and verify if it can affect the biochemical and behavioral mechanisms of physical stress in animals.

2. Materials and methods

2.1 Animals

Fifty-six male Swiss mice, approximately 45 days old, 30-35g. Animals were provided from our breeding colony and kept in plastic boxes containing a maximum of three animals per cage under controlled environment conditions (12:12h light-dark cycle, with the onset of light phase at 7:00, 25±1 °C, 55% relative humidity) with standard food and water *ad libitum*. Mice were acclimated for seven days before initiation of any procedures. All experiments were conducted following national and international legislation: Brazilian College of Animal Experimentation (COBEA) and the US Public Health Service's Policy on Human Care and Use of Laboratory Animals-PHS Policy and

with the approval of the local Ethics Committee (#038/2019).

2.2 Acute restraint stress (ARS)

The resource of having the availability of strains that exhibit different behavioral responses to established stress paradigms is valuable (Sathyanesan et al., 2017). The induction of acute resistance stress was carried out through individual confinement for two hours per day in 50 ml polycarbonate cylinders, with dimensions of 20cm in length and 6.5 cm in diameter adapted from Domingues et al., (2019).

2.3 Supplement

The compound used is in the process of patent registration and was initially called MultiCafé® (MC). It was synthesized elaborated and made available by the Federal University of Santa Maria - UFSM, partner of this project. It is a completely natural supplement enriched with L-carnitine tartrate base (79.9%), dry guarana extract (20%) and chelate selenium (0.1), in the following presentations: roasted guarana stone, flour of rice and Brazil nuts, all considered safe ingredients for consumption and having their properties easy to extract through aqueous processes from hot to cold and with acidic pH (Hedström et al., 2016).

The administered dose (maximum volume of 0.7 ml/animal/day) of each compound, was used as previously determined and used in humans trials (500 mg/Kg/day), which was converted for use in experimental animals (Ehling et al., 2015). In the study 2.63 mg/kg/day was used, orally gavage.

2.4 Experimental design

The animals were divided into four groups of 7 animals each: G1- Control, G2 – MC (MC administration), G3 - Stress, and G4 - (Stress plus MC administration). The process lasted a total of 10 days, where the groups that were exposed to the ARS went through this for 2 hours/day the entire period, whereas the control animals without stress were handled for 10 min per/day. Cage was cleaned and changed; then, they return to it. Two hours after the ARS, the animals were submitted to the behavioral tests executed by two trained observers blind to the treatments. Behavioral tests were evaluated every 2

days.

A different set of animals and protocol was used for biochemical analysis. For this, mice were euthanized by decapitation 24 hours after the last MC administration. Kidneys, liver, brain, and blood were removed and immediately frozen with nitrogen for storage for further analysis.

2.5 Behavioral tests

2.5.1 Y-Maze Test

Considered a simple test, it consists of two short-term spatial recognition tests, based on the innate tendency of exploration rodents (Rayatnia et al., 2011). This test was evaluated through an adaptation by Kraeuter et al., (2019), following the methodology: test divided into two stages, training, and tests, respectively, lasting 5 minutes interspersed with an interval of 90 minutes. During training, one of the arms of the apparatus was closed, so the animal did not have that restricted area. In the test period, the device was fully released, where two observers recorded and timed the entries and the permanence of the animals in each arm, respectively.

2.5.2 Elevated Plus-Maze Test

The test was chosen to check anxiety symptoms in animals, through the event of "conflict of approach and avoidance," evaluation methods according to Johnson, (1995). For this test, red light is required, so that the animals were placed in their housing boxes in the test room half an hour before the start, for the adjustment, the whole evaluation was carried out by two researchers who timed and noted the number of entries of animals in each segment and duration of time in them.

2.5.3 Splash Test

According to Isingrini et al., (2010), the test of choice for assessing depression, it is simple to spray 1ml of a 10% sucrose solution on the animal's back, which is placed individually in a clear acrylic box. Where it is evaluated by a person expecting the animal to exhibit self-cleaning behavior (grooming), the frequency and latency to initiate this behavior are calculated in 5 minutes.

2.6 Laboratory tests - biochemical tests for oxidative parameters

2.6.1 Reactive oxygen species (ROS)

This parameter was determined through the levels of 2'- 7 '- dichlorofluorescein (DCF) (Myhre et al., 2003). 50 µL of liver, kidney and brain homogenates were used for the test, these were added in Tris-HCl (10 mM) and 2 '7' -dichlorofluorescein diacetate (DCFH-DA) (1 mM), incubated by 1 hour in the dark. The fluorescence reading was performed with excitation at 488 nm, emission at 525 nm and opening at 1.5 nm, correction of the results of the protein levels and expressed as DFEH-DA fluorescence (Adaptation by Saccol et al., 2020).

