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Patrícia Molz

INFLUÊNCIA *IN VIVO* DE NUTRIENTES SOBRE A CITOTOXICIDADE E ESTABILIDADE GENÔMICA ASSOCIADAS À SOBRECARGA DE AÇÚCAR

Santa Cruz do Sul
2015

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Dissertação apresentada ao Programa de Pós-graduação Mestrado em Promoção da Saúde, Universidade de Santa Cruz do Sul – UNISC, como requisito parcial para a obtenção do título de Mestre em Promoção da Saúde.

Orientador**:** Drª Silvia Isabel Rech Franke
Co-orientador: Dr. Daniel Prá

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RESUMO

Existem evidências de que a hiperglicemia é o principal fator para origem e progressão do diabetes, esteja associada à genotoxicidade e a citotoxicidade. Além disso, há evidências de que certos minerais e vitaminas, tais, como o cromo (Cr), ferro (Fe) e vitamina C possam interferir no risco de desenvolver diabetes. O objetivo geral desta dissertação foi avaliar o efeito de nutrientes (cromo, ferro, vitamina C e açúcar invertido) na citotoxicidade, estabilidade genômica e estresse oxidativo associados à hiperglicemia *in vivo* (ratos *Wistar*). Após 17 semanas de ingestão de açúcar invertido, numa concentração de 32%, os ratos tornam-se obesos. Ao mesmo tempo, os ratos apresentaram um aumento significativo na gordura abdominal, possivelmente causada pelo aumento da ingestão calórica. Além disso, os ratos apresentaram tolerância à glicose diminuída, conforme avaliado pelo Teste de Tolerância à Glicose Intraperitoneal (ipGTT). Danos primários no DNA, avaliados pelo Ensaio Cometa, apresentaram-se aumentados no sangue, mas não no pâncreas. Não se observou aumento no estresse oxidativo no sangue, avaliado pela técnica de proteínas carboniladas. Além disso, nenhum aumento de danos cromossômicos ao DNA foi observado na medula óssea, conforme avaliado pelo teste de micronúcleos. A ingestão de Cr na forma de $ClCr_3$ (58,4 mg/L) reduziu a glicemia e melhorou a tolerância à glicose em ratos pré-diabéticos. Concomitantemente, o Cr auxiliou na redução do IMC e reduziu a quantidade de gordura peritoneal, dado de forma isolada ou associado ao açúcar invertido. Apesar da concentração sanguínea de Cr ser mais elevada nos animais suplementados com Cr, àqueles animais que receberam Cr mais açúcar invertido apresentaram uma depleção do micronutriente. No que diz respeito ao dano primário no DNA, os grupos suplementados com Cr apresentaram danos mais elevados no sangue e mais baixos no pâncreas comparados aos grupos controle e açúcar invertido. Pelo ensaio de proteína carboniladas ou micronúcleos de medula óssea não se observou toxicidade celular. O Cotratamento com vitamina C, associado ou não com açúcar invertido, contribuiu para uma redução do peso e da gordura peritoneal e, consequentemente promoveu uma diminuição do IMC do grupo que recebeu vitamina C e açúcar invertido, que se assemelhou ao grupo controle, ao final do experimento. A vitamina C reduziu a glicemia de jejum dos animais de ambos os grupos suplementados com Vitamina C e, normalizou a tolerância à glicose do grupo que recebeu concomitantemente a vitamina C e açúcar invertido, igualando ao grupo controle. A sobrecarga de Fe atuou na diminuição da gordura peritoneal e da glicemia de jejum, mas não influenciou na obesidade e na tolerância à glicose de ratos pré-diabéticos. A concentração sanguínea de Fe não diferiu entre todos os grupos, entretanto a concentração de Fe no pâncreas apresentou-se diminuída. O grupo tratado com Fe apresentou maiores índices de dano no DNA no sangue, mas não no pâncreas. A sobrecarga de Fe não induziu estresse oxidativo, nem mutagenicidade. Concluímos com este estudo que o modelo de pré-diabetes induzido por overdose de açúcar invertido foi eficaz, uma vez que simulou as características metabólicas de pré-diabetes em seres humanos, envolvendo alterações no metabolismo da glicose, sem afetar significativamente a funcionalidade do pâncreas ou induzir danos cromossômicos ao DNA. Cr melhorou o metabolismo da glicose, no entanto induziu danos primários no DNA no sangue, mas não no pâncreas. Contudo, o Cr não aumentou o estresse oxidativo por proteínas carboniladas ou danos cromossômicos. No caso da vitamina C, o seu uso apresentou efeitos anti-obesogênico e hipoglicemiante. Já a sobrecarga de Fe não influenciou na tolerância à glicose e nem na obesidade. Além disso, a ingestão de Fe apenas influenciou na genotoxicidade no sangue, conforme avaliado pelo ensaio Cometa.

Palavras-chave: prediabetes, açúcar invertido, cromo, ferro, vitamina C.

ABSTRACT

There is evidence that hyperglycemia is the main factor for the origin and progression of diabetes and it is associated with genotoxicity and cytotoxicity. Furthermore, there is evidence that some vitamins and minerals such as chromium (Cr), iron (Fe) and vitamin C can interfere in the risk of developing diabetes. The objective of this dissertation was to evaluate the effect of nutrients (Cr, Fe, vitamin C and invert sugar) on cytotoxicity, genomic stability and oxidative stress associated with hyperglycemia in vivo (*Wistar* rats). After 17 weeks of invert sugar intake in a concentration of 32%, the rats became obese and with prediabetes. At the same time, the rats presented a significant increase in abdominal fat, possibly caused by the increased caloric intake. Moreover, the rats presented an impaired glucose tolerance, assessed by Intraperitoneal Glucose Tolerance Testing (ipGTT). Primary DNA damage, assessed by the Comet assay,was increased in the blood, but not in the pancreas. It was not observed an increase in oxidative stress in the blood, measured by the protein carbonyls technique. Furthermore, no increase in chromosomal DNA damage was observed in bone marrow, as evaluated by the micronucleus test. Cr intake in the form of ClCr3 (58.4 mg/L) decreased blood glucose and improved glucose tolerance in the prediabetic rats. At the same time, Cr aided in decreasing the body mass index (BMI) and decreased then amount of peritoneal fat, when ingested alone or in combination with invert sugar. Despite the blood concentration of Cr be higher in animals supplemented with Cr, those animals receiving Cr in combination with invert sugar presented a depletion of this micronutrient in blood. In regards to the primary DNA damage, the groups that were supplemented with Cr had increased levels in the blood and lower levels in the pancreas in comparison to the control and invert sugar groups. Protein carbonylation assay or bone marrow micronucleus test showed no significant effects. The cotreatment with vitamin C, associated or not with invert sugar, contributed to a decrease in weight and peritoneal fat accumulation. Consequently, the group that received vitamin C and invert sugar promoted a decreased the BMI that resembled the control group in the end of the experiment. Vitamin C decreased the fasting glucose of both groups supplemented with Vitamin C and normalized glucose tolerance of group that received concomitant vitamin C and invert sugar treatment, matching the level of the control group. Fe overload decreased peritoneal fat and fasting glucose levels, but had no effect in obesity and glucose tolerance in the prediabetic rats. The Fe in blood concentrations did not differ between the groups, however, the Fe concentration in the pancreas decreased. The Fe group presented higher levels of DNA damage in blood, but not in pancreas. The Fe overload did not induce oxidative stress or mutagenicity. We conclude with this study that the prediabetes model induced by overdose of invert sugar was effective, because it simulated the metabolic characteristics of prediabetes in humans, involving changes in glucose metabolism without significantly affecting the functionality of pancreas or induced chromosomal damage to DNA. Cr improved glucose metabolism, however induced primary DNA damage in blood, but not in pancreas. However, Cr has not increased oxidative stress by protein carbonylation or chromosomal damage. The supplementation of vitamin C presented anti-obesogenic and hypoglycemic effects. Fe overload had no effect on glucose tolerance neither in obesity. Furthermore, the intake only influenced genotoxicity in the blood, measured by the comet assay.

Keywords: prediabetes, invert sugar, chromium, iron, vitamin C.

LISTA DE TABELAS

TABELAS DO PROJETO

TABELAS DO ARTIGO I

TABELAS DO ARTIGO III

TABELAS DO ARTIGO IV

LISTA DE FIGURAS

FIGURAS DO PROJETO

FIGURAS DO ARTIGO I

FIGURAS DO ARTIGO II

Figure 3. Effect of Cr concentration in blood of rats with prediabetes induced by invert sugar (IS) and treated with Cr. p: level of significance according to one-way ANOVA. C: in relation to control group according to Bonferroni post-hoc test at *p<0.05.

Figure 4. Dietary intake of Cr via diet (drinking water) in the rats treated with Cr and Cr+IS (IS). p: level of significance according to Student's t-test at *p<0.05.

FIGURAS DO ARTIGO III

Figure1. Evolution of Body Mass Index (BMI) between groups. r: correlation coefficient and p: significance level; according to Pearson's Test. The dashed line indicates the cutoff for classification of obesity in the rat BMI, according to Novelli et al.[19].

Figure 2. Blood glucose curves during 120 minutes ipGTT and respective area under the 120 minutes curve (AUC) for control group (n=8), invert group (n=8), Cr group (n=8) and Cr+invert sugar (n=8). Points depict mean±SD. One-way Analysis of Variance (One-way ANOVA), *p<0.01.

Figure 3. Level of DNA damage index in blood (a) and pancreas (b) cells, protein carbonylation (c) and Micronucleus bone marrow test between groups for 18 weeks. p: level of significance according to the One-way Analysis of Variance (One-way ANOVA), p<0.05.

FIGURAS DO ARTIGO IV

Figura 1. Evolução Índice Massa de Corporal (IMC) dos grupos Controle, AI, VitC e VitCAI. r: coeficiente de correlação e nível de significância de p; de acordo com o teste correlação de Pearson. A linha tracejada indica o corte para a classificação de obesidade em ratos IMC, segundo Novelli et al[21].

Figura 2. Curva glicêmica durante 120 minutos ipGTT e respectiva área sob a curva (AUC) de 120 minutos para os grupos C (n = 8), AI (n = 8), VitC (n = 8) e VitCAI (n = 8). Pontos retratam média±DP. Teste ANOVA (One-way ANOVA), *p<0,05 e *p<0,001.

LISTA DE ABREVIATURAS E SIGLAS

µg	Micrograma
µL	Microlitro
AA	Ácido ascórbico
AGEs	Produtos finais de glicação avançada
AI	grupo controle açúcar invertido
AngII	Angiotensina II
ATP	Adenosine triphosphate
AUC	Area under the curve
b.w.	Body weight
BHE	Barreira hematoencefálica
BMI	Body mass index
C	grupo controle água
Cal	Calorias
CBMNCyt	Ensaio de citoma de micronúcleos em células binucleadas
CEUA	Comissão de Ética no Uso de Animais
CFe	grupo controle Fe
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Dióxido de Carbono
Cr	Cromo/Chromium
Cr+IS	Chromium plus Inverted Sugar group
$CrCl_3$	Cloreto de cromo/ Chromium (III) chloride
CrPic/$Cr(pic)_3$	Cromo picolinato
DCFH	2',7'-diclorofluorescina-diacetato
DM	*Diabetes mellitus*
DM1	*Diabetes Mellitus* tipo I
DM2	*Diabetes Mellitus* tipo II
DMSO	Dimetil sulfóxido/dimethyl sulfoxide
DP/SD	Desvio padrão/ Standard deviation
DRIs	Dietary Reference Intakes
EDTA	Ethylenediaminetetraacetic acid
eNOS	Enzima óxido nítrico síntese endotelial
EROs	Espécies reativas de oxigênio

FAPERGS	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
FCS	Fetal calf serum
FD	Damage frequency
Fe	grupo Fe
FeAI	grupo açúcar invertido+Fe
FrucGluc	Fructose + Glucose
FTG/GTF	Fator de tolerância à glicose/glucose tolerance factor
FT-IR	Espectroscopia de Reflectância Difusa no Infravermelho com Transformada de Fourier
GLUT	Proteínas facilitadoras de transporte de glicose
HE	Hematoxylin & Eosin
ID/DI	Índice de dano/ Damage Index
IMC	Índice de Massa Corporal
ipGTT	Intraperitoneal Glucose Tolerance Testing
IS	Inverted Sugar group
Jac/STAT	Janus quinase (Jac) de sinal transdutor e ativador de transcrição (STAT)
Kg	Quilograma
LDL	Lipoproteína de baixa densidade
LMP	Low melting point agarose
LMWCr	Low-molecular-weight chromium-binding substance
MAPK	Proteína ativada por mitógeno quinase
MN	Micronúcleos
NAD(P)H	Nicotinamida adenina dinucleotídeo fosfato
NaOH	Hidróxido de Sódio
NBC	Cromo ligado à niacina
NBUD	Brotos nucleares
NF_kB	Fator de transcrição nuclear kB
NO	Óxido Nitrico
NPBs	Pontes nucleoplásmicas
O2-	Ânion radical superóxido
OECD	Organização para a Cooperação e Desenvolvimento Econômico
ONOO-	Peroxido nitrato
PBS	Phosphate buffered saline/Solução salina tamponada com fosfato
PCE	Polychromatic erythrocyte

PCK	Proteína quinase
PIXE	Particle Induced X-ray Emission
SCGE	Eletroforese em gel de célula única
SM	Síndrome metabólica
SNC	Sistema nervoso central
STAT	Sinal transdutor e ativador de transcrição
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling
UL	Tolerable Upper Intake Level
VitC	grupo controle vitamina C
VitCAI	grupo açúcar invertido+vitamina C

SUMÁRIO

APRESENTAÇÃO

Esta dissertação de mestrado está dividida da seguinte forma: projeto de pesquisa, relatório do trabalho de campo, artigos, nota para divulgação da pesquisa na imprensa e anexos.

O projeto de pesquisa contempla a justificativa para a execução deste trabalho, através do embasamento teórico que nos levou a formular a presente proposta de trabalho. Neste item, se encontra também o método de trabalho no qual a execução do experimento seguiria, assim como a determinação do tempo, orçamento e recursos humanos. Aborda ainda, os riscos e dificuldades para ser executado o projeto, bem como os resultados e impactos esperados.

O relatório do trabalho de campo descreve os passos para realização do experimento, desde o delineamento da pesquisa, a execução do experimento e as análises de dados que resultaram nos artigos desenvolvidos na presente dissertação.

O capítulo III apresenta os quatro artigos resultantes deste experimento. No Artigo I estão apresentados os resultados obtidos pela ingestão de superdose de açúcar invertido no metabolismo de ratos *Wistar* machos. O Artigo II avalia o poder da técnica de Particle Induced X-ray Emission (PIXE) para detectar níveis cromo no sangue e em tecidos, mais especificamente, no pâncreas. O Artigo III descreve os resultados encontrados quanto ao uso de compostos contendo cromo como reguladores da hiperglicemia (efeito no metabolismo da insulina) e o poder de gerar a instabilidade genômica. E, no Artigo IV estão apresentados os resultados encontrados a partir da ingestão de vitamina C como regulador do transporte celular de glicose no teste Intraperitoneal de tolerância à glicose.

A seção nota à imprensa descreve uma síntese do projeto em forma de texto de jornal, no qual a matéria expõe os resultados da pesquisa como retorno à sociedade, de forma que contribuirá para a qualificação e promoção da saúde e bem-estar do ser humano.

Esta dissertação foi defendida em março de 2015, perante banca constituída segundo as exigências da CAPES e normas regimentais do programa. Anteriormente, o projeto havia sido defendido ao PPG para então ser submetido ao CEUA e ser aprovado para execução da pesquisa (PROTOCOLO 14/2013). E, antes de ser defendida, realizou-se uma pré-banca no qual foi composta por examinadores internos do programa a fim de qualificar o presente trabalho.

CAPÍTULO I
PROJETO DE PESQUISA

Patrícia Molz

INFLUÊNCIA *IN VIVO* E *IN VITRO* DE NUTRIENTES SOBRE A CITOTOXICIDADE E ESTABILIDADE GENÔMICA ASSOCIADAS À SOBRECARGA DE AÇÚCAR

Projeto de pesquisa apresentado ao Programa de Pós-Graduação em Promoção da Saúde – Mestrado, Universidade de Santa Cruz do Sul.

Orientador: Drª Silvia Isabel Rech Franke
Co-orientador: Dr. Daniel Prá

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1 INTRODUÇÃO

A hiperglicemia crônica é identificada como um dos principais determinantes para o diabetes mellitus tipo II (DM2) (SANTILLI *et al*., 2010), provocando distúrbios no metabolismo dos carboidratos, lipídios e proteínas, devido à falta de insulina ou da incapacidade da insulina exercer adequadamente seus efeitos no organismo. Ainda, pode ocorrer a existência de hiperglicemia de grau suficiente para causar alterações funcionais ou patológicas por um longo período antes que o diagnóstico de DM2 seja estabelecido (PEREIRA *et al*., 2013). No Brasil, a ocorrência média de DM2 na população adulta é de aproximadamente 13.4 milhões de pessoas, correspondendo a aproximadamente 6.5% da população entre 20 e 79 anos de idade (IDF, 2012).

Vários aspectos da fisiopatologia do DM2 permanecem inexplorados. O estudo de alterações celulares no DM2 é importante, uma vez que há controvérsia na literatura. Há estudos que mostram que em pacientes DM2, a hiperglicemia causa aumento nos índices de danos e outros estudos que mostram que os danos no DNA não se alteram com a hiperglicemia, comparados aos indivíduos normais (ADAIKALAKOTESWARI *et al*., 2007; BAGATINI *et al*., 2008). Em estudo recente, foi observado que a citotoxicidade é um fenômeno marcante mesmo nos estágios iniciais do estabelecimento do DM2, condição conhecida clinicamente como pré-diabetes (PEREIRA *et al*., 2013).

A origem do DM2 está ligada à sobrecarga de sacarídeos. Um aumento na concentração de glicose no sangue provoca aumento do estresse oxidativo que contribui para o desenvolvimento e a progressão das complicações associadas ao DM2 (PAZDRO; BURGESS, 2010) e a ingestão excessiva de açúcar é um dos fatores de risco (MALIK *et al*., 2010). Há indícios de que a toxicidade dos sacarídeos provoca danos genotóxicos em células intestinais (HANSEN *et al*., 2008), mas a toxicidade para células nervosas permanece inexplorada, especialmente comparando-se os diferentes tipos de sacarídeos. A saber, células hipocampais são grandes consumidoras de glicose e há controvérsia na literatura se, no encéfalo, a glicose é tóxica (GOLD *et al*., 2007) ou não (HOYLAND; LAWTON; DYE, 2008), pois alguns estudos mostram benefícios da sobrecarga de glicose (OWEN *et al*., 2010). Por outro lado, alterações neurocomportamentais são descritas no DM2, a despeito da etiologia desconhecida (PASQUIER *et al*., 2006). Embora estejam bem estabelecidos os efeitos deletérios da hiperglicemia crônica, a toxicidade da glicose, dados bioquímicos precisos e mecanismos moleculares responsáveis pelo aumento de glicose induzida pela toxicidade continuam a ser investigados (GIACCARI; SORICE; MUSCOGIURI, 2009).

Existem evidências indicando que certos nutrientes, como minerais e vitaminas estão

associados ao desenvolvimento da hiperglicemia (FENECH, 2005), contudo não se sabe ao certo o papel da sobrecarga desses nutrientes no controle do DM2. Em vista disso, têm-se sugerido que o cromo possa auxiliar na melhora da hiperglicemia, através de seu efeito na ação da insulina (ABDOLLAHI *et al*., 2013). Outro nutriente considerado importante nas complicações da hiperglicemia é o ferro, que está sendo apontado como cofator do desenvolvimento do diabetes (PRA *et al*., 2012). Ainda, antioxidantes como a vitamina C podem estar relacionados como agente redutor do estresse oxidativo associado ao aumento da hiperglicemia, além de auxiliar na regulação do transporte celular da glicose (RAFIGHI *et al*., 2013). Apesar dessas evidências serem bem fundamentadas, nenhuma destas hipóteses foram confirmadas e merecem ser estudadas mais profundamente.

O Cromo (Cr) é um metal de transição que ocorre naturalmente, sendo mais comumente encontrados em valências, Cr (III) e Cr (VI). A bioquímica do Cr é muito complexa e contribui para a sua reatividade e disponibilidade em sistemas biológicos (WISE; WISE, 2012). Cr (III) é considerado um elemento essencial para a função da insulina e da eliminação de glicose na alimentação dos mamíferos. Assim, o Cr foi identificado como um elemento traço essencial necessário para manter a tolerância à glicose normal e tem sido denominado “fator de tolerância à glicose” (FTG). Com base na sua função biológica, a suplementação de Cr pode apresentar um fator chave para a intervenção dietoterápica na hiperglicemia (LAU *et al*., 2008). O papel do Cr na potencialização da insulina deve ser minuciosamente avaliado devido a estudos recentes indicarem que esse elemento pode causar instabilidade genômica e por não apresentar papel na promoção da estabilidade genômica (WISE; WISE, 2012).

O ferro (Fe) é considerando um dos elementos mais importantes para a manutenção do sistema humano (BAO *et al*., 2012). Entretanto, a sobrecarga de Fe tem sido associada à instabilidade do genoma, assim como ao aumento do risco de câncer, como se observa na hemocromatose hereditária (PRA *et al*., 2012). Estudos indicam que a hemocromatose pode estar relacionada com o DM (COOKSEY *et al*., 2010), influenciando o metabolismo da glicose (FERNANDEZ-REAL; LOPEZ-BERMEJO; RICART, 2002). O Fe pode ser um pró-oxidante (ZHAO *et al*., 2012; PASQUALE *et al*., 2013), pois participa da reação de Fenton (PROUSEK, 2007), gerando radicais livres (MINAMIYAMA *et al*., 2010), que se produzidos em quantidades maiores que a quantidade eliminada podem provocar o estresse oxidativo (KUNDU *et al*., 2013) e danos celulares (JIANG *et al*., 2004). No entanto, ainda não está claro se a sobrecarga de Fe pode aumentar o risco de se desenvolver DM2 entre indivíduos saudáveis.

Há evidências de que a vitamina C possa exercer papel protetor nas etapas de indução e

progressão do DM2 (STYSKAL *et al*., 2012), apresentando importantes efeitos no metabolismo da inibição do transporte de glicose *in vivo* (LI *et al*., 2006; CASTRO *et al*., 2008). Além disso, a Vitamina C pode atuar como antioxidante (CASTRO *et al*., 2009) ou pró-oxidante, particularmente na reação com metais de transição (HALLIWELL, 2001). A vitamina C pode desempenhar um papel central como um "limpador" de espécies oxidantes ou pode favorecer a redução de certos metais de transição (especialmente ferro), permitindo com que eles participem de reações que geram radicais livres. Em concentrações elevadas a vitamina C pode ter efeito de indução de apoptose (NAGAPPAN *et al*., 2012). Ainda são necessários mais estudos para elucidar os efeitos benéficos e nocivos da Vitamina C em diferentes patologias, como neste caso, o DM2.

Diante do exposto **pergunta-se:** qual a influência de nutrientes (vitamina C, ferro, cromo, glicose e frutose) na citotoxicidade e estabilidade genômica induzidas pela hiperglicemia, utilizando-se modelos *in vivo* (com ratos) e *in vitro* (cultivo celular)?

2 MARCO TEÓRICO

2.1 Hiperglicemia no Diabetes Mellitus

A hiperglicemia é caracterizada pelo aumento anormal da glicemia no sangue que provoca desordens metabólicas, que por sua vez, provoca danos em muitos dos sistemas do corpo (PRABHAKAR; DOBLE, 2011). Quando os níveis de glicose seguem alterados por longos períodos de tempo caracteriza-se por DM2 (ASILIOGLU; TURNA; PAKSU, 2011). Hoje, o DM2 é uma das doenças crônicas mais frequentes da atualidade, juntamente com a obesidade e as doenças cardiovasculares (VAN WIER *et al*., 2013). Associada a um custo elevado (DAKHALE; CHAUDHARI; SHRIVASTAVA, 2011), a patologia está entre as cinco principais causas de morte nos países desenvolvidos (HIGGINS, 2013) e é considerada uma epidemia nos países em desenvolvimento (LIU; YIN; MORRISSEY, 2012; PRADEEPA; PRABHAKARAN; MOHAN, 2012), tornando-se um problema de saúde pública mundial (CUEVAS; ALVAREZ; CARRASCO, 2011; LAM; LEROITH, 2012).

O DM2 é uma síndrome de disfunção das células β envolvendo deficiência relativa de insulina (ROBERTSON; HARMON, 2006), podendo causar alterações patológicas e funcionais em vários tecidos-alvo (RODEN, 2012). Responsável por mais de 90% dos casos de diabetes, é o resultado de complexas interações entre genética, ambiente e fatores demográficos, tipicamente diagnosticados em obesos ou pessoas de meia idade (KALLIKAZAROS, 2013). Segundo a Associação Americana de Diabetes (ADA, 2013) são considerados portadores de DM aqueles que apresentam níveis de glicemia de jejum $\geq$ 126 mg/dL (7,0 mmol/L), teste de tolerância a glicose (2 horas após a ingestão de 75g de glicose) $\geq$ 200 mg/dL (11,1mmol/L) e hemoglobina glicada (A1C) $\geq$ 6,5%.

Quando ocorrida em longo prazo, a hiperglicemia crônica do DM2 é associada à disfunção e/ou a falência de diferentes órgãos e tecidos, especialmente os olhos, tecido nervoso, coração, rins e vasos sanguíneos. Os sintomas da hiperglicemia acentuada incluem poliúria, polidipsia, perda de peso, às vezes com polifagia e visão turva, comprometimento do crescimento e suscetibilidade a determinadas infecções (ADA, 2013).

O controle da hiperglicemia é realizado pela insulina, que é o hormônio chave com papel crucial na fisiopatologia do DM2. A insulina é secretada pelas células β pancreáticas, cujo papel principal é o de controlar a homeostase da glicose, que consequentemente estimula o transporte de glicose no músculo e nas células adiposas, reduzindo a glicose hepática pelas vias gliconeogênese e glicogenólise. Ainda, a insulina também é necessária para a captação de ácidos aminados e de síntese de proteína (RAINS; JAIN, 2011).

Nas últimas décadas, a prevalência de DM2 nas sociedades do mundo ocidental tem

aumentado, principalmente devido ao aumento do teor calórico na dieta e à redução da atividade física (KALLIKAZAROS, 2013). Estudos epidemiológicos indicam que sobrepeso e obesidade são fatores de risco importantes para o DM2, especialmente pelo aumento da adiposidade em torno dos depósitos centrais (HU; MALIK, 2010). Em 2010, a prevalência de DM2 era de 6,6%, atingindo cerca de 285 milhões de portadores (BANDEIRA *et al.*, 2013). Pessoas com estado hiperglicêmico intermediário (pré-diabetes) têm maior propensão de desenvolver DM2 dentro dos próximos 10 anos, sendo que a identificação precoce é fundamental para iniciar o tratamento adequado e intervenções, a fim de prevenir ou retardar a progressão para DM2 (MURPHY; WINMILL, 2013), pois está previsto que em 2030, o número de portadores desta doença poderá chegar a 552 milhões de pessoas (BANDEIRA *et al.*, 2013).

2.1.1 Hiperglicemia e o Sistema Nervoso Central

O mecanismo da hiperglicemia através do diabetes ainda é complexo, pois pode causar complicações potencialmente fatais para o paciente (CLAPES; FERNANDEZ; SUAREZ, 2013). Entre as principais complicações relacionadas ao sistema nervoso central (SNC) estão as alterações na cognição, neuropsicologia, eletrofisiologia, neurocomportamento, estrutura neuroquímica e atividades apoptóticas (LI; SIMA, 2004). Os mecanismos celulares do diabetes associados à deficiência cognitiva são pouco conhecidos, contudo, sabe-se que muitos fatores estão associados; tais como a deficiência na sinalização da insulina, a desregulação do metabolismo da glicose, doença cardiovascular e as complicações microvasculares (SIMS-ROBINSON *et al.*, 2012).

A hiperglicemia crônica pode afetar negativamente o cérebro através de diferentes mecanismos que levam a diferentes déficits cerebrais. Por isso o diabetes está associado com deficiências na cognição, aumentando o risco de demência e alterações neurofisiológicas e estruturais no cérebro (KOMOROWSKI *et al.*, 2012). Além disso, o DM está associado com a atrofia cerebral e alterações eletrofisiológicas que podem resultar em déficits de aprendizagem, memória, atenção, função executiva e eficiência psicomotora (SIMS-ROBINSON *et al.*, 2012). Outra complicação decorrente da hiperglicemia e que esta relacionada a modificações morfológicas e de plasticidade hipocampal é a encefalopatia diabética (GOLD *et al.*, 2007) que está associada a apoptose neuronal, declínio cognitivo, deficiências neurofisiológicas e mudanças estruturais (LI; SIMA, 2004; SIMA; KAMIYA; LI, 2004).

O cérebro é o órgão que mais utiliza energia no organismo. Mesmo apresentando 2% da massa corporal, ele usa 25% da energia total do corpo e a atividade neuronal utiliza 80% da energia do cérebro. A maior parte dessa energia é utilizada para restaurar o potencial de repouso da membrana após a despolarização neuronal e também para a reciclagem de neurotransmissor e o transporte axo-dentrítico. Como a glicose é a fonte energética essencial para o cérebro, ela entra no cérebro através da barreira hematoencefálica (BHE) pelos GLUTs. Os GLUTs são proteínas facilitadoras de transporte de glicose que são reguladas para gerar ATP e fornecer carbono para reações biossintéticas em conjunto com atividades cerebrais funcionais locais (CASTRO *et al*., 2009).

2.1.2 Hiperglicemia e o Estresse Oxidativo

O estresse oxidativo é definido como um estado de desequilíbrio entre antioxidantes e pró-oxidantes, em favor dos últimos (BANDEIRA *et al*., 2013). As mitocôndrias, são as maiores fontes celulares de espécies reativos de oxigênio (EROs), especificamente radicais superóxido. Na presença de oxigênio molecular, os elétrons que vazam do metabolismo mitocondrial reagem formando os radicais livres superóxido, que são mediadores de inúmeras reações em cadeia oxidativas e são também precursores de muitas outras EROs. O excesso de produção de EROs prejudica gravemente a célula pela geração de dano a macromoléculas, incluindo ácidos nucléicos, lipídios e proteínas. Os DNAs, tanto nuclear e mitocondrial, são suscetíveis a oxidação podendo gerar mutações, que pode implicar na senescência celular, apoptose e o desenvolvimento de fenótipos de células cancerosas (STYSKAL *et al*., 2012).

A patogenicidade da hiperglicemia ainda não está totalmente estabelecida em relação às complicações do diabetes. Mas, sabe-se que o estresse oxidativo tem um papel primordial na patogênese de complicações do diabetes. Contudo, o estresse oxidativo parece ser causado por um aumento tanto da produção de radicais livres e/ou uma redução acentuada das defesas antioxidantes, induzindo um aumento de produtos da peroxidação lipídica e uma diminuição de superóxido-dismutase, catalase, os níveis de glutationa, ou glutationa peroxidase (MIRANDA *et al*., 2007).

