UNIVERSIDADE ESTATUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOTECNOLOGIA

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Efeito do tratamento de N-acetilcisteína sobre o metabolismo energético e estresse oxidativo no miocárdio de ratos diabéticos.

Dissertação apresentada ao Programa de Pós-graduação em Farmacologia e Biotecnologia do Instituto de Biociências de Botucatu da Universidade Estadual Paulista "Júlio de Mesquita Filho", para obtenção do título de Mestre em Farmacologia e Biotecnologia.

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BOTUCATU
2017

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Kaga, Anderson Kiyoshi.
Efeito do tratamento de N-acetilcisteína sobre o metabolismo energético e estresse oxidativo no miocárdio de ratos diabéticos / Anderson Kiyoshi Kaga. - Botucatu, 2017

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Ana Angélica Henrique Fernandes
Capes: 21000000

1. Metabolismo energético. 2. Stress oxidativo. 3. Antioxidantes. 4. Miocárdio - Doenças. 5. Rato como animal de laboratório. 6. Diabetes mellitus tipo 1.

Palavras-chave: Antioxidantes; Diabetes mellitus tipo 1; Estresse oxidativo; Metabolismo energético; Tecido cardíaco.

DEDICATÓRIA

Aos meus pais, Aimar e Kiyosi e a minha tia Ayako por me proporcionarem toda a instrução e recurso necessário para conquistar mais este passo em minha vida, a presença de vocês é de suma importância, pois vocês são a minha base, minha estrutura.

A minha orientadora Profª. Drª. Ana Angélica, por ter me recebido em seu laboratório, ter tido paciência comigo e pelo seu carinho. A sua orientação me proporcionou adquirir uma nova visão sobre o conteúdo abordado na área de bioquímica, bem como aprofundado todo o conhecimento teórico.

AGRADECIMENTOS

Aos meus pais, Aimar e Kiyosi e também a minha tia Ayako por participarem da realização deste sonho.

A minha orientadora Profª. Drª. Ana Angélica por ter compartilhado do seu tempo e saber, pelos auxílios fornecidos e permitido que eu pudesse navegar pelo imenso "mar" da Bioquímica.

A meus familiares de Botucatu, Lúcia e Nivaldo por terem me acolhido na chegada a cidade de Botucatu, este apoio foi fundamental para eu alcançar este objetivo.

A minhas amigas, irmãs de coração, Daniele, Fabiana, Gióia e Silvia, são as culpadas por eu alcançar este objetivo, mesmo que distantes, sempre estavam próximas me auxiliando e me acalmando nos piores momentos.

Aos meus amigos e companheiros de laboratório do departamento de Bioquímica, Nágilla, Pedro e Priscila, e também aos alunos de iniciação científica que passaram este período comigo, Bárbara, Lucas e Mariana.

Aos meus amigos e companheiros de pós-graduação no programa de Farmacologia e Biotecnologia, em especial, Daniel, Lilliam e Loyane.

As minhas amadas amigas, "irmãs" de Botucatu, Ketlin, Priscila e Talita, por me acompanharem, fornecerem o ombro, e também a me puxarem a orelha quando foi necessário.

Aos técnicos do departamento de Química e Bioquímica, Augusto Santana Nascimento, Danielle Fernandes da Silva e Fábio Henrique Fava, pessoas maravilhosas que me auxiliaram durante o desenvolvimento do meu experimento e sempre estiveram dispostos a ajudar.

A secretária do departamento de Química e Bioquímica, Gabriela Cristina Gomes Valim Athanazio por me auxiliar, principalmente naqueles momentos corriqueiros.

Ao departamento de Química e Bioquímica por fornecer a estrutura física necessária para o desenvolvimento do estudo.

A seção de pós-graduação e a coordenação do programa de pós-graduação em Farmacologia e Biotecnologia pelo suporte acadêmico.

A CAPES pelo apoio financeiro, ao conceder a bolsa de estudos para o desenvolvimento deste trabalho.

"Toda a arte de ensinar é apenas a arte de acordar a curiosidade natural nas mentes jovens, com o propósito de serem satisfeitas mais tarde"

(Anatole France)

SUMÁRIO

RESUMO

O diabetes mellitus é um grupo heterogêneo de distúrbios no metabolismo energético e consequente estresse oxidativo, fatores importante para o desenvolvimento de complicações diabéticas como cardiomiopatias. A utilização de antioxidantes tem contribuído para melhorar a hiperglicemia e também minimizar as complicações diabéticas. O N-acetilcisteína apresenta ação antioxidante e antihiperglicemiante, sendo capaz de contribuir na terapêutica desta patologia. Este estudo propôs avaliar o metabolismo energético e estresse oxidativo no miocárdio de ratos com diabetes mellitus tipo 1 tratados com N-acetilcisteína. Foram utilizados 32 ratos wistar, com peso corporal ±250g, e 60 dias de idade. Os animais foram distribuídos nos grupos experimentais (*n*=8): C (normais, controle); NAC (normais, tratados com N-acetilcisteína); DM (diabéticos, não tratados); DM-NAC (diabéticos, tratados com N-acetilcisteína). Os animais receberam ração e água *ad libitum*. O diabetes mellitus tipo 1 experimental foi induzido através da administração de estreptozotocina através da via intraperitoneal, em dose única, dose de 60mg Kg^{-1} de peso corporal. Os animais dos grupos NAC e DM-NAC receberam N-acetilcisteína, por via intragástrica com dose de 25mg $Kg^{-1}dia^{-1}$ durante 37 dias. Durante o período experimental (37 dias) foram avaliados consumo de água e ração. Após o período experimental os animais, em jejum de 12h, foram anestesiados (xilazina e cetamina) e eutanasiados. O sangue e tecido cardíaco (±100mg ventrículo esquerdo) foram coletados e armazenados em freezer -80°C. Os resultados foram analisados por ANOVA One-way seguido do teste de Tukey para comparação das médias. Os testes foram considerados significativos quando $p<0.05$. A ingesta alimentar e hídrica foi maior ($p<0.05$) no grupo DM, quando comparado aos demais grupos. Enquanto o peso corporal diminuiu ($p<0.05$) em animais diabéticos em relação a C e NAC, os quais não diferiram ($p>0.05$) de DM-NAC. O NAC reduziu ($p<0.05$) a glicemia nos ratos diabéticos, porém apresentou glicemia maior que os ratos normais (C e NAC). A insulina sérica no grupo DM-NAC esteve aumentada ($p<0.05$) quando comparada aos ratos do grupo DM. A energia ingerida e o consumo de carboidratos e proteínas aumentou ($p<0.05$) em ratos diabéticos não tratados (DM) comparativamente aos demais grupos, porém sem diferença significativa ($p>0.05$) entre C e NAC. A eficiência alimentar em DM foi menor ($P<0.05$) quando comparada a DM-NAC, os ratos normais (C e NAC) apresentaram valores maiores ($P<0.05$) que os diabéticos. A concentração cardíaca de proteínas totais e glicogênio em animais diabéticos (DM) foi inferior ($p<0.05$) aos

demais grupos, sendo que o tratamento com NAC melhorou ($p<0.05$) estes parâmetros. A atividade da fosfofrutoquinase no miocárdio de ratos diabéticos foi recuperada ($p<0.05$) pela administração de NAC alcançando valores dos grupos obtidos para C e NAC. Não houve diferença estatística na atividade da lactato desidrogenase sérica entre os grupos ($p>0.05$). O NAC reduziu ($p<0.05$) a concentração sérica de triacilgliceróis, colesterol total, VLDL-colesterol e LDL-colesterol e aumentou ($p<0.05$) a concentração de HDL-colesterol em ratos diabéticos em comparação ao grupo DM. O grupo NAC-DM apresentou menor ($p<0.05$) índice aterogênico em relação ao grupo DM. A atividade da lactato desidrogenase no tecido cardíaco de ratos não demonstrou diferença estatística entre os grupos. No miocárdio de ratos diabéticos a atividade piruvato desidrogenase diminuiu ($p>0.05$), e o NAC melhorou ($p>0.05$) em DM-NAC, porém sem alcançar os valores obtidos em ratos normais (C e NAC). A administração de NAC foi capaz de melhorar ($p>0.05$) atividade de β-hidroxiacil-CoA desidrogenase e citrato sintase no tecido cardíaco dos ratos diabéticos (DM-NAC). A relação da atividade da lactato desidrogenase/citrato sintase foi menor ($p<0.05$) no grupo DM que em outros, os quais não diferiram significativamente. Não houve diferença significativa na relação β-hidroxiacil-CoA desidrogenase/citrato sintase. A atividade da superóxido dismutase no miocárdio reduziu no grupo DM, enquanto os grupos que receberam NAC (NAC e DM-NAC) demonstraram valores intermediários entre C e DM. O NAC aumentou ($p<0.05$) a atividade cardíaca da catalase em ratos diabéticos, atingindo valores próximos aos grupos C e NAC. A atividade da glutationa peroxidase no tecido cardíaco aumentou no grupo DM-NAC em relação a DM, mas não diferiu ($p>0.05$) quando comparada ao grupo C e NAC. A concentração cardíaca de glutationa total e GSH diminuiu ($p<0.05$) em DM quando comparada com outros grupos. O grupo DM mostrou maiores valores para hidroperóxido de lipídeo no miocárdio, enquanto a administração de NAC melhorou este valor ($p<0.05$). O tratamento com NAC normalizou ($p<0.05$) a relação do hidroperóxido de lipídeo com as enzimas antioxidantes em ratos diabéticos. Diante dos resultados obtidos pode-se concluir que o DM1 promoveu alterações metabólicas em cardiomiócitos, favorecendo a oxidação de ácidos graxos em detrimento da oxidação de glicose com estresse oxidativo levando as disfunções cardíacas. A administração de NAC pode ser uma alternativa terapêutica, pois reverteu a desregulação do metabolismo energético induzido pelo

DM1 no músculo cardíaco através do seu efeito antiabetogênico, controlando a glicemia e consequentemente atenuou o estresse oxidativo. O NAC melhorou os parâmetros nutricionais e normalizou o perfil lipídico e o índice aterogênico.

ABSTRACT

Diabetes mellitus is a heterogeneous group of disturbances in energy metabolism and consequent oxidative stress, important factors for the development of diabetic complications such as cardiomyopathies. The use of antioxidants has contributed to improve hyperglycemia and also minimize diabetic complications. N-acetylcysteine has antioxidant and antihyperglycemic action, being able to contribute to the therapeutics of this pathology. This study aimed to evaluate the energetic metabolism and oxidative stress in the myocardium of rats with type 1 diabetes mellitus treated with N-acetylcysteine. 32 male wistar rats, with ±250g body weigth, 60 days old were distributed in groups (*n*=8): C (normal, control); NAC (normal, treated with N-acetylcysteine); DM (diabetic, untreated); DM-NAC (diabetic, treated with N-acetylcysteine). The rats received water and a rodent chow *ad libitum*. Experimental type 1 diabetes mellitus was induced by streptozotocin (60mg Kg^{-1} body weight, single dose, i.p.). Animals of the group NAC and DM-NAC received N-acetylcysteine (25mg Kg^{-1} day^{-1}) by gavage for 37 days. During the experimental period (37 days), food and water intake were evaluated. After this period the animals, in a 12h fast, were anesthetized (xylasin and ketamin) and euthanized. Blood and cardiac tissue (±100mg, left ventricle) were collected and stored in a freezer -80°C. Results were analyzed by One-way ANOVA followed by Tukey test for comparison of means. The results were considered significant when $p<0.05$. Food and water intake were higher ($p<0.05$) in the DM group, when compared to the other groups. While body weight decreased ($p<0.05$) in diabetic animals related to C and NAC, which did not differ ($p>0.05$) DM-NAC. NAC increased ($p<0.05$) glycemia in diabetic rats, but presented higher glycemia than normal rats (C and NAC). Serum insulin in DM-NAC group was increased ($p<0.05$) when compared to DM group rats. Energy intake and carbohydrate and protein consumption increased ($p<0.05$) in untreated diabetic rats (DM) compared to the other groups, but without significant difference ($p>0.05$) between C and NAC. Alimentary efficiency in DM was lower ($p<0.05$) when compared to DM-NAC, normal rats (C and NAC) had higher values ($p<0.05$) than diabetics. Cardiac concentration of total proteins and glycogen in diabetic animals (DM) were decreased ($p<0.05$) to the other groups, and treatment with NAC improved ($p<0.05$) these parameters. Phosphofructokinase activity in myocardium of diabetic rats was recovered ($p<0.05$) by the administration of NAC reaching values of the groups C and NAC. There was no statistical difference in serum lactate