2.6.2 Production thiobarbituric acid reactive substance (TBARS)

Lipid peroxidation biomarker, measured by the reaction with thiobarbituric acid, through the concentration of malonaldehyde (MDA) already described by Jentzsch et al., (1996). The reaction mixture consisted of 200 µL of liver, kidney and brain homogenate, 500 µL of Acetic acid buffer, 500 µL of 0.8% thiobarbituric acid (TBA), 200 µL of sodium dodecyl sulfate (SDS) 8.1% and 100 µL of H2O. The mixture was incubated at 95 ° C for 2 hours and then read on a spectrophotometer at 532 nm. TBARS levels were expressed in nmol MDA / mg of protein.

2.6.3 Catalase activity (CAT)

It was determined according to the method of Nelson and Kiesow (1972). It consists of adding 20 µL of liver, kidney and brain homogenate to a mixture of 2,000 µL of 50 mM potassium phosphate buffer (TFK) pH 7 and 100 µL of 0.3 M hydrogen peroxide (H2O2). he absorbance was recorded for 1 minute (every 15 seconds) at 240 nm and the results were given in nmol/mg protein/minute (adapted Saccol et al., (2020).

2.6.4 Total thiols determination (T-SH)

To determine the total levels of (T-SH), the method of Ellman (1959) was used with some changes. 200 µL of liver, kidney and brain homogenate, 750 µL of potassium phosphate buffer (TFK) 1 M pH 7.4 and 50 µL of 5.5′-dithio-bis (2-nitrobenzoic acid) (DTNB) 10mM were added. The reaction product was measured at 412 nm.

2.6.5 Acetylcholinesterase (Ache)

AChE activity was estimated using the Ellman method (Ellman et al., 1961) using acetylthiocholine iodide (ATC) as a substrate and etopropazine as a butyrylcholinesterase

(BChE) inhibitor (Worek et al., 1999). The following protocol was followed, 880 µl of 0.1M TFK buffer, added with 25 µl of homogenized studied tissue, were used and exposed to the water bath for 2 minutes at 30º C. Then, 50 µl of 9 mM ATC and 50 µl of 36 mM DTNB were added for readings at 412 nm at times 0, 60, and 120 seconds, (Ellman et al., 1961; Worek et al., 1999).

2.6.6 Total antioxidant capacity

Determined using the phosphomolybdenum method, based on the spectrophotometric determination of the reduction of Mo + 4 to Mo + 5, with subsequent formation of Mo + 5 phosphate, which shows maximum absorption at 695 nm (Prieto et al., 1999). Homogenized tissue samples (10 mg / mL), dissolved in distilled water (BSE) or 1% DMSO (fractions) were combined, in an Eppendorf tube, with 1 ml of the reagent solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The closed tubes were incubated at 95 ° C for 90 min. After cooling, at room temperature, absorbance at 695 nm was determined. Ascorbic acid was used as a standard.

2.6.7 Protein determination

The protein content was determined as described previously using bovine serum albumin (BSA) as standard.

2.7 Statistical analysis

The entire database obtained was subjected to statistical analysis performed by the statistical program GraphPad Prism v.8.01 (Graphpad inc.; La Jolla, USA), and the results expressed as mean ± standard error of the mean (SEM). For continuous data showing normal distribution, two-way ANOVA analysis of variance was applied, followed by Tuckey post-hoc, p values < 0.05 were considered significant. The data were corrected by the protein content and expressed as a percentage of control.

3. Results

Elevated Plus-Maze Test

For anxiety test behavior (Fig.1), on the third day of the research development, the animals in the control group remained for a significantly longer time in the open arm of the apparatus compared to Stress group ($p<0.05$). After that day, significance has

dropped and ceases to exist.

The other two behavioral tests (Y-Maze and Splash test) applied to the groups of animals did not present significant results (data not shown).