Evidências indicam que o estresse oxidativo pode ter um efeito inibidor sobre a sinalização da insulina, que está fortemente correlacionada com um ambiente pró-oxidante, podendo então, alterar significativamente a patogênese e progressão da doença. Sendo assim, os radicais livres e EROs desempenham um efeito negativo no sistema glicoregulatório, através da estimulação das vias de resposta ao estresse, por causa da disfunção celular ou

apoptose, fazendo com que a morte das células seja importante para a regulação da glicose insulina, tais como células β pancreáticas (STYSKAL *et al*., 2012).

Através de um controle adequado da glicemia, consegue-se reduzir as complicações crônicas do diabetes. Deste modo, terapias adjuvantes podem ser necessárias para auxiliar na prevenção ou no retardamento do aparecimento das complicações do diabetes (MIRANDA *et al*., 2007).

A Figura 1 apresenta a relação entre a hiperglicemia e a formação de EROs.

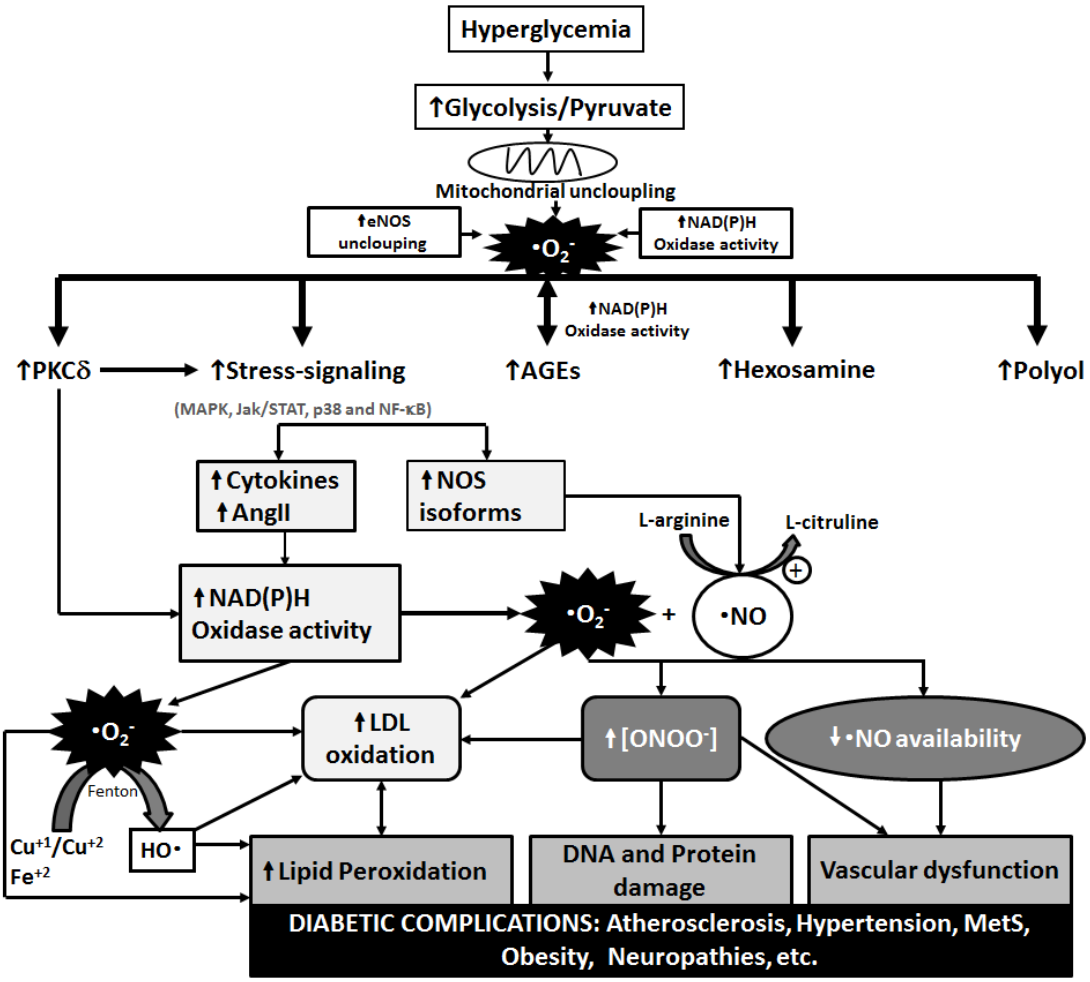

Figura 1. Participação da hiperglicemia no desencadeamento do estresse oxidativo de múltiplas vias no decurso do diabetes. AGEs: produtos finais de glicação avançada; AngII: angiotensina II; Cu+1: Cobre+1; Cu+2: Cobre+2; eNOS: síntese de óxido nítrico endotelial; Fe+2: Ferro+2/Ferro heme; Jac/STAT: Janus quinase (Jac) de sinal transdutor e ativador de transcrição (STAT); LDL: lipoproteína de baixa densidade; MAPK: proteína ativada por mitógeno quinase; NAD(P)H: nicotinamida adenina dinucleotídeo fosfato; NF-kB: fator de transcrição nuclear kB; -NO: óxido nítrico; NOS: síntese de óxido nítrico; O2-: ânion radical superóxido; OH: radical hidroxila; ONOO-: peroxido nitrato; PCKδ: proteína quinase C δ; SM: síndrome metabólica (BANDEIRA *et al*., 2013).

2.2 Efeito da Glicose e da Frutose na Hiperglicemia

O consumo de açúcares totais aumentou significativamente nos últimos 30 anos com mudanças nas fontes de adoçantes (LOWNDES *et al*., 2012). Antes de 1960, os refrigerantes eram adoçados com sacarose (50% de glicose e 50% de frutose), mas atualmente a principal fonte de adoçante na dieta é o xarope de milho rico em frutose (55% de frutose e 42%glicose) (MELANSON *et al*., 2007).

Bebidas adoçadas com açúcar desempenham um papel importante no aumento mundial na prevalência de sobrepeso e obesidade (SHELUDIAKOVA; ROONEY; BOAKES, 2012). Com isso, as Dietary Reference Intake (DRIs), orientaram para consumo de açúcar de até 25% do total de calorias diárias (TRUMBO *et al*., 2002). Essa recomendação deveu-se ao aumento do consumo de calorias proveniente de bebidas adoçadas com açúcares (4,8% para 10,3% para crianças e adolescentes com 2-19 anos e de 5,1% para 12,3% entre adultos com 19-39 anos) (HU; MALIK, 2010). Em 2009, a Associação Americana do Coração recomendou restrições significativas sobre o consumo de açúcares, sugerindo que o consumo diário por homens e mulheres respectivamente não excedam 150 e 100 calorias (JOHNSON *et al*., 2009). Esta recomendação é inferior à ingestão por 90% dos adultos do país (LOWNDES *et al*., 2012).

As ingestões de bebidas adoçadas com açúcar receberam recentemente a atenção como um potencial fator de risco para a síndrome metabólica (SANCHEZ-LOZADA *et al*., 2010) e o diabetes (JOHNSON *et al*., 2007; PALMER *et al*., 2008; MALIK *et al*., 2010). Estudos epidemiológicos têm identificado uma correlação positiva entre a ingestão de sacarose na dieta e a incidência de diabetes (KWON; KIM; KIM, 2008).

A glicose é essencial para todos os seres vivos e é consumida e/ou produzida pelos processos metabólicos das vias da glicólise e gliconeogênese (SRINIVAS *et al*., 2013). Ainda é o substrato primário para o metabolismo do cérebro (WANG *et al*., 2006), exercendo efeitos mais profundos sobre as regiões temporal medial, tais como a elevada densidade de receptores de insulina no hipocampo (MESSIER, 2004), que são conhecidos por promover a captação de glicose celular (OWEN *et al*., 2010). Evidências demonstraram que a administração de glicose melhora o desempenho da memória verbal de forma confiável em longo prazo (HOYLAND; LAWTON; DYE, 2008).

Entretanto, pesquisadores têm confirmado que as concentrações de glicose têm uma correlação linear com a citotoxicidade endotelial (XIAN-PEI *et al*., 2009), pois a hiperglicemia facilita as células endoteliais produzirem uma grande quantidade de EROs. No entanto, a influência da hiperglicemia no ciclo da célula endotelial ainda está em discussão

(HENG *et al*., 2013). A toxicidade da glicose pode também afetar outros passos importantes no caminho de insulina à expressão do gene para a liberação de insulina no sangue (ROBERTSON *et al*., 2003).

Já a frutose, ao contrário da glicose, tem sido considerada uma forma segura de açúcar por não estimular a secreção de insulina das células β do pâncreas (MELANSON *et al*., 2007; KWON; KIM; KIM, 2008). Com isso, acredita-se que a frutose é mais lentamente absorvida pelo trato gastrointestinal do que a glicose, por não apresentar mecanismo de absorção ativo na mucosa intestinal. Após a absorção, a frutose entra na circulação portal e é transportada para o fígado, onde poderá ser convertida em glicose (BRAY; NIELSEN; POPKIN, 2004).

Apesar de a frutose estar presente em quantidades significativas nas frutas, a maior fonte na dieta é sob a forma de frutose adicionada em sobremesas, bombons, refrigerantes e outras bebidas adoçadas (LINDQVIST; BAELEMANS; ERLANSON-ALBERTSSON, 2008). Com isso, dietas ricas em energia a partir de frutose podem provocar um aumento da ingestão calórica, contribuindo para o ganho de peso consequente da redução da insulina e leptina na sinalização no cérebro (STANHOPE; HAVEL, 2008).

2.3 Efeito do Cromo (Cr) na Hiperglicemia

Naturalmente o Cr é um metal de transição com estados de valência variando de -2 a 6, contudo, a atividade biológica do Cr é conferida pelo seu estado de valência (LAU *et al*., 2008), onde as valências mais comuns são o Cr (IV) (WISE; WISE, 2012) que é amplamente utilizado e liberado no ambiente por diversas indústrias e o Cr (III) é frequentemente utilizado como suplemento dietético (KUBRAK *et al*., 2010).

O Cr (III) é um elemento essencial para a saúde humana e as influências celulares são menos significativas porque é mais estável quimicamente do que o Cr (IV) (HORIE *et al*., 2013). A forma inorgânica de Cr (III) pode ser encontrada como cloreto de cromo ($CrCl_3$) que é excretado mais rapidamente do que com cromo ligado à niacina (NBC) ou picolinato (CrPic) (LAU *et al*., 2008; PREUSS *et al*., 2008; KUBRAK *et al*., 2010). O Cr (III) extracelular possui mais dificuldade em passar através da membrana celular do que o Cr (VI), indicando ser um composto menos citotóxico do que o Cr (IV) (HORIE *et al*., 2013). O Cr (VI) é um potente cancerígeno provocando peroxidação lipídica e danos no DNA, causando a morte celular (LAU *et al*., 2008).

A recomendação de ingestão diária de Cr pelas DRIs é de 20 a 35 µg/dia, dependendo da idade e sexo (TRUMBO *et al*., 2001). As fontes alimentares incluem frutos do mar, ostras, carne, queijo, fígado, cereais integrais, frutas, feijão verde, espinafre e brócolis (LAU *et al*.,

2008). Do Cr (III) ingerido, apenas 0,5-2% é absorvido (LAU *et al*., 2008). Em função da absorção ser restrita, alguns autores atribuem baixo nível de toxicidade ao Cr (III) (SHARMA *et al*., 2011).

O Cr (III) é um elemento essencial envolvido no metabolismo de carboidratos e lipídios. Existem evidências que o Cr (III) potencialize a ação da insulina em pacientes com intolerância à glicose, aumentando a sinalização do receptor de insulina. A deficiência de Cr (III) tem sido considerada como um possível fator de risco para o desenvolvimento de DM (BASAKI *et al*., 2012). Os estudos que investigam os efeitos benéficos da suplementação de Cr (III) (GUNTON *et al*., 2005; BALK *et al*., 2007; IQBAL *et al*., 2009; CEFALU *et al*., 2010) ainda são controversos e os efeitos observados, se existentes, parecem ser muito restritos (WISE; WISE, 2012).

As biodisponibilidades de vários compostos do Cr foram avaliadas e o papel biológico do Cr no nível molecular foram investigados (VINCENT, 2000). A capacidade do Cr de produzir efeitos saudáveis parece dependente de sua forma e a eficácia de estudos individuais que examinam a segurança do elemento, deve apontar o composto específico a ser utilizado (PREUSS *et al*., 2008).

O Cr (III) forma moléculas volumosas que são fracamente absorvidas pelas células (WISE; WISE, 2012). O Cr (III) é absorvido a partir do trato gastrointestinal e transportado pela transferrina, sendo transferido para vários tecidos, incluindo fígado, baço e rins (LAU *et al*., 2008; WANG; CEFALU, 2010). Já o Cr (VI) é encontrado como ânion cromato, que é rapidamente transportado pela membrana (WISE; WISE, 2012).

A absorção de Cr pode ser aumentada pela ingestão de vitamina C, aminoácidos ou oxalato, mas reduzido por antiácidos, fibras, fitatos ou ingestão de açúcares (WANG; CEFALU, 2010). Há estudos indicando não haver vantagem da suplementação com Cr (III) em indivíduos sem DM (WANG; CEFALU, 2010); contudo uma meta-análise com 41 estudos indicou que a suplementação com cromo melhorou os níveis de hemoglobina glicada e glicemia de jejum (BALK *et al*., 2007).

Os primeiros trabalhos avaliando o efeito do Cr no DM ocorreram nos anos de 1950, quando Schwarz e Mertz reportaram ao Cr o fator de tolerância à glicose (SCHWARZ; MERTZ, 1957). A maioria dos estudos indicou um efeito positivo do Cr na glicemia de jejum, evidenciando que o elemento facilita a sinalização da insulina (SHARMA *et al*., 2011). Esse ponto tem sido discutido, especialmente devido à toxicidade do Cr e de seu potencial de ciclar entre as formas Cr (III) e Cr (IV), mesmo quando o Cr (III) é administrado. Além da nutrição

inadequada, a incidência de DM2 pode estar relacionada à maior excreção de Cr (STUPAR; VRTOVEC; DOLINSEK, 2007).

O picolinato de cromo (CrPic) é uma das principais formas de suplementação com Cr. Um estudo com CrPic indicou um aumento dos níveis de Cr cerebrais e melhorou as medidas do metabolismo de carboidratos e as propriedades da serotonina (KOMOROWSKI *et al*., 2012). Outro estudo com CrPic indicou melhora significativa nas alterações metabólicas, incluindo alterações favoráveis na histopatologia do fígado, rim e pâncreas (SAHIN *et al*., 2007). Contudo, os mecanismos de ação do CrPic permaneceram não completamente conhecidos, apesar das múltiplas vias de ação que estão sendo propostos (CEFALU *et al*., 2002; JUTURU; GORMLEY, 2005).

Tem-se evidenciado que o Cr (VI) tem efeitos genotóxicos e pode induzir danos no DNA e mutações genéticas que levam ao câncer (URBANO; RODRIGUES; ALPOIM, 2008). O Cr (VI) é a forma mais potente de Cr no que diz respeito à toxicidade e carcinogenicidade e os mecanismos de indução do câncer ainda estão sendo elucidados (WISE; WISE, 2012).

Em visto disto, o estudo em relação ao Cr ainda permanece controverso quanto à suplementação para o controle glicêmico em pacientes diabéticos (WANG; CEFALU, 2010). Dessa forma, estudos avaliando o impacto do cromo em situações de hiperglicemia são necessários.

2.4 Efeito do Ferro na Hiperglicemia

O ferro (Fe) é o quarto elemento mais abundante na crosta terrestre, bem como o elemento de transição mais abundante nos organismos vivos (FERNANDEZ-REAL; LOPEZ-BERMEJO; RICART, 2002), tornando-o essencial, porque atua como um cofator ativando enzimas envolvidas na maioria dos principais processos metabólicos na célula (PRA *et al*., 2012). A ingestão adequada de Fe é de 7 a 18 mg/dia dependendo da idade e do gênero (TRUMBO *et al*., 2001), obtido principalmente a partir de fontes alimentares (BAO *et al*., 2012), como carne vermelha, peixe (PASQUALE *et al*., 2013), além de oleaginosas, como o feijão e a lentilha (LONNERDAL, 2009).

O Fe pode ser encontrado nas formas de Fe não-heme e Fe heme (PRA *et al*., 2012). A fonte predominante de ferro da dieta é o Fe não-heme, que apresenta uma biodisponibilidade baixa (3-10%) e é influenciado pelos outros alimentos. Já o Fe heme apresenta uma biodisponibilidade superior ao não-heme (20-30%), cuja absorção é menos influenciada por outros componentes da dieta (ANDERSON *et al*., 2005).

Como o Fe é um metal de transição redox-ativo, em quantidades excessivas ele é potencialmente perigoso, pois catalisa várias reações celulares que resultam na produção de EROs (BAO *et al*., 2012), por isso, ele é considerado um elemento pró-oxidante de primeira linha (FERNANDEZ-REAL; LOPEZ-BERMEJO; RICART, 2002; PASQUALE *et al*., 2013). Através do estresse oxidativo, o Fe induz a resistência à insulina, diminuindo a internalização da insulina e do aumento da síntese de ferritina, que está associada com o aumento do risco de DM (KUNDU *et al*., 2013).

Atualmente são discutidos diversos aspectos de uma doença genética chamada de hemacromatose hereditária (BAO *et al*., 2012), onde o Fe acumula-se no coração, fígado, pâncreas e outros órgãos com níveis circulantes de ferritina extremamente elevados (1000 a 10.000 ng/mL) (RAJPATHAK *et al*., 2009). Esses aumentos dos estoques de Fe foram associados com elevações significativas nos níveis de glicose no sangue e os níveis de insulina (HAAP *et al*., 2003).

Pessoas com sobrecarga de Fe associados à hemocromatose apresentam uma elevada prevalência de diabetes (22%) e intolerância à glicose (31%), que está associada com a diminuição da capacidade de secreção de insulina (COOKSEY *et al*., 2010). Em vista disso, está cada vez mais comprovado que o Fe influencia no metabolismo da glicose, associados com o desenvolvimento da intolerância à glicose (FERNANDEZ-REAL; LOPEZ-BERMEJO; RICART, 2002). Assim, a indução da resistência à insulina, inibe a absorção de glicose, o que resulta em um aumento anormal da produção da glicose hepática (ZHAO *et al*., 2012).

O Fe está intimamente ligado ao estresse oxidativo (FERNANDEZ-REAL; LOPEZ-BERMEJO; RICART, 2002), através da acumulação de Fe, que contribui para o aumento da formação de radicais livres (MINAMIYAMA *et al*., 2010), através da reação de Fenton (PROUSEK, 2007; ASLEH; LEVY, 2010). O baço, medula óssea e fígado são os principais órgãos envolvidos na reciclagem de ferro (PRA *et al*., 2012). Outra quantidade significativa de ferro é armazenada dentro da mitocôndria, participando em complexos enzimáticos utilizados para reações de fosforilação oxidativa, como as enzimas do citocromo (PRÁ *et al*., 2009). O ferro também participa da composição físico-química de enzimas envolvidas na síntese e reparação de DNA (PRA *et al*., 2012).

2.5 Efeito da Vitamina C na Hiperglicemia

A vitamina C também conhecida como ácido ascórbico (AA) é uma pequena molécula solúvel em água, sintetizada a partir de açúcares simples em plantas e animais. Tornou-se conhecida na década de 1930, quando foi identificada como possível remediadora do

escorbuto, doença aguda em tempos de guerra e fome. Os humanos não possuem a capacidade de sintetizá-la, devido a uma mutação no gene oxidase de L-gulonolactone (RIVAS *et al*., 2008), que codifica a enzima que catalisa a etapa terminal na via biosintética, precisando então adquirir vitamina C a partir da alimentação (VISSERS *et al*., 2013).

Segundo as DRIs, a recomendação diária de vitamina C é de 60 a 90 mg/dia para os homens e de 45 a 75 mg/dia para mulheres (TRUMBO *et al*., 2001), quantidades estabelecidas para garantir que haja um mínimo de excreção na urina e que ao mesmo tempo impeça o desenvolvimento do escorbuto (VISSERS *et al*., 2013). Preocupa-se também que a ingestão excessiva de vitamina C causaria toxicidade. Há evidências de que o consumo elevado é contraindicado para indivíduos com condições de sobrecarga de ferro, tais como a hemocromatose, ou com insuficiência renal. Esse tema é controverso, visto que alguns autores defendem que o excesso de vitamina C seja eficientemente eliminado na urina (PADAYATTY *et al*., 2010).

A vitamina C é um excelente antioxidante (OWU *et al*., 2012) hidrofílico no plasma, sendo degradada mais rapidamente do que os outros antioxidantes quando o plasma é exposto as EROs (PUNITHAVATHI; ANUTHAMA; PRINCE, 2008). Evidências apontam que a vitamina C possui inúmeros efeitos biológicos relevantes e efeitos associados ao acúmulo de radicais livres, quando em excesso (FRANKE *et al*., 2005). Também, desempenha um papel importante na proteção de danos causados pelos radicais livres, indicando uma diminuição basal dos danos do DNA (CHOI *et al*., 2005), e em concentrações extracelular acima podem ser citotóxico (KOH *et al*., 2007). Através da ação como um pró-oxidante, a vitamina C possui a capacidade de reduzir os íons de metais de transição, promovendo assim a reação Fenton, agindo sobre os peróxidos, produzindo radicais hidroxilas altamente reativas (AZQUETA *et al*., 2013).

Existe um aumento de evidências indicando que a vitamina C pode oferecer proteção contra algumas doenças crônicas (FRANKE *et al*., 2004). O DM está associada com níveis reduzidos de componentes do sistema de defesa antioxidante incluindo a vitamina C (STYSKAL *et al*., 2012). Há evidências da diminuição de vitamina C no soro de diabéticos. A causa permanece desconhecida, sendo atribuível à diminuição da ingestão, ao aumento da perda renal ou ao aumento da utilização em face do estresse oxidativo (GAEDE *et al*., 2001).

A vitamina C atua no metabolismo neuronal, pois é altamente concentrada em SNC e funciona como um antioxidante, neutralizando as EROs. Em nível intracelular, a vitamina C inibe a utilização da glicose e estimula captação de lactato pelos neurônios, podendo alterar o metabolismo de substratos utilizados nestas células (CASTRO *et al*., 2008; CASTRO *et al*.,

2009), aumentando a sua concentração extracelular, consequentemente, aumentando em resposta à atividade cerebral (MIELE; FILLENZ, 1996).

2.6 Marcadores de Citotoxicidade, Estabilidade Genômica e Estresse Oxidativo Associados à Hiperglicemia

A dieta é um fator chave na determinação da estabilidade genômica (FENECH, 2002). Já a instabilidade genômica refere-se há uma grande variedade de modificações genômicas, uma característica fundamental da maioria das formas de câncer, onde a integridade da estrutura dos cromossomas e o número estão usualmente comprometidos (WISE; WISE, 2012).

Os danos de DNA estão se tornando cada vez mais interligados com a alimentação inadequada e os principais biomarcadores utilizados em estudos com nutrição são ensaios genotóxicos, citotóxicos e estresse oxidativo (FENECH, 2010). As técnicas mais promissoras são: ensaio cometa alcalino (SCGE), ensaio de citoma de micronúcleos em células binucleadas (CBMNCyt), Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) e 2',7'-diclorofluorescina-diacetato (DFCH).

O Ensaio Cometa, eletroforese em gel de célula única (SCGE) é um método simples, que pode ser utilizado em qualquer célula para avaliar as quebras nos filamentos de DNA (LIAO; MCNUTT; ZHU, 2009; HARTLEY; SPANSWICK; HARTLEY, 2011). Ao longo dos anos, tem sido utilizado o SCGE como um dos métodos padrão para avaliar danos no DNA, com aplicações em testes de genotoxicidade, biomonitorização humana e epidemiologia molecular (COLLINS, 2004; FIKROVA *et al*., 2011), induzida por vários agentes numa variedade de células, *in vitro* e *in vivo* (TICE *et al*., 2000).

A versão alcalina (pH> 13) do SCGE detecta um amplo espectro de lesões de DNA, isto é, quebras simples ou duplas no DNA e sítios álcali-lábeis. Com isso, este tipo de SCGE está sendo identificado para mostrar maior sensibilidade para a detecção de danos ao DNA induzidos e tem sido recomendado para testes de genotoxicidade (SPEIT; ROTHFUSS, 2012). Além disso, o teste tem proporcionado uma imagem do nível do estresse oxidativo, que no DM está realçado pelo seu potencial impacto sobre a disfunção metabólica, pois o estresse oxidativo está rapidamente se tornando à palavra de ordem nutricional e médica para o século XXI (BALASUBRAMANYAM *et al*., 2010). Ao contrário do SCGE, o ensaio de CBMN Cyt é um marcador de dano cromossomal, utilizado com linhagens celulares, linfócitos do sangue periférico, eritrócitos e células bucais (Figura 2). MNs se originam a partir de fragmentos acêntricos do cromossomo ou cromossomos inteiros que se atrasam na

anáfase, durante a divisão nuclear. O ensaio mede a quebra de cromossomos, reparo incompleto de DNA, perda de cromossomos, amplificação do gene, não disjunção, necrose, apoptose e citostase, medir cromossomos dicêntricos (NPBs), um biomarcador de cromossomos dicêntricos resultantes de fusões-finais dos telômeros ou erros de reparo de DNA (FENECH, 2007).

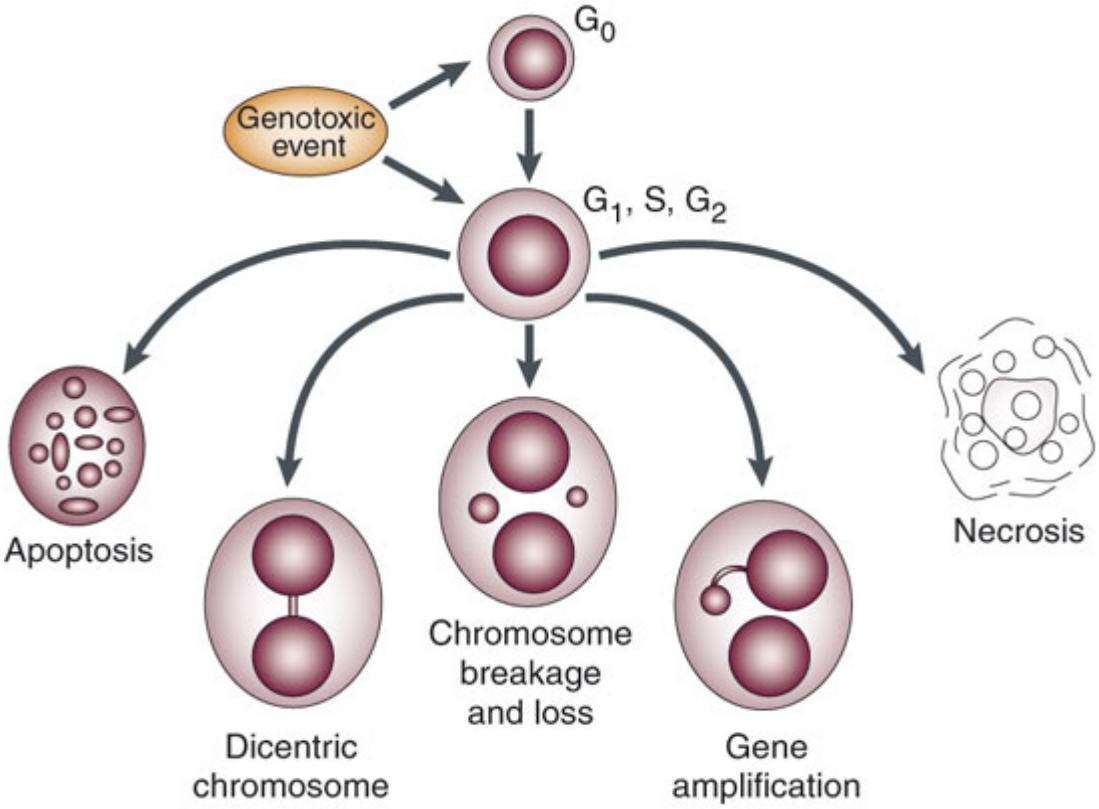

Figura 2. Os biomarcadores do ensaio de citoma de micronúcleos em células binucleadas: 1) micronúcleos (MN), biomarcadores de quebras cromossômicas ou perda; 2) pontes nucleoplásmicas (NPB), biomarcadores de cromossomos dicêntricos que se originam a partir de qualquer erro de reparo do DNA e de quebras de DNA ou fusões finais dos telômeros, e 3) brotos nucleares (NBUD), um biomarcador de amplificação do gene. A morte celular por necrose ou apoptose, é medida em função das alterações morfológicas no núcleo e no citoplasma. A proporção de células mononucleares para binucleadas também fornece uma medida da resposta mitogênica e citostática. G0, G1, S, G2 e referem-se a fases do ciclo mitótico (FENECH, 2007).

A técnica de TUNEL avalia a morte celular programada (apoptose) e foi projetado para detectar células apoptóticas que passam por extensa degradação do DNA durante os estágios finais da apoptose (KYRYLKOVA *et al.*, 2012). Está bem estabelecida que a geração EROs seja uma das causas da morte celular por apoptose, que é caracterizada por alterações morfológicas e bioquímicas. Estes incluem despolarização mitocondrial e alterações na assimetria da bicamada de fosfolipídio, a condensação da cromatina, fragmentação nuclear, e

a formação de vesículas de membranas ligadas, denominadas corpos apoptóticos (CURTIN; DONOVAN; COTTER, 2002).

Para avaliar o estresse oxidativo um bom marcador é o DFCH que avalia atividades antioxidantes. A célula tem evoluído diversos sistemas enzimáticos antioxidantes, a fim de se proteger contra EROs, que estão envolvidos em patologias como doenças neurodegenerativas como o DM (GIRARD-LALANCETTE; PICHETTE; LEGAULT, 2009). Ainda não se sabe ao certo as principais fontes de estresse oxidativo, o papel de antioxidantes naturais no controle do estresse oxidativo (CURTIN; DONOVAN; COTTER, 2002).

A despeito do grande emprego clínico do Cr no DM, uma revisão sobre os efeitos de Cr (III) apresenta resultados positivos para uma variedade de resultados genotóxicos, incluindo aumento de mutações (EASTMOND; MACGREGOR; SLESINSKI, 2008) não evidenciando que o Cr (III) possa contribuir negativamente para manutenção estabilidade genômica (WISE; WISE, 2012). O Fe desempenha um papel importante nos danos em biomoléculas principalmente através da reação de Fenton, consequentemente, provocando danos genotóxicos e mutagênicos (PRA *et al*., 2008). A vitamina C pode atuar como um antioxidante e um pró-oxidante (FENECH, 2010), e parece modular o reparo do DNA (FRANKE *et al*., 2005) e a resposta a agentes mutagênicos (FRANKE *et al*., 2004).