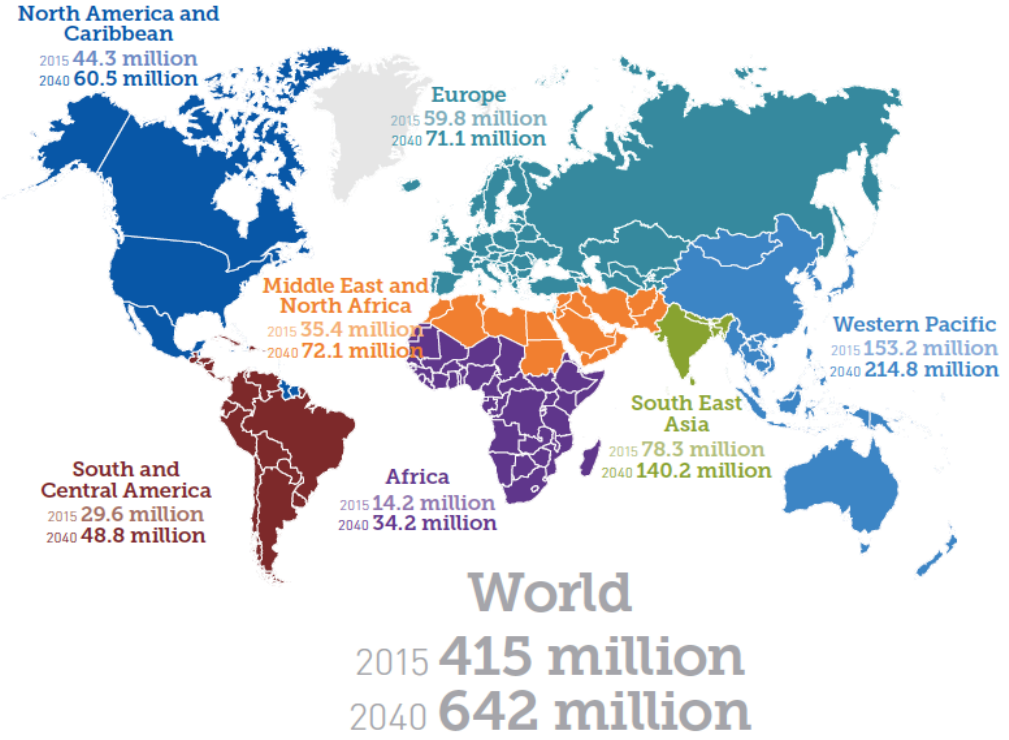
dehydrogenase activity between the groups ($p>0.05$). NAC decreased serum concentration of triacylglycerols, total cholesterol, VLDL-cholesterol and LDL-cholesterol ($p<0.05$) and increased HDL-cholesterol concentration in diabetic rats compared to the DM group ($p<0.05$). NAC-DM group had a lower ($p<0.05$) atherogenic index than the DM group. Lactate dehydrogenase activity in rat heart tissue did not show statistical difference ($p>0.05$) between the groups. In myocardium of diabetic rats pyruvate dehydrogenase activity decreased ($p<0.05$), and NAC improved ($p<0.05$) in DM-NAC, but did not reach values obtained in normal rats (C and NAC). NAC administration was able to improve ($p>0.05$) β-hydroxyl-CoA dehydrogenase activity and citrate synthase in cardiac tissue of diabetic rats (DM-NAC). The ratio Lactate dehydrogenase/citrate synthase was lower ($p<0.05$) in DM group than others, which did not differ significantly. There was no significant difference in ratio β-hydroxyl-CoA dehydrogenase/citrate synthase. Superoxide dismutase activity in myocardium was reduced ($p<0.05$) in DM, whereas groups receiving NAC (NAC and DM-NAC) demonstrated intermediate values between C and DM. NAC increased ($p<0.05$) cardiac catalase activity in diabetic rats, reaching values close to the C and NAC groups. Glutathione peroxidase activity in myocardium increased in DM-NAC group compared to DM, but did not differ ($p>0.05$) when compared to group C and NAC. The cardiac concentration of total glutathione and reduced glutathione activity decreased ($p<0.05$) in DM when compared to the other groups. DM group showed higher values for lipid hydroperoxide in myocardium, while the administration of NAC improved this value ($p<0.05$). NAC treatment normalized ($p<0.05$) the relationship between lipid hydroperoxide and activity antioxidants enzymes in diabetic rats. In view of the obtained results it can be concluded that type 1 diabetes mellitus promoted metabolic alterations in cardiomyocytes, favoring the oxidation of fatty acids in detriment of oxidation of glucose with oxidative stress leading to cardiac dysfunction. NAC administration may be a therapeutic alternative, since it reversed the deregulation of the energy metabolism induced by the DM1 in the cardiac muscle through its antidiabetogenic effect, controlling the glycemia and consequently attenuated the oxidative stress. NAC improved the nutritional parameters and normalized the lipid profile and the atherogenic index.

INTRODUÇÃO

O Diabetes mellitus é uma da doença crônica degenerativa que ocupa a quinta maior causa de morte em países desenvolvidos, ocasionando sério problema de saúde pública, sendo caracterizada uma das maiores emergências de saúde global do século (Bandeira *et al*., 2013; International Diabetes Federation - IDF , 2015).

A Federação Internacional de Diabetes em 2015 estimou que a população diabética mundial era de 415 milhões de pessoas no mesmo ano da publicação, sendo que este valor alcançará 642 milhões em 2040. Enquanto Wild *et al*. (2004) estimaram 366 milhões de indivíduos acometidos pela doença e Rochette *et al*. (2014) 430 milhões em 2030 (Figura 1).

Figura 1. Número estimado de indivíduos com diabetes mellitus (20 a 79 anos de idade) ao redor do mundo e por região em 2015 e 2040.



Este infográfico apresenta o número de indivíduos diabéticos em 2015, e a estimativa de indivíduos que terão diabetes em 2040, dados expressos em milhões. Os dados são demonstrados no geral no mundo, cinza, e depois por diferentes regiões do mundo, como: América do sul e central, vermelho; América do norte e Caribe, azul marinho; Europa, turquesa; Oriente médio e Norte da África, laranja; África, roxo; Sudoeste asiático, verde; Oeste pacífico, azul (fonte IDF, 2015).

O número de pessoas falecidas pelas complicações diabéticas em 2013 foi de 5,1 milhões, números superiores quando comparados a outras patologias como a

síndrome da imunodeficiência adquirida (SIDA) e a tuberculose, com 1,5 milhões óbitos em cada patologia (Figura 2) (IDF 2013, IDF 2015).

Figura 2. Número de adultos que morreram com diabetes, SIDA e tuberculose no mundo em 2013.

Este infográfico apresenta os dados de adultos que morreram ao redor do mundo em 2013 devido a diabetes, síndrome da imunodeficiência adquirida e tuberculose, dados expressos em milhões (Adaptado: IDF, 2013; IDF 2015).

O Diabetes mellitus é uma doença caracterizada pela produção insuficiente de insulina e/ou a deficiência na ação insulínica, promovendo um grupo heterogêneo de distúrbios no metabolismo de carboidratos, lipídeos e proteínas, levando a hiperglicemia (American Diabetes Association, 2012; IDF, 2015; Tao, 2015).

Esta patologia pode ser classificada em: diabetes mellitus tipo 1, diabetes mellitus tipo 2 e diabetes gestacional. O diabetes mellitus tipo 1 (DM1) é resultado de uma resposta auto-imune, que promove a destruição de células β-pancreáticas levando a uma deficiência na produção de insulina, o qual é responsável por estimular a translocação dos transportadores de glicose para a superfície da membrana plasmática para internalizar glicose da corrente sanguínea. Este tipo acomete principalmente crianças e adolescentes, porém pode ocorrer na fase adulta em menor incidência (American Diabetes Association, 2012; Atkinson *et al*., 2014)

O diabetes mellitus tipo 2 é desenvolvido devido a múltiplos fatores, como por exemplo a obesidade, a qual leva a resistência insulínica com isso prejuízo na internalização da glicose (American Diabetes Association, 2012; Marx, 2002). É frequentemente desenvolvida em indivíduos adultos, porém a incidência vem

aumentando em crianças e adolescentes, devido hábitos alimentares inadequados e ao sedentarismo (Marx, 2002; Winer & Sowers, 2004; Tao, 2015; IDF 2015). Porém indivíduos portadores do diabetes mellitus tipo 2 podem não mais produzirem insulina por perda de massa pancreática pela produção exacerbada de insulina e assim desenvolverem o DM1 (Sah, 2016).

A terceira classificação corresponde ao diabetes gestacional que ocorre quando os níveis glicêmicos se tornam elevados durante a gestação por volta de 24ª. semana e normaliza ao nascimento ou persisti por mais dois meses (American Diabetes Association, 2012; IDF, 2015).

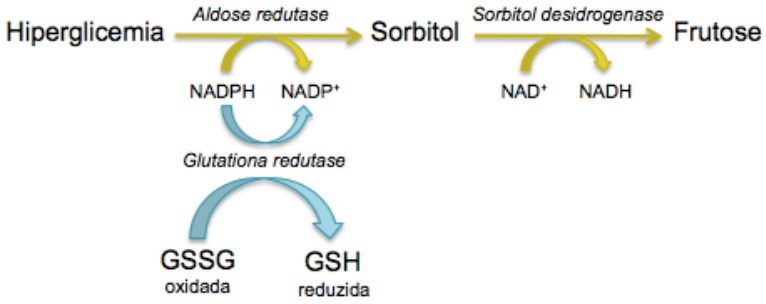
Entretanto o DM1 acomete cerca de 10% da população diabética (Gillespie, 2006; Tao, 2015) tendo incidência mundial de 3 a 5% ao ano, porém de 5 a 15% de adultos portadores de diabetes mellitus tipo 2 desenvolvem DM1 (Raskin & Mohan, 2010; Atkinson *et al*., 2014).

No DM1 a hiperglicemia crônica e persistente que promovem sinais clássicos como: polidipsia, poliúria, polifágia, perda de peso e cetoácidose; além de ser a causa primária das complicações diabéticas, dentre elas: nefropatia, neuropatias, retinopatias e complicações cardiovasculares (American Diabetes Association, 2012; Raskin & Mohan, 2010; Atkinson *et al*., 2014; Tao, 2015).

A recuperação da homeostase glicêmica pode ser obtida através da ativação de diferentes vias metabólicas tais como: via dos polióis – realizada com a finalidade de reduzir glicose a sorbitol, com diminuição dos níveis de NAPDH, o qual é substrato para a glutationa redutase realizar a conversão de glutationa oxidada a glutationa reduzida (figura 3); glicação avançada – glicação de lipídeos e proteínas com consequente aumento de mediadores pró-inflamatórios; proteína quinase C – contribui com o aumento da expressão de genes pró-inflamatórios e a via da hexosamina –

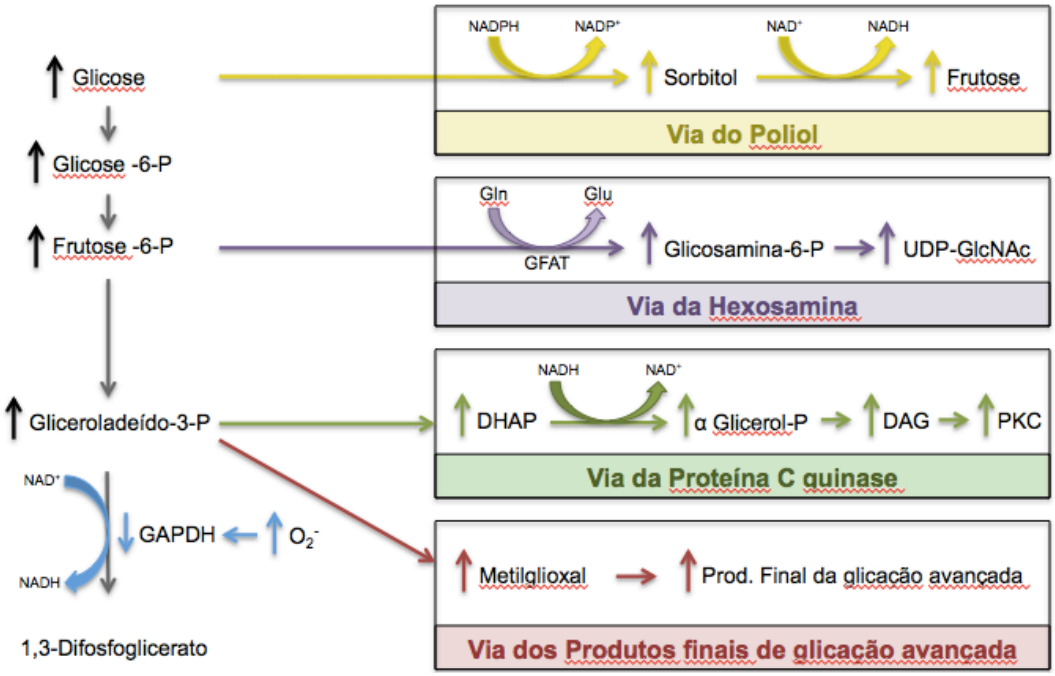
participa do aumento da transcrição de citocinas pró-inflamatórias (Figura 4) (Brownlee, 2001; Maritim *et al*., 2003; Valko *et al*., 2007; Pan *et al*., 2010).

Figura 3. Inter-relação da via dos polióis com glutationa redutase



A glicose é reduzida a sorbitol pela aldose redutase utilizando como co-fator o NADPH. Baixas concentrações de NADPH impedem com que a glutationa redutase reduza a glutationa oxidada (GSSG) em glutationa reduzida (GSH). O sorbitol é reduzido a frutose pela sorbitol desidrogenase usando como co-fator NAD⁺ (Adaptado: Reis et al., 2008).