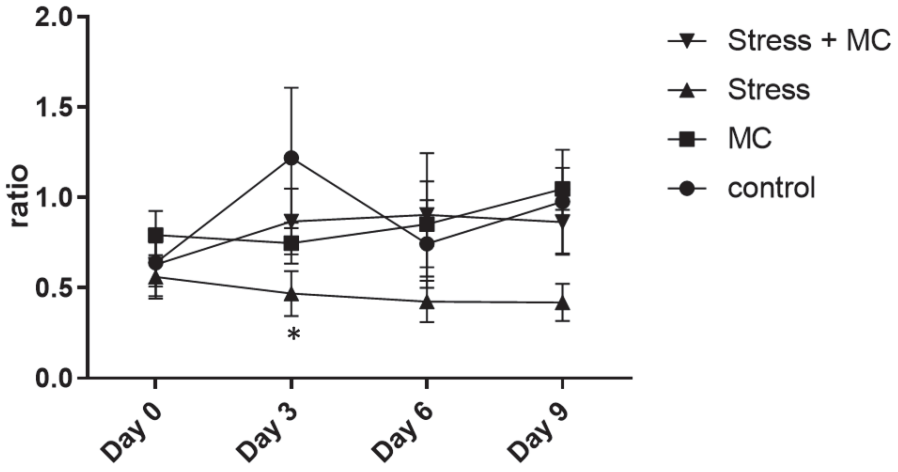


*Figure 1:The effects of the Multicafé® compound on the animals' behavior in relation to anxiety were evaluated using the elevated cross test. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. The vertical lines indicate the SEM and the symbols indicate: * different when compared to the control group.*

Biochemical analysis

Reactive oxygen species (ROS) and thiobarbituric acid reactive substance (TBARS) production

In the liver, ROS production increased in Stress group compared to all other groups (Figure 2A). However, Stress + MC decrease did not show differences when compared to control. Regarding the brain (Figure 2B) and kidney (Figure 2C), there was significantly decreased ROS content in the Stress + MC group when compared to the Stress group but without the significance of these two groups concerning the control group.

No significant changes in TBARS level were found between groups (Fig. 3).

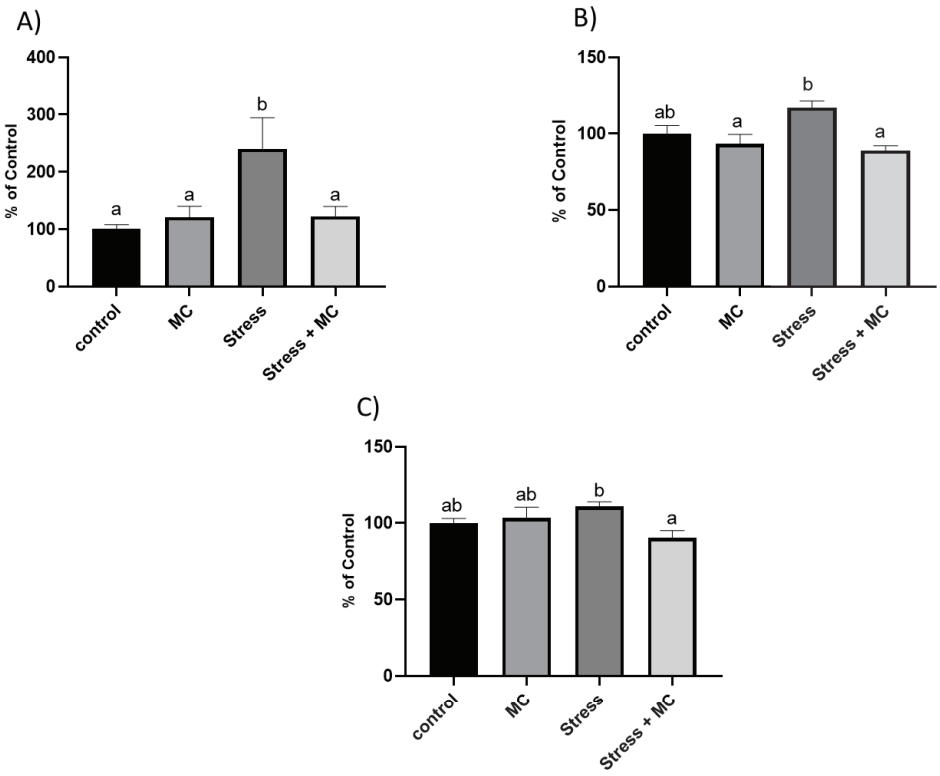


Figure 2: Effect of resistance stress and MC treatment on ROS production in the liver (A) kidney (B) and brain (C) of mice. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. Data are expressed as mean ± S.E.M (n = 8, p<0.05). Means for a variable with superscripts without a common letter differ.