Os fatores genéticos, estresse oxidativo, biogênese mitocondrial, e envelhecimento são fatores que podem afetar a função mitocondrial e levar à resistência à insulina (RAINS; JAIN, 2011). O estresse oxidativo envolve moléculas reativas que podem danificar ácidos nucléicos, lipídios e proteínas, aspectos proeminentes na etiologia e na progressão de DM (AL-AUBAIDY; JELINEK, 2011). Por isso, é importante identificar os fatores dietéticos que estão envolvidos na instabilidade genômica com o objetivo de diminuir o desenvolvimento de doenças.

2.7 Hiperglicemia na Interdisciplinaridade

O progresso científico tem sido visto como uma sucessão de refinamentos elementares (PAN *et al*., 2012), através de um crescente número de publicações sobre a caracterização e medição da interdisciplinaridade pelos pesquisadores (SAYAMA; AKAISHI, 2012). Essas mudanças estão ocorrendo, a partir de revoluções científicas com repercussões globais para avanços em campos específicos ou sub-campos da ciência (PAN *et al*., 2012).

A interdisciplinaridade pode ser descrita como uma forma de trabalhar a assistência à promoção da saúde em várias disciplinas, com seus respectivos métodos (SCHILLING; HAVERICH, 2012). Assim, novos paradigmas estão nascendo não só por causa de evidências

que contradizem teorias existentes, mas também porque novas perguntas estão surgindo e quadros teóricos estão aparecendo, impulsionados pelos avanços tecnológicos (PAN *et al*., 2012). Em vista disso, os cientistas estão cada vez mais dedicando recursos para contextualizar questões além da pesquisa de bancada (PENDERS; VOS; HORSTMAN, 2009), impondo-se cada vez mais como exigência imprescindível para se abordar as questões relacionadas com a saúde da população (RAYNAUT, 2002).

Pesquisas envolvendo diferentes especialistas estão direcionadas a uma abordagem multidisciplinar para resolver problemas complexos, através da promoção da saúde e redução de custos de saúde pública. A ciência da nutrição está mudando rapidamente e a nutrigenômica está cada vez mais presente com uma abordagem interdisciplinar (PENDERS; VOS; HORSTMAN, 2009), pois a prevenção de danos ao genoma deve ser uma prioridade na definição de orientações nutricionais, para as estratégias de saúde pública como ações preventivas da medicina geral (FENECH, 2010).

Assim, as pesquisas na área da nutrição tendem a auxiliar na resolução problemas com foco interdisciplinar, uma vez que as fronteiras científicas da nutrição estão alinhadas com os novos avanços na área da genética molecular. Nesse sentido, vários profissionais devem estar envolvidos nas pesquisas, entre eles podemos citar nutricionistas, biólogos, geneticistas, farmacêuticos, físicos e estatísticos (Figura 3).

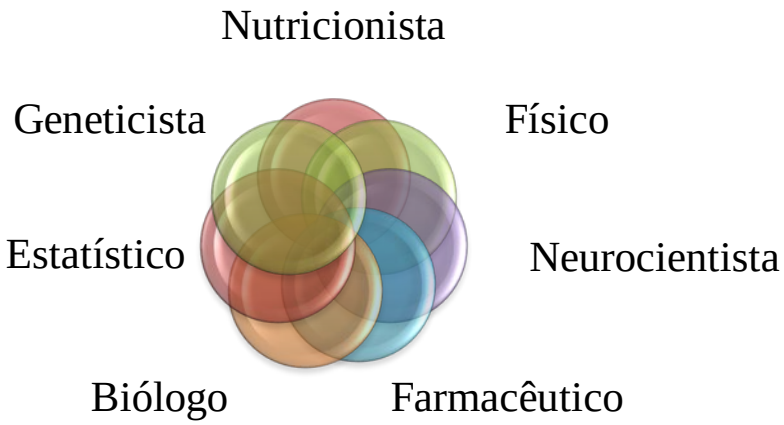

Figura 3. Atuação multiprofissional na nutrição moderna (FRANKE *et al*., 2006; MOLZ *et al*., 2013).

3 OBJETIVOS

3.1 Objetivo geral

Avaliar o efeito de nutrientes (vitamina C, ferro, cromo, glicose e frutose) na citotoxicidade, estabilidade genômica e estresse oxidativo associados à hiperglicemia, tanto *in vivo* (ratos *Wistar*) como *in vitro* (cultura celular).

3.2 Objetivos Específicos Primários do Experimento *in vivo* (ratos *Wistar*)

1. Avaliar a citotoxicidade (viabilidade celular, apoptose e necrose), genotoxicidade (dano primário no DNA, micronúcleos, brotos nucleares e pontes nucleoplásmicas) e estresse oxidativo (acumulação de 2′-7′-diclorofluoresceína: DCFH) *in vivo* (ratos *Wistar*), após o tratamento com glicose e frutose (açúcar invertido), ferro, cromo e vitamina C;
2. Avaliar o possível efeito protetor do cromo e da vitamina C na citotoxicidade, genotoxicidade e estresse oxidativo em ratos *Wistar*, após o tratamento com glicose mais frutose (açúcar invertido);
3. Avaliar o possível efeito potencializador do ferro na citotoxicidade, genotoxicidade e estresse oxidativo da glicose em ratos *Wistar*, após o tratamento com glicose mais frutose (açúcar invertido);

3.3 Objetivos Específicos Primários do Experimento *in vitro* (cultura celular)

1. Verificar a citotoxicidade (viabilidade celular, apoptose e necrose), genotoxicidade (dano primário no DNA, micronúcleos, brotos nucleares e pontes nucleoplásmicas) e estresse oxidativo (acumulação de 2′-7′-diclorofluoresceína: DCFH) induzidos pela glicose, frutose, ferro, cromo e vitamina C *in vitro* (cultura de células gliais);
2. Verificar o possível efeito protetor do cromo e da vitamina C na citotoxicidade, genotoxicidade e estresse oxidativo induzidas pela glicose (*in vitro*);
6. Verificar o possível efeito potencializador do ferro na citotoxicidade, genotoxicidade e estresse oxidativo da glicose (*in vitro*).

3.4 Objetivos Específicos Secundários para o experimento *in vivo* (ratos *Wistar*)

1. Classificar o estado nutricional, através do IMC específico, ratos *Wistar*, antes, durante e após o tratamento com glicose e frutose (açúcar invertido), ferro, cromo e vitamina C;
2. Avaliar a glicemia de jejum de ratos *Wistar*, antes, durante e após o tratamento com glicose e frutose (açúcar invertido), ferro, cromo e vitamina C;

3. Verificar o nível de insulina nos ratos *Wistar* após o tratamento com os nutrientes (vitamina C, ferro, cromo, glicose e frutose).

4. Avaliar a concentração elementar de ferro e cromo no sangue, fígado, baço, pâncreas e encéfalo dos ratos *Wistar*, após o tratamento com glicose e frutose (açúcar invertido), ferro, cromo e vitamina C;

5. Verificar alterações histopalógicas no fígado, baço e encéfalo em ratos *Wistar* após a intervenção com os nutrientes: vitamina C, ferro, cromo, glicose e frutose.

4 MÉTODO

A presente pesquisa será realizada em dois momentos. Primeiramente será realizado um estudo com animais no Biotério da Universidade de Santa Cruz do Sul (UNISC), após a aprovação da Comissão de Ética no Uso de Animais (CEUA), atendendo resolução do Conselho Nacional de Saúde e aos "Princípios éticos na experimentação animal do Colégio Brasileiro de Experimentação Animal" (ANEXO A). Conseguinte, será realizado um ensaio com cultura de células gliais no Laboratório de Nutrição Experimental da UNISC.

4.1 Amostra/população/sujeitos

4.1.1 Amostra

A amostra será composta por 64 ratos *Wistar* machos, que serão divididos aleatoriamente em 8 grupos (8 animais por grupo; Tabela 1), segundo as recomendações da Organização para a Cooperação e Desenvolvimento Econômico (OECD, 1997), onde os estudos devem incluir pelo menos cinco animais por grupo. Contudo, para obter-se melhor poder estatístico, serão incluídos mais 3 animais por grupo, o mesmo número utilizado em outros estudos do nosso grupo de pesquisa para avaliação de danos no DNA (FRANKE *et al*., 2005).

Tabela 1. Grupos de tratamento

Grupo	**N**
Controle negativo	8
Controle açúcar	8
Controle ferro	8
Controle cromo	8
Controle vitamina C	8
Açúcar + ferro	8
Açúcar + cromo	8
Açúcar + vitamina C	8
	Total = 64

4.1.2 População

Farão parte dos testes com animais, ratos *Wistar* machos com 100 dias de idade, pesando aproximadamente de 200 a 275 gramas. O uso de animais com tempo de vida mais avançado foi determinado pelo estilo de vida social dos animais comparados aos dos humanos (ANDREOLLO *et al*., 2012), devido à prevalência do DM2 ser mais comum em pessoas mais velhas (ADA, 2013).

4.1.3 Sujeitos

Os sujeitos da pesquisa serão 64 ratos *Wistar* machos, provenientes do biotério central da Universidade Federal de Santa Maria (UFSM), RS.

4.1.4 Critérios de inclusão

Serão incluídos na pesquisa, ratos *Wistar* machos com aproximadamente 100 dias de idade, pesando entre 200 a 275 gramas.

4.1.5 Critérios de exclusão

Serão excluídos:

- ratos *Wistar* fêmeas;

- ratos *Wistar* machos que apresentarem menos de 200 gramas ou mais de 275 gramas no início do experimento;

- ratos *Wistar* com qualquer tipo de afecção.

4.2 Delineamento metodológico

A pesquisa a ser realizada será de delineamento experimental através de pesquisa com animais e em células de cultura. Segundo Gil (2002), este é o tipo de experimento que representa o melhor exemplo de pesquisa cientifica, constituindo o delineamento mais prestigiado nos meios científicos.

De um modo geral, a pesquisa de natureza experimental visa determinar um objeto de estudo, selecionar as variáveis que seriam capazes de influenciá-lo (GIL, 2002), através da manipulação ou exercendo um controle efetivo onde o grupo será comparado com outro grupo mantido em condições habituais (controle) (GAYA, 2008).

Trata-se, portanto, de uma pesquisa em que o pesquisador é um agente ativo, e não um observador passivo, e ainda constituem o mais valioso procedimento disponível aos cientistas para testar hipóteses que estabelecem relações de causa e efeito entre as variáveis. Com a possibilidade de controle, os experimentos oferecem garantia muito maior do que qualquer outro delineamento metodológico (GIL, 2002).

4.3 Hipóteses e Variáveis

4.3.1 Hipóteses do experimento *in vivo* (ratos *Wistar*)

Hipótese 1: A ingestão de glicose, frutose e ferro induzem a citotoxicidade, genotoxicidade e estresse oxidativo em ratos *Wistar*.

Hipótese 2: Cromo e vitamina C têm efeito protetor contra a citotoxicidade, genotoxicidade e estresse oxidativo em ratos Wistar tratados com glicose e frutose.

Hipótese 3: O consumo de ferro potencializa a citotoxicidade, genotoxicidade e estresse oxidativo em ratos *Wistar* tratados com glicose e frutose.

Hipótese 4: A ingestão de glicose e frutose na forma de açúcar invertido induzem sobrepeso nos ratos *Wistar*.

Hipótese 5: Os tratamentos com ferro e cromo causam elevação na concentração elementar no sangue, fígado, baço e encéfalo dos ratos *Wistar* pela técnica de PIXE.

Hipótese 6: A ingestão de glicose e frutose como açúcar invertido causam elevação da glicemia de jejum e redução no nível de insulina nos ratos *Wistar*.

4.3.2 Hipóteses do experimento *in vitro* (cultura celular)

Hipótese 1: Glicose, frutose e ferro induzem a citotoxicidade, genotoxicidade e estresse oxidativo *in vitro* (cultura celular).

Hipótese 2: Cromo e vitamina C têm efeito protetor contra a citotoxicidade, genotoxicidade e estresse oxidativo induzidos pela glicose e frutose *in vitro* (cultura celular).

Hipótese 3: Ferro potencializa a citotoxicidade, genotoxicidade e estresse oxidativo induzido pela glicose e frutose *in vitro* (cultura celular).

4.3.3 Definições das Variáveis

4.3.3.1 Variáveis Dependentes

Marcadores de citotoxicidade: variável quantitativa contínua que indica a viabilidade celular das células em cultura celular e a quantidade de células apoptóticas, pelo ensaio de TUNEL, e de células necróticas, pelo ensaio de Micronúcleos.

Marcadores de genotoxicidade: variável quantitativa contínua que indica o índice e a frequência de dano ao DNA, obtidos a partir do teste cometa, e de aberrações cromossômicas e micronúcleos, obtidos a partir da técnica de micronúcleos em eritrócitos.

Marcador de estresse oxidativo: variável quantitativa contínua que indica a quantidade de EROs indicados pela acumulação de 2′-7′-diclorofluoresceína (DCFH).

4.3.3.2 Variáveis independentes

Concentração de ferro: variável quantitativa contínua, referente às reservas de ferro, obtida através do método de PIXE.

Concentração de cromo: variável quantitativa contínua, referente às reservas de cromo, obtida pelo método de PIXE.

Concentração de glicemia de jejum: variável quantitativa contínua, referente às concentrações de glicose no sangue, através glicômetro portátil.

4.3.3.3 Co-variáveis

Índice de Massa Corporal (IMC): variável categórica, referente à classificação do estado nutricional específico para animais.

Concentração de insulina: variável quantitativa contínua, referente às concentrações de insulina no sangue, através do kit Sensitive Rat Insulin RIA Kit.

4.4 Procedimentos Metodológicos e Cronograma de Execução

O presente trabalho será viabilizado através das etapas descritas a seguir:

-1ª etapa: aquisição dos animais;

-2 ª etapa: execução do experimento com ratos *Wistar* será conduzida da seguinte forma;

1. Realização da avaliação inicial dos animais (IMC, glicemia de jejum e dano ao DNA em sangue periférico);
2. Condução do tratamento diário com açúcar invertido, cromo, ferro e vitamina C, juntamente com a verificação do IMC dos animais (semanalmente) e da glicemia de jejum e danos ao DNA em sangue (quinzenalmente);

3. No último dia do experimento serão avaliados o IMC, glicemia de jejum e dano ao DNA em sangue;
4. Realização da eutanásia;

Após a eutanásia serão avaliados: dano ao DNA no encéfalo, micronúcleos em medula óssea, apoptose, estresse oxidativo, níveis de insulina, concentrações de cromo e ferro no sangue e órgãos e alterações histopatológicas nos órgãos;

-3ª etapa: digitação dos dados do experimento com animais em planilhas eletrônicas;

-4ª etapa: obtenção das células gliais;

-5ª etapa: treinamento de técnicas envolvendo cultura celular;

-6ª etapa: execução do experimento com cultura celular;

1. Semeação das culturas celulares com suas respectivas doses de tratamento (sacarose, cromo, ferro e vitamina C);
2. Avaliação da viabilidade celular;
3. Realização dos testes pós-tratamento (dano no DNA, micronúcleos, apoptose, estresse oxidativo).

-7ª etapa: digitação dos dados da cultura celular em planilhas eletrônicas;

-8ª etapa: análise dos resultados obtidos das técnicas de citotoxicidade, estabilidade genômica e estresse oxidativo, tanto do experimento com animais como do experimento de cultura celular;

-9ª etapa: organização, análise e discussão dos dados do experimento com animais e com cultura celular;

-10ª etapa: redação da dissertação;

-11ª etapa: apresentação dos resultados obtidos na banca de dissertação;

-12ª etapa: submissão dos artigos.

4.5 Técnicas e instrumento de coletas e análise de dados e procedimentos de intervenção do Experimento *in vivo*

4.5.1 Experimento *in vivo* (modelo de intervenção)

Conforme o protocolo, prescrito no Regulamento do Biotério da Universidade de Santa Cruz do Sul (UNISC), considerado na Lei nº. 11.794, de oito de outubro de 2008 (BRASIL, 2008), primeiramente os ratos serão confinados num período de ambientação (uma semana). A temperatura ficará constante (22 ± 23°C), com umidade de 60% e ração normoprotéica-calórica Presense Nestlé® e água *ad libitum*; também receberão iluminação adequada num

ciclo claro escuro de 12 em 12 horas. Após este período, os grupos serão formados aleatoriamente e divididos conforme a tabela 2.

Tabela 2. Relação dos grupos de tratamentos, substâncias e doses empregadas e número de ratos *Wistar*.

Grupo	Tratamento (concentração)	Co-tratamento (concentração)	N
Controle negativo	Água	--	8
Controle açúcar	Açúcar invertido (320 g/L)	--	8
Controle ferro	Ferro (12,8 mg/L como sulfato)	--	8
Controle cromo	Cromo (58,4 mcg/L como cloreto)	--	8
Controle vitamina C	Vitamina C (60 mg/L)	--	8
Açúcar + ferro	Açúcar invertido (320 g/L)	Ferro (12,8 mg/L como sulfato)	8
Açúcar + cromo	Açúcar invertido (320 g/L)	Cromo (58,4 mcg/L como cloreto)	8
Açúcar + vitamina C	Açúcar invertido (320 g/L)	Vitamina C (60 mg/L)	8
			Total = 64

A condução do experimento com os ratos *Wistar* será da seguinte forma: primeiro os animais serão avaliados no seu estado inicial (IMC, concentração de glicose em jejum e dano ao DNA), depois eles serão tratados com as superdoses de açúcar invertido, cromo, ferro e vitamina C. Durante o período de tratamento de 4 meses, os ratos serão aferidos, semanalmente, quanto ao IMC e, quinzenalmente, quanto à glicemia de jejum e quanto ao índice de danos ao DNA. No final do experimento, um dia antes do sacrifício (eutanásia), os animais serão avaliados quanto ao IMC, danos de DNA no sangue e glicemia de jejum. Durante o sacrifício serão coletadas amostras de sangue e tecidos para avaliação da insulina, concentração sanguínea de minerais, apoptose, estresse oxidativo, histopatologia e também será coletada a medula óssea dos animais para o teste de micronúcleos.

4.5.2 Alimentação, substâncias testes e doses

Durante 4 meses, os animais receberão ração *ad libitum*. Quanto à ingestão de líquidos, esta será limitada a 220 mL/kg de peso corporal por dia por animal. Esse nível garantirá ingestão adequada de líquido, visto que esse nível estará 2 desvios padrão acima da ingestão média, conforme observado por Wade *et al*. (2002) (199,0 ± 9,3 mL/kg de peso corporal por dia). Tanto a ingestão de ração, como a de líquidos serão monitoradas frequentemente de modo a calcular o aporte das substâncias testadas.

Serão oferecidas juntamente com a água de beber os nutrientes. As doses serão administradas das seguintes formas: glicose e frutose serão preparadas em forma de açúcar invertido e em concentração de 320 g/L (GLENDINNING *et al*., 2010); o cromo será

oferecido em forma de cloreto de cromo 58,4 mcg/L (Sigma 27096) e se assemelhará aos níveis já disponíveis na literatura (PORTER; RAYMOND; ANASTASIO, 1999; VINCENT, 2000; WANG; CEFALU, 2010) em virtude de não haver valores referentes ao limite máximo tolerável por um ser humano saudável sem riscos de efeitos adversos (Tolerable Upper Intake Level, UL) (TRUMBO *et al*., 2001); as diluições contendo ferro (Sigma 215422) e vitamina C (Sigma A4544) serão preparadas em concentrações 12,8 mg/L e 60 mg/L, respectivamente baseando-se na UL para adultos, transpostos para os pesos médios dos ratos durante os tratamentos (TRUMBO *et al*., 2001).

4.5.3 Coleta e preparação de amostras de sangue

Amostras de sangue periférico (cerca de 20 µL) serão coletadas no início da manhã dos animais em jejum, por meio de uma picada na cauda dos mesmos, afim de não trazer sofrimento aos ratos. O procedimento é similar a uma picada no dedo em humanos (sem uso de anestésicos) para avaliação de glicemia de jejum com o uso de glicômetro portátil. No primeiro dia, antes de ser iniciado o tratamento com os nutrientes, os ratos serão avaliados quanto à glicemia de jejum inicial e quanto ao índice de danos basal ao DNA em sangue. Durante os quatro meses de experimentação, quinzenalmente, serão coletadas amostras de sangue para o acompanhamento da glicemia de jejum e do índice de danos ao DNA (ensaio cometa). Para a mensuração da glicemia de jejum, o sangue periférico coletado da cauda será imediatamente mensurado por meio de glicômetro portátil e as amostras de sangue utilizadas para a realização do ensaio cometa serão mantidas a 4ºC no escuro e serão transportadas ao laboratório de Nutrição Experimental em microtubos com anticoagulante heparina.

4.5.4 Ensaio Cometa de sangue

O Ensaio Cometa de células do sangue será realizado de acordo com o protocolo padrão do grupo (FRANKE *et al*., 2006; PRA *et al*., 2011).

Serão utilizadas lâminas pré-revestidas com agarose, onde 5 µL de sangue serão adicionados a 95 µL de agarose de baixo ponto de fusão (LMP) (0,75%). Uma lamínula será colocada delicadamente sobre a lâmina e durante 5 minutos ela será resfriada a 4ºC para permitir a solidificação do gel. Após as lâminas se solidificarem, as lamínulas serão removidas e as células serão lisadas em elevadas concentrações de sal e detergente (NaCl 2,5 M, EDTA 100 mM, Tris 10 mM, pH 10,2, em que 1% de Triton X-100 e 10% de DMSO) e colocadas em uma cuba de eletroforese, para submeter-se a corrente elétrica adicionada de uma solução alcalina (NaOH 300 mM, EDTA 1 mM, pH> 13), para o desenrolamento do

DNA e para a expressão de sítios alcalino lábeis como quebras de cadeia simples. Depois, as lâminas serão lavadas com tampão de neutralização (0,4 M de Tris, pH 7,5), e fixadas com nitrato de prata.

Para cada amostra de sangue, 100 células serão selecionadas aleatoriamente e analisadas (50 por lâminas, duas lâminas por indivíduo), em microscópio óptico convencional com uma ampliação de 200X, onde será obtido pela avaliação visual das classes de dano (de 0-4). Os núcleos intactos aparecerão redondos (classe 0 – sem dano), já as células lesadas foram classificadas entre classes um (dano mínimo) a quatro (dano máximo). O índice de danos (DI) será obtido pela soma das células individuais classificados, que varia de 0 (nenhum dano: 0 x 100 células) a 400 (dano máximo: 4 x 100 células). A frequência de dano (FD), em percentagem (%) será calculada pela razão entre o número de células com danos e o total de células da amostra. Núcleos de células não-detectáveis (cabeça e cauda claramente separados) não serão avaliados.

4.5.5 Parâmetro bioquímico – Glicemia de jejum

Para avaliar o estado de hiperglicemia, será avaliada quinzenalmente em jejum a glicose de jejum dos ratos pelo uso de um glicômetro portátil e serão considerados hiperglicêmicos, quando atingirem glicemia de jejum de pelo menos 150 mg/dL, conforme protocolo da Animal Models of Diabetic Complications Consortium (AMDCC, 2003).

4.5.6 Avaliação antropométrica *in vivo*

Verificar-se-á, semanalmente, o peso e o comprimento dos animais para classificação do IMC. Assim, os animais serão classificados quanto ao estado nutricional de acordo com IMC específico para ratos, conforme Novelli *et al*. (2007).

4.5.7 Eutanásia

No final dos 4 meses de tratamento, será realizada a eutanásia pelo método de decapitação, utilizando-se a guilhotina, segundo as normas do Biotério, considerado na Lei nº. 11.794, de oito de outubro de 2008 (BRASIL, 2008). Durante o procedimento será coletado sangue para verificar os níveis de insulina e para as técnicas de DCFH, TUNEL e do método PIXE. O encéfalo será retirado para, respectivamente, avaliar-se o dano no DNA pelo ensaio cometa e determinação dos elementos pelo método PIXE. Serão também elaboradas lâminas histológicas do encéfalo, fígado e do baço.

O método escolhido para a eutanásia será a decapitação, porque é um procedimento que não ocorre interferência de outros agentes. Assim, é considerado um método eficiente, ocorrendo a perda imediata do reflexo palpebral e produzindo mínimas mudanças fisiológicas (ANDERSEN *et al*., 2004).

4.5.8 Coleta e preparação de amostras depois da eutanásia

As amostras de sangue serão coletadas da veia jugular no momento do sacrifício para verificar-se a glicemia de jejum e realizar os experimentos de ensaio cometa, PIXE, apoptose e estresse oxidativo. Também serão coletadas amostras de tecidos (encéfalo, fígado e baço) para os procedimentos de ensaio cometa do hipocampo e para a confecção de lâminas histológicas. Durante o sacrifício amostras de medula óssea serão coletadas para a realização do ensaio de micronúcleos.

4.5.9 Ensaio Cometa de hipocampo

O Ensaio Cometa de hipocampo será baseado do protocolo padrão do grupo (FRANKE *et al*., 2006; PRA *et al*., 2011) para experimentos com ratos. Para obtermos as suspensões de células do hipocampo, o encéfalo será removido imediatamente após o sacrifício e seccionados em junção entre o bulbo e a medula espinhal. Posteriormente, o encéfalo será transferido para uma placa de Petri em gelo para extrair o hipocampo. O hipocampo será identificado com as coordenadas descritas por Paxinos, Watson e Emson (1980), usando uma placa de acrílico para a diferenciação das áreas do encéfalo (Insight®, Brasil). Os cortes obtidos serão dissecados com o auxílio de uma agulha calibre 14 para a obtenção de tecido do hipocampo e serão armazenadas em microtubos contendo solução salina tamponada com fosfato (PBS), que será adicionada 10% de dimetil sulfóxido (DMSO), onde o tecido será dissociado e 60 µL serão destinados para o Ensaio do Cometa. Todos os procedimentos serão realizados sob luz amarela fraca para evitar a indução de danos ao DNA pela radiação ultravioleta.

Assim como realizado em amostras de sangue, as amostras do hipocampo serão realizadas em duplicata, onde 100 células serão selecionadas aleatoriamente e analisadas (50 por lâminas, duas lâminas por indivíduo), em microscópio óptico convencional com uma ampliação de 200X, onde será obtido pela avaliação visual das classes de dano (de 0-4). Os núcleos intactos aparecerão redondos (classe 0 – sem dano), já as células lesadas foram classificadas entre classes um (dano mínimo) a quatro (dano máximo). O índice de danos (DI) será obtido pela soma das células individuais classificados, que varia de 0 (nenhum dano: 0 x

100 células) a 400 (dano máximo: 4 x 100 células). A frequência de dano (FD), em percentagem (%) será calculada pela razão entre o número de células com danos e o total de células da amostra. Núcleos de células não-detectáveis (cabeça e cauda claramente separados) não serão avaliados.

4.5.10 Ensaio de micronúcleos em eritrócitos da medula óssea

Ao final do experimento, os fêmures dos animais serão removidos e suspensões de medula óssea serão preparadas com o uso de soro bovino fetal; dois esfregaços serão preparados para cada animal. As lâminas serão fixadas por imersão em metanol: ácido acético (3:1) e coradas com azul de metileno (5%), e codificadas para posterior análise "cega" em microscópio. Os micronúcleos serão avaliados em 2000 PCE como marcadores de dano cromossomal (permanente). A razão entre a quantidade de eritrócitos policromáticos e (PCE) eritrócitos normocromáticos (PCN) foi avaliada em 1000 células, como marcador de toxicidade dos compostos (DA SILVA *et al*., 2002; PRA *et al*., 2008).

4.5.11 Análise de ciclo celular e determinação do nível de estresse oxidativo pela técnica de 2',7'-diclorofluorescina-diacetato (DCFH) por citometria de fluxo

O nível de estresse oxidativo será avaliado pelo uso de DCFH-DA de acordo com o protocolo de Girard-Lalancette, Pichette e Legault (2009). As células serão lavadas com PBS e incubadas por 30 min com HBSS contendo DCFH-DA (Sigma–Aldrich, Oakville, ON). Ao final do tratamento com DCFH-DA, as células serão lavadas novamente com PBS. A fluorescência será avaliada imediatamente em um leitor de placas, utilizando excitação de 485 nm e emissão de 530 nm. Para as células em cultura será calculado o IC50, utilizando regressões logarítmicas das curvas dose-resposta após a subtração das fluorescências do branco e das amostras. Os IC50 serão calculados a partir de 3 determinações.

4.5.12 Avaliação de apoptose pela técnica de Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL)

A determinação de apoptose por TUNEL será realizada de acordo com (DARZYNKIEWICZ; GALKOWSKI; ZHAO, 2008).

Primeiramente será verificado a marcação de quebras no DNA com Br-dUTP para análise por citometria de fluxo, onde será suspendida 106 células em 0,5 mL PBS. Depois esta suspensão será transferida com uma pipeta Pasteur para um tubo de polipropileno contendo 4,5 mL de formaldeído gelado (1% em PBS), mantendo o tubo por 15 minutos no gelo. A

seguir as células serão centrifugadas a 3000rpm por 5 min, ressuspendidas em 5 mL de PBS e novamente serão centrifugadas e ressuspendidas em 0,5 mL de PBS.

A suspensão será transferida com uma pipeta Pasteur para um tubo de polipropileno contendo 4,5 mL de etanol 70% gelado. Se necessário, as células poderão ser estocadas em etanol por diversas semanas a -20ºC. Depois, o próximo passo será a centrifugação a 2000 rpm por 3 min, então o etanol será removido e se ressuspenderá as células em 5 mL de PBS e centrifugadas novamente a 3000 rpm por 5 min.

A seguir o pellet será ressuspendido em 50 µL de solução contendo 10 µL do tampão de reação TdT 5•X, 2,0 µL da solução estoque de Br-dUTP, 0,5 µL (12,5 U) de TdT, 5 µL da solução de CoCl2, 33,5 µL de H2O destilada. Por conseguinte as células serão incubadas por 40 min a 37 ºC e após esse período, será adicionado 1,5 mL do tampão de lavagem e centrifugado a 3000 rpm por 5 min. O pellet novamente será ressuspendido em 100 µL da solução anti-Br-dU FITC- (ou Alexa Fluor 488) mAb conjugado, incubado a temperatura ambiente por 1 h, adicionado 1 mL da solução de coloração de iodeto de propídio (PI) e novamente incubado por 30 min a temperatura ambiente ou 20 min a 37 ºC no escuro.

Para a análise por citometria de fluxo, as células serão excitadas com a fluorescência de luz azul (linha de laser de 488 nm ou filtro de excitação BG12) e então serão medidas a fluorescência verde do FITC- (ou Alexa Fluor 488) anti-Br-dUAb a 530 ± 20 nm e a fluorescência vermelha do PI a >600 nm.

4.5.13 Parâmetro bioquímico – Insulina

No final do experimento, será coletado sangue periférico da cauda dos animais para verificar os níveis de insulina de modo a avaliar a resistência à insulina, que será desenvolvido com o Sensitive Rat Insulin RIA Kit (Linco Research, Inc.) (COOKSEY *et al*., 2004).

4.5.14 Particle Induced X-ray Emission (PIXE)

Durante o sacrifício, serão coletadas amostras de sangue (1mL), o fígado, o baço e o hipocampo para a determinação de minerais pelo método PIXE. As amostras coletadas serão colocadas em microtubos e liofilizadas. Depois, serão peletizadas com cerca de 13 mm de diâmetro e colocadas em um porta-amostras para as medições específicas para o PIXE.