Figura 4. Vias metabólicas que auxiliam na redução de hiperglicemia e promovem o aumento na produção e EROs



As quatro vias que auxiliam na homeostase de glicose. O aumento de superóxido inibe parcialmente a via glicolítica por inibir a atividade da gliceraldeído 3-fosfato desidrogenase (GAPDH). Assim o há um aumento da gliceroldeído 3-fosfato, que promove o aumento de metilglioxal principal precursor dos produtos finais de glicação avançada. O aumento de gliceroldeído-3-fosfato favorece no aumento de dihidroxiacetona fosfato (DHAP) e consequentemente de diacilglicerol (DAG), o qual ativa a proteína c quinase (PKC). Com a elevação da concentração de frutose-6-fosfato favorecendo a formação de UDP-N-acetilglicosamina (UDP-GlcNAc) aumenta a modificação de proteínas. O aumento de fluxo da via dos polióis, consome NADPH o que impede de restaurar os níveis de glutationa reduzida. GFAT, Glutamina:frutose-6-fosfato aminotransferase; Gln, Glutamina; Glu, Glutamato; NAD⁺, Dinucleótido de nicotinamida; UDP, Uridina difosfato (Adaptado: Ceriello & Testa, 2009).

Embora sejam vias que tendem a diminuir a glicemia trazem prejuízos por aumentarem a produção de radicais livres derivados do oxigênio molecular, denominadas de espécies reativas de oxigênio (EROs) (Bennett & Seefeldt, 2010).

Por outro lado, a deficiência de produção insulina promove diminuição na razão insulina:glucagon, o que torna possível o entendimento pelo qual o metabolismo em indivíduos diabéticos torna-se catabólico com características de desnutrição, desta forma a lipólise, degradação de proteínas gliconeogênese e glicogenólise estão acentuadas (Herbert & Nair,2010; Rains & Jain, 2011).

Na lipólise ocorre a hidrólise dos triacilgliceróis no tecido adiposo em ácidos graxos livres para a corrente sanguínea, os quais são β-oxidados em moléculas de acetil-CoA que são oxidadas no ciclo do ácido cítrico com produção de equivalentes redutores $FADH_2$ e NADH, os quais serão re-oxidados na cadeia transportadora de elétrons com produção de energia. A elevação da concentração de equivalentes redutores aumenta o fluxo de elétrons na cadeia transportadora de elétrons, o que promove a hiperpolarização da membrana mitocondrial interna através durante a fosforilação oxidativa. Estes eventos favorecem a redução parcial do oxigênio molecular às principais formas de EROs, tais como, o ânion superóxido ($\bullet O_2^-$) e peróxido de hidrogênio (H_2O_2) (Rolo & Palmeira, 2006; Valko *et al*., 2007; Sivitz & Yorek, 2010; Rains & Jain, 2011).

Os EROs também podem ser denominados radicais livres, moléculas ou fragmentos moleculares que possuem um ou mais elétrons despareados em sua orbita molecular, com aumento no seu grau de reatividade (Valko *et al*., 2007).

O ânion superóxido também pode ser produzido através de reações enzimáticas, pela via NADPH oxidase, xantina oxidase. As EROs também podem ser produzidas através de reações não enzimáticas pela reação do oxigênio com compostos orgânicos (Pham-Huy *et al*., 2008).

Estudos experimentais e clínicos revelaram que a hiperglicemia estabelecida no diabetes mellitus está associada à produção aumentada de EROs, caracterizando o

estado de estresse oxidativo (Johansen *et al*., 2005).

O estresse oxidativo é caracterizado por desequilíbrio entre espécies reativas e antioxidantes, com favorecimento de espécies reativas (Sies, 1997), possivelmente devido a falha na defesa endógena antioxidante (Valko *et al*., 2007).

Elevados níveis de radicais livres provocam danos aos componentes celulares como proteínas, lipídeos de membrana e ácidos nucléicos, resultando em disfunção e, eventualmente morte celular, devido ao desequilíbrio entre a produção e a neutralização das EROs, cenário denominado de estresse oxidativo (Gillespie, 2006; Small *et al*., 2012; Rajendran *et al*., 2014). Isto implica na etiologia de grande número de doenças crônicas degenerativas, incluindo aterosclerose, câncer, diabetes e envelhecimento (Bray, 2000), que apresentam as estruturas celulares danificadas (Pham-Huy *et al*., 2008; Small *et al*., 2012; Rajendran *et al*., 2014).

Portanto as alterações metabólicas encontradas no estado diabético, provenientes da produção insuficiente de insulina e consequente hiperglicemia, estabelecem um quadro de estresse oxidativo que é um dos fatores desencadeantes das complicações diabéticas como cardiomiopatias, e em especial a aterosclerose (Asghar *et al*., 2009; Kim, *et al*., 2011).

O miocárdio é caracterizado por elevada capacidade oxidativa (Stanley & Sabbah, 2005) com grande quantidade de mitocôndrias e, possui elevada taxa de consumo de oxigênio, uma vez que utiliza os ácidos graxos como principal substrato para produção de energia (Lopaschuk *et al*., 2002). E, portanto maior produção de equivalentes redutores (NADH e $FADH_2$), contribuindo para aumentar a fonte de EROs.

Além disso a oxidação completa de ácidos graxos seguido da fosforilação oxidativa no tecido cardíaco exige alto consumo de oxigênio para produção de ATP,

diminuindo a razão P:O (moléculas de ATP produzidas / Oxigênio consumido) (Lopaschuk *et al*., 2010; Gray *et al*., 2014). Desta forma, aumenta a possibilidade de geração de EROs neste tecido, uma vez que torna-se altamente oxigenado.

A dislipidemia, distúrbio que acomete cerca de 97% dos pacientes diabéticos (Dokken, 2008), é caracterizada por elevação no nível plasmático de triacilgliceróis e de colesterol total, promovendo aumento na concentração das lipoproteínas de muito baixa densidade (VLDL-colesterol) e a de baixa densidade (LDL-colesterol), e diminuição da concentração da lipoproteína de alta densidade (HDL-colesterol) (Grundy, 1999; Kim, *et al*., 2011).

A hiperglicemia observada no DM1 está intimamente relacionada com a dislipidemia o que leva ao aumento do risco de doenças isquêmicas cardiovasculares e cerebrovasculares. Por outro lado, a susceptibilidade da LDL a glicação e oxidação parece estar relacionada ao controle glicêmico inadequado e pela redução no sistema de defesa antioxidante, observada no DM1. Desta forma, o controle da glicemia é determinante como fator benéfico, pois diminui o estresse oxidativo e reduz a aterogênese diabética (Johansen *et al*., 2005).

Estudos demonstraram que o aumento de LDL-colesterol relaciona-se diretamente com o desenvolvimento da aterosclerose, através do processo inflamatório desencadeado pela oxidação das LDL-colesterol, as quais são fagocitadas por macrófagos na camada sub-endotelial, com diferenciação em células esponjosas que entram na constituição da placa de ateroma e consequentemente aterosclerose (Valko *et al*., 2007; Dokken, 2008; Schwartz & Reaven, 2012; Schilling, 2015).

Diversos estudos têm demonstrado que a hiperglicemia crônica e persistente, e estresse oxidativo podem explicar os mecanismos de diversas complicações diabéticas (Niedowicz & Daleke, 2005; Bandeira *et al*., 2013).

A neutralização das EROs e portanto o controle do estresse oxidativo é realizado por antioxidantes. Existem basicamente dois sistemas antioxidantes: enzimático e não-enzimático (Halliwell, 1996; Sies, 1997). O primeiro corresponde às enzimas catalase (CAT), superóxido dismutase (SOD) e glutationa peroxidase (GSH-Px). Os agentes não-enzimáticos correspondem principalmente, a glutationa, vitaminas C e E (Kakkar *et al*., 1995; Maritin, 2003).

O sistema antioxidante enzimático tem como a primeira linha de defesa a atividade da SOD, responsável pela dismutação do ânion superóxido ($\bullet O_2^-$) a peróxido de hidrogênio (H_2O_2), enquanto a CAT e a GSH-Px reduzem o H_2O_2 em água através de mecanismos distintos. A CAT é uma enzima NADPH-dependente, desta forma há necessidade de NADPH para que haja a reação de redução. Enquanto a GSH-Px necessita de glutationa reduzida (GSH) como substrato para converter o H_2O_2 em água com liberação de glutationa oxidada (GSSG), a qual requer a atividade da glutationa redutase (GR), na presença de NADPH, para retorna na forma reduzida (GSH) (Niedowicz & Daleke, 2005; Bennett & Seefeldt, 2010).

Um dos principais mecanismos que tentam explicar o estresse oxidativo está relacionado com a redução do conteúdo celular de GSH e/ou aumento da GSSG. Assim, pode-se atribuir ao estresse oxidativo a deficiência e/ou redução da concentração de GSH ou até mesmo a deficiência de seu precursor, o aminoácido cisteína (Atkuri *et al*., 2007).

A utilização de substâncias naturais com propriedades tanto antioxidantes como hipoglicemiantes têm sido extensivamente estudadas. Estudos vêm

demonstrando que a suplementação de antioxidantes é capaz de atenuar as doenças crônico-degenerativas como diabetes mellitus, bem como as complicações a ela associada (Pham-Huy *et al*., 2008; Sadi *et al*., 2012; Sindhi *et al*., 2013, Rajendran *et al*., 2014).

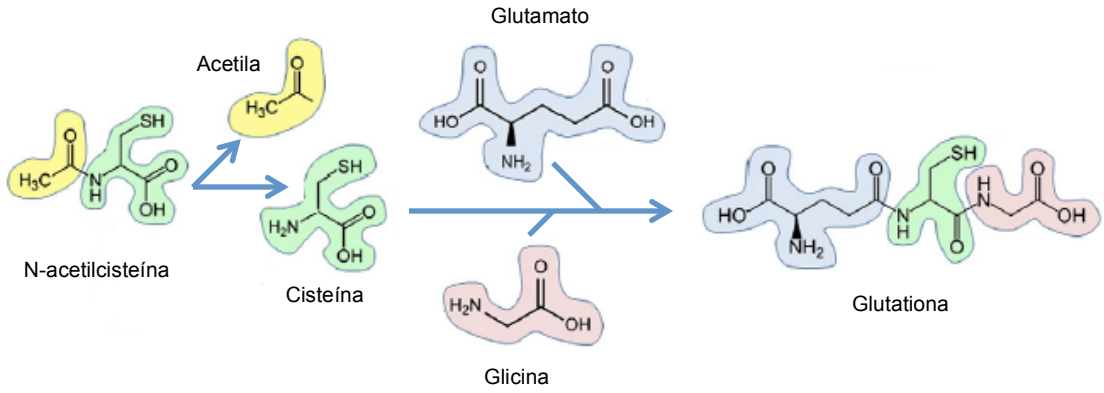
Deste modo, estudos com intervenção farmacológica baseada em compostos antioxidantes sobre as enfermidades crônicas-degenerativas como o diabetes mellitus são de grande interesse. Os antioxidantes são substâncias estáveis que atuam como agentes redutores, capazes de reduzir as substâncias oxidadas tornando-as estáveis, desta forma tendo a função de reparar a estrutura das macromoléculas celulares (Lobo *et al*., 2010; Carocho & Ferreira, 2013).

Além disso, torna-se notório a atuação dos antioxidantes ao neutralizar as EROs, com o impedimento da propagação do estresse oxidativo (Ceriello & Testa, 2009) de tal forma, que poderiam prevenir e/ou retardar o desenvolvimento das complicações diabéticas (Ceriello & Motz, 2004). Desde que o sistema antioxidante torna-se ineficaz ao compensar à produção excessiva de EROs, favorecendo, portanto, o estresse oxidativo, a administração de antioxidantes pode ser efetiva no controle do diabetes mellitus. Estudos epidemiológicos estabeleceram que a ingestão de antioxidantes encontra-se relacionada inversamente com a ocorrência de doenças promovidas pelas EROs (Sadi *et al*., 2012).

A N-Acetilcisteína (NAC, C_5H_9-NO_3S) tem sido usada na prática clinica como agente mucolítico, devido a reduzir as pontes dissulfeto de proteínas presentes no muco. Também pode ser utilizado como antidoto para overdose de paracetamol, uma vez que o NAC é capaz de restaurar os níveis de glutationa (figura 5), o qual está depletado nesta condição. Atualmente, o NAC é utilizado extensivamente na prática clinica na prevenção da nefropatia, causada por contraste radioativo, toxicidade de

quimioterápicos, desordens psiquiátricas e cardiotoxicidade da doxorrubicina (Giustarini *et al*., 2012; Samuni *et al*., 2013; Rushworth & Megson, 2014).

Figura 5. N-acetílcisteína como precursor de Cisteína na síntese de Glutationa.



A N-acetilcisteína sofre hidrólise em acetila e o aminoácido cisteína, o qual é conjugado com glicina e glutamato respectivamente, para formação da glutationa. (Adaptado: Rushworth & Megson, 2014).

O efeito antioxidante do NAC está bem estabelecido, ele exerce efeito antioxidante direto por sequestrar EROs através do radical sufidril presente na sua estrutura química (Sivitz & Yorek, 2010). E exerce efeito antioxidante indireto que após sofrer hidrólise fornece o aminoácido cisteína, o qual é precursor da GSH (figura 4), que é substrato para a GSH-Px converter o peróxido de hidrogênio em água (Atkuri *et al*., 2007; Seiva *et al*., 2009; Sivitz & Yorek, 2010).