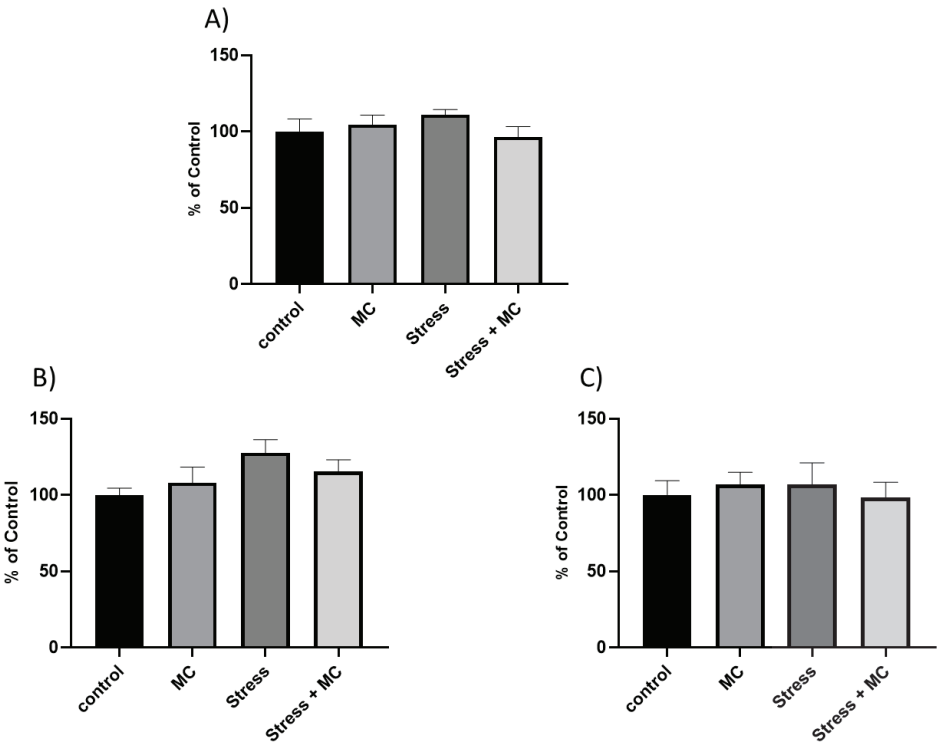


Figure 3: Effect of resistance stress and MC treatment on lipid peroxidation (TBARS levels) in the liver (A) kidney (B) and brain (C) of mice. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. Data are expressed as mean ± SEM (n=8, p<0.05).

Catalase activity (CAT)

Liver (Figure 4A) and kidney (Figure 4B) CAT enzyme activity increased in the Stress group compared to the control group. However, Stress + MC treatment decreased its activities at levels similar to the control group. There were no significant results in brain CAT activity analysis (Figure 4B).