As medições das amostras serão realizadas no Laboratório de Implantação Iônica do Instituto de Física da Universidade Federal do Rio Grande do Sul. As amostras serão irradiadas em 10-6 mbar em uma câmara de vácuo, com um feixe de protões de 2MeV e uma

corrente média de 3,5 nA, durante 400 segundos. A característica de raios-x será detectada com detector de Si (Li) (Sirius 80 e2v). A resolução em energia do detector é de 150 eV em 5,9 keV. O sistema será calibrado e padronizado com fígado bovino (NIST, material de referência 1577b) padrão (ROHR *et al*., 2013). A quantificação espectros PIXE será realizada usando o software GUPIXWIN (CAMPBELL *et al*., 2000).

4.5.15 Análise Histológica dos Órgãos

Serão analisados os seguintes órgãos: encéfalo, fígado e baço. Esses órgãos serão coletados durante o sacrifício e submetidos aos processamentos para posterior análise microscópica, objetivando-se o reconhecimento de possíveis lesões tissulares. A metodologia aplicada no processamento seguirá os protocolos tradicionais de histologia (JUNQUEIRA; CARNEIRO, 1999; DE TOLOSA *et al*., 2003; GARTNER; HIATT, 2007; BANCROFT; GAMBLE, 2008), incluindo as etapas de coleta e fixação, desidratação, diafanização, inclusão, microtomia, distensão, coloração e análise histológica, conforme detalhado a seguir.

a) Coleta e fixação

A fixação será iniciada imediatamente após a coleta do tecido, para que durante a análise microscópica as estruturas celulares se mostrem preservadas ao máximo. As amostras a serem fixadas não deverão apresentar grandes tamanhos, caso contrário a penetração do fixador no interior dos tecidos não será completa. Recomenda-se que as amostras sejam coletadas com um tamanho máximo de $1cm^3$. O tempo de fixação varia de acordo com o tipo de tecido, podendo levar de 6 a 24 horas em tecidos moles, como os que serão processados no presente projeto.

O processo de fixação possui quatro objetivos principais: parar a lise celular, impedir o desenvolvimento microbiano, enrijecer os tecidos para posterior microtomia e aumentar a afinidade do tecido aos corantes histológicos. Existe uma variedade muito grande de fixadores, sendo que cada um deles é indicado para fixar uma gama de tecidos ou estruturas diferentes. O fixador comumente usado na rotina de laboratórios de anatomia patológica é a solução de formaldeído a 10% tamponado, pois é considerado de baixo custo e um dos mais práticos, já que após a fixação em formol os tecidos podem ser transferidos diretamente para a bateria de desidratação. Por esses motivos, esse será o fixador utilizado no processamento das amostras.

b) Desidratação

A desidratação dos tecidos, e posterior diafanização, possuem como objetivo preparar as amostras para serem impregnadas em parafina histológica (ou outros meios) para então serem montados pequenos blocos de parafina contendo amostras em seus interiores, para posterior corte no micrótomo. O álcool etílico é o produto mais comumente usado para a desidratação por ser barato e apresentar ótimos resultados. O processo de desidratação deverá ser lento e gradual. Dessa forma, será realizada a imersão das amostras em soluções de álcool etílico em concentrações de 70ºGL, 80ºGL, 90ºGL e 100ºGL. Duas imersões em álcool absoluto serão necessárias para a remoção completa da água de todas as estruturas celulares. Os tecidos deverão ser mantidos no mínimo por uma hora em cada solução de álcool. Ao término dessa etapa o álcool será ser substituído por um solvente miscível em parafina (xilol), processo conhecido como diafanização.

c) Diafanização

A diafanização, também conhecida como clarificação (pois torna os tecidos translúcidos), será realizada com um solvente miscível em parafina, o xilol. Os tecidos serão imersos nesse solvente e então todo o álcool utilizado na desidratação e presente no interior das células será automaticamente substituído pelo xilol. A troca de álcool por xilol será realizada também de maneira lenta e utilizando três trocas de xilol, para que a remoção de álcool seja completa.

d) Inclusão

Nessa etapa, o xilol utilizado na diafanização será substituído por um meio apropriado para cortes em micrótomo. A parafina histológica é o meio de inclusão mais barato e largamente utilizado em laboratórios clínicos e de pesquisa no mundo. Essa etapa compreende desde o processo de impregnação dos tecidos na parafina fundida até o momento de montagem dos blocos com as amostras. A impregnação dos tecidos animais durará três horas e deverá ser conduzida com a parafina fundida a temperatura média de 60ºC, pois temperaturas superiores a essas causam muitas alterações nas estruturas celulares.

e) Microtomia

A microtomia dos tecidos incluídos em parafina será realizada em micrótomo de rotação a temperatura ambiente. Os cortes não distenderão totalmente, caso a temperatura da água estiver muito baixa, e, se a temperatura estiver muito quente, a parafina derreterá,

dificultando a adesão e ajuste dos tecidos sobre as lâminas. A seleção da navalha mais adequada para cada tipo de tecido, assim como a ângulo de disposição da navalha também será realizada de acordo com o tecido a ser cortado.

f) Distensão

Após cortados, os selos de parafina contendo as amostras serão distendidos antes de serem aderidos às lâminas de microscopia. Esse processo será realizado colocando-se os cortes em água aquecida em temperatura média de 45ºC. Caso a temperatura da água esteja muito baixa os cortes não distenderão totalmente e se a temperatura estiver muito quente a parafina derreterá, dificultando a adesão e ajuste dos tecidos sobre as lâminas. Uma fina camada de albumina glicerinada será colocada sobre as lâminas para aumentar a adesão dos cortes às mesmas.

g) Coloração

Antes dos cortes serem corados, o excesso a parafina será removida dos tecidos utilizando-se xileno. A hidratação dos tecidos será iniciada logo após a remoção da parafina. Utilizar-se-á soluções graduais de álcool etílico (100ºGL até 70ºGL) seguido por água destilada. Já hidratados os tecidos serão corados, sendo que a coloração universal para amostra histológicas normais ou patológica é a Hematoxilia & Eosina, coloração por esse motivo selecionada para este trabalho. A Hematoxilina se comporta como uma solução básica, corando as estruturas ácidas das células, como o núcleo e o RNA do retículo endoplasmático granuloso e confere a cor azul/roxa a essas estruturas. Já a Eosina é um corante ácido e possui afinidade por estruturas básicas, como o citoplasma, conferindo a elas a coloração rosa. Após corados, os tecidos serão desidratados também utilizando álcool etílico, porém agora em concentrações crescente (50ºGL até 100ºGL). Depois de desidratados as lâminas serão submergidas em xileno, solvente miscível com a maioria dos meios de montagem.

h) Montagem

Depois de coradas as lâminas de microscopia serão montadas com lamínula, que será aderida à lâmina com uma resina específica para essa finalidade, sendo que neste trabalho será usado o Entellan (Merck). Essa resina de montagem propiciará um meio adequado ao corte histológico, fazendo-o se conservar por longos períodos, para posteriores análises.

i) Análise histológica

A análise das lâminas será realizada por um patologista e por um biólogo. Na análise serão consideradas lesões tissulares e celulares. Serão investigadas a presença/ausência das seguintes lesões: edema, hemorragia, inflamação, aberrações nucleares, atrofia, displasia, hipertrofia (especifica para fígado e baço) e espessura da camada granular (específica para encéfalo) (ABBAS; VINAY KUMAR; FAUSTO, 2010; FRANCO *et al*., 2010). A presença de outras lesões específicas também será registrada se forem detectadas.

4.6 Técnicas e instrumentos de coleta e análise de dados e procedimentos de intervenção do Experimento *in vitro*

4.6.1 Experimento *in vitro* (modelo de intervenção)

O ensaio de cultivo celular será realizado segundo metodologia usual para cultivo celular (UMEGAKI; FENECH, 2000), sendo mantidas em RPMI-1640, contendo 10% de soro bovino fetal. As culturas serão semeadas até atingir a confluência necessária. Em seguida, as células serão tratadas com doses crescentes de glicose ou frutose (3 concentrações cada), combinadas com 3 substâncias: ferro ou cromo ou vitamina C (3 concentrações cada). Na Tabela 3 estão apresentadas as concentrações a serem empregadas no estudo.

Tabela 3. Concentrações dos nutrientes testados nos experimentos *in vitro*. Valores baseados em níveis fisiológicos para humanos.

Nutriente	**Concentração (motivo)**		
	0	1	2
Glicose e frutose (mg/mL)	1 (euglicemia)	1,4 (glicemia levemente aumentada)	2,0 (glicemia acima do limite desejado para diabéticos)
Ferro (µg/mL)	0,6 (limite de corte para a anemia)	1,2 (concentração intermediária)	2,4 (limite para risco de hemocromatose)
Cromo (µg/mL)	0,01 (limite mínimo no soro)	0,015 (concentração intermediária)	0,03 (limite máximo no soro)
Vitamina C (µg/mL)	3 (limite mínimo no soro: baixa ingestão)	6 (concentração ideal no soro)	12 (limite máximo no soro)

Os tratamentos serão mantidos por 7 dias. O meio será trocado no 3º dia. A densidade celular será monitorada em câmara de Neubauer. Os experimentos serão conduzidos em triplicatas, sendo repetidos 3 vezes.

4.6.2 Técnicas e instrumentos de coleta do Experimento *in vitro*

Ao final dos tratamentos, serão realizados os ensaios cometa, o citoma de micronúcleos em células binucleadas, o DCFH e o TUNEL, técnicas já descritas anteriormente.

4.7 Processamento e análise de dados/Estatística

Os dados serão tabulados no programa Excel (Microsoft Office 2010) e analisados no software Statistical Package for Social Sciences (SPSS) versão 20.0. Para o desenho plotagem dos gráficos será utilizado o programa GraphPadPrism 5.01 (GraphPad Software, Inc; San Diego, CA). Os dados serão expressos como média e desvio padrão. Para comparação dos grupos antes e após a condução dos experimentos será realizado ANOVA de medidas repetidas. A comparação entre os grupos no momento pós-experimento será realizada por meio de uma ANOVA de uma via, seguida do teste post hoc apropriado para verificar as diferenças entre os grupos. A interação entre o efeito do tempo e o efeito dos nutrientes será analisada por meio de ANOVA mista. A análise de correlação de Pearson ou Spearman também será empregada para correlacionar o peso, IMC, concentrações elementares, valores de glicose jejum e insulina com os marcadores citotóxicos, genotóxicos e de estresse oxidativo. Todos os dados serão verificados quanto à normalidade e homocedasticidade. Nos casos em que os dados não apresentarem homocedasticidade e/ou normalidade, uma transformação logarítmica será empregada. Valores de probabilidade $<0,05$ serão empregados como nível de significância.

4.8 Considerações éticas

Conforme descrito anteriormente, o experimento será primeiramente submetido à aprovação do CEUA e as execuções dos ensaios atenderão a resolução do Conselho Nacional de Saúde e aos “Princípios éticos na experimentação animal do Colégio Brasileiro de Experimentação Animal”. Para o ensaio de cultura celular não será necessário à aprovação do Comitê de Ética.

5 CRONOGRAMA DE EXECUÇÃO

Ano	**2013**							**2014**												**2015**	
ETAPAS/MESES	**J**	**J**	**A**	**S**	**O**	**N**	**D**	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**	**D**	**J**	**F**
Encontro com o orientador	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Revisão bibliográfica sobre o tema proposto	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Submissão ao CEUA	**X**	**X**																			
Condução dos estudos *in vivo* (tratamento com sobrecarga de sacarose, cromo, ferro e vitamina C)			**X**	**X**	**X**	**X**	**X**														
Análise dos ensaios de citotoxicidade, genotoxicidade, apoptose e estresse oxidativo			**X**	**X**	**X**	**X**	**X**														
Digitação dos dados do experimento com animais em planilha Eletrônica			**X**	**X**	**X**	**X**	**X**														
Organização, análise e discussão preliminar dos dados coletados							**X**	**X**	**X**	**X**											
Condução dos experimentos *in vitro*								**X**	**X**	**X**											
Análise dos ensaios de citotoxicidade, genotoxicidade, apoptose e estresse oxidativo								**X**	**X**	**X**											
Digitação dos dados do experimento com cultura celular em planilha Eletrônica										**X**	**X**										
Organização, análise e discussão final dos dados coletados											**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**			
Redação dos artigos com experimentos de animais e com cultura celular											**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**			
Pré-defesa em banca dos resultados dos projetos																			**X**		
Defesa da banca dos resultados do projeto																					**X**
Encaminhamento dos artigos confeccionados referentes aos resultados do projeto																					**X**

6 RECURSOS HUMANOS e INFRAESTRUTURA

A execução dos experimentos do projeto será efetuada principalmente pela pesquisadora principal que já possui experiência na prática de estudos similares. O experimento com animais será realizado no biotério da UNISC com o auxilio dos professores orientadores e do mestrando da UFRGS. O ensaio de cultura celular será realizado no Laboratório de Nutrição Experimental, após a aquisição dos equipamentos necessários, sob a orientação do prof. Guido Lenz, coordenador do Laboratório de Sinalização Celular da UFRGS, experiente na técnica.

O teste cometa e micronúcleos serão realizados no laboratório de Nutrição Experimental (experimento com animais e de cultura celular), as técnicas histopalógicas serão realizadas no laboratório de histologia e patologia da UNISC, o PIXE no Laboratório de Implantação Iônica do Instituto de Física da UFRGS e as técnicas de TUNEL e DCFH no Laboratório de Nutrição Experimental, também sob orientação do prof. Guido Lenz. A análise da concentração de insulina será realizada num laboratório de análises clínicas.

Participantes do projeto de pesquisa

Membro	Afiliação	Função no projeto
Silvia Isabel Rech Franke	PPG Promoção da Saúde (Unisc)	Orientadora
Daniel Prá	PPG Promoção da Saúde (Unisc)	Co-Orientador
Patrícia Molz	PPG em Promoção da Saúde (Unisc)	Mestranda – bolsista Fapergs
Guido Lenz	Centro de Biotecnologia (UFRGS)	Colaborador
Deivis de Campos	Depto Biologia e Farmácia (Unisc)	Colaborador
João Antonio Pêgas Henriques	Centro de Biotecnologia (UFRGS)	Colaborador
Johnny Ferraz Dias	Instituto de Física (UFRGS)	Colaborador
Carla E. I. dos Santos	Instituto de Física (UFRGS)	Colaboradora
Paulo Jobin	Instituto de Física (UFRGS)	Colaborador
Joel Henrique Ellwanger	PPG em Biologia Celular e Molecular (UFRGS)	Mestrando

Laboratórios e período de utilização

LOCAL	Semestre 2013/2	Semestre 2014/1
Biotério da Unisc	X	
Laboratório de Nutrição Experimental da Unisc	X	X
Laboratório de Genética e Biotecnologia da Unisc	X	X
Laboratório de Implantação Iônica do Instituto de Física da UFRGS	X	X

7 ORÇAMENTO/RECURSOS MATERIAIS

CUSTEIO			
Itens a serem financiados (Especificações)	**Valor Unitário (R$)**	**Valor Total (R$)**	**Fonte viabilizadora**
Ratos *Wistar* (64 animais)	15,00	960,00	CNPq
Ração (230kg)	134,00	1340,00	CNPq
Maravalha estéril (50kg)	0,92	550,00	CNPq
Substâncias testes (Cr, Fe, Vitamina C, adoçantes)	–	2.500,00	CNPq
Material para o ensaio cometa (p.ex. agarose de baixo ponto de fusão, ácido tungstosilícico, nitrato de prata e reagentes de uso geral)	–	2.751,00	CNPq
Material para o ensaio de micronúcleos de eritrócitos	–	3.000,00	CNPq
Material para o teste de citoma de micronúcleos em células binucleadas (soro bovino fetal, rpmi, fitohemaglutinina e citocalasina B, KCl, formol, metanol, ácido acético.	–	5.000,00	CNPq
Material para cultivo celular (meios de cultura, frascos e placas para cultura)	–	2.000,00	CNPq
Materiais para dissecação do hipocampo (tesoura e pinça cirúrgica, solução tampão PBS (100 mM de Tris; 0.9% de NaCl em água; pH 7.5), gelo seco)	–	50,00	Pesquisador
Material para confecção de lâminas histo-patológicas (lâmina, lamínula, álcool, xilol, parafina histológica, hematoxilina de harris, eosina, entellan, navalha para micrótomo e formalina)	10,50	672,00	CNPq
Material para a técnica TUNEL	–	3.000,00	CNPq
Material para a técnica de Apoptose	–	1.500,00	CNPq
Kit para determinação de insulina *Sensitive Rat Insulin RIA Kit* (Linco Research, Inc.)	–	1.200,00	CNPq
Fitas para determinação de glicemia	–	800,00	CNPq
Fita métrica	5,00	5,00	Pesquisador
Material de escritório (folha para impressão, CDs, pendrives)	2,00	200,00	Pesquisador
TOTAL GERAL		**24.028,00**	

O projeto será custeado:

- Verba do edital CHAMADA UNIVERSAL MCTI/CNPq Nº 14/2013 FAIXA A (Professora Silvia Franke 2013 e 2015 R$ **23.122,00**). Destinados ao projeto R$ 23.122,00aos experimentos descritos

Órgão financiador: edital Chamada Universal - MCTI/CNPq Nº 14/2013 (Auxílio a Doutora Silvia Franke: Nº Processo: 479554/2013-7 Título do projeto “Influência *in vivo* e *in vitro* de nutrientes sobre a citotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar”.

8 RESULTADOS E IMPACTOS ESPERADOS

Com o presente estudo pretende-se aumentar a compreensão dos mecanismos fisiopatológico desencadeador da origem e progressão do diabetes associados à hiperglicemia através: i) da avaliação detalhada da citotoxicidade, genotoxicidade e potencial de induzir acúmulo de radicais livres pela sobrecarga de glicose e frutose, em células gliais e em diferentes tecidos de ratos; ii) da obtenção de dados novos sobre o papel do cromo na modulação do dano causado pela hiperglicemia, possivelmente ajudando a refutar as fracas evidências científicas que suportam o papel benéfico do nutriente no diabetes como protetor contra o diabetes e potencializador da ação da insulina; iii) do entendimento de como a vitamina C age frente à sobrecarga dos monossacarídeos; iv) de alguns entendimentos que possibilitem avaliar o mecanismo molecular associado à potencialização do risco de diabetes pela sobrecarga de ferro, embora as evidências epidemiológicas estejam aumentando nos últimos anos.

Nesse sentido, o conhecimento do impacto das concentrações fisiológicas dos nutrientes, avaliados pela sobrecarga de glicose e frutose, permitirá que os profissionais de saúde que atuam diretamente e indiretamente na nutrição possam desenvolver estratégias para o manejo clínico-dietético do diabetes. Sejam elas, aumentando a ingestão de nutrientes de forma protetora ou reduzindo a ingestão de nutrientes que possam possivelmente apresentar impacto negativo na doença.

Os experimentos *in vitro* e *in vivo* permitirão entender mais detalhadamente estes mecanismos, de uma forma que com seres humanos seriam inviáveis. Assim, este estudo abordando a nutrigenômica, espera avaliar a instabilidade genômica e promover a estabilidade genômica, relacionando com as concentrações sanguíneas destes nutrientes e a hiperglicemia, afim de facilitar o planejamento e a condução de intervenções futuras.

9 RISCOS/ DIFICULDADES/LIMITAÇÕES

Como esse projeto não prevê o envolvimento de seres humanos nas amostras/grupos da pesquisa, não existem riscos para esse grupo. Os riscos potenciais que existem sobre os animais que serão utilizados no modelo experimental são os inerentes a incorreta administração dos nutrientes e manejo dos animais, que poderiam causar desconforto aos mesmos ou até mesmo a morte, o que dificultaria o poder da análise estatística. Todos esses são riscos inevitáveis, entretanto, eles serão diminuídos pelo fato de que os participantes do projeto possuem experiência com o manejo de animais, administração de nutrientes e no manejo de modelos de doenças metabólicas. Ainda, todos os procedimentos serão rigorosamente estudados e planejados antes de serem executados. Porém, uma dificuldade na execução deste experimento em particular é referente a problemas burocráticos relacionados a infraestrutura do biotério que não poderão ser sanados por nós, os pesquisadores do projeto.

Os experimentos com culturas celulares também poderão apresentar risco pela baixa viabilidade celular provocando a diminuição do poder estatístico, devido à incorreta suplementação dos nutrientes. Contudo, como os participantes do experimento *in vitro* serão guiados por um professor capacitado e com grande experiência na técnica, acreditamos queos riscos e dificuldades na execução do experimento serão mínimos. Ainda, os participantes deste específico experimento já estão sendo capacitados para evitar quais dificuldades durante a execução.

Quanto aos riscos existentes para os pesquisadores durante a realização das atividades experimentais são pequenos, levando em consideração que os protocolos de boas práticas laboratoriais dos locais onde os testes serão realizados são bastante rígidos, assegurando a proteção dos indivíduos durante a pesquisa. A experiência e histórico dos pesquisadores desse projeto com pesquisas envolvendo animais também será um fator importante na minimização dos riscos inerentes a execução do projeto.

A escolha da realização de um trabalho experimental foi selecionado devido a utilização de superdose de nutrientes que podem provocar toxicidade. Outra limitação deste projeto é a utilização de animais ao invés de seres humanos. Contudo, os trabalhos com animais permitem um controle do tratamento realizado que não é possível com humanos, devido à falta de comprometimento dos pacientes. Assim, de um uma forma mais segura, este estudo conseguirá mimetizar os efeitos nos seres humanos.

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CAPÍTULO II
RELATÓRIO DO TRABALHO DE CAMPO

RELATO DE CAMPO

Este foi um estudo experimental, dividido em duas partes, no qual, primeiro realizou-se um estudo *in vivo* com animais de experimentação. A amostra envolveu 64 ratos *Wistar* machos, que foram divididos aleatoriamente em 8 grupos, a fim de atender as recomendações da Organização para a Cooperação e Desenvolvimento Econômico (OECD) e obter-se melhor poder estatístico da amostra.

Primeiramente o projeto foi submetido à Comissão de Ética no Uso de Animais (CEUA) da UNISC, aprovado pelo Protocolo 14/2013 e depois ao Edital Universal - MCTI/CNPq Nº 14/2013, a fim de arrecadar recursos financeiros para a realização da pesquisa, no qual foi contemplado. A pesquisa iniciou-se ainda em 2013, quando os ratos para uso de experimentação foram adquiridos do Biotério Central da Universidade Federal de Santa Maria (UFSM), sendo estes transportados, segundo as normas do biotério da UNISC, sob supervisão da veterinária responsável pelo biotério. Após a chegada dos animais no biotério da Unisc, eles foram acomodados aleatoriamente em caixas individuais e identificados e ambientados por uma semana.

O experimento somente iniciou-se após o período de ambientação dos ratos e da permissão da coordenação do Biotério, administrados pelos cursos de Educação Física e de Medicina da UNISC. Ao todo, a execução do experimento durou 18 semanas, no qual, semanalmente e preferencialmente nas segundas-feiras os animais eram pesados e medidos, para avaliação antropométrica. A alimentação dos ratos também era semanalmente pesada, no caso da ração e, medida, no caso dos líquidos, para que fosse possível contabilizar o consumo efetivo. Durante o dia da pesagem e medição, também eram preparados as soluções líquidas contendo a) açúcar invertido, b) vitamina C, c) vitamina C e açúcar invertido, d) cloreto de cromo, e) cloreto de cromo e açúcar invertido, f) sulfato ferroso e g) sulfato ferroso e açúcar invertido, e então distribuídos nas mamadeiras, conforme o grupo específico de tratamento. A cada 15 dias, os animais eram submetidos a jejum de 6h para a verificação da glicemia e concomitantemente era coletado sangue sob pouca luz para a realização do Ensaio cometa. Ainda durante o experimento, foi autorizada, pela coordenação do Biotério, a realização da manutenção dos animais durante os finais de semana e feriados, desde que fosse a mestranda acompanhada pela professora orientadora.

Depois de 9 semanas de nossa experimentação animal, ocorreu a invasão ao Instituto Royal, em São Roque (SP), no qual ativistas resgataram os animais do Instituto para fins de experimentação científica e, a partir de então, ficamos apreensivos quanto a possíveis invasões no biotério da Unisc. Contudo, terminamos o experimento sem presenciar invasões.

Depois das 18 semanas de experimentação, os animais foram sacrificados. Para a realização dos experimentos deste último dia, auxiliaram nos procedimentos bolsistas do Laboratório de Histopatologia, acadêmicos da biologia e bolsistas do Laboratório de Nutrição Experimental da Unisc. Os animais novamente foram submetidos a jejum de 6h para as realizações dos procedimentos. Durante o sacrifício, foi coletado sangue para o Ensaio Cometa, PIXE, FT-IR e para a determinação de insulina e estresse oxidativo. O encéfalo foi coletado e pesado, em seguida parte do hipocampo foi retirado para a realização do Ensaio Cometa e PIXE. Também foram pesados e coletados o fígado, o baço e o pâncreas sendo os mesmos acondicionados em formol tamponado a 10% para a realização de lâminas histológicas e uma parte de cada um desses órgãos foi separada e reservada em outro frasco para a determinação do PIXE. A gordura peritoneal dos animais foi apenas pesada, sem ser armazenada. Parte do pâncreas coletado foi utilizada para a realização do Ensaio Cometa. Uma técnica do Genotox Royal participou do sacrifício, para a realização do Micronúcleos de medula óssea.

Depois da finalização do experimento, executaram-se as metodologias propostas para obtenção dos resultados: as lâminas do Ensaio cometa foram confeccionadas logo depois do experimento, assim como as lâminas de micronúcleos de medula óssea. Ambas as leituras das lâminas foram analisadas por pessoas capacitadas. As amostras de sangue e de tecidos separadas para a determinação de minerais pela técnica de PIXE foram liofilizadas e transportadas para o Laboratório de Implantação Iônica do Instituto de Física da UFRGS, onde foram pastilhadas e estão sendo analisadas. As amostras de sangue coletadas para determinação metabolômica pelo FT-IR, foram liofilizadas e estão sendo analisadas. Os órgãos coletados para a confecção de lâminas histológicas foram confeccionados pelo Laboratório de Histopatologia da UNISC e foram analisadas por um patologista experiente na área. Amostras de sangue coletadas para determinação de insulina foram processadas por um Laboratório de Análises Clinicas de Santa Cruz do Sul, contudo como tentou-se reproduzir a técnica de HSU e colaboradores (2013), no qual determinou-se a insulina de ratos com o kit de imunoensaio comercial de detecção de insulina do Laboratório Abbott. Entretanto, a determinação não foi possível, pois não houve sensibilidade do equipamento para a determinação de insulina nas amostras. Outra amostra sanguínea de soro foi enviada ao Laboratório de Estresse Oxidativo e Antioxidantes da UCS para fins de avaliação de Proteínas Carboniladas. Juntamente com esta amostra de soro, também foi enviada uma amostra de cada solução oferecida aos ratos para a realização do teste Capacidade de Varredura do DPPH.

A determinação de Proteínas Carboniladas, realizada em parceria com o Laboratório de Estresse Oxidativo e Antioxidantes da UCS, aconteceu devido a não afirmação da parceria com o Laboratório de Sinalização Celular da UFRGS. Na UFRGS seriam realizadas as técnicas de DCFH por citrometria de fluxo, a fim de avaliar estresse oxidativo e de TUNEL para avaliar apoptose via cerebral.

A segunda parte do trabalho constituiria do experimento *in vitro* a ser realizado em cultura celular. Células de gliomas seriam tratadas com doses crescentes de glicose ou frutose (3 concentrações cada), combinadas com 3 substâncias: ferro ou cromo ou vitamina C (3 concentrações cada). Para tal, necessitar-se-ia para a execução do experimento uma sala de cultivo celular equipada com os equipamentos necessários. Primeiramente a estufa de CO2 foi contemplada no Edital Pró Equipamentos Edital 2012, porém a divulgação da liberação do recurso ocorreu em Outubro de 2013 e a estufa foi adquirida em 2014, depois de um demorado processo de compras através do SICONV. Uma adequação do Laboratório de Nutrição Experimental necessitaria ser realizada para que a estufa de CO_2 pudesse ser instalada com segurança. Contudo, devido a entraves burocráticos de centros financeiros da instituição, até o momento a estufa não foi instalada. Desta forma, por este motivo, não se realizou até então esta parte do experimento.

CAPÍTULO III
ARTIGO I

A simple rat model of prediabetes induced by invert sugar overdose in drinking water

Running title: Rat prediabetes model by invert sugar overdose

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ABSTRACT

Prediabetes is associated with intermediate hyperglycemia and impaired glucose tolerance, often concomitantly with obesity. So far, there is no animal model mimicking prediabetes in humans. In this study we aimed to validate a simple and affordable method to induce prediabetes in male *Wistar* rats. After 17 weeks with 32% invert sugar in drinking water, the rats became obese and had a significant increase in abdominal fat, possible caused by increased caloric intake. Moreover, the rats presented impaired glucose tolerance as evaluated by the Intraperitoneal Glucose Tolerance Testing. Primary DNA damage as evaluated by the comet assay was increased in blood but not in pancreas. No protein carbonylation was seen in serum. Moreover, no increase in permanent DNA damage was seen in bone marrow as evaluated by the micronucleus test. The histological evaluation indicated that some rats had liver steatosis and that the pancreatic islets were enlarged but not statically significantly. Therefore, the model of prediabetes induced by invert sugar overdose was effective in simulating the metabolic characteristics of prediabetes in humans that typically involve alterations in glucose metabolism without significantly affecting the functionality of the pancreas or inducing permanent DNA damage. Given the model´s simplicity and affordability, it can be used to evaluate strategies to prevent diabetes.

Keywords: prediabetes, invert sugar, obesity, glucose tolerance.

1 Introduction:

Diabetes Mellitus (DM) is a metabolic disorder resulting from defects in insulin secretion, insulin action, or both [1]. The number of people with DM increased in the last years worldwide. Currently, there are 378 million people living with DM [2], and among them 90 to 95% have Type 2 Diabetes Mellitus (T2DM) [1]. Epidemiological studies indicate that overweight, unhealthy diet, physical inactivity, age, and impaired glucose tolerance are important risk factor for T2DM [3, 4]. The first stage to the development of T2DM is Prediabetes (intermediate hyperglycemia: glycemia between 100-125 mg/dL, or glucose tolerance test between 140-199 mg/dL after 2h of 75 g oral glucose intake), being sufficient to cause functional pathologic alterations for a long time before the diagnosis of T2DM can be established [5].

The use of animal models has provided an opportunity to study various types of disease and metabolic dysfunctions. Although in literature there are many types of animal models of DM, mostly these studies do not appear to mimic the real clinical situation of the DM in humans [6, 7]. To date, there is no specific animal model of prediabetes. A prediabetes animal model can be very useful for evaluating strategies to prevent T2DM.

In this context, the use of genetically modified animals as well as being expensive is not easily available [7]. Genetic modified animals usage may not always be satisfactory, as well as DM-inducing drugs such as streptozotocin and alloxan induce Type 1 Diabetes Mellitus (T1DM), causing the destruction of pancreatic cells [6], but this type of DM affects only 5-10% of the population [1]. In contrast, the induction of T2DM is more connected to changes in lifestyle, with emphasis in dietary factors [8].