Sheela *et al*. (1995) administraram de N-acetilcisteína sulfóxido, isolado e purificado da cebola, em ratos com diabetes induzido por aloxana e observaram redução na concentração sérica de glicose, enquanto que a quantidade de glicogênio hepático aumentou consideravelmente em relação àqueles diabéticos não tratados com N-acetilcisteína sulfóxido. Outros estudos demonstraram que o NAC é capaz de reduzir os níveis glicêmicos, devido provavelmente à sua capacidade de provocar a produção e secreção de insulina (Fulghesu *et al*., 2002; Rushworth & Megson, 2014).

A NAC reduziu tanto a glicemia como a concentração sérica e hepática de triacilgliceróis e de colesterol, ao diminuir a atividade das enzimas lipogênicas, tais

como, a ácido graxo sintase, HMG-CoA redutase, respectivamente (Hsu *et al*., 2004). Além disso, há relatos do NAC atenuar a severidade da aterosclerose, ao estabilizar o desenvolvimento da placa aterosclerótica (Samuni *et al*., 2013). Outros autores como Diniz *et al*., (2006); Novelli *et al*., 2009 verificaram que administração de NAC diminuiu a concentração tanto de colesterol, e suas frações lipoproteicas, como de triacilgliceróis, enquanto o nível sérico de HDL-colesterol aumentou na presença de dieta hipercalórica.

HIPÓTESE E OBJETIVOS

HIPÓTESE

O tratamento com NAC apresenta efeito antidiabetogênico, melhora o perfil lipídico, normaliza o metabolismo energético e controla o estresse oxidativo no tecido cardíaco de ratos com DM1, induzido experimentalmente.

OBJETIVO GERAL

Este estudo avaliou os efeitos do NAC sobre as alterações metabólicas e o estresse oxidativo no tecido cardíaco de ratos com DM1.

OBJETIVOS ESPECÍFICOS:

- Determinar a glicemia e a dislipidemia promovida pelo diabetes mellitus;
- Evidenciar o efeito do diabetes mellitus sobre o estresse oxidativo e metabolismo energético no tecido cardíaco;
- Analisar o efeito anti-diabetogênico do NAC sobre a concentração sérica de glicose, peso corporal, controle alimentar e de água em ratos com diabetes mellitus;
- Identificar os efeitos da administração do NAC sobre a dislipidemia, metabolismo energético e estresse oxidativo (biomarcadores) em miocárdio de ratos diabéticos.

CAPÍTULO 1

Evaluation of energetic metabolism and oxidative stress in cardiac tissue of diabetic rats: favorable effect of NAC
Journal of Biomedical Sciences

Evaluation of energetic metabolism and oxidative stress in cardiac tissue of diabetic rats: favorable effect of NAC.

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Background: Type 1 diabetes mellitus (T1DM) is complex metabolic syndrome derived from autoimmune destruction of pancreatic β-cells leading insulin deprivation and hyperglycemia, which contributes to oxidative stress that is associated pathogenesis of diabetic complications such as cardiomyopathy. The aim of present study was evaluate the effect of N-acetylcysteine (NAC) on energy metabolism and cardiac oxidative stress in streptozotocin (STZ)- induced diabetes.

Methods: 32 male Wistar rats (60-days-old) weighting 250g were randomly distributed in into four groups (*n*=8): C: normal rats; NAC: normal rats treated with NAC; DM: diabetic rats; DM-NAC: diabetic rats treated with NAC. T1DM was induced by STZ (60 mg kg^{-1} b.w., ip; single dose) and NAC (25 mg kg^{-1} b.w. day^{-1}) was administrated by gavage, during 37 days. The animals received diet and water *ad libitum*. After experimental period was collected blood and cardiac tissue samples to analysis of energetic metabolism and oxidative stress.

Results: After experimental period the classic symptoms of DM such as low insulin, hyperglycemia, polydipsia, polyuria and body weight loss were evidenced in DM group. NAC administration in diabetic rats (DM-NAC) improved these parameters triggered by STZ. DM group had metabolic shifting in cardiac tissue in favor of fatty acids oxidation in detriment oxidation glucose in myocardium. Significant increase

glucose oxidation and reduction fatty acids oxidation was observed in diabetic rats treated with NAC (DM-NAC). Lipid hidroperoxide increased while the activity of antioxidant enzymes, including catalase, superoxide dismutase and glutathione peroxidase decreased in cardiac tissue. Oral administration of NAC to diabetic rats (DM-NAC) showed a significant decline of stress oxidative. Conclusion: These results suggest antidiabetogenic effect of NAC to enhance the level of insulin and control hyperglycemia. NAC prevented the metabolic shifting and consequently attenuated the oxidative stress in cardiac tissue of STZ-diabetic rats.

KEYWORDS

type 1 diabetes mellitus, N-acetilcysteine, energy metabolism, oxidative stress, cardiac tissue.

BACKGROUND

Type 1 diabetes mellitus (T1DM) is considered a complex syndrome caused by autoimmune-mediated destruction of pancreatic β-cells, leading in absolute insulin deficiency and disturbance in metabolism of carbohydrate, lipid and protein. These metabolic abnormalities alters the glicemic homeostasis characterized by chronic and persistent hyperglycemia [1,2].

Diabetes mellitus (DM) is one of the most common chronic diseases, being the fifth cause of death around the world. In 2013, International Diabetes Federation estimated that there were 382 millions of people living with diabetes in the world, and this amount will reach 592 millions in 2035. T1DM primarily affects childhood and adolescence, approximately in 10% of all cases of diabetes, with increasing prevalence of around 3 to 5% per year [2,3,4,5].

In general, DM is a potent risk factor for cardiac dysfunction, known as diabetic cardiomyopathy that occurs in approximately 30% of type 1 diabetic patients

[6,7]. The diabetic cardiomyopathy is accompanied by deregulation on energy metabolism in myocardium with increased of fatty acids that contribute to the decrease in glucose metabolism [8].

The excessive β-oxidation of fatty acids increases acetyl-CoA concentrations, which is oxidized in citric acid cycle with more production of reducing equivalents, FADH (flavin adenine dinucleotide, reduced) and NADH (nicotinamide adenine dinucleotide, reduced) that re-oxidize in electron transport chain (ETC) [9,10]. Excess electron flux in ETC, the higher concentration of NADH and FADH hyperpolarize the inner mitochondrial membrane through the enhance of oxidative phosphorylation, favoring the generation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), main reactive oxygen species (ROS), which trigger the oxidative stress (imbalance between ROS production and antioxidant defense system) [4,9,11]. Excess electrons flux also contributes to mitochondrial ROS generation, which alters cell redox state [12,13].

Interesting to note the low relative P/O ratio (corresponds the number of molecules of ATP produced/consumed oxygen in the oxidative phosphorylation) during the complete oxidation of fatty acids, because are highly reduced molecules when compared to glucose molecule. Thus the glucose oxidation produces relatively more ATP per reduced oxygen atom in oxidative phosphorylation, when compared to the fatty acids oxidation [14,15]. Besides, during the glycolysis, process independent of oxygen supply, also produces ATP in substrate level phosphorylation.

Study demonstrates greater metabolic inflexibility in the myocardium in favor of the fatty acids oxidation under diabetic condition, may cause cardiac dysfunction and increased probability of heart failure [16,17].

In addition, numerous studies have reported the link between hyperglycemia, established in DM, and oxidative stress through various mechanisms, such as, polyol

pathway and decreased of pentose phosphate pathway – leads decrease of NADPH, needed to reduce the gluthatione; advanced glycation end products – increase proinflammatory mediators; protein kinase C pathway and hexosamine pathway – increase the expression and transcription of proinflammatory genes [11,18,19].

Peroxidation in protein and lipid, irreversible damage and cell death, triggered by oxidative stress, have implications on alterations in the cardiac function consequence from DM [4,20,21,22].

Despite the fact that metabolic abnormities may be implicated in the etiology of diabetic cardiomyopathy and oxidative stress, the supplementation of antioxidants compounds could control this unfavorable effect caused by diabetic state. Moreover, becomes important to devise strategies of therapeutic intervention in particular to favor glucose oxidation and reduce the oxidation of fatty acids and hence improve the energy metabolism in cardiac muscle.

Many studies have shown beneficial effect of antioxidants compounds as possible pharmacological agents both the treatment and prevention of DM [12,23]. Since the endogenous antioxidant defense system is impaired and there is a decrease of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) specifically in myocardial tissue in diabetic state, the use of antioxidants, substances able to scavengers ROS, becomes important in the prevention of oxidative stress [11,21,24,25,26].

N-acetylcysteine (NAC, C_5H_9-NO_3S) has been used in clinical practices for more than 50 years. It began in these practices as a mucolytic agent, due to reduce disulfide bridges of proteins in mucus. After approximately 10 years, a new important role for NAC was discovered, it started to be used as an antidote to acetaminophen overdose, once restore glutathione level, depleted in this condition. Currently, NAC

has other uses in clinical practices including radio-contrast-induced nephropathy, chemotherapy-induced toxicity, psychiatric disorders, doxorubicin-induced cardiotoxicity [27,28,29].

The antioxidant effect of NAC has been well established, because after hydrolysis provides cysteine necessary as precursor during the synthesis of reduced glutathione, substrate of GSH-Px allowing the activity this enzyme [12,30,31]. The NAC may also exert its antioxidant effect scavenging ROS by sulfhydryl group present in its chemical structure [12].

However studies have demonstrated the ability of NAC in improve insulin secretion by pancreatic, exercising antidiabetogenic effect [29,32].

Considering that DM triggers a series of metabolic abnormalities leading to oxidative stress, was formulated the following hypothesis: the NAC presenting antioxidant/antidiabetogenic properties may favor glucose uptake and its utilization as metabolic fuel, equilibrate the myocardial energy metabolism and attenuate oxidative stress in diabetic rats.

Thus, the purpose of the present study was to evaluate the effect of NAC on energy metabolism and oxidative stress in myocardium of rats with T1DM.

MATERIALS AND METHODS

Animal care and maintenance

Male Wister, 32 rats, weighing 250g, 60 days of age, were maintained in polypropylene cages at room temperature (23°C) with constant 12-h light/12-h dark cycle and a relative humidity of 60±5%. The animals had free access to water and standard chow diet (Purina, Campinas, Brazil), containing 22.0% protein, 3.8% fat, 44.5% carbohydrate and 3.0kcal/g of metabolizable energy. All experimental procedures were approved by the Ethics Committee on the Use of Animals (CEUA) at

the Institute of Biosciences of Botucatu, University of São Paulo State (UNESP). (Approval number 706).

Experimental groups and induction of type 1 diabetes mellitus

The T1DM was induced experimentally with an intraperitoneal injection of streptozotocin (STZ) in single dose at 60 mg kg^{-1} b.w. After 48h STZ administration, blood samples were obtained from the tail for determination of glycemia with the aid of glucometer (Boehringer Mannheim, Eli Lilly Ltda, Brazil). The animals having glycemia ≥ 250 mg dL^{-1} were considered diabetics as previously demonstrated [33]. The NAC was given at the concentration of 25 mg kg^{-1} b.w. day^{-1}, via intragastric, for 37 days [34]. The experimental animals were distributed in a randomly into four groups (*n*=8). Control group (C): non-diabetic rats; NAC group (NAC): non-diabetic rats with NAC; diabetic group (DM): STZ-induced diabetic rats and diabetic group and NAC (DM-NAC): STZ-induced diabetic rats with NAC (DM-NAC). The food and water consumption was evaluated daily at the same time (8:00-9:00 am). Body weight was measured weekly.

Preparation of serum and cardiac samples

At the end of the experimental period (37 days) and after an overnight fast (12-14h), the animals were anaesthetized (0.1mL $100g^{-1}$ b.w. of the 2:1 solution of ketamina chloride and xylazine chloride, i.p.) and euthanized by decapitation. The serum was separated by centrifugation (1,400g/10min) and approximately 100mg of cardiac tissue (left ventricle) were homogenized with aid in of a motor-driven teflon glass *Potter Elvehjem*, in the presence of in 0.1M phosphate buffer pH 7.4, and centrifuged at 10,000xg/15min at 4°C. In the resultant supernatant was determined enzymatic activity and oxidative stress.

Measurements of biochemical parameters

Serum level of glucose was measured using enzymatic colorimetric method, after incubation with glucose oxidase/peroxidase according [35]. Insulin concentration was determination by enzyme immune assay kit (EIA kit, Cayman Chemical, USA).

Lactate dehidrogenase (LDH) activity was determined in the presence of tris-HCl buffer 50mM pH 7.5, pyruvate and reduced nicotinamide adenine dinucleotide (NADH), which oxidation was proportional to enzymatic activity [36]. Pyruvate dehydrogenase (PDH) activity was assayed during the conversion of pyruvate to acetyl-CoA in reactive mixture containing potassium phosphate buffer (50mM, pH 7.4), coenzyme A, dithiothreitol, $MgCl_2$, nitrobluetetrazolic (NBT), phenazine methosulfate, coenzyme A, nicotinamine adenine dinucleotide (NAD), sodium pyruvate, and thiamine pyrophosphate [37]. The ability of citrate synthase (CS) catalyze the condensation of acyl group with oxaloacetate was determined using tris-HCl buffer 50mM, pH 8.0), acetyl-CoA and oxaloacetate as substrates, and 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB). Activity of β-hydroxyacil coenzyme A dehydrogenase (OHADH) was assayed in tris-HCl buffer (50mM, pH 7.0) containing EDTA, acetoacetyl coenzyme A and NADH [37].