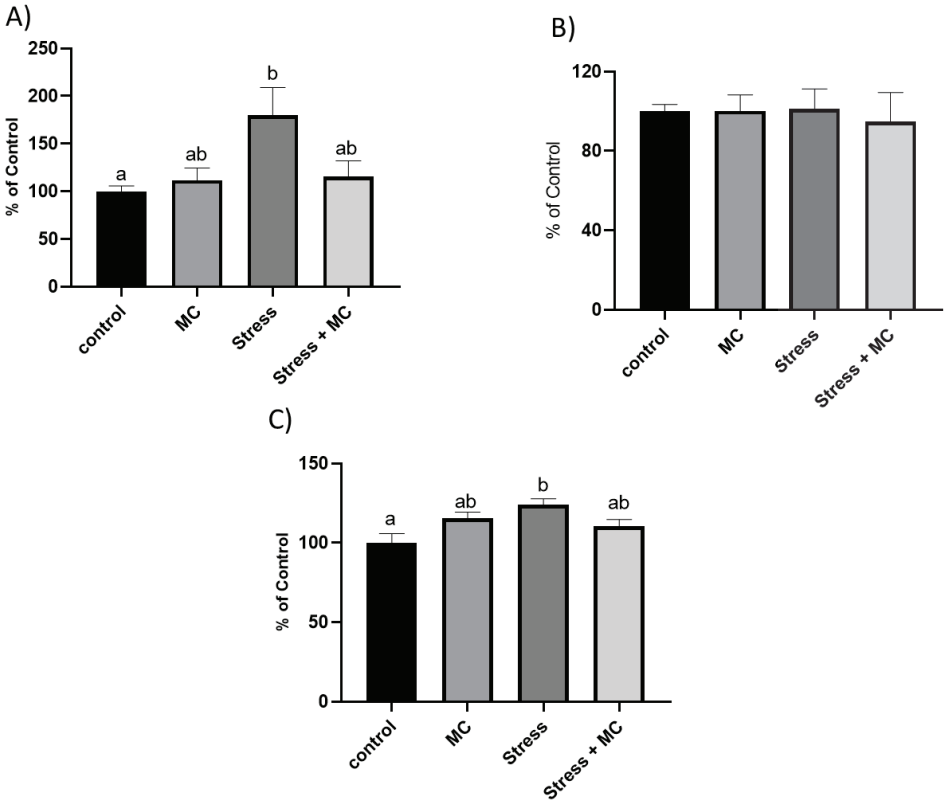


Figure 4: Effect of resistance stress and MC treatment on catalase activity in the liver (A) kidney (B) and brain (C) of mice. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. Data are expressed as mean ± S.E.M (n = 8, p<0.05). Means for a variable with superscripts without a common letter differ.

Acetylcholinesterase (Ache)

Liver and Kidney AChE activity increase in the Stress group when compared to the control group. However, Stress + MC decreased its activity (Figure 5A and 5C, respectively). No significant changes between the groups in brain AChE activity was observed (Figure 5B).

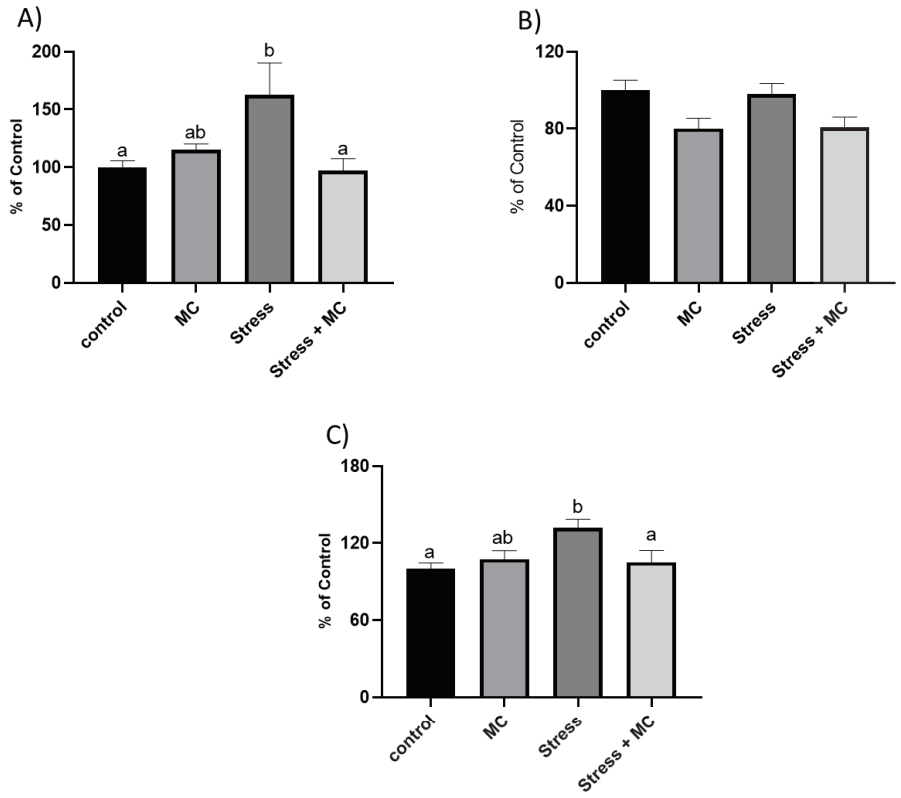


Figure 5: Effect of resistance stress and MC treatment on AChE activity in the liver (A) kidney (B) and brain (C) of mice. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. Data are expressed as mean ± S.E.M (n = 8, $p<0.05$). Means for a variable with superscripts without a common letter differ.

Total non-protein thiols determination (T-SH)

Total non-protein liver thiols are demonstrated in Figure 6. In the liver (Fig 6A), brain (Fig 6B) and kidney (Fig 6C) the stress group decreased the Total Non-protein T-Sh levels significantly when compared to the control group. However, the Stress + MC treated group blunt this effect in brain and kidney.