T2DM models induced by high-energy intake (high carbohydrates and/or fatty), or cafeteria-like diets might show more similarity with the dietary habits taken by individuals with high risk for T2DM [3, 8]. Sugar overload in animals can induce metabolic syndrome, increase plasma concentrations of insulin, leptin, triglycerides, glucose and free fatty acids, and lead to glucose intolerance [9].

Sugar overload in human diet is typically associated to sweetened foods and beverages. Sweeteners have been used in food and drinks industries as the main ingredient in forms of “free sugars” (high sugars: glucose, fructose, and sucrose, sugar sweetened beverages [SSB], sugar sweetened soft drinks [SSSD], and high fructose corn syrup [HFCS]) [10]. One of the most commonly used sweeteners is the invert sugar, a syrup made on of glucose, fructose and residual sucrose obtained by the hydrolysis reaction or inversion of sucrose (between 10 to 100% of inversion) that while in chemical equilibrium has an equal proportion of glucose,

fructose and sucrose (33% each). With 20% sweetening power in comparison to pure sucrose, invert sugar is used in many types of foods, is normally more hygroscopic, and does not crystallize easily [12, 13]. Fructose has had a lower influence on insulin concentrations than glucose, and no influence on plasma glucose levels [11]. Excessive blood glucose (hyperglycemia) has been shown to lead to diabetes-associated complications such as oxidative damage [5, 12].

Therefore, the objective of this study was to validate a simple method to induce prediabetes in male *Wistar* rats by overdose intake of invert sugar, evaluating obesity and oxidative damage.

2 Methods:

This study was approved by the Animal Ethics Committee of the University of Santa Cruz do Sul (Protocol nº 14/2013), and all procedures adopted followed guidelines according to the Brazilian regulations for animal studies (Law No. 11794/1999).

2.1 Animals and Experimental design

Sixteen male *Wistar* rats (100 days of age), weighting approximately 300 g, kept in individual cages, under a controlled climate (light/dark cycle of 12 h, 22 ± 3 C and 60% of humidity), receiving water *ad libitum* and normoprotein-caloric Nuvilab® were used in the study. Before the beginning of the treatment, rats were acclimatized during seven days. Then, the rats were randomly assigned into 2 groups and treated for 18 weeks, as 1) control group (n=8): no experimental intervention (received chow and water *ad libitum*), and 2) Invert sugar group (n=8): experimental intervention (chow and water *ad libitum*, inverted sugar (320 g/L) added to the drinking water). The intake of the chow and water were measurement daily. At the end of the experiment the rats were sacrificed by decapitation, using a guillotine specific for rodents.

2.2 Obesity evaluation: Body Mass Index (BMI) and peritoneal fat weighing

Rats of both groups were weighted and measured (nose-anus length) weekly. The BMI specific for rats was obtained and interpreted according Novelli and collaborators [13] and Obesity was classified when the BMI was higher than 0.68 g/cm^2. For weighing of peritoneal fat, after sacrifice the peritoneal fat was dissected and weighed. Peritoneal fat excision was realized according to the descriptions of Cinti [14].

2.3 Disglycemia evaluation: Fasting blood glucose and Intraperitoneal Glucose Tolerance Testing (ipGTT)

To evaluate the hyperglycemic state, glucose was measured biweekly, after fasting of 6 hours with a glucometer-ACCU-CHEK Active Meter® (Roche Diagnostics GmbH, Mannheim, Germany). For the ipGTT [15], the first blood sample (time 0 - baseline) was measured as obtained from a small cut in the tails of the rats. Then, 1 mg/kg b.w. glucose (Equiplex®) was administered and the blood glucose was measured at 5, 15, 30, 60 and 120 minutes afterwards. The area under the curve (AUC) was calculated based in the results of the ipGTT test of each rat.

2.4 Histopathological analysis

The liver and pancreas samples were fixed in 10% formalin solution, dehydrated with ethanol, diaphanized with xylene, and embedded in paraffin. Paraffin sections were sectioned at 7µm of thickness using a microtome (Leica, Nussloch, Germany) and mounted on microscope slides. These histological cuts were deparaffinized with xylene, rehydrated and stained with Hematoxylin & Eosin (HE). Then, the slides were dehydrated with ethanol, cleared with xylene, and covered with Entellan® and coverslips.

In liver histopathological analysis was performed for determination of hepatic steatosis. Additionally, Ishak hepatic activity index was used to evaluate: i) Periportal or periseptal interface hepatitis (piecemeal); ii) Confluent necrosis; iii) Focal (spotty) lytic necrosis, apoptosis and focal inflammation; iv) Portal inflammation. In pancreas the following analysis was performed: 1 – quantification of the pancreatic islets (one area of interest: 32X magnification); 2 - quantification of β cells (one area of interest: 32X magnification); 3 - investigation of the occurrence of hyperplasia, inflammation, fibrosis, and necrosis of the β cells.

2.5 The Comet Assay (blood and pancreas samples)

For the Comet Assay, blood samples were collected from the animals tails (except at the end of treatment when blood was collected from jugular vein) and mixed with heparin. A small piece of the pancreas was dissected at the end of the experiment and was placed in phosphate buffered saline (PBS) with dimethyl sulfoxide (DMSO), and then dissociated with the aid of forceps to obtain a cell suspension.

The Comet Assay of blood and pancreas cells was performed according to Franke et al. [16]. Five microliters of blood embedded in 95.00 µL of low melting point agarose (LMP) (0.75%) or 20.00 µL of pancreas cell suspension added to 80.00 µL of LMP were placed over a slide pre-coated with agarose and a coverslip was gently placed over the slide. After the mixture solidified, the coverslips were removed and the slides were put in freshly prepared lysis solution containing high salt and detergent concentrations (2.50 M NaCl, 100.00 mM EDTA, 10.00 mMTris, pH 10.20, with freshly added 1% Triton X-100 and 10% DMSO) for a minimum of 1 h under refrigeration, order to lyse the cells, removing the nuclear membrane and the cytoplasmic contents.

Subsequently, the slides were exposed to an alkaline solution (300.00 mM NaOH, 1.00 mM EDTA, pH>13) for DNA unwinding and to express the alkali-labile sites as single strand breaks. The slides were then immediately submitted to an electrical current (electrophoresis in

the same solutions at 300mA and 25 V (0.90 V/cm) for 15 min at 4 ºC to induce the migration of DNA fragments in the direction of the current. After, the slides were washed with neutralization buffer (Tris 0.40M, pH 7.5), fixed and silver-stained. All procedures were conducted under dim yellow light to prevent DNA damage induced by ultraviolet radiation.

For each rat, two slides (either for blood or pancreas) were prepared and 100 cells were randomly selected and analyzed (50 per slide, 2 slides per animal) under a conventional optical microscope with 200X magnification. The DNA migration damage was classified into five classes, from class 0 (no DNA migration) to class 4 (maximal migration) according to tail size and intensity. The damage index (DI) was obtained by the sum of the individual cells sorted, ranging from 0 (no harm: cells 100 x 0) to 400 (maximum damage: 100 x 4 cells). The damage frequency (FD), in percentage terms (%) was calculated by the ratio of the number of damaged cells among the 100 cells analyzed. Cells with non-detectable nuclei (head and tail clearly separated) were not evaluated.

2.6 Protein carbonylation

Protein oxidative damage was measured by the determination of the carbonyl group in a reaction with dinitrophenylhydrazine. The results were expressed as nmol of DNPH/mg of protein. Total Proteins kit from Labtest (Protein Kit, LabtestDiagnostica S.A., Brazil) was used for the determination of total protein content [17].

2.7 Micronucleus bone marrow test

Bone marrow sample were collected from both femurs according to Picada and collaborators [18]. After extraction of bone marrow, the samples were mixed with fetal calf serum (FCS) obtaining a cell suspension. Subsequently, the smears were prepared and the cells were stained with 5% Giemsa. The polychromatic erythrocyte (PCE)/normochromatic erythrocyte ratio was scored in 1,000 cells. Micronuclei were evaluated as per 1000 PCE [19].

2.8. Data analysis

Data was analyzed with the aid of GraphPad Prism 5.01 software (GraphPad Software, Inc.; San Diego, CA). All results were checked for normality and homoscedasticity. The Student's t and Mann Whitney test was employed for comparisons between groups. The significance level was set at $p<0.05$.

3 Results

The intake of invert sugar in concentration of 320 g/L by 17 weeks increased in about 30% the caloric intake of the invert sugar group (p<0.001). The invert sugar group drank more liquids (p=0.004), but ate less chow (p<0.0001) (Table 1).

This excessive caloric intake influenced the weight gain of the rats that drank inverted sugar. The rats had a weight gain of 32% compared to the animals of the control group (p=0.01), meaning an average weight gain of 187.29 g b.w. throughout the study or 1.49 g b.w./day. The gain of abdominal fat was 162% higher in the invert sugar group than in the control group (p<0.0001) (Table 1).

The invert sugar group had higher BMI than the control group throughout the experiment. A suddenly sharp increase in BMI was observed around the 12th week of treatment. At the end of the experiment, the rats of the invert sugar group were classified as obese (0.69±0.03 g/cm^2 *vs* 0.62±0.04 g/cm^2; p=0.01) (Figure 1).

During the experiment, the animals of both groups presented a slight increase in glycemia and at the end of the experiment its levels were similar in both groups (invert sugar group = 123.38±7.74 mg/dL vs control group = 129.75 ±11.25 mg/dL; p=0.21). The results of the ipGTT (Figure 2) indicated that the invert sugar group presented AUC about 41% higher compared with the control group (p=0.01). The invert sugar group showed higher glycemia at 5 (p<0.01), 15 (p=0.03), 30 (p<0.01), and 120 minutes (p=0.04) after the glucose injection in relation to control group. Moreover, the glycemia did not return to the initial values after 120 minutes in invert sugar group, possibly due to insulin resistance.

Concerning histology, 25% of rats of invert sugar intake and no rat of the control group presented hepatic steatosis. The Ishak hepatic activity index does not indicated significant results regarding the lesions evaluated (p>0.05). In relation to pancreas, it was observed a similar number of β cells between the groups and a slight increase β cells hyperplasia in the invert sugar group, although not significantly (p=0.47 and p=0.45, respectively). Other types of injury evaluated in pancreas (inflammation, fibrosis, and necrosis) were not found (Figure 3).

In relation to Comet Assay, it was observed a higher level of DNA damage in the blood (Figure 4a, b) of the invert sugar group in relation to the group control either at the 12th week (p<0.05) or at the end of the treatment (p>0.01) In pancreas, the DNA damage level evaluated at the end of treatment (18ª week) was lower in invert sugar group (although not significantly; p>0.05) (Figure 4c, d).

Regarding protein oxidative damage by carbonylation, there was no difference between the groups (control group = 0.45 ± 0.14; invert sugar = 0.44 ± 0.28; p=0.95). In micronucleus test, groups also did not differ significantly (control group = 4.81 ± 2.63 vs invert sugar = 4.42 ± 0.38; p=0.36).

4 Discussion

The result of the present study indicate the possibility of the establishment of the first prediabetes model in rats by sugar intake overload, similarly to the dietary pattern associated to the establishment of prediabetes in humans. We used rats with older age in relation to most studies. While most studies generally start the treatment with 21 days old rats we started with rats with 100 days of age (3.1 month which would be equivalent to 9 years in humans). This age was chosen based in the increase of diabetes incidence in childhood [20]. The duration of our treatment was longer than most studies with a time span of 4 moths or 12 human-equivalent years (rats with 7.3 month which would correspond to 21 years in humans). There is a consensus that diabetes is a slow progressing condition and cover a larger time span could be helpful to understand diabetes progression [20]. The observed turning point of the metabolic alterations after 12 weeks (equivalent to 7 years of glucose overload in humans brings new evidence in this direction of the diabetes establishment versus lifespan. Indeed, we consider that our method is an excellent model of prediabetes, because is an inexpensive model, can be induced in relatively short time and is potentially easily reproducible.

Our experimental model reproduced the typical metabolic alterations observed in prediabetes, such as obesity and impaired glucose tolerance [21]. This model is likely to be superior than other available in literature as most animal models already available are not faithful to reproduce many characteristics of prediabetes in humans (Table 2) [5, 21-24].

We used an overload of invert sugar because it is of easy acquisition and an inexpensive sugar that has been used by food industries as the main ingredient in forms sugars, such as in beverages [9]. Moreover, diet is one of the main risk factors for the development of prediabetes in humans [3, 8]. Sugar-sweetened beverages have been shown to induce similar dietary alterations to those induced by invert sugar in our study [10, 25, 26], as sugar when consumed as liquid stimulates more energy intake than from solid foods [27] due to low satiety and incomplete compensation of liquid calories [25, 28-31] as well as stimulates appetite [32].

Moreover, sweetened-beverages can induce large increases in visceral fat [10, 29]. The same concentration of invert sugar (32% or 4.40 kcal/mL) as used in our study was previously shown to induce obesity and adiposity in rats treated for 10 weeks [33]. Obesity and excess body fat are characteristics of prediabetes [21, 23, 24].

Fructose seems to be the more obesogenic than glucose [34], possibly by promoting the lipogenesis as well as leading to triglycerides increasing [35, 36]. In agreement, we observed fat accumulation in central adiposity in the rats treated with sucrose/fructose by invert sugar

(162% higher than control group). Such results are similar to a study that reported 95% in fat accumulation in mice having free access to fructose-sweetened in relation to the control group as well as that fructose (15% solution in water) induced more adiposity than sucrose (10% solution in water) during approximately 10 weeks [37].

Invert sugar treatment did not induce T2DM, as the glycemia did not exceed the DM2 threshold for rats [150 mg/dL, as determined by Animal Models of Diabetic Complications Consortium [15]]. Invert sugar in concentration of 32% lead to only small increase in blood glucose (14.71 mg/dL, equivalent to 0.82 mmol/L) after the 18 weeks of treatment. Powel and collaborators [38], similarly to our study, did not observe changes in fasting glucose of rats with 68% sucrose in chow for 51 weeks. However, Wilson and Hughes [39] treated rats for 1 week with 35% sucrose in drinking water and observed a significant increase in glycemia of approximately 1.5 mmol/L.

According to Wilson and Hughes [39], an increase of 1 mmol/L in serum glucose is estimated to prejudice insulin secretion. We observed glucose tolerance impairment as shown by the ipGTT test. The AUC increased in our study is in agreement with the study of Sheludiakova, Rooney and Boakes [26] that evaluated rats treated with 10% sucrose and fructose and glucose in water drinking and verified AUC higher compared with control group. The level of glycemia was about 19% higher in the invert sugar group at 120 minutes, suggesting that the rats presented impaired glucose tolerance and possibly insulin resistance typical of prediabetes.

The impaired glucose tolerance has been associated with metabolic disorders caused by excessive consumption of refined carbohydrates, mainly containing fructose [36]. When fructose and glucose are intake together, as in inverted sugar, the absorption of fructose is increased in about 29% [40]. We choose a prediabetes-inducing model to mimic excessive intake of sweetened beverage and, therefore, simulate prediabetes, similar to that of humans [41]. Invert sugar, such as the sweetened beverages, could contribute to insulin resistance, β cell dysfunction in pancreas and inflammation [42].

In the present study, 25% of the animals that had received invert sugar presented hepatic steatosis. Probably it was the installation of insulin resistance could intensify the accumulation of peritoneal and ectopic fat, which could be directly associated with fructose intake and stimulation of hepatic lipogenesis [29, 43]. Still, there was also a decrease in the number of pancreatic islet and an increase in hyperplasia of β cells, although not significantly, indicative of insulin resistance caused by hyperinsulinemia [44, 45].

Another factor that may play an important role in the development of diabetes and its complications is the oxidative stress [46]. Oxidative stress is defined as a state of imbalance between antioxidant and pro-oxidants, in favor of the later [47]. The obesity is a state of chronic oxidative stress [48], and the obesity is strongly associated with oxidative stress, similarly to occurred in prediabetes stage [49]. We did not observe an increase in oxidative stress in serum according to the Protein carbonylation method. Differently of our study, evidences suggested that obesity, insulin resistance and development of T2DM (prediabetes stage), caused oxidative stress related to pathologies with significant involvement of protein carbonyls [50]. As in our study, the animals were prediabetics and beginning the process of insulin resistance (visualized by impaired glucose tolerance). The slight increase in carbonylation of the protein can be due to the increase of obesity since both BMI, as abdominal adiposity were increased.

In our study, the DNA damage evaluated by Comet Assay in blood of animals of the invert sugar group was higher at the end of the experiment, and mainly in the 12^{th} week. At the same time (12th week), a significant increase in BMI was observed, but not in blood glucose. Leffa and collaborator [51] in their study evaluating Cafeteria diet by 4 weeks also verified an increased damage index than group control. The authors still suggested that the genotoxic results found were correlated with the increased levels of oxidative stress due to obesity that could be induced an inflammatory response due to depletion of antioxidants, which may be reflected in increased damage DNA.

DNA damage evaluated by the Comet Assay in pancreas did no differ significantly between groups in the present study. Evidence suggests that oxidative DNA damage appear to play a role in pancreatic dysfunction [52]. Unlike our study, two other studies evaluating the DNA damage in the pancreas by the Comet Assay found increased damage index in the pancreas of diabetic rats compared to controls [53, 54]. However, it is important to highlight that these both studies used drugs to induce DM, and that promote the destruction of β cells in the pancreas, leading the DM1, and also induce extensive DNA damage to several organs [55]. However, the reason why there was no significant increase in DNA damage in pancreas in our study remains to be investigated. A possible hypothesis for this found is the own state of prediabetes, still not causing damage in cellular level in pancreas.

In micronucleus test also there was not verified difference in DNA damage between the groups. Despite evidence suggesting that the level of damage increases markedly the loss of glycemic control (confirmed in our study by increased DNA damage in the blood; Figure 3a), cell damage occurs in the early stages of the development of T2DM (prediabetes stage), but

may be masked to some extent by intrinsic cytotoxicity hyperglycemia. Though, the nature of the chromosomal DNA damage in DM is not completely understood. Also evaluating the frequency of bone marrow micronucleus in rats receiving the cafeteria diet, Leffa and colleagues [51] found increased the frequency this type of damage, possible induced by stress of obesity. In our study it is not clear why the frequency of micronuclei was not associated with disglycemia. One possible justification is that the metabolic alterations of prediabetes are not intense enough to induce the formation of micronuclei, which is likely to occur only when T2DM is already installed [5].

5 Conclusion:

The model of prediabetes induced by invert sugar overdose in drinking water in rats simulated the metabolic characteristics of previous stages to diabetes development. We treated rats with invert sugar (mixture of glucose and fructose), substance widely used in sweetened beverages. The treatment of DM2 induction was associated with obesity and increased visceral fat, and the disglycemia caused glucose tolerance by AUC in ipGTT, step to the development of insulin resistance. However, our model was effective in inducing prediabetes, without damaging substantially the pancreas of the animals. Despite an increase in whole blood primary DNA damage, no increase was observed in protein carbonylation or bone marrow micronucleus. Indeed, no substantial increase in genomic stability was observed, in agreement with several studies with prediabetes or metabolic controlled T2DM human studies. The model of prediabetes induction utilized in this study can be easily reproducible and contribute to future strategies for intervention in prediabetes.

Conflicts of interest

The authors declare they have no conflicts of interest.

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FIGURE LEGENDS

Table 1. Weight gain and food intake of control group versus invert sugar group. p: significance level; according to Pearson's Test.

Table 2. Summary of general characteristics prediabetes in human compared to ours rats prediabetes models.

Figure 1. Evolution of Body Mass Index (BMI) of control and invert sugar groups. r: correlation coefficient and p: significance level; according to Pearson's Test. The dashed line indicates the cutoff for classification of obesity in the rat BMI, according to Novelli et al.[13].

Figure 2. Blood glucose curves during 120 minutes Intraperitoneal Glucose Tolerance Testing (ipGTT) (a) and respective area under the 120 minutes curve (AUC) (b) for control group (n=8) and invert group (n=8). Points depict mean±SD. *$p<0.05$, **$p<0.01$ control group versus invert sugar group.

Figure 3. Photomicrographs of liver (a) and pancreas (b, c). Percentage distribution of hepatic steatosis (d), number of β cells (e) and β cells hyperplasia (f) p: level of significance according to the Mann Whitney test. *Statistical difference between groups in the same week ($p<0.05$).

Figure 4. Evolution of the DNA damage index in blood cells according to the damage index in 0, 12th and 18th weeks (a). Level of DNA damage index in the blood pancreas (b), Micronucleus bone marrow test (c) and Protein carbonylation d) of rats invert sugar intake for 18 weeks. p: level of significance according to the Student-t test. *Statistical difference between groups in the same week ($p<0.05$).

Table 1. Weight gain and food intake of control group versus invert sugar group. *p*: significance level; according to Pearson's Test.

Parameters	Groups		*p*
	Control	Sugar Invert	
Number of animals	8	8	
Initial body weight (g)	332.50±12.15	333.50±28.12	0.46
Final body weight (g)	471.88±34.24	526.43±41.81	0.001
Body weight gain (g)	139.38±22.08	187.29±38.56	<0.01
Body length (cm)	27.56±0.56	27.86±0.48	0.15
Body Mass Index (g/cm^2)	0.62±0.04	0.69±0.03	0.01
Food consumption (g/day)	28.70±3.68	15.00±1.60	<0.001
Energy intake (Cal/day)	111.36±14.28	145.19±14.23*	<0.001
Water intake (mL/day)	48.10±10.08	62.92±6.72	<0.01
Peritoneal fat (g)	4.29±1.43	11.48±73.00	<0.001

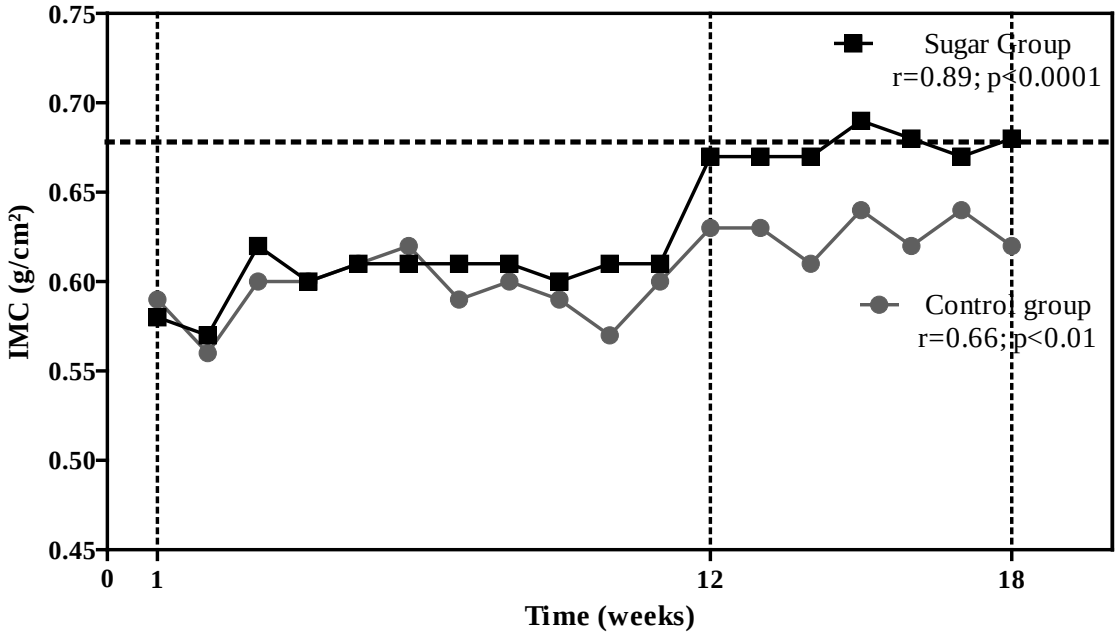
Values are mean±standard deviation of the groups

*Energy intake from food and water sucrose intake

Table 2. Summary of general characteristics found in humans with prediabetes compared to our prediabetes' model in rats

	Human PD*	**Rat PD**
Underlying cause	Impaired glucose tolerance	Impaired glucose tolerance
Age of onset	Postmaturity	Postmaturity
Body weight	Increased	Increased
Abdominal Fat	Increased	Increased
BMI	Increased	Increased
Diet	Hypercaloric	Hypercaloric
Pancreas	Mildly Increased islets	Mildly Increased islets

* [21][22][8][5].



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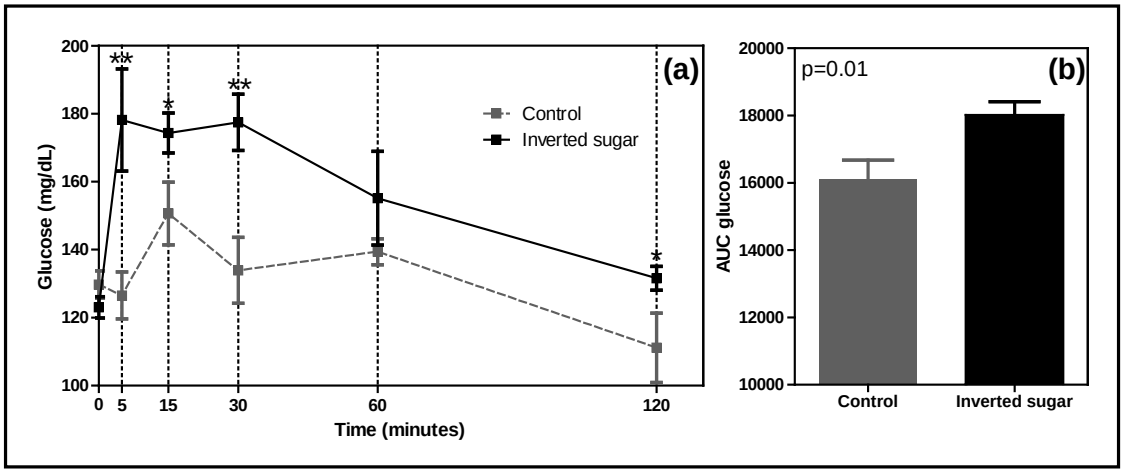

Figure 2.

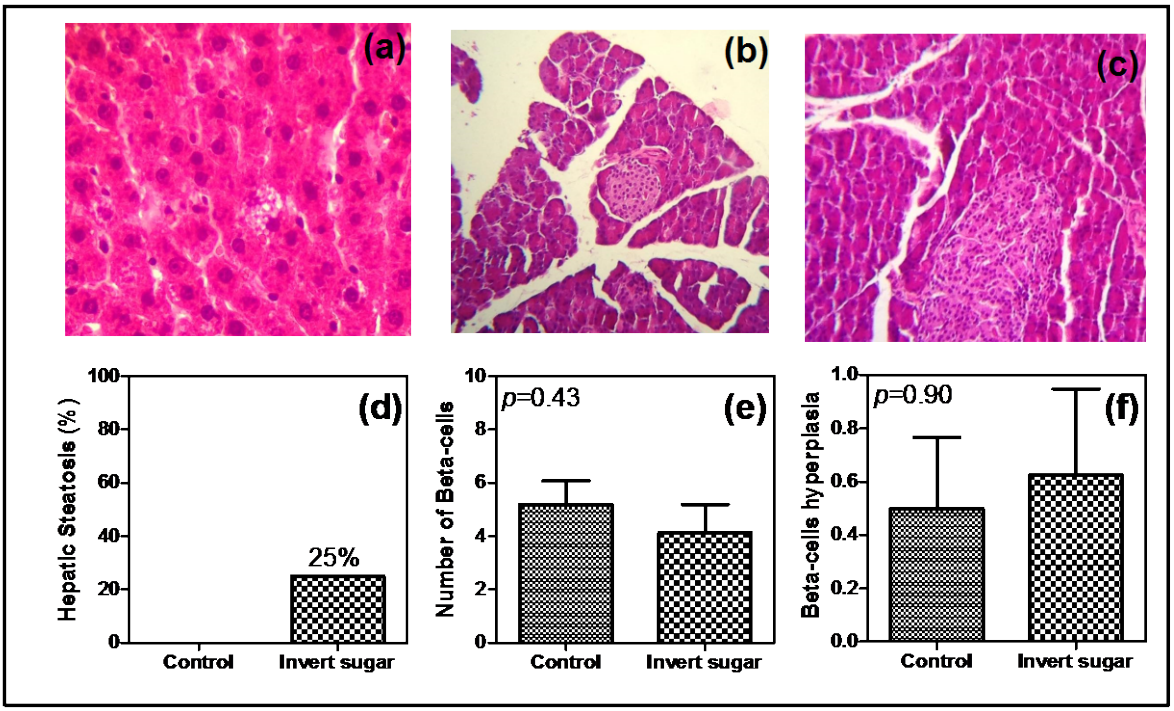

Figure 3.

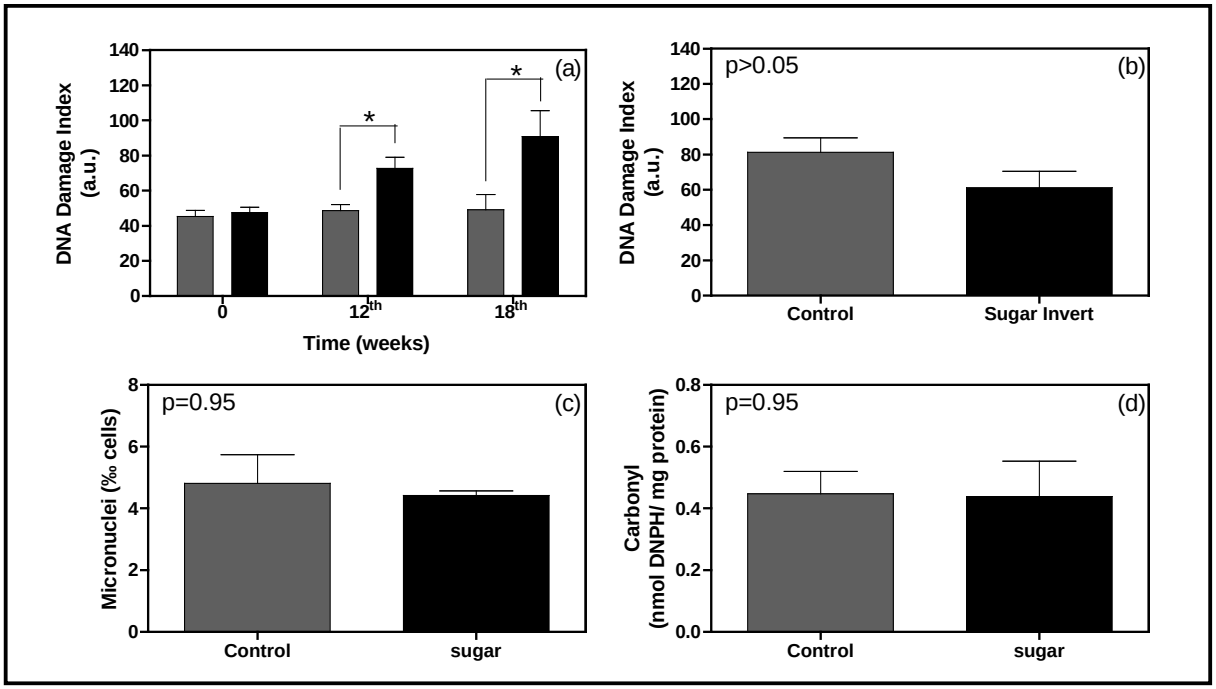

Figure 4.