The cardiac concentration lipid hydroperoxide (LH) was estimated based principle of oxidation of Fe^{2+} obtained from ammonium ferrous sulphate, in the presence sulfuric acid, butylated hydroxytolueno (BHT) and xylenol orange in solution of the methanol (90%, v/v), incubated for 30min at room temperature according to previous study [38]. For the determination of glutathione peroxidase (GSH-Px) activity provoked NADPH oxidation in reactive mixture with sodium phosphate buffer (0.1M; pH 7.4), EDTA, glutathione reductase, sodium azida,

reduced glutathione (GSH) and oxygen peroxide (H_2O_2). The GSH-Px activity is proportional to the reduction of oxidative glutathione, measured by rate of $NADPH_2$ [39]. The detection of inhibitory rate of the reduction of nitro blue tetrazolium (NBT) for free radical generated by hydroxylamine in presence phenazine and sodium phosphate buffer (50 mM; pH 7.4), allowed to obtain the superoxide dismutase (SOD) activity second method described previously [40]. Catalase (CAT) activity was determined in phosphate and sodium buffer (50mM; pH 7.0) containing hydrogen peroxide [41]. Reduced glutathione (GSH) was measured with a kinetic assay in reaction media containing 100 mM phosphate buffer pH 7.4, 5 mM EDTA, 0.2 mM NADPH, 2 mM 5,59-dithiobis-(2-nitrobenzoic) acid (DTNB) and 2U of glutathione reductase [42]. And total glutathione was assayed with Tris-HCl buffer (0.1 M; pH 8.0) containing, 0.6 M DTNB, 0.5 mM EDTA and 1U of glutathione reductase [42].

The activities of the enzyme were obtained, at temperature 25°C, using a ELISA micro-plate reader (EON μQuant Bio-Tek Instruments, USA) coupled to computer system control with Gen5 2.0 software.

Statistical Procedure

The data were submitted to analysis of variance (ANOVA) and Tukey's test to evaluate statistical differences between the experimental groups. The significant level adopted was of 5% of probability. All values are presented as mean ± standard deviation (SD) [43].

RESULTS

Table 1- Main characteristics in the end of experimental period

PARAMETERS	GROUP			
	C	NAC	DM	DM-NAC
Insulin *ηg/dL*	2.02 ± 0.27[c]	2.21 ± 0.30[c]	0.49 ± 0.14[a]	1.09 ± 0.15[b]
Glycemia *mg/mL*	92.71 ± 14.41[a]	98.10 ± 14.07[a]	351.04 ± 27.94[c]	128.84 ± 12.87[b]
Body weight *g*	361.08 ± 31.32[b]	376.34 ± 19.54[b]	267.05 ± 45.90[a]	337.93 ± 32.70[b]
Food intake *g/day*	25.74 ± 0.39[a]	25.88 ± 0.52[a]	42.06 ± 2.39[c]	37.65 ± 4.94[b]
Water intake *mL/day*	34.96 ± 0.77[a]	35.34 ± 0.99[a]	171.66 ± 8.49[c]	128.79 ± 31.55[b]

Data are mean ± SD (n=8). a, b, c In each row, means followed by different letter indicates statistically significant difference (p<0.05). Control rats (C); N-acetylcysteine treated rats (NAC); diabetic rats (DM); diabetic rats treated with N-acetylcysteine (DM-NAC).

The glycemia, water and food intake were higher (p<0.05) in DM group when compared with the DM-NAC group, while body weight decreased (p<0.05). Nevertheless body weight in diabetic rats treated with NAC did not differ from C and NAC groups. The decrease in serum insulin (p<0.05) was accompanied by the reduction in glycemia (p<0.05) in untreated diabetic rats (DM), while supplementation of NAC resulted in elevated serum insulin and reduction of glycemia (p<0.05) (Table 1).

Table 2 Energetic metabolism in myocardium

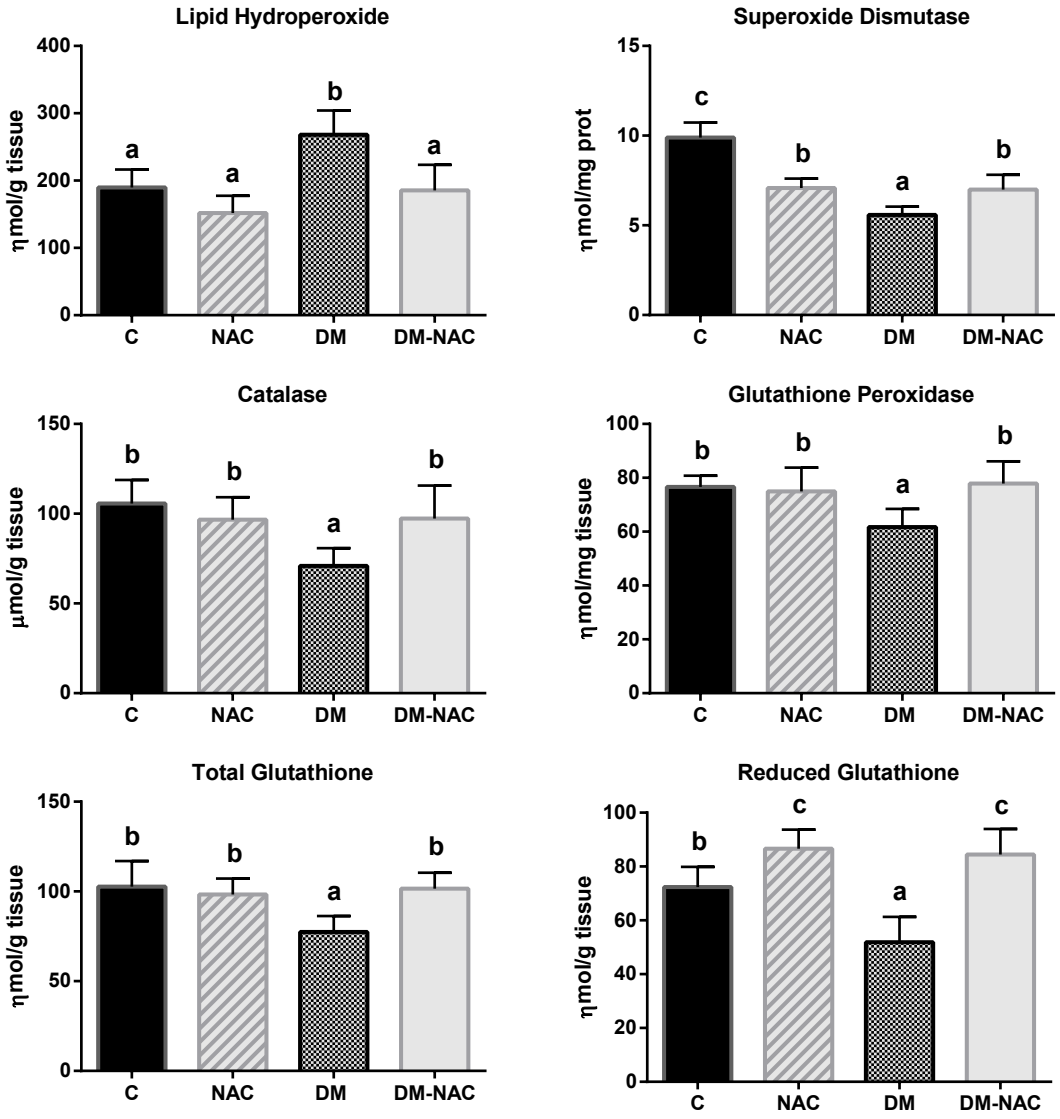
PARAMETERS	GROUP			
	C	NAC	DM	DM-NAC
LDH *ηmol/mg tissue*	1.47 ± 0.26	1.43 ± 0.27	1.52 ± 0.30	1.43 ± 0.31
PDH *ηmol/mg tissue*	0.44 ± 0.06b	0.47 ± 0.05b	0.36 ± 0.04a	0.47 ± 0.02b
OHADH *ηmol/mg tissue*	55.72 ± 7.15a	68.17 ± 8.11ab	118.34 ± 12.08c	72.78 ± 8.93b
CS *ηmol/mg tissue*	89.39 ± 15.62a	88.70 ± 14.05a	144.57 ± 10.29b	94.46 ± 13.81a
LDH/CS *ηmol/mg tissue*	0.017 ± 0.004b	0.016 ± 0.002b	0.011 ± 0.002a	0.016 ± 0.005b
OHADH/CS *ηmol/mg tissue*	0.63 ± 0.09	0.79 ± 0.15	0.82 ± 0.10	0.79 ± 0.22

Data are mean ± SD (n=8). a, b, c In each row, means followed by different letter indicates statistically significant difference (p<0.05). Lactate dehydrogenase (LDH); pyruvate dehydrogenase (PDH); β-hydroxyacyl coenzyme-A dehydrogenase (OHADH); citrate syntase (CS). Control rats (C); N-acetylcysteine treated rats (NAC); diabetic rats (DM); diabetic rats treated with N-acetylcysteine (DM-NAC).

There were no significant alterations in cardiac activity of LDH among the groups. Untreated diabetics rats had lower (p<0.05) PDH in myocardium, while NAC treatment (DM-NAC) normalized the activity of this enzyme. DM animals increased (p<0.05) the cardiac activity of both OHADH and CS, in compared to the other

experimental groups. In contrast, the DM-NAC group decreased significantly ($p<0.05$) the activity OHADH and CS. The ratio LDH/CS was lower ($p<0.05$) in DM group than others, which did not differ significantly. There was no significant difference in ration OHADH/CS (Table 2).

Figure 1 Myocardial oxidative stress



Data are mean ± SD (n=8). a, b, c In each row, means followed by different letter indicates statistically significant difference ($p<0.05$). Control rats (C); N-acetylcysteine treated rats (NAC); diabetic rats (DM); diabetic rats treated with N-acetylcysteine (DM-NAC).

Table 3 Relationship between lipid hydroperoxide and antioxidant enzymes

PARAMETERS	GROUP			
	C	NAC	DM	DM-NAC
LH/SOD *μmol/g tissue*	19.19 ± 3.32[a]	21.43 ± 4.41[a]	48.52 ± 6.71[b]	26.72 ± 6.51[a]
LH/CAT *ηmol/mg protein*	1.82 ± 0.43[a]	1.58 ± 0.34[a]	3.87 ± 0.72[b]	1.95 ± 0.51[a]
LH/GSH-Px *ηmol/mg tissue*	2.48 ± 0.46[a]	2.02 ± 0.29[a]	4.43 ± 0.85[b]	2.40 ± 0.60[a]

Data are mean ± SD (n=8). a, b, c In each row, means followed by different letter indicates statistically significant difference ($p<0.05$). Lipid hydroperoxide (LH); Superoxide dismutase (SOD); Glutathione peroxidase (GSH-Px). Control rats (C); N-acetylcysteine treated rats (NAC); diabetic rats (DM); diabetic rats treated with N-acetylcysteine (DM-NAC).

Lipid hydroperoxide concentration increased ($p<0.05$) while the cardiac activity SOD, CAT, GSH-Px, concentration of total glutathione and GSH decreased ($p<0.05$) in the DM group. On the other hand, NAC treatment normalized both LH as activity of SOD, CAT, GSH-Px and Total glutathione. NAC administration increased GSH concentration in NAC and DM-NAC when compared with C and DM (Figure 1). NAC treatment improved LH/SOD, LH/CAT and LH/GSH-Px ratio in the cardiac tissue of diabetic rats (DM-NAC) (Table 3).

DISCUSSION

Streptozotocin is a substance of diabetogenic characteristic since it selectively promotes the destruction of β-pancreatic cells due to the similarity of its chemical structure to that of the glucose molecule, facilitating thus cellular internalization by means of a glucose transporter (GLUT-2) [44,45]. The major mechanism of action of STZ lies in the alkalinization and fragmentation of the DNA molecule, that is repaired by poly-ADP ribose polymerase activation, decreasing NAD^+ cellular concentration and ATP production, which leads to energy deficit, followed by cell death and insulin synthesis inhibition [46]. In addition, STZ can release nitric oxide, a free radical that enhances its toxic action by contributing to damages in the DNA [45].

Therefore, STZ administration consists in a classic model to experimentally induce T1DM since it impairs insulin release. In the present study, animals of group DM had a reduction in the insulin concentration and consequently in the glucose

uptake by cells responsive to the action of this hormone, leading to hyperglycemia (Table 1).

Insulin deficit induces the catabolic metabolism, such as lipolysis and proteolysis, processes that lead to the degradation of triacylglycerols and muscular and hepatic proteins, respectively, those biochemical pathways contributes to gluconeogenesis [47]. Tissue/structural protein degradation releases α-ketoacids which can be oxidized in the citric acid cycle and provide carbonic chains to support gluconeogenesis, contributing to increase the blood glucose.

Fat and muscular mass loss might have been responsible for decreasing the body weight of diabetic animals (DM). These animals also had an increase in food intake probably due to inadequate use of calories caused by the unavailability of glucose in the cell (Table 1). These results are consistent with studies in the literature which have reported body weight loss in diabetic state [33,48].

In diabetes the maximal capacity of glucose reabsorption by the renal tubules reaches its limit; therefore, the osmotic volume increases in the attempt to dilute the glomerular filtrate, consequently losing excessive liquid through the urine [49,50] and leading to greater water intake, as shown for diabetic animals (DM) (Table 1).