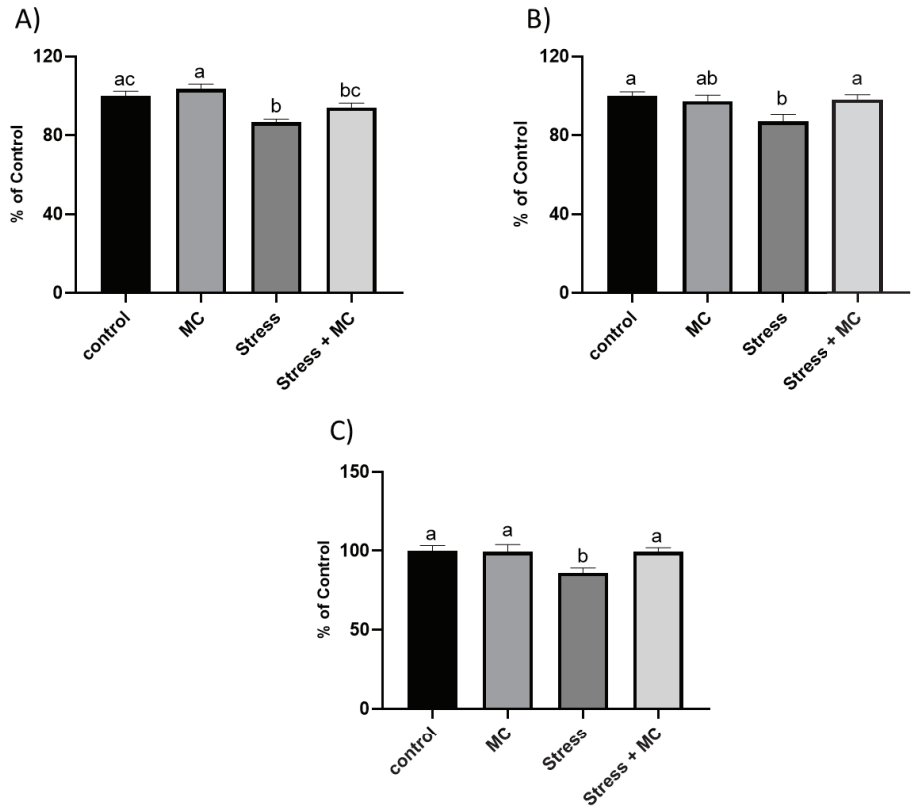


Figure 6: Effect of resistance stress and MC treatment on T-SH levels (A) kidney (B) and brain (C) of mice. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. Data are expressed as mean ± S.E.M (n = 8, p<0.05). Means for a variable with superscripts without a common letter differ.

Total antioxidant capacity (TAC)

The liver TAC was decreased in both Stress and Stress + MC groups when compared to control (Figure 7A). No changes were observed in this parameter in brain tissue (Figure 7B). The kidney TAC was decreased in Stress and group when compared to control, but not in Stress + MC, which blunted this effect (Figure 7 C).