ARTIGO II

Determination of chromium by PIXE technique in a rat model of prediabetes

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Abstract

Cr intake has been suggested as an alternative to the conventional treatment of type 2 diabetes (DM2), however this aspect remains controversial. To date very few studies using Cr to attenuate DM2 evaluated the concentration of the element either in blood or in organs. In this study, we induced prediabetes (early stage in diabetes establishment) by invert sugar administration in rats. Cr was administered concomitantly with invert sugar as a strategy to prevent DM2 establishment. The levels of Cr in blood and liver were determined by PIXE technique. The results indicated that the supplementation with Cr associated with invert sugar blocked the establishment of prediabetes. Cr intake differed significantly ($p<0.01$) between groups supplemented with Cr. Blood Cr concentrations also differed significantly between the groups, but besides the highest intake of Cr in rats treated with invert sugar plus Cr such animals did not present significantly increased levels of blood Cr in relation to control. This aspect might indicate that prediabetes depletes blood Cr level. PIXE technique was not capable of detecting the hepatic concentrations of Cr. In conclusion, PIXE technique was effective in detecting the Cr level fluctuations in blood, but not in liver in this prediabetic rat model.

Keywords: Prediabetes, chromium supplementation, glycemia, glucose tolerance, PIXE, elemental concentration

1. Introduction

Chromium (Cr) is a transition metal that occurs naturally in environment and is most commonly found in the Cr^{3+} and Cr^{6+} valences. The biological activity of Cr is conferred by its valence state [1]. Additionally, Cr^{3+} is an essential element for human health and has low toxicity because the Cr in this valence is more chemically stable than Cr+6 [2].

Dietetic form Cr^{3+} has a low absorption rate (0.1-2.5%), and foods have low amounts of Cr^{3+} [3, 4]. Moreover, there is evidence that refined foods and simple sugars might decrease Cr absorption. Cr chloride, the most common inorganic form of Cr commercialized, has a much lower bioavailability, because its inorganic form is less absorbed than organic forms [5].

Little is known about the nutritional effects of Cr on mammal's metabolism. Still, Cr has been related as an essential element to carbohydrates and lipids metabolism [6], and Cr deficiencies have been associated to insulin resistance and type 2 diabetes (DM2) [7]. Thus, Cr has been suggested as a relevant alternative to diet therapy of the DM2 [1], but this aspect remains controversial [5, 6].

In spite of the potential beneficial effect of Cr in DM2 and prediabetes (intermediate state between a healthy state and DM2), there are very few studies evaluating the Cr concentration in tissues such as blood or in organs. The Particle Induced X-ray Emission (PIXE) technique can be an essential tool for assessing the blood and organs Cr concentration [8].

In this context, the aim of study was to determine Cr concentrations in blood and liver by PIXE technique in a rat model of prediabetes induced by invert sugar overload and simultaneously treated with Cr. A set of rats were divided into four sub-sets: i) one without any treatment; ii) one treated with invert sugar; iii) one treated with Cr; iv) one treated with Cr plus invert sugar.

2. Material and methods

The study procedures followed the recommendations of the Brazilian regulations for animal studies (Law No. 11794/1999), and the study protocol was approved by the Animal Ethics Committee from the University of Santa Cruz do Sul (protocol 14/2013). Thirty-two

male *Wistar* rats with about 100 days of age, weighting approximately 300 g were used in the experiment. The rats were kept in individual cages, under a light/dark cycle of 12 hours, 22±3 ºC and 60% of humidity, receiving normoprotein-caloric Nuvilab® chow, and treatment/control tap water *ad libitum* throughout the experiment. Before start the treatment, the rats were acclimatized to the lab conditions for one week.

2.1. Treatment, Cr intake calculation and assessment of prediabetes stage

The rats were randomly assigned in the following groups (Figure 1): Group 1: Control (C) (n=8). Animals without any treatment; Group 2: Inverted Sugar (IS) (n=8). Animals treated with invert sugar (32%) diluted in drinking tap water; Group 3: Chromium (Cr) (n=8). Animals treated with a solution of 58.4 mg/L Cr chloride (CrCl3·6H2O) in drinking tap water; Group 4: Chromium plus Inverted Sugar (Cr+IS) (n=8). Animals treated with a solution of 58.4 mg/L Cr chloride plus inverted sugar (32%) in drinking tap water.

Along the treatment, the water intake was registered daily. The average intake of Cr was calculated based in the daily intake of drinking water of the groups. At the end of the treatment (after 18 weeks), the rats were sacrificed with the aid of a guillotine specific for rodents. Prediabetes stage was evaluated by Intraperitoneal Glucose Tolerance Testing (ipGTT) according with Animal Models of Diabetic Complications Consortium [9] and the result was express by AUC, calculated based in a plot of blood glucose concentration in blood against versus time.

2.3. PIXE technique

1mL of whole blood and liver sample were collected for PIXE analysis. The samples were lyophilized, homogenized, pressed into pellets. of 13 mm diameter and placed at a specific sample holder for PIXE measurements [8]. The blood samples were measured by

PIXE at the Ion Implantation Laboratory of the Physics Institute of the Federal University of Rio Grande do Sul. A 3 MV Tandetron accelerator provided a 2 MeV proton beam to irradiated the samples with an average current between 0.5 and 1.0 nA during 600 s. The characteristics X-rays were detected by a Si (Li) detector placed at 135º with respect to the beam direction. This detector works with an energy resolution of 160 eV at 5.9 keV approximately. A 431 μm thick mylar filter with a fractional hole size of 2.7% covered the beryllium window to avoid excessive count rates due to low energy X-rays. PIXE spectra were quantified using GUPIXWIN software [10]. For quantification, this study made use of the Micromatter® thin films standard (NIST reference material 1577b). Additionally, to fit the spectra as thick samples, the blood matrix composition determined previously by Rutherford Backscattering Spectrometry (RBS) was 0.725 of carbon, 0.135 of nitrogen, 0.075 of oxygen and 0.065 of fluor.

2.4. Statistical analysis

Data were analyzed using GraphPad Prism 5.01 software (GraphPad Software, Inc.; San Diego, CA). All results were checked for normality and homoscedasticity. Student's T-test or One-way Analysis of Variance (One-way ANOVA) followed by Bonferroni post-hoc test. The significance level was considered $p<0.05$. Results are presented as average ± standard deviation (SD).

3. Results

Invert sugar intake was similar between groups (20.1±2.1 g per day in IS group vs 20.5±2.5 g per day in Cr+IS group) and did not differ significantly ($p=0.327$).

In regards to the AUC, it was observed significant difference between groups ($p=0.026$; Figure 2). The IS group presented higher AUC than the C group and the AUC of Cr+Invert

sugar was very similar to that of the C and the Cr groups, indicating the Cr treatment can block prediabetes induction.

The blood Cr concentration (Figure 3) differed significantly between the groups ($p=0.042$). The Cr group presented significantly higher blood Cr concentration than the C group. The Cr group presented higher blood Cr concentration than the Cr+IS group, although it did not differ significantly. This occurred in spite of a higher intake of the Cr of the Cr+IS group. The Cr average intake was 1.19±0.16 mg Cr/b.w. per day in Cr group and 1.43±0.16 mg Cr/b.w. per day in Cr+IS group, being significantly higher in the later ($p<0.01$, Figure 4).

Regarding liver Cr concentrations, the PIXE technique was not capable to detect the element in the samples.

4. Discussion

The treatment with invert sugar induced prediabetes in the animals (assessed by AUC [11]), and the co-treatment with Cr in the form of CrCl3 was capable of blocking prediabetes induction.

The relation between Cr and DM2 is not fully understood yet. The main hypothesis for this association is linked to the potentiation of insulin action, influencing glucose metabolism [5]. In a review in which were evaluated animal studies [12], 6 of the 12 studies indicated that Cr supplementation had a positive effect in improving glucose tolerance, in agreement with our results. Differently to our study, we must emphasize that none of these reviewed studies evaluated the Cr concentration in any organ or tissue.

The Cr concentration in the tissue can contribute to understanding of the real effect of Cr on glucose [6]. However, few studies can perform this assessment, mainly because Cr is low absorbed [3, 5]. Some studies suggest that DM2 individuals have decreased levels of Cr in tissues [5, 13]. In our study, the levels of blood Cr of the IS group was similar to the C

group, indicating that Cr depletion might not be seem in the blood during prediabetes. One might recall that prediabetes is the early stage of DM2 establishment and many of the biological alterations observed in DM2 are not present yet.

In our study, Cr concentration in blood increased substantially after supplementation, although the literature reports that Cr has low absorption [6]. The dietary intake of Cr was 22% higher in the Cr+IS group than the Cr group, but the Cr blood concentration was 7% lower in the Cr+IS group in relation to the Cr group. Invert sugar possibly partially depleted blood levels of Cr, probability by its biological use, acting in glucose metabolism [5]. Another hypothesis is that the invert sugar interfered in Cr absorption, increasing its excretion in the urine [3]. Both hypotheses deserve further testing.

It has been described in the literature that Cr can be distributed in different organs of the body, with the highest concentration in the liver [3]. However, in our study we did not found Cr in the liver by PIXE technique in none of the Cr-treated groups nor in the control groups, possibly by low limit of the detection of the system [14].

5. Conclusions

Our results indicated that supplementation with Cr in $ClCr_3$ form influenced positively the metabolism of glucose in the tested rat model of prediabetes, improving the hyperglycemia (AUC), possibly through its effects on insulin. The results presented showed that Cr intake was depleted when associated invert sugar, possibly being used for potentiating of insulin action. This hypothesis deserves further investigation.

Finally, it was observed that PIXE can be a useful technique to determination of Cr concentration in blood. However, the PIXE seems not to be sensitive enough to detect the hepatic levels of Cr. Moreover, more detailed studies to evaluate Cr supplementation in

pancreas (the organ that produced insulin) of rats can improve the understanding of the effect of Cr on glucose metabolism.

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Legend to figures

Figure 1. Outline of the methodological procedures.

Figure 2. Effect of Cr over the Area Under the Curve (AUC) of glycemia along time in the Intraperitoneal Glucose Tolerance Testing (ipTGG) in rats with prediabetes induced by invert sugar (IS) and treated with Cr. p: level of significance according to one-way ANOVA. C: in relation to control group according to Bonferroni post-hoc test at *p<0.05

Figure 3. Effect of Cr concentration in blood of rats with prediabetes induced by invert sugar (IS) and treated with Cr. p: level of significance according to one-way ANOVA. C: in relation to control group according to Bonferroni post-hoc test at *p<0.05.

Figure 4. Dietary intake of Cr via diet (drinking water) in the rats treated with Cr and Cr+IS (IS). p: level of significance according to Student's t-test at *p<0.05.

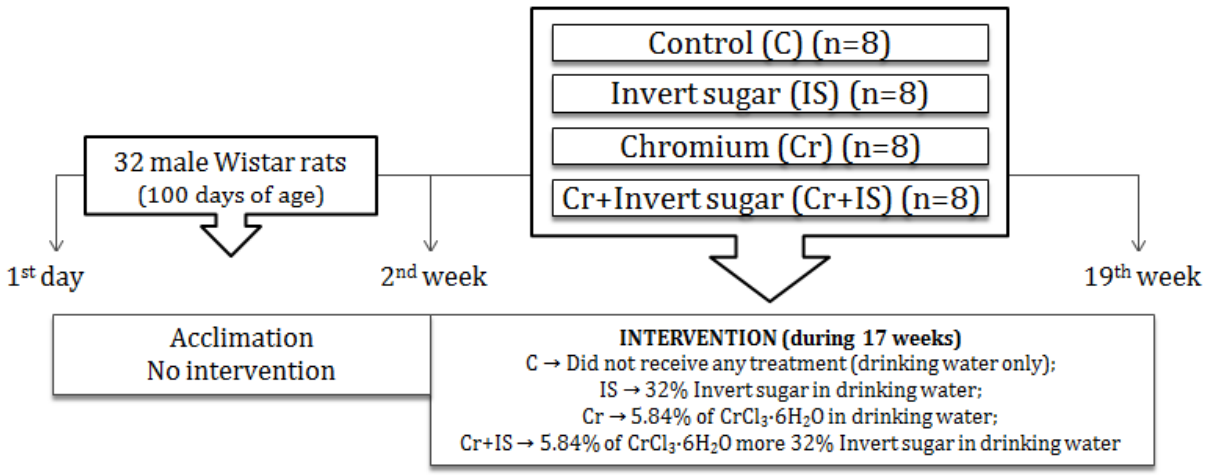

Figure 1.

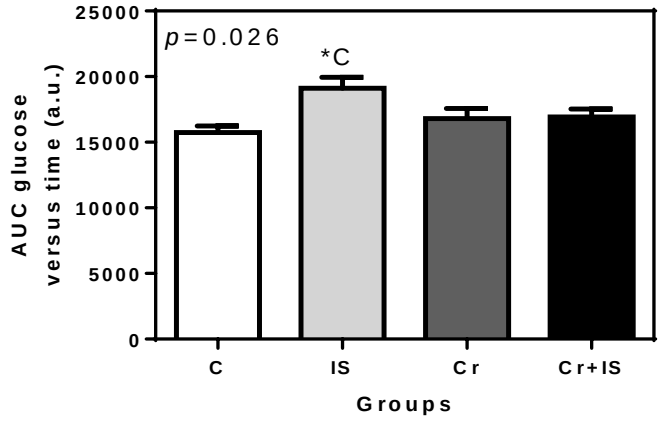

Figure 2.

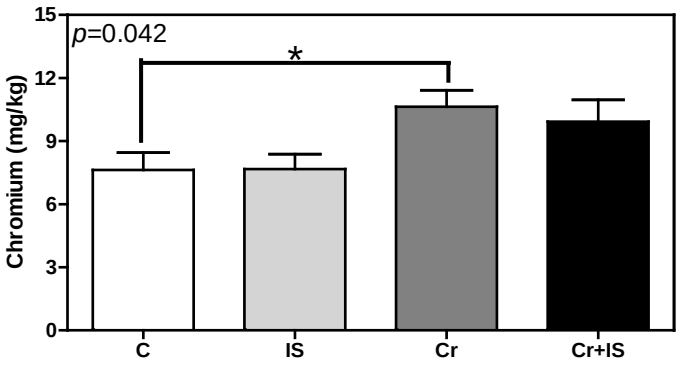

Figure 3.

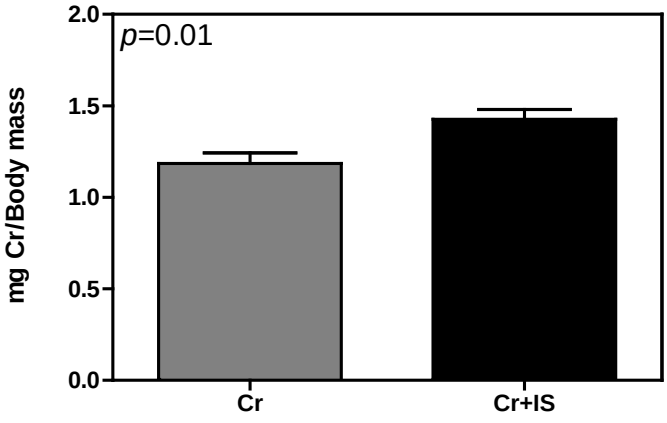

Figure 4.

ARTIGO III

Chromium intake normalizes dysglycaemia and induces primary DNA damage but not chromosomal damage in a prediabetes rat model

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Running title: Chromium intake in prediabetes and genomic stability

Authorship: P.M., D.P. and S.I.R.F. conceived and designed the experiment. P.M. performed the experiments and laboratory tests. PM. analyzed the data, wrote and provided intellectual input on the paper. D.P. and S.I.R.F. supervised all experiments and the manuscript preparation. All authors revised the final version of the article.

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ABSTRACT

Objective: to evaluate the effect of Chromium (Cr) as dietetic supplementation on glucose metabolism and genomic stability in a prediabetes rat model.

Research Methods & Procedures: 36 *Wistar* rats were divided in four groups (8 rats each): control, invert sugar; Cr; Cr+invert sugar. Invert sugar (32%) and $CrCl_3$ (58.4 mg/L) were offered in the drinking water. Intraperitoneal Glucose Tolerance Testing (ipGTT) test was used as dysglycemia marker. Obesity was assessed by the body mass index (BMI) and by peritoneal fat content. Elementary Cr was determined by the Particle-Induced X-ray Emission (PIXE). The Comet Assay evaluated primary DNA damage in blood and pancreas. Bone marrow micronucleus was assessed chromosomal DNA damage in bone marrow. Carbonylated protein measurement evaluated the level of oxidative stress in blood.

Results: Invert sugar intake induced prediabetes because it promoted dysglycaemia the rats. In ipGTT, it was observed a lower area under the curve (AUC) in Cr + invert sugar group than in the invert sugar group, although not significantly (p=0.25). The Cr+invert sugar group presented lower BMI than the invert sugar group (p<0.001). Cr concentration in blood was significantly higher (p=0.04) in the Cr group than in the Cr+invert sugar. Regarding to primary DNA damage, Cr and Cr+invert sugar groups presented significantly higher levels in blood (p=0.03), but significantly lower levels in pancreas (p=0.04). No significant differences between the groups were observed for carbonylated protein measurement. Chromossomal damage was not was observed as evaluated by micronucleus test.

Conclusion: In this experimental model of prediabetes, Cr improved glucose metabolism however induced primary DNA damage in blood, but not in pancreas. It did not increase oxidative stress or chromosomal damage. Therefore, Cr usage in diabetes in the conditions tested can not be downplayed. Further studies are needed to elucidate the toxicity of chromium if it exists.

Keywords: prediabetes, invert sugar, chromium, insulin resistance, DNA damage, glucose tolerance.

Introduction

Chronic hyperglycaemia has been identified as one of the key determinants to type 2 diabetes mellitus (DM2) [1], and excessive intake of sugar is among the principal risk factors for it (MALIK et al., 2010). In addition, hyperglyacemia can induce functional or pathologic alterations during a long time until the DM2 diagnosis can be established [2].

An increase in blood glucose concentration is shown to promote oxidative stress that contributes to development and progression of complications associated to the DM2 [3]. Studies with DM2 patients have associated hyperglyacemia with increased DNA damage [4, 5]. Cytotoxicity has also been observed in the early stages of the development of DM2 [2].

Over fifty years, chromium (Cr) in $^{3+}$ valence has been considered as an essential trace element due to be involved in carbohydrate and lipid metabolism [6]. In addition, Cr has been considered a key factor to diet therapy in hyperglyacemia [7], suggesting that Cr could be associated in control of hyperglyacemia, due to the effect on insulin action, through the "glucose tolerance factor" (GTF) [8]. However, this association has been widely discussed in the literature and findings show mixed results [8-11]. Moreover, studies have also reported that Cr can promote genomic instability [12].

Then, the aim of this study was evaluate the effect of Chromium (III) chloride ($CrCl_3$) dietetic supplementation in prediabetes male *Wistar* rats on glucose metabolism and genomic stability.

Material and Methods

Animals and experimental design

This experiment was approved by the Animal Ethics Committee of the University of Santa Cruz do Sul (Protocol 14/2013) and followed the Brazilian regulations for animal studies (Law No. 11794/1999). Thirty-six male *Wistar* rats (100 days of age), weighting 336.58 ± 23.43 g were accommodated in standard laboratory conditions: light/dark cycle of 12 h, 22 ± 3 ºC and 60% of humidity. The animals received water and chow (normoprotein-caloric Nuvilab®, Quimtia, Colombo, Brazil) *ad libitum*.

The rats were acclimated to laboratory conditions for a period of one week. Afterwards, the animals were randomly assigned in four treatment groups, organized as follows: Group 1: control group (n=8); Group 2: control invert sugar (n=8); Group 3: control Cr (n=8); Group 4: Cr + invert sugar (n=8). The rats were treated for 18 weeks and at the end of the experiment they were sacrificed by decapitation, using a guillotine specific for rodents.

Prediabetes induction and Cr administration

To induce prediabetes, the rats received an invert sugar overload (32%) added in drinking water. It was opted to induce animals to DM2 by carbohydrate dietary overload to mimic the installation of DM2 in humans. Many sugar-sweetened beverages have approximately 34% sugar [13], concentration similar to the 32% sugar used in the present study. There is evidence that sucrose overload impairs glucose tolerance and promotes obesity, two known risk factors for DM2 progression [14, 15].

For Cr supplementation Chromium (III) chloride ($CrCl_3 \cdot 6H_2O$) (Sigma Aldrich) was used in concentration of 58.4 mg/L in drinking water. Cr was diluted alone or concomitantly with invert sugar (32%). The concentration of Cr was based in previous studies evaluating Cr and its effect on glucose metabolism [16-18].

Chow and water (control or supplemented with invert sugar and chromium alone or in combination) were provided *ad libitum*. The daily intake of invert sugar and Cr was calculated based in the intake of the period 24 hour.

Body Mass Index (BMI) for rats and weighing of peritoneal fat

Rats were weighted and measured (nose-anus length) weekly. The BMI specific for rats was obtained and interpreted according to Novelli and collaborators [19]. After sacrifice of the animals, the peritoneal fat (posterior subcutaneous, mesenteric, and retroperitoneal regions) was dissected and weighed. The excision of each part of the peritoneal fat was realized according to the descriptions of Cinti [20].

Intraperitoneal Glucose Tolerance Testing (ipGTT)

At the end of study, the ipTGG was measured [21]. Firstly, fast glucose was evaluated (time 0 - baseline) after fasting of 6 hours. Subsequently, 1mg/kg of body weight glucose (Equiplex®) was administered intraperitoneally in animal and blood was collected and glucose measured at times 5, 15, 30, 60 and 120 minutes. Glucose was measured using a glucometer-ACCU-CHEK Active Meter® (Roche Diagnostics GmbH, Mannheim, Germany). Blood was obtained by a small cut in tail’s tip. The area under the curve (AUC) was calculated for the ipGTT test.

The Comet Assay (blood and pancreas cells)

The blood and pancreas samples were collected at the end of the treatment. Blood samples were collected from jugular vein and mixed with heparin. Pancreas samples were

dissected, mixed in phosphate buffered saline (PBS) plus dimethyl sulfoxide (DMSO) and then gently dissociated to obtain a cell suspension.

The comet assay was executed according to Franke et al. [22]. For each blood or pancreas simple, two slides were prepared and 100 cells were randomly selected and analyzed (50 cell per slide and 2 slides per simple) under a conventional optical microscope with 200X magnification. The DNA migration damage was classified into five classes, from class 0 (no DNA migration) to class 4 (maximal migration) according to tail size and intensity. The damage index (DI) was obtained by the sum of the individual cells sorted, ranging from 0 (no harm: cells 100 x 0) to 400 (maximum damage: 100 x 4 cells). The damage frequency (FD), in percentage terms (%) was calculated by the ratio of the number of damaged cells among the 100 cells analyzed. Cells with non-detectable nuclei (head and tail clearly separated) were not evaluated.

Micronucleus test

The techniques for bone marrow sampling followed the recommendations of Picada and collaborators [23]. The bone marrow samples (both femurs) were mixes with fetal calf serum (FCS) to obtain a cell suspension and smears were prepared. The cells were stained with 5% Giemsa. Micronuclei were evaluated as per 1000 polychromatic erythrocyte (PCE). The PCE/normochromatic erythrocyte ratio was also scored as a marker of toxicity [24].

Oxidative stress by carbonyl proteins

Protein oxidative damage was assessed according Lanine and collaborators [25], using Total Proteins kit from Labtest (Protein Kit, Labtest Diagnostica S.A., Brazil). The determination of the carbonyl groups in a reaction with dinitrophenylhydrazine and the results were expressed as nmol of DNPH/mg of protein.

Cr microelement determinations

The Cr microelement concentration was determinate by Proton Induced X-ray Emission (PIXE) technique [26] carried out at the Ion Implantation Laboratory of the Physics Institute of the Federal University of Rio Grande do Sul. While the blood samples for PIXE was taken after the sacrifice, in which were collected 1 mL blood sample and lyophilized. The samples were then pressed into pellets of approximately 13 mm of diameter and placed at a specific sample holder for PIXE measurements.

The samples were irradiated in a 10-6 mbar vacuum chamber, with a 2 MeV proton beam and an average current of 3.5 nA, during 400 s per sample. The characteristic X-rays were detected with a Si (Li) detector (Sirius 80 e2 V). The energy resolution of the detector is 150 eV at 5.9 keV. The system was calibrated and standardized with bovine liver (NIST, reference material 1577b) standard [27]. The PIXE spectra quantification was performed using GUPIXWIN software [28].

Statistics analysis

Data was analyzed with the aid of GraphPad Prism 5.01 software (GraphPad Software, Inc.; San Diego, CA). All results were checked for normality and homoscedasticity. The Student's t and One-way Analysis of Variance (One-way ANOVA) followed by Bonferroni's Multiple Comparison Test employed for comparisons between groups. Pearson's correlation analysis was also used. The significance level was set at $p<0.05$.

Results

After 18 weeks of treatment with invert sugar in drinking water (concentration of 32%) a dysglycemic stage was reached, as characterized by higher adiposity and glucose intolerance.

Table 1 presents the anthropometrics and dietetics parameters, according to the treatment group. The invert sugar group presented higher body weight gain than the other groups ($p<0.01$), while Cr supplementation resulted in a lower weight gain in the Cr and the Cr+Invert sugar groups. In relation to peritoneal fat, it was also verified a significant difference between the groups ($p<0.001$). It was observed lower peritoneal fat (minus 9%, $p<0.0001$) in the Cr+invert sugar in relation to the invert sugar group.

A similar effect to the peritoneal fat was observed for the BMI (Figure 1). It was verified a significant difference between the groups ($p<0.001$). The Cr group presented lower BMI (0.60 ± 0.03 g/cm^2) than the rats of control group (0.62 ± 0.04 g/cm^2). In the Cr+invert sugar group (0.63 ± 0.04 g/cm^2), it was observed a reduction in BMI in relation to the invert sugar group (0.69 ± 0.04 g/cm^2).

The Cr+invert sugar group had a higher liquid ($p<0.001$) and caloric ($p<0.001$) intake (Table1). The group Cr present lower caloric intake, promoting a lower BMI. The Cr intake was higher ($p<0.001$) in the Cr+invert sugar group (1.57 ± 0.20 mgCr/Body mass) than in the Cr group (1.22 ± 0.18 mgCr/Body).

Cr concentration in blood (Table 1) was similar between the control and the invert sugar groups (8.31±2.58; 7.67±1.89 mg/kg, respectively), but higher in the Cr group (10.64±2.20 mg/kg, p=0.04). The Cr+invert group had about 6% less Cr in blood (9.92±2.94 mg/kg) than the Cr group (p=0.04).

In ipGTT test (Figure 2), the baseline (time 0) fasten glucose was significantly lower (p=0.01) in Cr+invert sugar group (115.78 ± 9.31 mg/dL) than the invert sugar group (123.38 ± 7.74 mg/dL). We observed a higher AUC in the invert sugar group (18743 ± 2116.68) than other groups, but not significantly (p=0.02). The rats of the Cr+invert group presented values of the AUC (17490 ± 2333.89) lower than the Cr group (17704 ± 3177.31). Glucose levels differed significantly between groups at 5 (p=0.01), 30 (p=0.04) and 120 minutes (p=0.02). Moreover, the glycaemia of the Cr+invert sugar group almost returned to the initial values after 120 minutes, possibly due to role of Cr on insulin resistance.

In relation to the Comet Assay results in blood (Figure 3), it was observed lower DI in the Cr+invert sugar (67.11 ± 16.33) than the Cr (84.88 ± 18.82) group and the invert sugar group (90.86 ± 38.69), significantly (p=0.03). DNA damage in pancreas was higher in the control group in relation to other groups (p=0.04). Regarding protein oxidative damage by carbonylation, there was no difference between the groups (control group = 0.45 ± 0.14; invert sugar = 0.44 ± 0.28; Cr + invert sugar =0.39 ± 0.04; Cr 0.34 ± 0.14; p=0.95). In micronucleus test, groups also did not differ significantly (control group = 4.81 ± 2.63 vs invert sugar = 4.42 ± 0.38; Cr =5.75 ± 0.56, Cr + invert sugar 4.56 ± 3.12; p=0.36).

Discussion

In the present study, invert sugar intake induced dysglycemia (observing impaired glucose tolerance), characterized as the first stage in the development of DM2 (prediabetes) [29, 30]. The co-treatment of Cr+invert sugar (1.65 mg Cr/kg/day in drinking water containing 32% invert sugar) was effective in improving metabolic parameters, promoting a lower weight gain and peritoneal fat, also acted in improving glucose tolerance. Still, it was observed that the Cr+invert sugar group had a BMI similar to the control group, indicating that Cr supplementation promoted a limited weight gain and assisted in the regulation of appetite, even with a high caloric intake. Unlike our study, Urmila Shinde and colleagues [31] using a dose of 8.41 and 20.78 μg Cr/kg/day of $CrCl_3$ oral for 6 weeks and Yoshimoto and colleagues [32], using a dose of 20 μg Cr/kg/day of $CrCl_3$ (ip) for 4 weeks, observed no attenuation in weight gain. It is important to highlight that both studies tested Cr in a streptozotocin-induced DM model which differs to our because this type of drug induce DM1

via destruction of the pancreatic β-cells [33] and our study aimed to simulate DM2 early stages.

We used longer treatment and higher concentrations of Cr compared to other studies that evaluated the effect of Cr in animal genetically modified or drug-induced diabetes. Król and Krejpcio [34], using 0.1-1 mg Cr/kg (Cr (pic)3 form) daily for 8 weeks found no improvement in glucose in rats treated with high-fructose diet (up to 60%). Our concentration (1.65-16.50x higher than in the studies cited above) administered for 18 weeks concomitantly with sugar in the drinking water was effective in attenuating weight gain, fat accumulation and improved dysglycemia in rats.

The Cr concentration used did not induce no adverse effects in our experiment, partly due to its low absorption and also because in the inorganic form, Cr still less absorbed [35, 36]. It should be mentioned that the dosage used in rodents were extrapolated to humans, and it is concluded that it would be substantially higher (4592-3306 times) than the recommended daily intake for humans (25 to 35 μg Cr/day or 0.36 to 0.50 Cr μg/kg/day for a person weighing 70 kg). It also should be emphasized that the dietary recommendation for humans is still preliminary, since the biological role of Cr is little known and is has controversial effects [10]. It is important to mention that a maximum tolerable limit (UL) has not been set for Cr in humans [37], possibly because no evidence of toxicity was found in literature in relation to the dietary intake of the micronutrient. In agreement, no adverse effects were found in the study of Anderson and collaborator [38] evaluating concentrations of 0.5, 25, 50 and 100 mg/kg as Cr in CrCl form in Harlan Sprague Dawley rats for 24 weeks.