In the present study, administration of NAC to diabetic animals (DM-NAC) improved the serum level of insulin and consequently reduced the glycemia (Table 1). These results are according to previous study [51], which observed an increase in insulin concentration in the presence of NAC for obese db/db rats. Those authors attributed the beneficial effect of NAC on the preservation of β-pancreatic cells to adequate insulin release. Previous studies [52,53] also observed beneficial effect of NAC on in vitro insulin secretion.

The increased insulin secretion can be related with reduced hyperglycemia; the better use of circulating glucose by the cells will raise the renal glucose threshold, so there is enhanced of water reabsorption and, hence decreased hydric consumption, as observed in DM-NAC group (Table 1).

The binding of insulin to its membrane receptor triggers an intracellular response that results in the translocation of the glucose transporter (GLUT 4) to the outer surface of the plasmatic membrane, facilitating glucose internalization in the muscular, hepatic and adipose cells [54] and the maintenance of glycemia at normal levels. In the present study, higher insulin concentration in NAC-treated diabetic animals (DM-NAC) reflected greater glycemia control and consequently lower water intake by these animals. According to previous study [55] also observed reduced water consumption with NAC supplementation under diabetic conditions.

Although diabetic animals subjected to NAC treatment had lower food intake, they had greater body weight, compared to non-treated diabetic animals (Table 1). This is due to the inhibition of degenerative processes such as lipolysis and proteolysis, and consequently gluconeogenesis, by insulin, which preserves the tissues and consequently the body weight by means of its anabolic action.

Diabetic cardiomyopathy is seen as a result of complex relationships between metabolic abnormalities that accompany the DM and its cellular consequences, with decreased ATP levels, leading to cardiac dysfunction and, consequently impairment in contractile function [6].

Considering that heart requires a very high energy demand, the study of energy metabolism becomes relevant in DM. The energy metabolism can be evaluated through the activity key enzyme, thereby, obtain information on the capacity of important metabolic pathways and the preference of the oxidizable substrate by the

cell [56].

There is considerable evidence that depending of the pathophysiological state the cardiac cell may utilize one metabolic fuel or other to produce energy [57]. Indeed, it was observed that diabetic state, in present study, induced myocardial metabolic shifting, indicating the preference to oxidize fatty acids in detriment of glucose oxidation. This is in agreement with early findings that related low value for respiratory quotient, confirming special metabolic condition in T1DM that were unable to use glucose as tissue substrate and used lipids from adipose tissue as fuel for energy production [33,58].

This scenario can be evidenced by lower PDH (responsible for the regulation of glucose oxidation and so control the flux of intermediaries metabolites of glucose) in myocardium in the DM group (Table 2), causing impairment in glucose oxidation, since this enzymatic complex irreversibly catalyzes the oxidative decarboxylation of pyruvate, derived from glycolysis, to form acety-CoA in mitochondrial matrix. Besides increasing the activity OHADH (biomarker of β-oxidation) and CS (regulates the flux of metabolites for citric acid cycle) was observed in the cardiac tissue of the DM group (Table 2).

The high activity of these enzymes, observed in this study, can be explained since the hypoinsulinemia is a state associated with enhanced plasmatic fatty acids and accelerated delivery to heart, favoring the β-oxidation indicate by increased OHADH and consequently the influx the acyl group to be oxidized in the citric acid cycle accelerated this metabolic pathway in the mitochondrial matrix. The CS catalyzes condensation of acyl group from acetyl-CoA with oxaloacetate to formation citrate (intermediate of citric acid cycle). Thus, elevated activity of CS may be a consequence of to the high demand of acetyl-CoA from β-oxidation in myocardium

of diabetic animals. The exacerbated β-oxidation can compromise the ATP synthase activity and impair the generation of ATP [59]. In addition, excessive uptake of fatty acid might also decrease levels of the insulin-response glucose transporter (GLUT4) [60], resulting in low oxidation of glucose.

Studies have indicated an association between systolic cardiac dysfunction and excessive β-oxidation of fatty acids in myocardium [61]. However worsening in cardiac function has been observed in presence the ketone bodies, products of excessive β-oxidation of fatty acids, combined with suppression of oxidation glucose in T1DM [62].

Since adipose tissue lipolysis is enabled in the T1DM, the high flux of the acid fatty to heart increases, the metabolism of glucose is aggravated [12,14,63] with consequent inhibition of cardiac glycolysis, which is responsible for providing ATP during the transport of ions in the sarcoplasmic reticulum, therefore worsening myocardial contractility [6,62,64].

On the other hand, the decreased in PDH activity, observed in this study (Table 2), may be due to excessive presence of acetyl-CoA, which promote phosphorylation of PDH and inhibit its activity in the cardiac tissue thus impairing glucose oxidation, as described previously [62].

The results of the Table 2 clearly demonstrate that treatment with NAC prevented both decreased PDH activity such as excessive increase in OHADH and CS activity in diabetic animals (DM-NAC), when compared with DM group.

However the administration of NAC was effective to reverse metabolic abnormalities in energy metabolism caused by DM.

This beneficial effects can be attributed to ability of the NAC in promote the release of insulin in DM-NAC group. These observations are in agreement with other

studies that have reported increased of plasmatic insulin in presence of the NAC. Previous studies have showed that NAC prevented a pancreatic β cells [32,51,52].

A number of studies have suggested a link between increase the insulin and inhibition hormone sensitive lipase from adipose tissue [54]. The antilipolytic effect the insulin decrease fatty acid flux to heart and consequently lower oxidation rate this lipid [7] and concomitantly greater to internalization of glucose by cardiomyocytes in response to insulinic action.

NAC enhanced the glucose oxidation, demonstrated by increases of the PDH activity in the DM-NAC group (Table 2). This observation is consistent with previous studies which evidenced that pyruvate oxidation was favored by suppression oxidation fatty acids [62]. The greater control β-oxidation pathway favored PDH activity in DM-NAC rats, since high concentration of acetyl-CoA, end-product of the β-oxidation, inhibits activity of this enzyme [15].

In addition, changes in metabolic fuel selection is controlled by entry de glucose in cell, and when uptake and utilization of glucose rises, there is decreased of fat oxidation, confirmed by the diminish OHADH, thus explaining the higher control β-oxidation in DM-NAC rats that in non-treated diabetics rats.

Cytosolic pyruvate is reduced to lactate by LDH in final step of anaerobic glycolysis, which generates a limited amount of NAD^+, but sufficient to provide continuity glycolysis during hypoxia. In the present study, diabetic state had no effects on cardiac LDH activity (Table 2), indicating preservation of mitochondrial oxidative processes of myocardium. These results are according to previous study [33] that reported normal LDH activity in cardiac tissue of STZ-induced diabetic rats and maintenance of aerobic capacity in these animals. Normal LDH activity is indicative of improvement by mitochondrial oxidation [65].

The lower LDH/CS ratio and maintenance of the OHADH/CS ratio reveals that elevated CS activity was due to greater β-oxidation pathway than glycolysis, constituting substantial evidence that DM induced myocardial metabolic shifting these animals. NAC improved glucose utilization by the cell, as demonstrated by elevation LDH/CS ratio in DM-NAC group (Table 2). High rates of glucose oxidation result in a concomitant reduction in fatty acid oxidation [62]. These observations indicated that NAC supplementation protects the metabolic shifting in myocardium of DM rats, suggesting beneficial effects with recovery of cardiac function. Therefore, NAC minimizes the T1DM impairment in carbohydrate oxidation through reduction of fatty acid oxidation in the mitochondria, restoring the equilibrium between carbohydrate and lipid catabolism.

Since the control of myocardial fatty acids oxidation and restoration of cardiac energy metabolism increase cardiac efficiency, administration of NAC constitutes a class of compounds with antioxidant and antidiabetogenic properties that protects the heart from exacerbation of fatty acid oxidation for elevates the release of insulin and hence the hyperglycemia.

In general, the literature relates the association between DM and oxidative stress (failure to neutralize the ROS by the endogenous antioxidant system), which stands out as one main cause of etiology of diabetic complications such as cardiac dysfunction [66]. The alterations in cardiac energy metabolism contribute to overproduction of ROS in diabetes state, since elevated fatty acid oxidation rate increase the delivery of the electron derived from reducing equivalents (NADH and $FADH_2$) generated in the citric acid cycle and β-oxidation pathways, to electron-transport chain (ETC) [67,68,69]. Indeed, there is a link between ROS (superoxide anion radical and hydrogen peroxide) generation in the mitochondria [15] and β-

oxidation [70].

However, excess of electrons in ETC in the inner mitochondrial membrane can cause leakage and partially reduce oxygen, leading mitochondrial ROS formation such as superoxide anion radical ($O_2^{\cdot-}$), main ROS produced during the oxidative phosphorylation [12,71,72].

In the present study, oxidative stress could be confirmed by increased lipid peroxide (biomarker of lipid peroxidation) and reduced activity of antioxidant enzymes in the cardiac tissue of the diabetic group (figure 1). These results are described previously [33], which observed higher cardiac concentration of lipid hydroperoxide in T1DM.

Lipid hydroperoxide is an oxidative stress biomarker from cell membrane lipid peroxidation, leading to cell damage [73]. Oxidation of biological membrane phospholipids is caused by ROS in several oxidative stress-mediated pathologies, including DM; therefore, lipid hydroperoxide concentration is related to pathological complications [74,75]. Studies have reported enhanced lipid peroxidation in several tissues, such as the sciatic nerve [76], hepatic tissue [34] and blood plasma in humans [77].

The endogenous antioxidant system is responsible for the neutralization of ROS and is represented by the enzymes SOD, CAT and GSH-Px. SOD constitutes the first line of defense against ROS since it catalyzes the dismutation of the anion superoxide into hydrogen peroxide, which is totally reduced to water by CAT and GSH-Px but by different mechanisms. CAT requires NADPH as coenzyme to supply H^+ protons during reduction, while GSH-Px uses GSH as substrate in the reduction of hydrogen peroxide. Studies have demonstrated the importance of maintaining the intracellular

concentration of NADPH, which is necessary for glutathione reduction by glutathione reductase [21,74].

Previous studies have demonstrated decreased activity of antioxidant enzymes in DM [18,20,74,75]. The low activity of CAT and GSH-Px is associated to the decrease in the pentose phosphate pathway, in which glucose is converted into ribose 5-phosphate by glucose 6-phosphate dehydrogenase (G6PDH), which requires NADP as coenzyme with release of $NADPH_2$ [19].

The diabetic rats present decreased of GSH and total glutathione concentrations. The cell to maintanance of redox state actively transports accumulated GSSG out of the cell [78]. In this way there is decreased of the total glutathione in the accentuated state of oxidative stress, fact this, also observed in this study, since it is corroborated with a previous study that analyzed the concentration of total glutathione in the liver of diabetic rats [79].

Administration of NAC was efficient at normalizing lipid hydroperoxide level in the cardiac tissue of diabetic rats (DM-NAC) (Figure 1), indicating that the cell damage caused by oxidative stress-mediated DM in the myocardium of these animals was attenuated. Other authors have reported decreased LH concentration in the presence of NAC [33,80,81].

NAC is capable of scavenger free radicals [27], exerting thus its antioxidant activity by means of the thyol group present in its chemical structure [12,27]. However, some researchers noted an improvement in the antioxidant enzymes SOD, CAT and GSH-Px in the tissues of the sciatic nerve, kidneys and liver [81,82,83], showing ability of NAC to restore the activity of antioxidant enzymes and improving the signs of oxidative stress.

In this study was observed that normal rats that received NAC treatment had a reduction in the activity of SOD (figure 1). This finding diverges from others described previously [81], which observed no change in the activity of SOD in the sciatic nerve of normal rats after NAC administration. However, previous study [83] detected reduced activity of hepatic SOD in normal rats treated with NAC.

With the re-establishment of intracellular concentration of glucose, in the presence of NAC, the pentose phosphate pathway acts assuring adequate levels of NADPH; thus, the activity of CAT is normalized (figure 1) since it is a NADPH-dependent enzyme [74]. As the activity of GSH-Px depends on the reduction of oxidized glutathione in the presence of NADPH, the catalysis of this enzyme is favored whenever the GSSG/GSH ratio is decreased [84]. In addition, by supplying cysteine to the synthesis of GSH, NAC also contributes to increase GSH concentration and consequently elevated GSH-Px activity, besides regulating the concentration of a total glutathione concentration in myocardial of diabetic rats (figure 1); this reinforces the antioxidant action of NAC by regulating the endogenous antioxidant cellular redox state, which is improved.

Since SOD catalyses the dismutation of the anion superoxide into hydrogen peroxide, which is totally reduced to water by CAT and GSH-Px, higher LH/SOD, LH/CAT and LH/GSH-Px ratios indicate an imbalance between the excessive production of ROS and the low activity of antioxidant enzymes and therefore the oxidative stress in diabetic rats. Thus, there is lipid peroxidation and consequent increase in LH due to the low neutralization of ROS by the activity.

Treatment with NAC diminished these ratios by increasing the activity of antioxidant enzymes and consequently decreasing LH in the cardiac tissue of diabetic animals (DM-NAC) (Table 3).

In conclusion, T1DM promoted metabolic abnormalities in cardiomyocytes favoring free fatty acids oxidation in detriment the glucose oxidation and, therefore oxidative stress that leads cardiac dysfunction. Treatment NAC provides an alternative therapeutic strategy to revert the deregulation in energy metabolism induced by T1DM on cardiac muscle through its antibiabetogenic effect and consequently attenuated oxidative stress.