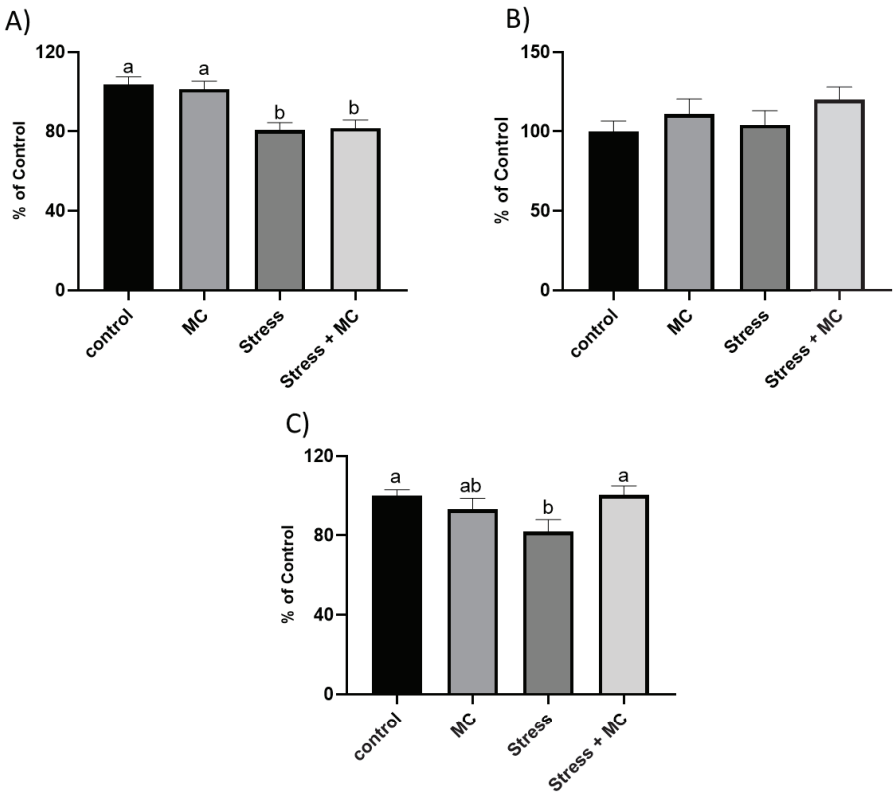


Figure 7: Effect of resistance stress and MC treatment on total antioxidant capacity in the liver (A) kidney (B) and brain (C) of mice. The animals were divided into four groups: Control, MC, Stress, and Stress + MC. Data are expressed as mean ± S.E.M (n = 8, p<0.05). Means for a variable with superscripts without a common letter differ.

4. Discussion

It is known that the massive consumption of drinks containing caffeine since antiquity is due to its stimulating effect. Only after that, interest in this product turns to health benefits, such as the prevention of some diseases (Gökcen and Şanlier, 2019). One of the objectives of this research was to evaluate the possible anxiolytic effect of the MC compound. We were able to observe here (Figure 1) that it agrees with previous reports, where it appears that stress decreases the animal's exploratory behavior (Çakır et al., 2017). Furthermore, that the anxiolytic effect of caffeine in animals is not yet well defined, as it may depend on high or low doses, and even on the type of stress suffered if chronic or acute (Barcelos et al., 2020; Çakır et al. , 2017). Also, through biochemical analyzes, we observed that MC had a positive effect in preventing the generation of oxidative stress. Which corroborates with Kelek et al., (2019), which indicates as one of the main effects of L-carnitine, the antioxidant action.

ROS, generated by intracellular aerobic metabolism and environmental stimuli such as anionic radical (O_2•) and hydroxyl radical (OH•), are the leading cause in humans/animals of oxidative damage and severe inflammation. Thus evidencing that there is a close link between chronic diseases and injuries from excessive oxidative stress (Cadet, 2016; Matsuzawa et al., 2005). These statements are in agreement with the results found in our research, where we observed that there was an increase in reactive oxygen species in all tissues analyzed in the animals of the Stress group. Another important observation was that the animals that were submitted ARS, but treated with MC, did not show that increase and yet, maintained its level similar to control. It makes us comfortable in stating that the compound, which has caffeine at its main constituents, has an antioxidant and anti-inflammatory effect, which as already described by (Kleber Silveira et al., 2018).

In this line, we observed TBARS as an oxidative stress parameter; however, we did not find significant variations on the tissues analyzed. We can attribute this lack of results at this because TBARS is an evaluation that indicates lipid peroxidation, an end-product of several oxidative stages, and because it might not show the mechanism of antioxidant action, but the monitoring of the effectiveness of an antioxidant molecule (Ghani et al., 2017). This way, we believe that the time of the ARS was too short to assess the significance of its results.

Likewise, antioxidant enzymes constantly seem in all tissues of the body. Catalase is one of them, which has its functional capacity activities that vary between different tissues (Christine N Scaglione et al., 2016). This characteristic was also observed in our study. The increase in liver and kidney' catalase activity on animals that suffered stress is in line with the descriptions that stress induces, triggering the activation of an antioxidant system, aiming to eliminate oxygen-derived free radicals overproduced, through the degradation of hydrogen peroxide radical. Even though ARS did not change the catalase activity in the brain, we attribute this effect to a need for a more prolonged time exposure to stress at this tissue, as chronic stress models (Samarghandian et al., 2017). Casaril et al., (2019) mentioned that CAT is considered a pro-oxidative indicator, also describes that where there is excessive production of peroxide, it favors the Fentom reaction and, consequently, the generation of hydroxyl radical, in turn, leading to lipid peroxidation. So, we could expect to see no changes or even a drop in CAT production in the brain.