The elementary Cr measurement in tissue is an important instrument to better understand the role of Cr on glucose [6]. Few studies evaluated concentration of Cr in tissues, principality in blood. Furthermore, Cr presented low absorbed [35, 39] and in DM, studies indicate that there is an increase depletion of Cr by refined foods, such as sugars [35]. Consequently, diabetic individuals have decreased levels of Cr, and blood and other tissue Cr concentration has been considered problematic because of the low concentration of Cr in these compartments [17, 35].

In our study, Cr concentration in blood increased substantially after supplementation, besides Cr low absorption reported in literature [6]. However, the dietary intake of Cr was 22% higher, but the blood concentration of Cr was 7% lower in the Cr+Invert sugar group in relation to the control group. Invert sugar possibly partially depleted Cr in blood, probability by its biological use, acting in glucose metabolism, in that Cr acted by chromudulin Cr seems modulate cellular glucose transport [35]. Other hypotheses to explain the depletion of Cr in

blood in Cr+Invert sugar group could be explain by widely distributed of Cr by body, in higher concentrations in liver, spleen and kidney [39]. The PIXE technique was sensitive to detect Cr in blood but not in the liver of the rats. Further studies are needed to elucidate the distribution of Cr between different tissues.

We must highlight that the IS group presented fasting blood glucose levels inferior than 150 mg dL [21], indicating that the rats of our experiment did not become diabetic. However, when evaluating glucose tolerance, the IS group had impaired glucose tolerance, as occurs in prediabetes [40]. Cr in the form of $CrCl_3$ positively decreased glycaemia concentration, reducing fasting glycaemia in relation the levels of IS group. At the same time, $CrCl_3$ promoted an improvement glucose tolerance, since glucose peaks were lower in the co-treated rats than in the rats that received only invert sugar.

The relation between Cr and DM is not fully understood yet. The main hypothesis for this association is linked to potentiation of insulin action, influencing glucose metabolism [35]. Balk and collaborators [41], in their review evaluated humans studies and did not find an effect of Cr supplementation in nine studies with impaired glucose tolerance and found no effect in majority of studies that evaluated Cr in blood. In this same review, specifically evaluating the studies that reported action of $CrCl_3$, they also indicated no effect of it on fasting glycaemia, as in glucose tolerance. In a review with animals [42], of the 32 experiments evaluating the effect of fasting glucose, 18 trials found positive effect on blood glucose. Evaluating the AUC of glucose in the same revision, half of the 12 trials observed improve in glucose tolerance after Cr supplementation. In our study, Cr associated with invert sugar showed a decrease in fasting glucose and improved glucose as observed by the fact that AUC of the Cr+Invert sugar was similar a control group ipTGG test.

The mechanisms associated with Cr and its potentiation of insulin action have yet to be investigated, primarily due to different DM induction models, such as the use of drugs or genetically modified animals, other than that used in our experiment diet [12]. Drugs for induction of DM as streptozotocin and alloxan induce DM1, by killing pancreatic β cells, destroying their ability to produce insulin [33], which is different from DM2 which is caused by defects in the secretion or insulin action [43]. Our model with invert sugar does not induce histological alterations in the β cells of the pancreas (data not shown) possibly because diabetes has not been installed yet. In prediabetes stage, firstly occurs impaired glucose tolerance, beginning the process of insulin resistance, which causes a dysfunction of beta cells without histopathological alterations [40, 44, 45].

The association found in our study, in which the Cr served in glucose metabolism, possibly is the biologically-active form has been suggest, low-molecular-weight chromium-binding substance (LMWCr), also called cromodulin [6]. It has been shown that Cr+3 is absorbed in the gastrointestinal tract by passive diffusion. In the bloodstream, Cr binds to serum proteins, but nearly 80% Cr binds to transferrin as a carrier [39]. When released, Cr binds to LMWCr (to make their active biological activity LMWCr need to connect to 4 atoms of Cr) and participate in insulin signal amplification system stimulating tyrosine kinase activity, and consequently activating the kinase of the insulin and therefore enhancing the action of insulin receptor. Thus, in our study, it is hypothesized that Cr could have modulated both the insulin action mechanism, such as insulin binding, increasing the number of insulin receptors, internalization of insulin sensitivity and β cells, thereby increasing glucose uptake and utilization of blood cells [7, 9, 35]. It must emphasized that we did not evaluate insulin concentration. This aspect should be addressed in future studies.

Studies evaluating Cr supplementation effects on glucose metabolism and/or Cr genotoxicity and mutagenicity are limited and there is no evidence to suggest that the Cr+3 causes genomic instability, but has no role in promoting genomic stability [12]. However, Eastmond [46] in his review showed that Cr^{+3} can generate genotoxicity, including mutations. Results from *in vitro* tests are quite variable, regardless of the chemical form of the compound tested Cr. In addition, most studies evaluating the inorganic Cr *in vivo* have largely negative results for genotoxic [46]. In our study, $ClCr_3$ seems to have the potential to generate primary DNA damage in blood, but not in pancreas. Conversely to our results, Staniek [47] also evaluated the comet assay in blood of *Wistar* rat after supplementation of Cr as CrProp for 4 weeks and did not found genotoxic effects. It must be emphasized the Cr toxicity might be dependent of its chemical form. Cr(Pic)3 is the form of Cr more studied and has been associated with toxicity and mutagenicity than other commercial forms, like $ClCr_3$ [6].

Thus, we opted to use the $CrCl_3$ due to its low toxicity [12]. Furthermore, the comet assay is the damage can be repaired or not [48], we also used the micronucleus test in bone marrow because it is as marker of carcinogenesis risk [49]. In the latter test, there was a slightly higher frequency of micronuclei in rats that ingested Cr, although not significantly. In their pilot study, Andersson and collaborators [50] evaluated the toxicity of ip doses of CrPic (0.75, 1.5 or 3 mg/kg b.wt) for 42 hours and found no positive effects for the comet assay. Another study assessing chromosomal micronuclei in the bone marrow damage, Greenberg [51] found no increase in the frequency of bone marrow micronucleus after oral dose of up to 2000 mg/kg body mass of Cr(Pic)3 for 18 or 42 h.

Finally, Cr supplemented rats did have increased levels of carbonylated proteins, which tend to be lower than rats that were not supplemented with Cr, but was not significantly, indicating that the $CrCl_3$ was not likely to induce the generation of oxidative stress. Vasylkiv and colleagues [52] evaluated the toxicity of Cr in fish after exposure for 96 hours $ClCr_3$, and observed an increase in protein carbonyl in the plasma (marker of oxidative stress) and only a tendency of increase in frequency of micronucleus. In our study we also observed a tendency of increased micronucleus frequency and no increase in oxidative stress in the Cr group.

Conclusion

Our results showed that Cr in the form of $CrCl_3$ positively influence the metabolism of glucose in rats models of prediabetes, improving glucose tolerance verified by the AUC in ipGTT test. Our results suggested that $CrCl_3$ has the potential to generate DNA damage in blood, but not in pancreas of the rats. Additionally, $CrCl_3$ also did not generate chromosomal damage as evaluated by micronucleus assay. Moreover, no negative effects were observed in protein carbonylation. These results suggest that $CrCl_3$ in concentrations used were unable to produce cellular toxicity. Of the many studies on the issue, little has been studied the relationship between Cr intake and genomic instability. In this sense, our study may contribute to a better understanding of the Cr effect in $CrCl_3$ form which has been studied.

Conflicts of interest

The authors declare they have no conflicts of interest.

Acknowledgments

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FIGURE LEGENDS

Table 1. Weight gain and food intake between groups. p: significance level; according to One-way Analysis of Variance (One-way ANOVA) and Student's t.

Figure1. Evolution of Body Mass Index (BMI) between groups. r: correlation coefficient and p: significance level; according to Pearson's Test. The dashed line indicates the cutoff for classification of obesity in the rat BMI, according to Novelli et al.[19].

Figure 2. Blood glucose curves during 120 minutes ipGTT and respective area under the 120 minutes curve (AUC) for control group (n=8), invert group (n=8), Cr group (n=8) and Cr+invert sugar (n=8). Points depict mean±SD. One-way Analysis of Variance (One-way ANOVA), *$p<0.01$.

Figure 3. Level of DNA damage index in blood (a) and pancreas (b) cells, protein carbonylation (c) and Micronucleus bone marrow test between groups for 18 weeks. p: level of significance according to the One-way Analysis of Variance (One-way ANOVA), $p<0.05$.

Table 1. Weight gain and food intake between groups. *p*: significance level; according to One-way Analysis of Variance (One-way ANOVA).

Parameters	Groups				
	Control	Invert Sugar	Cr	Cr + invert Sugar	*p*
Number of animals	8	8	8	8	
Initial body weight (g)	332.50±12.15	333.50±28.12	340.78±20.31	345.00±19.18	0.55
Final body weight (g)	471.88±34.24	526.43±41.81[a]	471.75±44.64[b]	512.22±48.08	<0.01
Body weight gain (g)	139.38±26.10	187.29±38.56[a]	128.38 ± 31.65[b]	167.22 ± 29.30	<0.01
Body length (cm)	27.56±0.56	27.86±0.48	28.13±0.52	28.61±1.17[d]	<0.001
BMI (g/cm²)	0.62±0.04	0.68±0.04[a]	0.60±0.03[b]	0.63±0.04[b]	<0.001
Food consumption (g/day)	28.70±3.68	15.00±1.60[a]	28.41±1.09[b]	16.82±1.87 [a,c]	<0.0001
Energy intake (Cal/day)	111.36±14.28	145.19±14.23[a]	110.23±4.22[b]	153.68±15.80 [a]	<0.0001
Wather intake (mL/day)	48.10±10.08	62.92±6.72[a]	48.15±7.77[b]	63.97±7.94[a,c]	0.0001
µg Cr/Body mass intake	-	-	1.22±0.18	1.57±0.20	<0.001[*]
Cr blood (mg/kg)	8.31±2.58	.67±1.89	10.64±2.20	9.92±2.94	0.04
Peritoneal fat (g)	4.29±1.43	11.23±2.81[a]	5.05 ± 2.78[b]	10.23 ± 4.13[a]	<0.0001

Values are shown in mean ± SD.
[a]Difference of control group
[b]Difference of invert sugar group
[c]Difference of Cr group
[d]All groups different of Cr+invert sugar
[*]Student's *t* test was employed

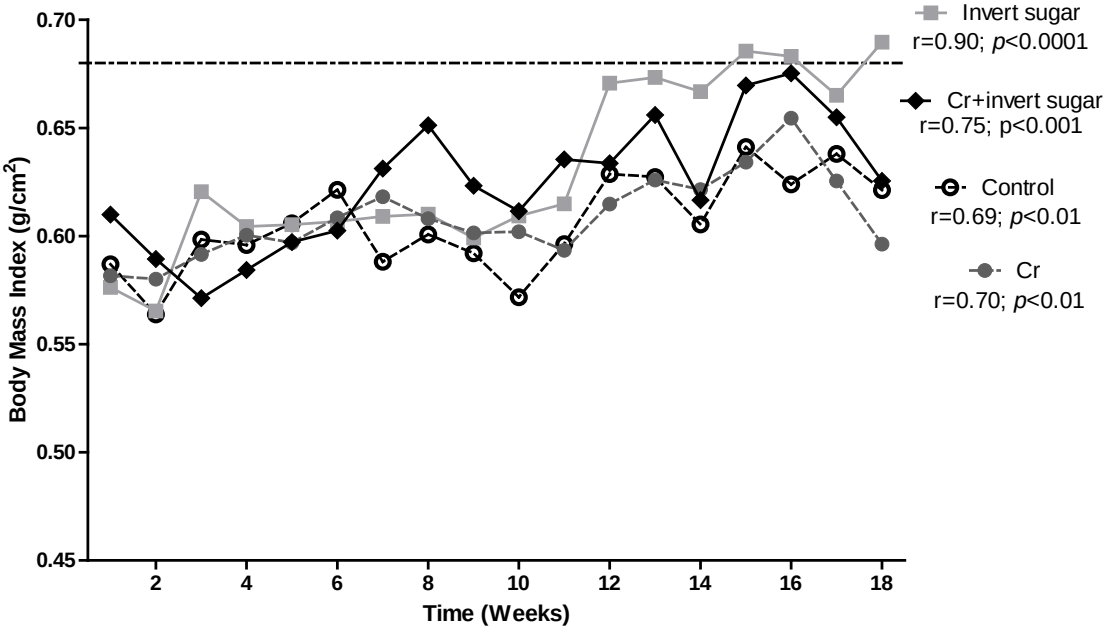

Figure 1.

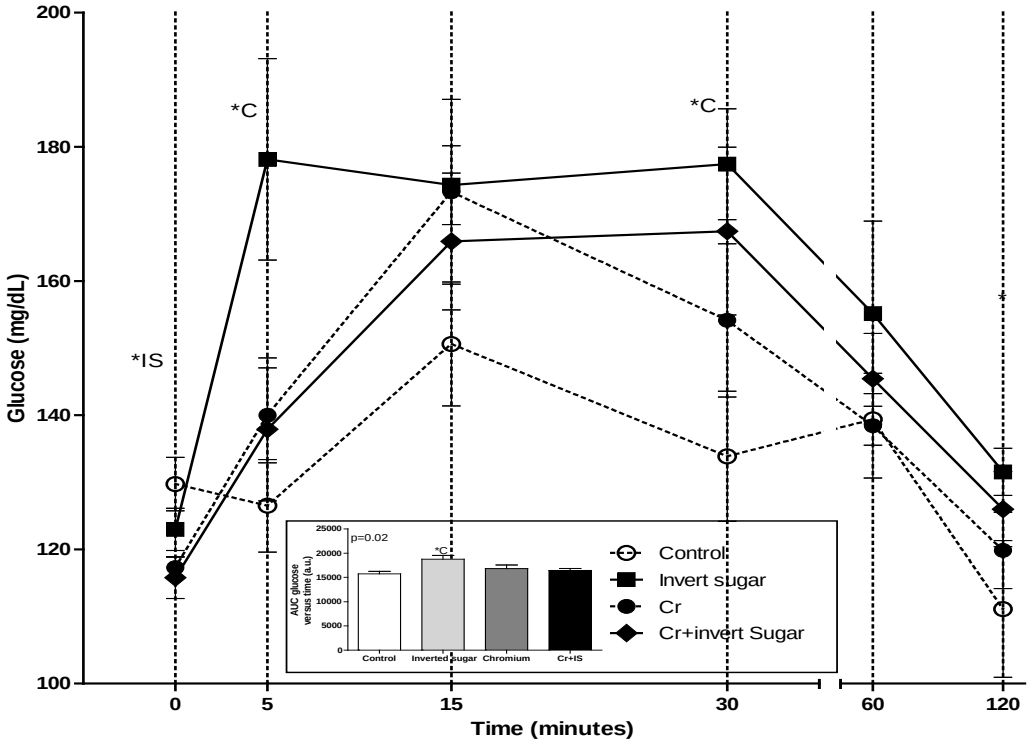

Figure 2.

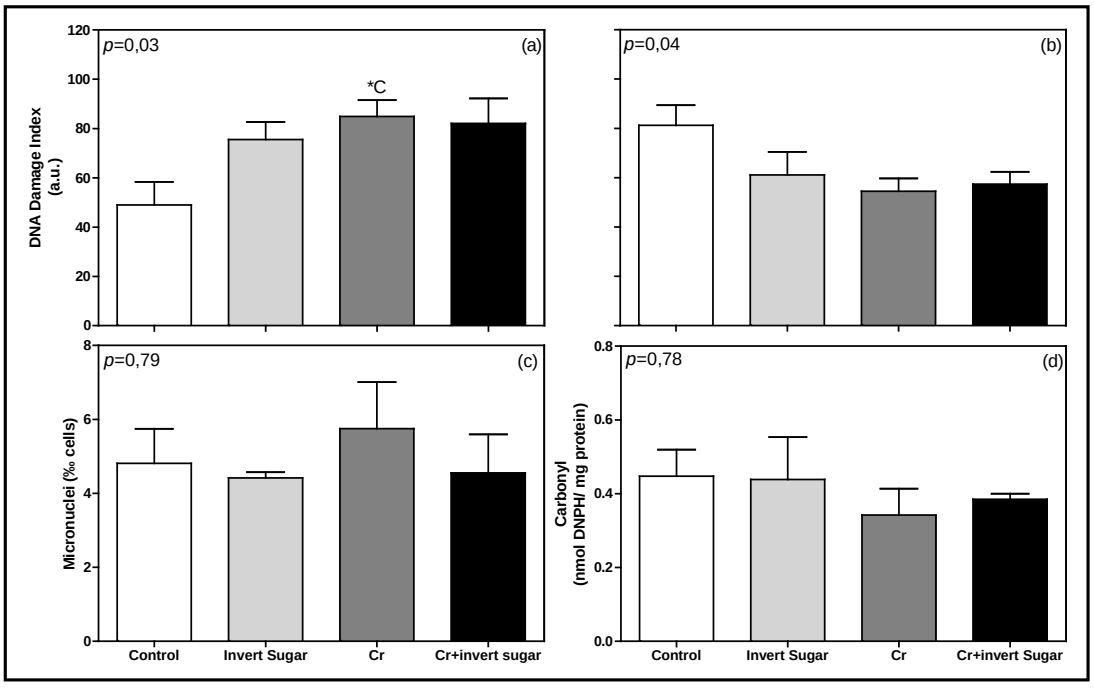

Figure 3.

ARTIGO IV

Vitamina C reduz o efeito obesogênico e hiperglicemiante de bebidas adoçadas em ratos pré-diabéticos

Efeito da vitamina C no pré-diabetes

Effect of vitamin C in prediabetes

Categoria: artigo original

Área temática: nutrição experimental

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Resumo:

Objetivos: O objetivo deste estudo foi avaliar o efeito da vitamina C sobre a obesidade e glicemia em ratos pré-diabéticos.

Métodos: Ratos *Wistar* foram tratados com sobrecarga de açúcar invertido a uma concentração de 32% na água de beber, como método de indução de pré-diabetes. Concomitantemente, vitamina C (60 mg/L) foi adicionada à água de beber como estratégia de prevenção da indução do pré-diabetes. O tratamento foi executado durante 17 semanas, sendo os ratos alocados nos seguintes grupos: controle água (C), controle açúcar invertido (AI), controle vitamina C (VitC) e açúcar invertido+vitamina C (VitCAI). A obesidade foi avaliada pelo IMC e pela quantidade de gordura peritoneal. O estágio de prediabetes foi avaliado pelo Teste de Tolerância à Glicose Intraperitoneal (ipGTT).

Resultados: Os grupos VitC e VitCAI tiveram redução significativa do peso (p= p=0,035) e da gordura peritoneal (p<0,001) em relação ao grupo AI. Em consequência, houve diminuição do IMC do grupo VitCAI, que assemelhou-se ao do grupo C ao final do experimento. A vitamina C reduziu a glicemia de jejum dos animais de ambos os grupos suplementados com Vitamina C. Além disso, a vitamina normalizou a intolerância à glicose do grupo VitCAI, igualando a AUC a do grupo C.

Conclusão: A suplementação de vitamina C teve efeito anti-obesogênico e hipoglicemiante, podendo ser indicada como agente preventivo de pré-diabetes. Estudos futuros são necessários para entender os mecanismos anti-obesogênico e anti-hiperglicemiante da vitamina C no pré-diabetes.

Termos de indexação: Pré-diabetes, vitamina C, obesidade, hiperglicemia.

Introdução

A vitamina C, também conhecida como ácido ascórbico, é uma das principais vitaminas da dieta e está presente em diversos tipos de alimentos como frutas e vegetais[1]. A vitamina C é um antioxidante natural cuja ação tem sido estudada por apresentar inúmeros efeitos biológicos relevantes[2].

Dentre os possíveis efeitos benéficos da vitamina C está a sua ação inversa com a obesidade[3]. Estudos tanto em humanos[4] quanto em animais[5] demostraram que a suplementação com vitamina C tem sido efetiva para a redução do peso corporal. Ainda nesse contexto, sugere-se que a vitamina C também pode exercer papel protetor nas etapas de indução e progressão do diabete mellitus tipo 2 (DM2)[6], auxiliando na regulação do transporte celular da glicose[7], e consequentemente, contribuindo para a melhoria do controle glicêmico na DM2.

Antes da instalação da DM2 ocorre o pré-diabetes, que é o estado hiperglicêmico intermediário, no qual os indivíduos apresentam uma tolerância à glicose diminuída, iniciando uma resistência à insulina[8]. E, é durante este estágio que ocorre uma maior propensão para o desenvolvimento de alterações funcionais ou patológicas, antes que o diagnóstico de DM2 seja estabelecido[9]. É durante este estágio está intimamente associado ao aumento do sobrepeso e obesidade, um preditor para o desenvolvimento DM2 e suas complicações[10, 11]. O excesso de consumo calórico e um estilo de vida sedentário são os principais fatores de risco para a obesidade e o desenvolvimento da DM2. Nesse contexto, a ingestão excessiva de açúcar desempenha um papel importante no aumento mundial na prevalência das duas patologias[12-14]. Com o aumento da carga glicêmica dietética, o balanço energético torna-se positivo e, ocorre o aumento da adiposidade, particularmente em torno do abdômen, concomitantemente levando à resistência à insulina[15].

Ainda é necessária a realização de mais estudos para elucidar os efeitos benéficos da Vitamina C na obesidade e prediabetes, uma vez que esse ainda é um tema pouco explorado. Portanto, o objetivo deste trabalho foi avaliar o efeito da vitamina C na prevenção da obesidade e glicemia em ratos sendo tratados com overdose de açúcar invertido para indução de pré-diabetes.

Metodologia

Animais

Este estudo foi aprovado pela Comissão de Ética no Uso de Animais da Universidade de Santa Cruz do Sul sob o protocolo nº 14/2013. Ratos *Wistar* Machos, com idade média de 100 dias de vida e peso corporal médio de 336,58 ± 23,43 g foram alocados no estudo. Os ratos foram mantidos em gaiolas individuais no biotério da Universidade, numa

temperatura ambiente de 22 ± 3°C, com fotoperíodo claro/escuro de 12 h e 60% de umidade.

Tratamento e substancias testes

Durante sete dias, os animais passaram por um período de ambientação, recebendo ração normoprotéica-calórico (Nuvilab®, Quimtia, Colombo, Brasil) e água *ad libitum*. Após este período, os animais foram divididos em quatro grupos, conforme os seguintes tratamentos: i) grupo C (controle água), ii) grupo AI (controle açúcar invertido), iii) grupo VitC (controle vitamina C), iv) grupo VitCAI (vitamina C + açúcar invertido).

Água foi utilizada como veículo para a administração da vitamina C e açúcar invertido, e os tratamentos foram oferecidos ad libbitum aos animais. Para indução do pré-diabetes, os ratos receberam uma sobrecarga de açúcar invertido a uma concentração de 32%[16]. Os grupos submetidos à suplementação de vitamina C (Sigma A4544) receberam o nutriente a uma concentração de 60 mg/L. A ingestão alimentar e ingestão hídrica foram mensurados semanalmente.

O ensaio teve duração total de 17 semanas e o método escolhido para sacrificar os animais foi à decapitação, utilizando a guilhotina.

Diagnóstico de pré-diabetes

Para induzir os animais ao estágio de pré-diabetes, optou-se por utilizar uma sobrecarga de açúcar a fim de promover desordens metabólicas, hemodinâmicas, estruturais e funcionais em roedores[17], aumentando as concentrações plasmáticas de insulina, leptina, triglicerídeos, glicose e ácidos graxos livres e levar a intolerância de glicose[9].

Utilizou-se o açúcar invertido para a indução de pré-diabetes, porque é um é constituinte típico de bebidas adoçadas com, sendo uma mistura de glicose, frutose e sacarose invertida em partes iguais (33% cada, enquanto que em 33% de inversão)[18, 19]. O diagnóstico de pré-diabetes foi realizado pelo teste de tolerância sobre glicose Intraperitoneal (ipGTT) avaliando a tolerância à glicose diminuída.

Marcador hiperglicemiante

Para a avaliação da tolerância a glicose diminuída realizou-se o Teste de tolerância à glicose intraperitoneal (ipGTT) no final do experimento[20]. A curva de ipGTT foi aferida nos animais utilizando-se um glicômetro portátil Accu-Chek®, coletando-se sangue da veia da cauda após 6h de jejum (tempo 0) e, após, a aplicação intraperitoneal de 1 mg de glicose por g de peso de rato nos tempos 5, 15, 30, 60 e 120 minutos. As alterações nas

concentrações de glicose durante o ipGTT foram expressas como a área sob a curva (AUC) de glicose.

Marcadores obesogênicos

Para a avaliação da obesidade, utilizou-se o Índice de Massa Corporal (IMC) específico para roedores, mensurado a partir da relação entre peso e comprimento do animal e calculado semanalmente segundo as recomendações de Novelli e colaboradores[21]. Foram considerados obesos, os animais que apresentaram IMC>0,67 g/cm^2.

No final do experimento coletou-se a gordura corporal dos animais de acordo com Cinti[22] para a pesagem a avaliação da quantidade de gordura peritoneal.

Análise estatística

Os dados foram tabulados e analisados no programa GraphPad Prism 5.01 (GraphPad Software, Inc; San Diego, CA). Os dados foram analisados pelo teste de ANOVA (One-way ANOVA), seguido pelo teste pós hoc de Bonferroni. A análise de correlação de Pearson ou Spearman também foi empregada. Todos os dados foram expressos como média e desvio padrão e foram verificados quanto à normalidade e homocedasticidade. O nível de significância considerado foi de $p<0,05$.

Resultados

As alterações metabólicas provocadas pela ingestão de açúcar invertido e a suplementação de vitamina C por 17 semanas estão descritos na Tabela 1.

Tabela 1. Peso corporal, gordura peritoneal e ingestão dietética dos ratos após o tratamento por 17 semanas com açúcar invertido e Vitamina C. p: nível de significância; de acordo com o teste ANOVA (One-way ANOVA).

	Grupos				
Parâmetros	Controle	AI	VitC	VitCAI	*p*
Numero de animais	8	8	8	8	
Peso inicial (g)	332,50±12,15	333,50±28,12	343,13±21,49	337,00±20,78	0,752
Peso final (g)	471,88±34,24	526,43±41,81[a]	473,63±32,57	495,57±42,87	0,035
Comprimento (cm)	27,56±0,56	27,86±0,48	27,63±0,44	28,08±0,80	0,346
Ingestão de ração (g/dia)	28,70±3,68	15,00±1,60[a]	27,85±2,12	15,98±2,96[a]	<0,001
Ingestão de líquidos (mL/dia)	48,10±10,08	62,92±6,72[a]	53,13±9,56[b]	62,35±7,39[a,c]	0,003
Ingestão energética (Cal/dia)	111,36±14,28	145,19±14,23[a]	108,07±8,23[b]	148,20±19,53[a,c]	<0,001
Ingestão efetiva de Vitamina C	-	-	7,48±1,66	8,93±1,76	0,013*

Gordura peritoneal (g)	4,29±1,43	11,23±2,81[a]	8,26±6,58[b]	8,14±3,47 [a,c]	<0,001

Valores são mostrados em média ± desvio padrão.
[a]Diferença do grupo controle
[b]Diferença do grupo açúcar invertido
[c]Diferença do grupo VitC
* Teste t de Student foi empregado

Observou-se uma diferença significativa entre os grupos em relação ao peso final dos animais (p=0,035), no qual, o grupo que recebeu a bebida adoçada apresentou um maior peso final dentre os grupos. A ingestão de vitamina C pelo grupo VitCAI (8,93±1,76 mg/kg) promoveu uma redução de 6% no peso dos animais. Um efeito similar foi encontrado referente à gordura peritoneal, observando-se uma diferença ainda mais significativa (p<0,001), no qual o grupo VitCAI apresentou uma redução de 28% em comparação ao grupo AI (8,14±3,47 g *vs* 11,23±2,81 g), (Tabela 1).

Em relação ao IMC, verificou-se diferença significativa entre todos os grupos (p=0,005), sendo observada maior evolução no IMC no grupo que recebeu açúcar invertido (IMC final médio = 0,69±0,03 g/cm^2). O cotratamento com vitamina C apresentou uma redução de 9% do IMC, ao final das 17 semanas, em relação ao grupo que ingeriu o açúcar invertido. Os ratos do grupo VitC apresentaram um IMC semelhante ao do grupo controle ao final do tratamento. Ao final das 17 semanas o grupo VitCAI apresentou uma redução de 9% do IMC em relação ao grupo que ingeriu o açúcar invertido. Os ratos do grupo VitC apresentaram um IMC semelhante ao do grupo controle ao final do tratamento. Ao final do experimento os três grupos (VitCAI, VitC e Controle) não apresentaram diferença significativa quanto ao IMC (p=0,641), enquanto que os grupos VitCAI e AI apresentaram diferença significativa entre eles (p=0,007) (Figura 1).

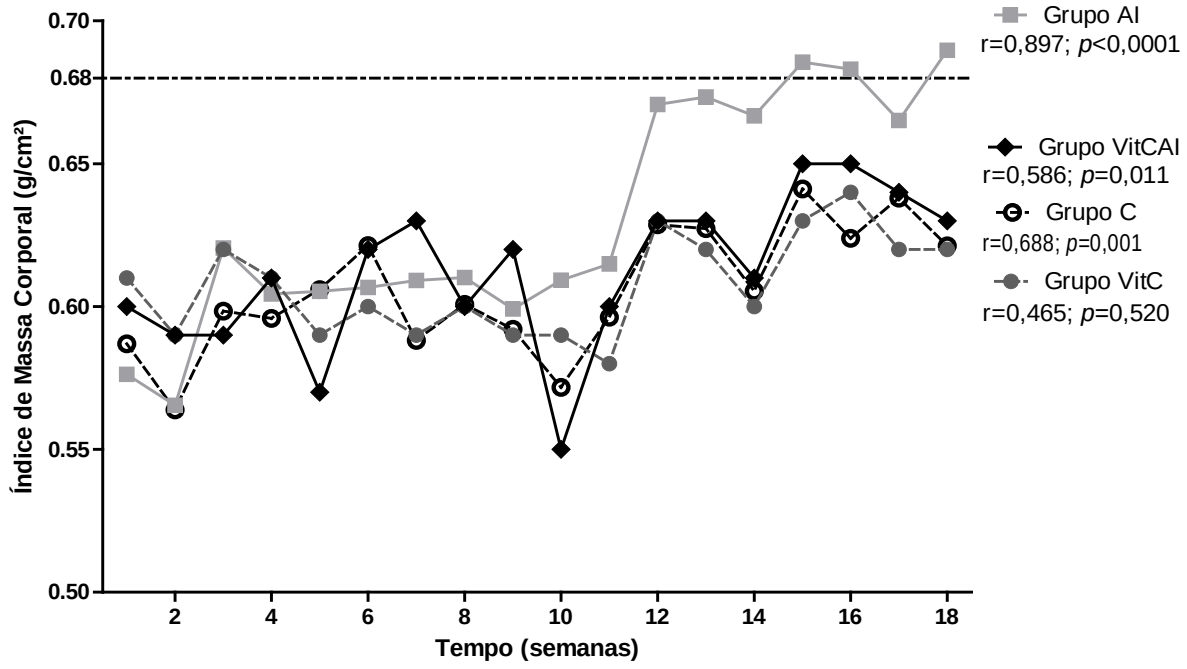

Figura 1. Evolução Índice Massa de Corporal (IMC) dos grupos Controle, AI, VitC e VitCAI. r: coeficiente de correlação e nível de significância de p; de acordo com o teste correlação de *Pearson*. A linha tracejada indica o corte para a classificação de obesidade em ratos IMC, segundo Novelli et al[21].