ABBREVIATIONS

ATP: Adenosine triphosphate; BHT: Butylated hydroxytolueno; CAT: Catalase; CS: Citrate synthase; DM: Diabetes mellitus; DNA: Deoxyribonucleic acid; DNTB: 5,5'-dithiobis-(2-nitrobenzoic) acid; EDTA: Ethylenediamine tetraacetic acid; ETC: Electron transport chain; FAD: Flavin adenine dinucleotide; FADH: Flavin adenine dinucleotide, reduced; $FADH_2$: Dihydroflavine-adenine dinucleotide; Fe^{2+}: Ferrous ion; GLUT-2: Glucose transporter 2; GLUT-4: Glucose transporter 4; GSH: Reduced glutathione; GSH-Px: Glutathione peroxidase; GSSG: Glutathione oxidized; H_2O_2: Hydrogen peroxide; LDH: Lactate dehidrogenase; LH: Lipid hydroperoxide; $MgCl_2$: Magnesium chloride; NAC: N-acetylcysteine; NAD: Nicotinamide adenine dinucleotide; NAD^+: Nicotinamide adenine dinucleotide, oxided; NADH: Nicotinamide adenine dinucleotide, reduced; NADPH: Nicotinamide-adenine dinucleotide phosphate, reduced; NADPH2: Dihydronicotinamide adenine dinucleotide phosphate; NBT: Nitro blue tetrazolium; O_2^-: Superoxide anion; OHADH: β-hydroxyacil coenzyme A dehydrogenase; PDH: Pyruvate dehydrogenase; Poly-ADP: Poly-adenosine diphosphate; ROS: Reactive oxygen species; ANOVA: Analysis of variance; SD: Standard deviation; SOD: Superoxide dismutase; STZ: Streptozotocin; T1DM: Type 1 diabetes mellitus; Tris-HCl: Tris hydrochloride.

ACKNOWLEDGEMENTS

This study was received financial support by Coordination for the Improvement of Higher Level -or Education- Personnel (CAPES).

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AUTHORS' CONTRIBUTIONS

AKK, AAHF, POB and LROR participated in the experimental period, biochemical analysis and data analysis. AKK and AAHF participated in experimental design and manuscript drafting. AKK, AAHF, NOLC, BMB, MGB and PMQ participated in the discussion and part of manuscript writting. AAHF coordinate this study. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experiments using animals investigation were performed in accordance with legislation (law 11,794/2008 and decree 6,899/2009) and was approved by the Ethics Committee on the Use of Animals (CEUA) at the Institute of Biological Sciences, University of São Paulo State (UNESP).

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CAPÍTULO 2

Lipid profile of diabetic rats STZ-induced treated with N-acetylcysteine
OMICS: A Journal of Integrative Biology

Lipid profile of diabetic rats STZ-induced treated with N-acetylcysteine

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Background: Type 1 diabetes mellitus (T1DM) is characterized by deficient insulin production leading to hyperglycemia, which is associated with diabetic complications such as cardiovascular diseases. Antioxidants have been showing good alternative to diabetes complications, N-acetylcysteine (NAC) has antioxidants characteristics. In this study was evaluate the effect of NAC on lipid profile and atherogenic index (AI) in rats streptozotocin (STZ)-induced diabetes.

Methods: 32 male Wistar rats (60 days of age) weighting ±250g were randomly distributed in into four groups (n=8): G_I: normal rats; G_{II}: normal rats treated with NAC; G_{III}: diabetic rats; G_{IV}: diabetic rats treated with NAC. T1DM was induced by STZ (60 mg/kg, ip; single dose), NAC (25 mg/kg/day) was administrated by gavage, during 37 days. The animals received chow and water *ad libitum*. After experimental period was collected blood and cardiac tissue samples to analysis energetic metabolism, lipid profile and AI.

Results: NAC decreased ($p<0.01$) glycemia, energy intake, carbohydrate and protein consumption in diabetic rats (G_{IV}), when compared with G_{III}. While alimentary efficiency was improved ($p<0.01$) in treated diabetic rats (G_{IV}). Diabetic rats treated with NAC decreased ($p<0.01$) lipid profile and AI in diabetic rats (G_{IV}) when compared G_{III}.

Conclusion: NAC improves lipid profile decreasing AI in rats diabetic STZ-induced.

KEYWORDS

type 1 diabetes mellitus, N-acetilcysteine, lipid profile, atherogenic index.

INTRODUCTION

In 2015, the International Diabetes Federation estimated a global diabetic population of 415 million, with expected to reach 642 million by 2040. Approximately 10% of the diabetic population suffers from type 1 diabetes mellitus (Gillespie, 2006; Tao, 2015), it is increasing by 3% every year (Raskin & Mohan, 2010; Atkuri, 2007).

Type 1 diabetes mellitus (T1DM) is a pathology mediated by an autoimmune response that destroys the pancreatic β-cells responsible for insulin production, resulting in a deficiency of this hormone. Because insulin promotes glucose uptake in the cellular environment, individuals with T1DM suffer from hyperglycemia (Atkinson *et al.,* 2014).

Glucose is the main oxidizable substrate in various cell types. In a diabetic state, catabolic pathways such as gluconeogenesis and glycogenolysis are activated in an attempt to maintain homeostasis (Herbert & Nair, 2010). In this scenario, lipolysis is also increased, which can trigger dyslipidemia, a risk factor for the development of atherosclerosis that affects 97% of diabetic patients (Dokken, 2008; Asghar *et al*., 2009; Kim, *et al*., 2011).

Dyslipidemia is characterized by elevated levels of triacylglycerol plasma and total cholesterol, increasing the concentration of very low density lipoproteins (VLDL-cholesterol) and low density lipoproteins (LDL-cholesterol), and decreasing the concentration of high density lipoproteins (HDL-cholesterol) (Grundy, 1999; Kim, *et al*., 2011).

Studies have shown that an increase in LDL-cholesterol is directly related to the development of atherosclerosis, the inflammatory process triggered by the oxidation of LDL-cholesterol that occurs due to the release of oxidizing agents during the metabolic pathways (Advanced glycation end products, protein kinase C pathway and hexosamine pathway) activated by hyperglycemia (Valko *et al*., 2007; Schilling, 2015).

Oxidized LDLs are phagocytosed by macrophages in the subendothelial layer, thereby transforming into foam cells that contribute to the formation of atherosclerotic plaque and, consequently, atherosclerosis (Dokken, 2008; Schwartz & Reaven, 2012).

The use of natural substances with antioxidant and hypoglycemic properties has been studied extensively. Studies have shown that antioxidant supplementation is able to alleviate chronic degenerative diseases such as diabetes mellitus and its associated complications (Pham-Huy *et al*., 2008; Sindhi *et al*., 2013, Rajendran *et al*., 2014).

Antioxidants are stable substances that act as reducing agents capable of reducing oxidized substances and making them stable, thus repairing the structure of the cellular macromolecules (Lobo *et al*., 2010; Carocho & Ferreira, 2013).

N-acetylcysteine (NAC) has antioxidant characteristics because its structure includes a sulfhydryl radical, which is responsible for the direct scavenging of oxidizing agents. It can also act indirectly by providing cysteine for glutathione synthesis, which is involved in the neutralization of reactive oxygen species (Sivitz & Yorek, 2010; Yi *et al*., 2016; Samuni *et al*., 2013).

Studies have shown that NAC is capable of lowering blood glucose levels, probably due to its ability to induce the production and secretion of insulin (Fulghesu *et al*., 2002; Rushworth & Megson, 2014). Furthermore, there are reports that NAC

reduces the severity of atherosclerosis by stabilizing the development of atherosclerotic plaque (Samuni *et al*., 2013). Diniz *et al*., (2006) and Novelli *et al*., (2009) showed that administration of NAC decreased the concentration of cholesterol and its lipoprotein fractions and triacylglycerols. Meanwhile, the authors reported that HDL-cholesterol levels increased in the presence of a high calorie diet.

The following hypothesis was thus formulated: NAC improves nutritional parameters, and reduces dyslipidemia and blood sugar levels in the diabetic state.

The purpose of this study was to evaluate the beneficial effects of NAC on the lipid profile and atherogenic index in rats with experimentally induced T1DM.

MATERIAL AND METHODS

Animals and Experimental procedures

This study was conducted according with protocol approved (number 706) by the Ethics Committee on the Use of Animals (CEUA) at the Institute of Biosciences of Botucatu, University of São Paulo State (UNESP). 32 male Wistar rats aged 60 days with ±250g body weight, were housed in polypropylene cages under acclimatized room (relative humidity 55±5%; 23±2°C and a dark-light cycle of 12h). All animals received commercial feed (Purina, Campinas, Brazil; contained 22.0% protein, 3,8% fat, 44.5% carbohydrate and 3.0 kcal/g of metabolizable energy) and water *ad libitum* during the experimental period of 37 days. Control of water and food intake was daily every time between 9:00 and 10:00 a.m.

The animals were randomly divided into 4 experimental groups (n=8): G_I: normal control rats; G_{II}: normal rats treated with N-acetylcysteine (NAC); G_{III}: diabetics rats; G_{IV}: diabetics rats treated with N-acetylcysteine (NAC).

In groups G_{III} and G_{IV}, T1DM was experimentally induced by administration of streptozotocin (STZ; 60 mg kg^{-1} b.w in single dose, ip.), diluted in 0.1 M sodium

citrate buffer, pH 4.5. After 48 hours the animals with blood glucose concentration ≥ 250 mg dL^{-1} were considered diabetics according to Santos *et al*. (2014). G_{II} and G_{IV} animals received NAC (25 mg kg^{-1} b.w. day^{-1}) via gavage, for 37 days (Ribeiro *et al*., 2011).

Nutritional parameters

The nutritional parameters were estimated the considering the formulas according Kawahara *et al*. (2014): energy intake = mean food consumption x dietary metabolizable energy (3.8 kcal/g); alimentary efficiency = weight gain/energy intake; carbohydrate consumption = mean daily consumption of food x percentage of food carbohydrate; protein consumption = mean daily consumption of food x percentage of food protein.

Preparation of Serum and cardiac samples

At the end of the experimental period, the animals were fasted overnight of 12h, anaesthetized with mixture (v/v) of ketamine chloride (10%)/xylazine chloride (2%) and euthanized by cervical decapitation. Blood Serum was obtained by centrifugation (1,400g/10min) for determination of glycemia and lipid profile. Two samples of 100mg each from left ventricle of cardiac tissue were separated for biochemistry determinations.

Analytical procedure

Glycemia, lipid profile and dehydrogenase lactate activity

The concentration of glucose was determined by the method enzymatic-colorimetric method, after incubation (37°C) with glucose oxidase/peroxidase and obtaining the final product quinoneimine (Trinder, 1969).

Serum triacylglycerol was assayed by previous hydrolysis with release of free fatty acids and glycerol, which under action of glycerol quinase and peroxidase

becomes in quinoneimine what, is directly proportional to the triacylglycerol levels in the sample (Soloni, 1971).

Total cholesterol levels were measured in presence of the cholesterol esterase/ oxidase according Moura (1982). VLDL-cholesterol (very low density lipoprotein cholesterol) was obtained by Friedewald formula (Friedewald *et al*., 1972). HDL-Cholesterol (high density lipoprotein cholesterol) was measured after selective precipitation of the serum lipoproteins (LDL-cholesterol and VLDL-cholesterol) by phosphotungstic acid and despised by centrifugation (Princen *et al*., 1992).

Dehydrogenase lactate activity was determined by enzymatic kinetic method, according to the oxidation rate of NADH (Reitman & Frankel, 1957).

Atherogenic index was calculated as demonstrated by Takasaki (2005), AI = (Cholesterol total - HDL-Cholesterol)/HDL-Cholesterol.

Samples of the left ventricle were homogenized in presence sodium phosphate buffer (0.1M, pH 7.4) and centrifuged (10,000xg/15min). In the supernatant determined the activity phosphofructokinase in media containing glyceraldehyde 3-phosphate dehydrogenase, aldolase, triose phosphate dehydrogenase, NADH, ATP and fructose 6-phosphate (Bass *et al*., 1969).

The total protein concentration was determined using colorimetric method in presence of biuret reagent with release of color product, whose intensity was measured by spectrophotometer (Moura, 1982).

For myocardial glycogen determination, sample of approximately 100 mg were homogenized in pherchloric acid and supernatant was submitted to treatment with amyloglucosidase according with method Roehring & Allred (1974).

Statistical analysis

All data were statistically analyzed using one way ANOVA and Tukey's test to compare the means of the groups. A probability of 0.05 or less indicated significantly different (Zar, 1996).

RESULTS

Table 1 Glycemia, energy intake, alimentary efficiency, carbohydrate and protein consumption in rats diabetic treated and not treated with NAC.

PARAMETERS	GROUPS			
	G_I	G_{II}	G_{III}	G_{IV}
Glycemia *mg/mL*	92.71 ± 14.41[a]	98.10 ± 14.07[a]	351.04 ± 27.94[c]	128.84 ± 12.87[b]
Energy intake *Kcal/day*	98.33 ± 1.50[a]	98.88 ± 2.02[a]	160.66 ± 9.27[c]	143.82 ± 19.12[b]
Alimentary efficiency *g/Kcal*	1.44 ± 0.11[c]	1.56 ± 0.12[c]	0.33 ± 0.21[a]	0.65 ± 0.17[b]
Carbohydrate consumption g/*day*	22.09 ± 0.34[a]	22.21 ± 0.45[a]	36.09 ± 2.08[c]	32.31 ± 4.30[b]
Protein consumption *g/day*	0.29 ± 0.01[a]	0.30 ± 0.01[a]	0.48 ± 0.33[c]	0.43 ± 0.06[b]

Data are mean ± SD (*n*=8). [a, b, c] In each row, means followed by different letter indicates statistically significant difference ($p<0.05$). Control rats (G_I); N-acetylcysteine treated rats (G_{II}); diabetic rats (G_{III}); diabetic rats treated with N-acetylcysteine (G_{IV}).