In the same vein, AChE activity plays an essential role in the rapid hydrolysis of

acetylcholine and modulates the concentration of this neurotransmitter in synapses (Pezzementi et al., 2011). In our study, there was an increase in its activity in the group Stress in the liver and kidney, in line with the statements by Cadet, (2016), when he mentions that stressful and traumatic events cause significant changes in the cognitive, neurotransmitter and neuroendocrine systems of models humans and animals. When we observed that AChE activity in the Stress + MC group decreased to levels similar to those in the control group, we can interpret that the caffeine-based compound can reduce AChE activity. In addition, as previously noted in our previous work Barcelos et al., (2014), this event may indicate an anti-inflammatory role of MC, since acetylcholine would be more available and because it has anti-inflammatory functions and suppress the production of pro-inflammatory cytokines. On the other hand, Pohanka and Dobes (2013) described that caffeine intake in the form of chocolate, coffee, energy drinks or tea does not have biological effects related to AChE. However, if there is a simultaneous intake of coffee and energy drink or pills containing caffeine with a significant concentration of caffeine, AChE activity can be inhibited (Pohanka and Dobes, 2013), and this is in line with our study. Caffeine-based products have dose-dependent effects and can have positive or adverse results, involving the double redox capacity of caffeine (Barcelos et al., 2020). Failure to obtain results on this parameter in the brain corroborates the research by Ngoupaye et al., (2018), who highlights that to obtain results on AChE in the brain, a stress model with a prolonged chronic period (21 days).

Finally, the total non-protein thiols group is a useful biological marker in the relationship between free radicals and the action of antioxidant defenses in the body, and their reduced levels are indicative of mild oxidative stress (Lozano-Paniagua et al., 2018). Here, in all three tissues (Liver, Brain, and Kidney), the animals of the Stress group had reduced T-SH levels, as an indicator of redox balance impairment (Ayar et al., 2019). And the fact that the groups that received the MC have approximate values to the control group may meet the reasons that we described earlier concerning the antioxidant effect of caffeine and L-carnitine, thus bringing the redox balance again.

Similarly, the total antioxidant activity is also a marker of the effectiveness of the body's antioxidant defense (Lozano-Paniagua et al., 2018). Thus, its low levels can indicate an oxidative stress environment, as we observed in the liver and kidney from the Stress group. However, only in the kidney, animals in the Stress + MC group were able to recover from the harmful effects of ARS. Affirming the idea that oxidative stress causes liver damage, decreasing effective liver defenses (Kim et al., 2016). The fact that we have

no differences in the brain can be attributed to the fact that the ARS period was shorter to affect this organ for prolonged periods (Ngoupaye et al., 2018).

5. Conclusion

The present study confirmed that the Multicafé® supplement has an antioxidant effect and can be used as a preventive or treatment in stress-related contention diseases. Thus, we conclude that this compound has a promising future for this cause. In addition, as its composition products are natively known, produced on a large scale worldwide, their production can be highly affordable. And finally, its all-natural composition can generate less unwanted effects when compared to other drugs or the current pharmacological treatment.

Author contributions

Cibele F Teixeira, Verônica F. Azzolin, Ivana BMda Cruz, provided the MultiCafé® compound object of the study. Rômulo P. Barcelos supervised the study and draft paper submission. Joana G Moretto and Rômulo P Barcelos wrote the manuscript. Joana G Moretto, Julia S da Silva, Aline Z Pasinato, Jeferson L Kunz, and Paola Marengo conducted the behaviour and laboratory tests. All authors heard and approved the final manuscript.

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4. CONCLUSÃO

O presente estudo confirmou que o suplemento Multicafé® tem efeitos antioxidantes e que pode ser usado como preventivo ou como tratamento em doenças relacionadas ao estresse de restrição. Então, podemos dizer que esse composto tem um futuro promissor para essa causa. E, como seus produtos de composição são mundialmente reconhecidos, sua produção é mais acessível.

5. CONSIDERAÕES FINAIS

Para o desenvolvimento desta pesquisa levantamos o questionamento a respeito se um composto formulado com produtos naturais, com sua base na cafeína poderia na prevenção ou redução do estresse oxidativo. E este questionamento foi resposndido de forma positiva através dos resultados obtidos. Levando em consideração que os componentes deste composto são conhecidos, produzidos em larga escala mundialmente, e tem seus bioativos sendo alvos de pesquisa por muitos cientístas. Então teremos o MultiCafé® como um produto acessível a população. Pois são produtos logisticamente viáveis e com fácil extração de seus componentes.

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