The glycemia, energy intake, carbohydrate and protein consumption were increased ($p<0.05$) in G_{III}, when compared with the others. Alimentary efficiency in diabetic rats not treated (G_{III}) was lower ($p<0.05$) than G_{IV}, normal rats (G_I and G_{II}) showed higher values than diabetic rats (G_{III} and G_{IV}).

Table 2 Biochemical characteristics in rats diabetic treated and not treated with NAC

PARAMETERS	GROUPS			
	G_I	G_{II}	G_{III}	G_{IV}
Total protein *mg/100mg tissue*	33.70 ± 1.40[c]	30.47 ± 0.98[b]	25.37 ± 1.08[a]	31.01 ± 1.43[b]
Glycogen *mg/g tissue*	34.50 ± 3.40[c]	33.04 ± 3.60[c]	16.16 ± 2.58[a]	27.01 ± 3.76[b]
Phosphofructokinase *ηmol/g tissue*	1.67 ± 0.18[b]	2.27 ± 0.71[c]	0.69 ± 0.10[a]	1.45 ± 0.30[b]
Lactate dehydrogenase *U/L*	67.29 ± 10.59	67.80 ± 9.86	66.78 ± 11.65	59.70 ± 6.40

Data are mean ± SD (*n*=8). [a, b, c] In each row, means followed by different letter indicates statistically significant difference ($p<0.05$). Control rats (G_I); N-acetylcysteine treated rats (G_{II}); diabetic rats (G_{III}); diabetic rats treated with N-acetylcysteine (G_{IV}).

Concentration of total proteins and glycogen in diabetic animals (G_{III}) decreased ($p<0.05$), and treatment with NAC improved ($p<0.05$) these parameters. Phosphofructokinase activity in myocardium of diabetic rats was recovered ($p<0.05$) by NAC administration, reaching values of the groups G_I and G_{II}. There was no

statistical difference in serum lactate dehydrogenase activity between the groups ($p>0.05$).

Table 3 Lipid profile in rats diabetic treated and not treated with NAC

PARAMETERS	GROUPS			
	G_I	G_{II}	G_{III}	G_{IV}
Triacylglycerol *mg/L*	153.46 ± 25.47[a]	161.30 ± 25.72[a]	312.70 ± 33.67[b]	171.30 ± 26.79[a]
Total cholesterol *mg/L*	98.24 ± 10.84[a]	98.62 ± 15.27[a]	150.38 ± 18.64[b]	109.33 ± 10.01[a]
VLDL-cholesterol *mg/L*	30.69 ± 5.09[a]	32.26 ± 5.14[a]	62.54 ± 6.73[b]	34.26 ± 5.36[a]
LDL-cholesterol *mg/L*	29.07 ± 8.17[a]	30.13 ± 10.43[a]	68.66 ± 16.14[b]	30.52 ± 10.45[a]
HDL-cholesterol *mg/L*	38.48 ± 6.59[b]	36.23 ± 8.49[b]	19.18 ± 4.28[a]	44.54 ± 7.82[b]
Atherogenic index	1.60 ± 0.47[a]	1.66 ± 0.30[a]	7.11 ± 1.63[b]	1.39 ± 0.19[a]

Data are mean ± SD (n=8). [a, b, c] In each row, means followed by different letter indicates statistically significant difference ($p<0.05$). Control rats (G_I); N-acetylcysteine treated rats (G_{II}); diabetic rats (G_{III}); diabetic rats treated with N-acetylcysteine (G_{IV}).

NAC administration decreased serum concentration of triacylglycerols, total cholesterol, VLDL-cholesterol and LDL-cholesterol ($p<0.05$) and increased HDL-cholesterol concentration in diabetic rats (G_{IV}) compared to the G_{III} group ($p<0.05$). G_{IV} group had a lower ($p<0.05$) atherogenic index than the G_{III} group.

DISCUSSION

Streptozotocin administration is a classic model for experimentally inducing T1DM, because it selectively destroys β-pancreatic cells (Rees & Alcolado, 2005; King, 2012). It reaches the intracellular environment by glucose transporters and promotes the alkalization and subsequent fragmentation of DNA, which is repaired by poly ADP-ribose polymerase, thus reducing NAD^+ levels and depleting cellular ATP, causing cell death (Lenzen, 2008).

However, insulin release is reduced and consequently the establishment of hyperglycemia, confirming the elevated glucose serum levels in diabetic animals in this study (G_{III}). These results were corroborated by Santos *et al*., 2014 and Braga *et al*., 2013.

The increased energy intake in diabetic rats (G_{III}) is related to the increased consumption of carbohydrates and protein, indicating higher feed consumption.

Although more feed was consumed, there was no increase in body weight, implying a decrease in feed efficiency, which suggests a lower utilization of oxidizable substrates and could contribute to the hyperglycemia observed in the diabetic animals (Braga *et al*., 2013).

NAC administration in diabetic rats (G_{IV}) was shown to reduce hyperglycemia. Ammon *et al*., (1986) and Roma *et al*., (2011) showed that administration of NAC *in vitro* improved insulin production and secretion in pancreatic β-cells. Hyperglycemia decreased in treated diabetic rats (G_{IV}), and consequently improved the utilization of nutritional intake with a reduced energy intake and increased feed efficiency, accompanied by a reduced consumption of carbohydrates and proteins.

In this study, it was possible to observe a cardiac reduction in the concentration of total proteins and glycogen in animals from group G_{III}, due to the severe catabolic state caused by T1DM. These results corroborate with the existing literature, which describes a special metabolism in the diabetic state where glucose is not used as an oxidizable substrate, thus activating the biochemical process gluconeogenesis, which is supported by a supply of α-keto acids (Herbert & Nair, 2010; Fernandes *et al*., 2011; Roul & Recchia, 2015).

The enzyme phosphofructokinase (PFK) catalyzes the irreversible conversion of fructose 6-phosphate into fructose-1-6-bisphosphate during glycolysis, making it a key enzyme for evaluating the speed of this metabolic pathway (Hasawi *et al*., 2014).

PFK activity was lower in group G_{III}, indicating reduced glycolysis in the diabetic state, even with less glycogen in these animals, probably due to the reduced synthesis of this polysaccharide by the cardiac tissue (Nishizawa & Bornfeldt, 2012; Braga *et al*., 2016).

Lactate dehydrogenase (LDH), a widely distributed enzyme found in the cytosol, catalyzes the reduction of pyruvate to lactate and is of fundamental importance in processes involving glycidic metabolism in anaerobiosis. Elevated serum levels in the plasma are important indicators of heart lesions, which cause serums to leak into the bloodstream (Fernandes *et al*., 2011; Oliveira *et al*., 2013).

In this study, LDH serum activity showed no change, indicating that a diabetic state does not damage heart tissue enough to increase the activity of this enzyme in the extracellular environment. However, other studies have identified increased LDH activity in blood plasma under diabetic conditions (Adeva-Andany *et al*., 2014)

The administration of NAC promoted an increased concentration of proteins and glycogen in diabetic animals, probably due to greater cellular utilization and internalization of glucose, since insulin release is higher in the presence of NAC (Ammon *et al*., 1986; Roma *et al*., 2011).

The improved glucose oxidation in diabetic and non-diabetic animals treated with NAC is proven by the increased PFK in the heart tissue.

In this paper, there was an increase in total cholesterol, LDL-cholesterol, triacylglycerols, VLDL-cholesterol and low HDL-cholesterol in diabetic rats (G_{III}), confirming the development of dyslipidemia in this pathology, which is supported by previous studies (Almeida *et al*., 2012; Ayyasamy & Leelavinothan, 2016).

T1DM causes an insulin deficiency, resulting in increased lipolysis and subsequent β-oxidation of acetyl-CoA, which can be used in lipogenesis together with HMG-CoA reductase, a key enzyme in cholesterol biosynthesis (Ayyasamy & Leelavinothan, 2016), promoting the hepatic formation of VLDL-cholesterol and consequently increasing serum levels of cholesterol and LDL-cholesterol. Fatty acids

that are not β-oxidated are esterified into triacylglycerols, which are incorporated into VLDL-cholesterol in the liver and exported to the bloodstream (Kim *et al*., 2011).

These metabolic events increase lipoproteins to a value above normal, identifying dyslipidemia as a risk factor for the development of atherosclerosis (Ayyasamy & Leelavinothan, 2016).

There is a correlation between dyslipidemia and hyperglycemia because LDL-cholesterol is easily glycated and is more susceptible to oxidation in the subendothelial layer (Ou *et al*., 2003).

Glycated LDL-cholesterol crosses the endothelial layer and interacts with oxidizing agents, turning it into oxidized LDL, identified as an invasive agent that promotes the recruitment of macrophages, resulting in foam cells and the consequent formation of atherosclerotic plaque (Dokken, 2008; Schwartz & Reaven, 2012).

In addition, the regulatory processes of hyperglycemia produce oxidizing agents and inflammatory mediators that are contributing factors in the formation of atherosclerotic plaque (Valko *et al*., 2007; Schilling, 2015).

Considering the (total-cholesterol-HDL-Cholesterol):HDL-cholesterol ratio, the atherogenic index was elevated in diabetic rats (G_{III}) due to the dyslipidemia that is characteristic of this pathology.

Administration of NAC decreased serum levels of triacylglycerols, VLDL-cholesterol and LDL-cholesterol in diabetic rats (G_{IV}), while increasing the concentration of HDL-cholesterol.

Considering that cell recognition between apolipoproteins and membrane receptors depends on their structure being intact, the antioxidant action of NAC can prevent oxidation of proteins and ensure endocytosis of lipoproteins, decreasing its serum concentration (Korou *et al*., 2010).

The improved dyslipidemia in diabetic rats treated with NAC (G_{IV}) can be explained by the greater insulin secretion in the presence of this antioxidant, since insulin decreases blood sugar levels and lipolysis in adipose tissue. Studies have shown that NAC increases insulin secretion (Roma *et al*., 2011 e Ammon *et al*., 1986). On the other hand, the presence of insulin increases the activity of lipoprotein lipase, which catalyzes the breaking of triacylglycerol ester bonds, increasing the clearance of VLDL-cholesterol (Yang *et al*., 2006; Ayyasamy & Leelavinothan, 2016).

In this paper, HDL-cholesterol levels increased in diabetic rats treated with NAC (G_{IV}). These results are corroborated by Almeida *et al*., 2012. In addition, the increased insulin level elevates the activity of lecithin-cholesterol acyltransferase (LCAT), the enzyme responsible for extracellular cholesterol esterification, thus increasing the efficiency of reverse cholesterol transport, indicating an inverse correlation with cardiovascular accidents (Dokken, 2008; Ayyasamy & Leelavinothan, 2016).

Studies have identified a relationship between antioxidants and reduced cholesterol levels, due to inhibition of HMG-CoA reductase activity and cholesterol biosynthesis (Braga *et al*., 2013, Ayyasamy & Leelavinothan, 2016).

Thus, the NAC in diabetic rats was able to normalize the atherogenic index because it was possible to control the lipid profile, a finding that is corroborated by Yang *et al*. (2006), who noted that an increased atherogenic index is related to low antioxidant activity.

Thus, NAC can help to reduce the formation of atherosclerotic plaque by lowering blood glucose levels, the glycation of LDL-cholesterol, and its consequent oxidation. Additionally, Sung *et al*. (2012) reported that NAC is capable of reducing

the expression of gene CD36, the gene involved in the formation of foam cells, another factor that can help to reduce the development of atherosclerotic plaque.

CONCLUSSION

Deficient production/action of insulin in T1DM promoted hyperglycemia and altered nutritional parameters, and provokes dyslipidemia and consequently increased the atherogenic index. NAC treatment improves the glycemia and nutritional parameters induced by T1DM, and this antidiabetogenic agent contributes to reduce diabetic complications such as dyslipidemia and atherosclerosis by improve atherogenic index.

ACKNOWLEDGEMENTS

This study was received financial support by Coordination for the Improvement of Higher Level -or Education- Personnel (CAPES).

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DISCLOSURE

The authors declare that they have no competing interests.

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CONCLUSÃO

A deficiente produção/ação da insulina no DM1 promove a hiperglicemia e alterações nos parâmetros nutricionais, e também causa alterações no metabolismo energético nas células cardíacas favorecendo a oxidação de ácidos graxos em detrimento a oxidação de glicose, aumentando o estresse oxidativo com disfunção cardíaca, além de provocar a dislipidemia e consequentemente o índice aterogênico. A administração do NAC é uma alternativa terapêutica que melhora a glicemia, parâmetros nutricionais e regula o metabolismo energético alterado pelo DM1 no tecido cardíaco através de seu efeito antidiabetogênico e antioxidante atenuando o estresse oxidativo, e portanto, reduz as complicações diabéticas tais como dislipidemia e aterosclerose por melhorar o índice aterogênico.

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