A ingestão alimentar e hídrica apresentaram uma diferença significativa entre os grupos (p<0,001; p=0,003). Ao mesmo tempo, observou-se uma maior ingestão energética no grupo VitCAI (148,20±19,53 Cal) em relação aos demais grupos (p<0,0001). Entretanto, o grupo que recebeu somente vitamina C apresentou uma ingestão calórica 37% menor que o grupo VitCAI e 3% menor que o grupo C (Tabela 1).

A glicemia de jejum (tempo 0) apresentou diferença significativa entre todos os grupos (p=0,002), sendo que os grupos cotratados com vitamina C (VitCAI = 113,28±9,86 mg/dL; VitC = 110,75±5,99 mg/dL) apresentaram os menores valores de glicemia comparados ao grupo C (129,75 ± 11,25 mg/dL). Ao verificarem-se as glicemias, após infusão da glicose nos tempos determinados, observou-se que os animais tratados com a bebida adoçada (grupo AI) apresentaram maior curva glicêmica em relação aos demais grupos de forma significativa (p=0,001), segundo a AUC. Os ratos de ambos os grupos que receberam a vitamina C apresentaram uma melhora na glicemia após a carga de glicose nos tempos 5 (p<0,001),15 (p=0,003), 30 (p=0,001) e 60 minutos (p=0,160). No tempo 120 minutos (p=0,002) pode-se observar que o grupo VitCAI apresentou uma melhora na tolerância à glicose em relação ao grupo que recebeu açúcar invertido, ao mesmo tempo que a glicemia do grupo VitC se igualou ao grupo C (Figura 2).

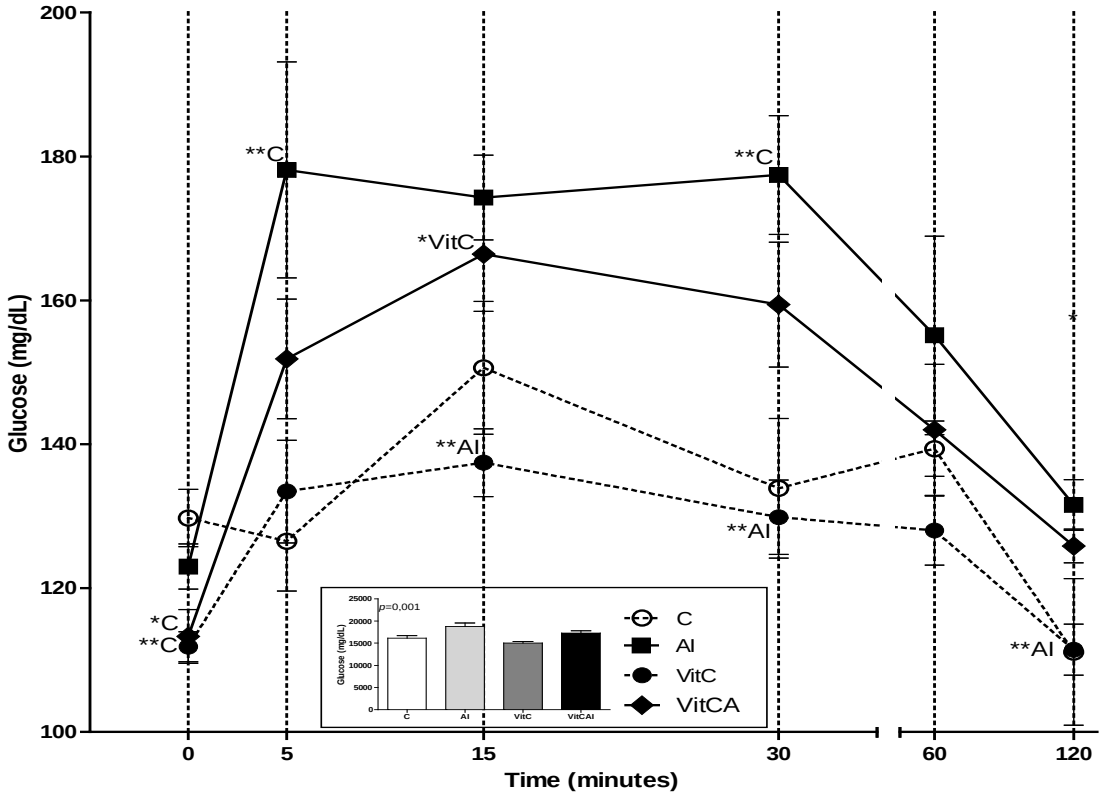

Figura 2. Curva glicêmica durante 120 minutos ipGTT e respectiva área sob a curva (AUC) de 120 minutos para os grupos C (n = 8), AI (n = 8), VitC (n = 8) e VitCAI (n = 8). Pontos retratam média±DP. Teste ANOVA (One-way ANOVA), *p<0,05 e **p<0,001.

Discussão

O tratamento, utilizando superdose de açúcar invertido a uma concentração de 32%, foi capaz de reproduzir o estágio de pré-diabetes nos ratos, uma vez que os induziu à tolerância à glicose diminuída (avaliado pelo teste de ipTGG). O cotratamento com vitamina C foi capaz de reduzir o ganho de peso induzido pela ingestão de açúcar invertido. Ao mesmo tempo, observou-se que a vitamina C foi efetiva para reduzir o acúmulo de gordura peritoneal. Tanto a redução do ganho de peso, quanto à redução de gordura visceral no grupo cotratado com vitamina C e açúcar invertido assemelharam-se ao grupo controle. Ambos os parâmetros (ganho de peso e gordura visceral) influenciaram efetivamente na diminuição do IMC dos ratos do grupo VitCAI. A avaliação do IMC é uma importante ferramenta para avaliar a obesidade[21], visto que é um dos fatores de risco para o desenvolvimento da DM2[11, 23].

A obesidade também está associada intimamente com uma alimentação inadequada[12, 14]. Estudos têm reportado que a alta ingestão calórica é o principal fator responsável pelo excesso de peso[23]. Bebidas adoçadas estimulam mais o apetite que alimentos sólidos, devido à baixa saciedade e compensação incompleta de calorias líquidas, sendo um importante contribuinte para o aumento da deposição de gordura[24, 25]. De fato, os grupos que receberam açúcares (Grupos AI e VitA) apresentaram as maiores ingestões de líquidos e menores de ração. Quanto à ingestão energética esses dois grupos apresentaram-se similares, entretanto o ganho de peso dos ratos cotratados com vitamina C foi 16% menor que aqueles que receberam somente açúcar. Possivelmente a vitamina C contribuiu para a redução do ganho de peso.

Al-Shamsi e colaboradores[5] evidenciaram que a redução do peso em ratos *Wistar* diabéticos (indução por streptozotocina) após a suplementação de vitamina C foi relacionada com a dose oferecida. Eles verificaram que doses moderadas e altas (50 mg/kg; 100 mg/kg) de vitamina C por 10 dias, tiveram um maior efeito redutor sobre o ganho de peso corporal, especialmente após o aparecimento do DM. Ainda neste contexto, Leffa e colaboradores[26] avaliando 1 mg/kg de vitamina C em ratos tratados com dieta de cafeteria durante 13 semanas, verificaram uma leve redução no peso destes animais, entretanto deve-se ressaltar que a ingestão calórica foi menor que os grupos controle e que recebiam dieta de cafeteria (biscoitos de chocolate, bolachas, marshmallows, salsichas, batatas fritas de queijo, batatas fritas, chips de bacon Doritos®, doce de amendoim, mocotó e refrigerantes). Embora o estudo de Al-Shamsi e colaboradores[5] tenha utilizado doses moderadas e altas (50 mg/kg; 100 mg/kg) de vitamina C para obter um efeito redutor sobre o ganho de peso corporal, o tempo utilizado (10 dias) foi bem menor que no nosso estudo. Por outro lado, Leffa e colaboradores[26] utilizaram uma dose de vitamina C (1 mg/kg) bastante inferior do que a testada neste trabalho, mas o tempo do experimento utilizado por eles foi

de apenas 13 semanas. Devemos ressaltar que no nosso estudo foi observada uma discrepância no ganho de peso dos animais cotratados com vitamina C e açúcar invertido em relação ao grupo que recebeu apenas açúcar invertido a partir da 12ª semana.

Assim como os mecanismos de ação da vitamina C na redução do ganho de peso não estão completamente estabelecidos, também se desconhece o efeito da vitamina C na DM[5]. Menos ainda sabe-se sobre os efeitos da vitamina C no prediabetes. No nosso estudo, ambos os grupos que receberam vitamina C apresentaram uma redução significativa na glicemia de jejum em relação ao grupo controle, mostrando um efeito hipoglicemiante da vitamina. Da mesma forma, a curva de AUC da glicose mostrou-se semelhando no grupo VitC e grupo controle. Quando associado com açúcar invertido a vitamina C apresentou uma redução na curva de AUC em relação ao grupo que recebeu apenas açúcar invertido. Ambos os estudos, avaliando a curva da AUC da glicose, mostraram que a vitamina C melhorou a tolerância à glicose. No estudo de Leffa e colaboradores[26], a suplementação com vitamina C ou com sucos que continham vitamina C não induziu diferença significativa na curva de AUC da glicose comparando esses tratamentos ao grupo de dieta de cafeteria. Similar aos nossos resultados, Al-Shamsi e colaboradores[5] encontraram um efeito redutor da glicemia de jejum e na curva de AUC na menor dose estudada de vitamina C por eles (10 mg/kg). É importante enfatizar que os mesmos autores não encontraram efeito hipoglicemiante na maior dose testada (100 mg/kg). Doses muito elevadas de vitamina C podem apresentar efeitos adversos à saúde, tais como, formação de cálculos renais e efeito pró-oxidantes[2].

Conclusão

A ingestão de vitamina C associada à overdose de açúcar em ratos pré-diabéticos foi anti-obesogênica e anti-hiperglicemiante. Este resultado é importante, considerando que dados epidemiológicos têm mostrado índices alarmantes de obesidade e, ao mesmo tempo mostrado um aumento crescente no consumo de bebidas adoçadas. Tais índices têm favorecido o aumento da população pré-diabética e, a suplementação com vitamina C pode ser utilizada como uma estratégia para prevenir o desenvolvimento do diabetes, sem a necessidade de altas doses. Estudos adicionais são necessários para entender os mecanismos anti-obesogênico e anti-hiperglicemiante da vitamina C no pré-diabetes.

Conflitos de interesses

Os autores declaram que não há conflitos de interesse.

Agradecimentos

Este estudo foi financiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brasil), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e o Laboratório de Nutrição Experimental da UNISC. Os autores agradecem aos colegas do Laboratório de Histologia e Patologia e do Laboratório de Biotecnologia Unisc pela ajuda.

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CAPÍTULO IV
NOTA À IMPRENSA

NOTA À IMPRENSA

Pesquisadores da Universidade de Santa Cruz do Sul, do Programa de Pós-graduação em Promoção da Saúde, realizaram um estudo com animais e comprovaram que ingerir doses adequadas de Vitamina C todos os dias pode melhorar o controle glicêmico no prediabetes.

A mestranda Patrícia Molz, sob a orientação da Dra. Silvia Isabel Rech Franke e co-orientação do Dr. Daniel Prá, liderou a pesquisa ao lado de uma equipe interdisciplinar, composta por nutricionistas, geneticista, médicos, patologista, químico, bioquímico, físicos e acadêmicos dos cursos de nutrição e de biologia. A pesquisa contou também com parcerias do Instituto de Física da Universidade Federal do Rio Grande do Sul (UFRGS) e do Programa de Biotecnologia da Universidade de Caxias do Sul (UCS), além de ser financiado pelo Edital Universal - MCTI/CNPq Nº 14/2013. Além de avaliarem o efeito da Vitamina C no prediabetes, eles pesquisaram mais dois nutrientes, o Cromo, considerado potencializador da insulina e, o Ferro, que em excesso tem sido associado ao desenvolvimento do diabetes.

Primeiramente eles sugeriram um modelo animal para indução de prediabetes, por meio do oferecimento, por via oral, de superdoses de um tipo de açúcar amplamente utilizado na indústria, o chamado açúcar invertido. Com a utilização do açúcar invertido não se necessitou usar drogas para induzir nos animais o estado pré-diabético, pois o uso de drogas os levariam a desenvolver o diabetes tipo 1, que não foi o foco do nosso estudo. É na prediabetes que se dá os primeiros estágios para o desenvolvimento da DM2 e quando associado a uma hiperglicemia crônica pode levar em cerca de 3 a 5 anos para o desenvolvimento do diabetes tipo 2 se não for controlado, mostrando o quão importante são as intervenções, pois podem retardar ou prevenir a doença.

Além de desenvolver em ratos, o pré-diabetes, que é o primeiro estágio para desenvolvimento do diabetes, doença que atinge cerca de 285 milhões de pessoas em todo o mundo, foram realizados, também, vários procedimentos padronizados pelo grupo, como avaliação antropométrica e classificação do estado nutricional e avaliação dos efeitos genotóxicos (agentes que causam danos ao DNA) causado pelos nutrientes.

CAPÍTULO V
CONSIDERAÇÕES FINAIS E PERSPECTIVAS

CONSIDERAÇÕES FINAIS

Este estudo avaliou o efeito de nutrientes (cromo, ferro, vitamina C e açúcar invertido) na citotoxicidade, estabilidade genômica e estresse oxidativo associados à hiperglicemia *in vivo* (ratos *Wistar*), no qual se pode concluir que:

- ✓ O modelo de pré-diabetes induzida por overdose de açúcar invertido foi eficaz, pois mimetizou as características metabólicas do pré-diabetes em humanos. Esse modelo conseguiu mimetizar as alterações metabólicas provocadas pelas substâncias amplamente utilizadas nas bebidas adoçadas.
- ✓ O tratamento com overdose de açúcar invertido induziu a obesidade e provocou o aumento de gordura visceral e disglicemia, causando tolerância à glicose diminuída, início para o desenvolvimento da resistência à insulina.
- ✓ O modelo de indução de pré-diabetes não danificou substancialmente o pâncreas dos animais, sendo que nesses animais apenas observou-se um aumento no dano do DNA primário no sangue e não no pâncreas. Nenhum aumento de micronúcleo de medula óssea ou proteína carboniladas foi observado.
- ✓ O modelo de indução de pré-diabetes utilizado neste estudo pode ser facilmente reproduzido e contribuir para futuras estratégias de intervenção no pré-diabetes.
- ✓ A suplementação de Cr sob a forma de $CrCl_3$ influenciou no metabolismo da glicose, em modelos de ratos de pré-diabetes, melhorando a tolerância à glicose.
- ✓ A ingestão de $CrCl_3$ possui o potencial de gerar dano de DNA no sangue, mas não no pâncreas dos ratos. Além disso, o $CrCl_3$ não gerou danos cromossômicos, conforme avaliado pelo ensaio de micronúcleo, nem aumentos de danos oxidativos pelo ensaio de proteínas de carboniladas.
- ✓ Este experimento pode contribuir para uma melhor compreensão do efeito do Cr (na forma $CrCl_3$), sobre o metabolismo da glicose e instabilidade genômica.
- ✓ A ingestão de vitamina C apresentou um efeito anti-obesogênico e anti-hiperglicemiante após a overdose de açúcar em ratos pré-diabéticos.
- ✓ A suplementação com vitamina C pode ser utilizada como uma estratégia de intervenção para prevenir o desenvolvimento do diabetes.
- ✓ A sobrecarga de Fe não influenciou na tolerância à glicose e na obesidade de ratos pré-diabéticos.
- ✓ O Fe apenas influenciou na genotoxicidade, sem ser mutagênico, na dose testada.

PERSPECTIVAS

A partir do estudo que se pretendia avaliar os efeitos dos nutrientes (cromo, ferro, vitamina C e açúcar invertido) na citotoxicidade, estabilidade genômica e estresse oxidativo associados à hiperglicemia, tanto *in vivo* (ratos *Wistar*) como *in vitro* (cultura celular), poder-se-ia:

- ✓ Compreender os mecanismos que atuam no metabolismo da glicose após a suplementação de Cr.
- ✓ Entender os mecanismos anti-obesogênico e anti-hiperglicemiante da vitamina C no pré-diabetes.
- ✓ Avaliar a concentração elementar de Fe e Cr no encéfalo dos ratos *Wistar*, após o tratamento com açúcar invertido, ferro, cromo e vitamina C;
- ✓ Verificar alterações histopalógicas no fígado, baço e encéfalo em ratos *Wistar* após a intervenção com os nutrientes: vitamina C, ferro, cromo.
- ✓ Avaliar o possível efeito potencializador do ferro na citotoxicidade, genotoxicidade e estresse oxidativo da glicose em ratos *Wistar*, após o tratamento com açúcar invertido;
- ✓ Avaliar a citotoxicidade (viabilidade celular, apoptose e necrose), *in vivo* (ratos *Wistar*), após o tratamento com glicose e frutose (açúcar invertido), ferro, cromo e vitamina C;
- ✓ Verificar a citotoxicidade (viabilidade celular, apoptose e necrose), genotoxicidade (dano primário no DNA, micronúcleos, brotos nucleares e pontes nucleoplásmicas) e estresse oxidativo (acumulação de 2′-7′-diclorofluoresceína: DCFH) induzidos pela glicose, frutose, ferro, cromo e vitamina C *in vitro* (cultura de células gliais);
- ✓ Verificar o possível efeito protetor do cromo e da vitamina C na citotoxicidade, genotoxicidade e estresse oxidativo induzidas pela glicose (*in vitro*);
- ✓ Verificar o possível efeito potencializador do ferro na citotoxicidade, genotoxicidade e estresse oxidativo da glicose (*in vitro*).

ANEXOS

ANEXO A – Artigo V

Este artigo está em preparação para ser submetido a Revisita *BioMetals (Springer)* – QUALIS INTERDISCIPLINAR – B2, Impact Factor: 2.689

Sobrecarga de Ferro induz a genotoxicidade, mas não a mutagenicidade em ratos pré-diabéticos

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Resumo

O papel da sobrecarga de ferro (Fe) e o risco de desenvolver DM2 ainda não são completamente compreendidos. Entretanto, sabe-se que a sobrecarga de Fe tem sido associada à instabilidade do genoma, e como um pró-oxidante pode provocar estresse oxidativo e danos celulares. Neste estudo, avaliamos, em ratos pré-diabéticos, o efeito da sobrecarga de Fe no metabolismo da glicose, na genotoxicidade e na mutagenicidade. Ratos *Wistar* machos foram tratados por via oral durante 17 semanas com sobrecarga de Fe (12,8 mg/L sob forma de sulfato ferroso) ou concomitantemente com 32% de açúcar invertido. Os ratos foram alocados nos seguintes grupos: controle água (C), controle açúcar invertido (AI), controle Fe (CFe) e açúcar invertido+Fe (FeAI). A obesidade foi avaliada pelo índice de massa corporal (IMC) e pela quantidade de gordura peritoneal. O estágio de prediabetes foi avaliado pelo Teste de Tolerância à Glicose Intraperitoneal (ipGTT). Fe elementar foi determinado por *Particle-Induced X-ray Emission* (PIXE). Danos primários ao DNA no sangue e no pâncreas foram avaliados pelo Ensaio Cometa. Danos cromossômicos foram determinados pelo ensaio de micronúcleos de medula óssea. A técnica de proteínas carboniladas foi utilizada para avaliar o nível de estresse oxidativo em sangue. A ingestão de Fe apontou diminuição da gordura peritoneal e da glicemia de jejum, mas não na obesidade e tolerância à glicose de ratos pré-

diabéticos. Ao mesmo tempo, o Fe no pâncreas apresentou-se diminuído, possivelmente, não influenciando na capacidade de secreção de insulina, uma vez que a concentração sanguínea de Fe não diferiu entre todos os grupos. O Fe mostrou-se genotóxico no sangue, mas não no pâncreas, conforme avaliado pelo ensaio cometa. Também não se observou geração de danos oxidativos, segundo avaliado pela formação de proteínas caboniladas no sangue, após sobrecarga de Fe, nem na mutagenicidade, conforme avaliado pelo teste do micronúcleo em medula óssea. Conclui-se que no estágio de prediabetes, a sobrecarga de Fe apenas influenciou na genotoxicidade, sem ser mutagênico, na dose testada.

Palavras chaves: Pré-diabetes, sobrecarga de ferro, tolerância à glicose, obesidade, concentração elementar, genotoxicidade, mutagenicidade

ANEXO B – Termo de Sigilo e Confidencialidade

UNISC
UNIVERSIDADE DE SANTA CRUZ DO SUL

TERMO DE SIGILO E CONFIDENCIALIDADE

Sr.(a) abaixo assinado, compromete-se a manter sigilo em relação a informações a que tiver acesso na qualidade de membro da banca examinadora constituída para análise de dissertação intitulada **Influência *In Vivo* e *In Vitro* de nutrientes sobre a citotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar** desenvolvido pelo **Programa de Pós-Graduação em Promoção da Saúde – Mestrado**, da Universidade de Santa Cruz do Sul - UNISC.

O abaixo assinado compromete-se ainda a usar as informações a que tiver acesso apenas com o propósito de avaliação da **dissertação**, não as revelando a qualquer titulo ou sob nenhum pretexto a terceiros.

A obrigação de sigilo não prevalece sobre informações que estejam sob domínio público antes da data de assinatura do presente instrumento ou que se tornaram públicas pelo Instituto Nacional de Propriedade Industrial-INPI ou por instituto competente em âmbito internacional.

O compromisso de sigilo é válido até que os direitos de propriedade intelectual relativos a **dissertação** tenham sido devidamente protegidos pela UNISC em âmbito nacional e internacional, perdurando até que o objeto da proteção caia em domínio público.
Caso as informações transmitidas na defesa da dissertação não sejam patenteáveis ou registráveis, ainda assim o signatário deve manter sigilo sobre elas, sendo o compromisso de sigilo válido por um período de 20 anos.

Caso o abaixo assinado descumpra quaisquer das obrigações previstas no presente Termo, a UNISC impetrará a(s) ação(ões) judicial(ais) cabível(eis) junto à autoridade competente, que aplicará as sanções de cunho civil e criminal pertinentes.

E PARA TODOS OS EFEITOS, firma o presente termo na presença das testemunhas abaixo assinadas.

Santa Cruz do Sul, 06 de abril de 2015.

_____

EXAMINADOR

Nome legível:

CPF:

Testemunhas:

1-_____ 2-_____

Nome: Nome:

CPF: CPF:

ANEXO C – Aprovação da Comissão de Ética no Uso de Animais (CEUA) da UNISC

UNIVERSIDADE DE SANTA CRUZ DO SUL – UNISC

PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO - PROPPG

COMISSÃO DE ÉTICA PARA USO DE ANIMAIS – CEUA/UNISC

PARECER DA CEUA - UNISC

Prezado Professor,

Após análise da atividade acadêmica submetida como projeto de pesquisa, intitulada "Influência *in vivo* e *in vitro* de nutrientes sobre a citotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar." e protocolada sob o número 14/2013, a Comissão de Ética no Uso de Animais – CEUA/UNISC considera o **protocolo aprovado** no que se refere o cumprimento do disposto na legislação nacional aplicável à utilização de animais para o ensino e a pesquisa.

Atenciosamente,

Coord. Comitê de Ética no Uso de Animais
UNISC

Santa Cruz do Sul, 15 de Julho de 2013.

ANEXO D – Autorização do Laboratório de Nutrição Experimental

UNISC
UNIVERSIDADE DE SANTA CRUZ DO SUL

Pró-reitoria de Pesquisa e Pós-graduação
Comissão de Ética para Uso de Animais - CEUA
Carta de Autorização do Coordenador do Laboratório

Eu, Silvia Isabel Rech Franke

Coordenador do Laboratório de Nutrição Experimental da UNISC , declaro para os devidos

fins que estou ciente e de acordo que a atividade acadêmica intitulada
Influência IN VIVO e IN VITRO de nutrientes sobre a citotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar.

e coordenada pelo(a) Prof. Drª Silvia Isabel Rech Franke

seja desenvolvida no laboratório supracitado.

Estou ciente também de que a mesma só poderá iniciar após aprovação da Comissão de Ética para o Uso de Animais - CEUA/UNISC.

Santa Cruz do Sul, 28 de maio de 20 13 .

Coordenador do Laboratório

Observação: quando o Coordenador do Laboratório for também o responsável legal pela atividade, solicitar assinatura do Chefe do Departamento o qual o mesmo está vinculado.

Data de Criação 17.12.2010 Md.9.01.005

ANEXO E – Autorização do Laboratório de Histologia e Patologia

UNISC
UNIVERSIDADE DE SANTA CRUZ DO SUL

Pró-reitoria de Pesquisa e Pós-graduação
Comissão de Ética para Uso de Animais - CEUA
Carta de Autorização do Coordenador do Laboratório

Eu, Juarez Alaor Schimidt ,

Coordenador do Laboratório de Histologia e Patologia da UNISC , declaro para os devidos fins que estou ciente e de acordo que a atividade acadêmica intitulada Influência IN VIVO e IN VITRO de nutrientes sobre a ciotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar,

e coordenada pelo(a) Prof. Drª Silvia Isabel Rech Franke ,

seja desenvolvida no laboratório supracitado.

Estou ciente também de que a mesma só poderá iniciar após aprovação da Comissão de Ética para o Uso de Animais - CEUA/UNISC.

Santa Cruz do Sul, 28 de maio de 2013 .

ISTO OK!
S.H.G.

Coordenador do Laboratório

Observação: quando o Coordenador do Laboratório for também o responsável legal pela atividade, solicitar assinatura do Chefe do Departamento o qual o mesmo está vinculado.

Data de Criação 17.12.2010 Md. 5.01.196

ANEXO F – Autorização do Laboratório de Genética e Biotecnologia

UNISC
UNIVERSIDADE DE SANTA CRUZ DO SUL

Pró-reitoria de Pesquisa e Pós-graduação
Comissão de Ética para Uso de Animais - CEUA
Carta de Autorização do Coordenador do Laboratório

Eu, Alexandre Rieger ,

Coordenador do Laboratório de Genética e Biotecnologia da UNISC , declaro para os devidos fins que estou ciente e de acordo que a atividade acadêmica intitulada Influência IN VIVO e IN VITRO de nutrientes sobre a citotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar.

e coordenada pelo(a) Prof. Drª Silvia Isabel Rech Franke ,

seja desenvolvida no laboratório supracitado.

Estou ciente também de que a mesma só poderá iniciar após aprovação da Comissão de Ética para o Uso de Animais - CEUA/UNISC.

Santa Cruz do Sul, 28 de maio de 2013.

Coordenador do Laboratório

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Data de Criação 17.12.2010 Mod. 5.01.195

ANEXO G – Autorização do Laboratório de Implantação Iônica da UFRGS

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UNIVERSIDADE DE SANTA CRUZ DO SUL

Pró-reitoria de Pesquisa e Pós-graduação
Comissão de Ética para Uso de Animais - CEUA
Carta de Autorização do Coordenador do Laboratório

Eu, Johnny Ferraz Dias

Coordenador do Laboratório de Implantação Iônica da UFRGS , declaro para os devidos fins que estou ciente e de acordo que a atividade acadêmica intitulada Influência IN VIVO e IN VITRO de nutrientes sobre a citotoxicidade e estabilidade genômica associadas à sobrecarga de açúcar.

e coordenada pelo(a) Prof. Drª Silvia Isabel Rech Franke

seja desenvolvida no laboratório supracitado.

Estou ciente também de que a mesma só poderá iniciar após aprovação da Comissão de Ética para o Uso de Animais - CEUA/UNISC.

Santa Cruz do Sul, 28 de maio de 2013.

Prof. Johnny Ferraz Dias
Lab. de Implantação Iônica
Instituto de Física - UFRGS

Coordenador do Laboratório

Observação: quando o Coordenador do Laboratório for também o responsável legal pela atividade, solicitar assinatura do Chefe do Departamento o qual o mesmo está vinculado.

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ANEXO H – Instrução para autores – Journal of Diabetes Research

Journal of Diabetes Research (formerly titled Experimental Diabetes Research) Author Guidelines

AUTHOR GUIDELINES

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This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described.

Results and Discussion

This section may each be divided by subheadings or may be combined.

Conclusions

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ANEXO I – Instrução para autores – NIMB: Nuclear Instruments and Methods in Physics Research B

Nuclear Instruments and Methods in Physics Research B, Author Guidelines

GUIDE FOR AUTHORS

.Your Paper Your Way

INTRODUCTION

Nutrition provides an international forum for professionals interested in the **applied and basic** biomedical **nutritional sciences, and publishes papers both of clinical interest and of scientific import.** Investigators are encouraged to submit papers in the disciplines of nutritionally related biochemistry, genetics, immunology, metabolism, molecular and cell biology, neurobiology, physiology, and pharmacology. Papers on nutrition-related plant or animal sciences which are not of direct relevance to man, whereas occasionally of interest are not the main focus of the Journal.

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Papers representing concise and original studies of scientific importance are considered. In the cover letter the author should justify the request for Rapid Communication. The review process is 10 days, authors are allowed one revision if accepted, and the final version of the paper appears in the next available issue of the journal.

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A Research Letter contains new data or a clinical observation, in a format that allows for rapid publication.

Review Article (up to 5000 words including tables, figures and references)
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Case Report (up to 2500 words including tables figures, and references) Case Reports include case studies of 4 or fewer patients that describe a novel situation or add important insights into mechanisms, diagnosis or treatment of a disease.

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Hypothesis (up to 3000 words including tables, figures and references)

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BEFORE YOU BEGIN

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State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

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This should include 1) title of paper (**use no abbreviations, limit: 120 characters with spaces**), 2) running head of fewer than 55 characters with spaces, 3) full names of all authors with highest academic degree(s); 4) affiliations of all authors; 4) role of each author in the

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ANEXO K – Instrução para autores – Brazilian Journal of Nutrition

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Scientific note: partial unpublished data of an ongoing research (maximum limit of 4 thousand words).

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Authors should indicate the article's category and subject area, namely: food and social sciences, nutritional assessment, nutritional biochemistry, nutrition, nutrition education, epidemiology and statistics, micronutrients, clinical nutrition, experimental nutrition, nutrition and geriatrics, nutrition, maternal and infant nutrition in meal production, food and nutrition policies and health.

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Preparation of the manuscript

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Article with one author

Burlandy L. A construção da política de segurança alimentar e nutricional no Brasil: estratégias e desafios para a promoção da intersetorialidade no âmbito federal de governo. Ciênc Saúde Coletiva. 2009; 14(3):851-60. doi: 10.1590/S1413-81232009000300020.

Article in electronic media

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Book

Alberts B, Lewis J, Raff MC. Biologia molecular da célula. 5ª ed. Porto Alegre: Artmed; 2010.

Electronic book

Brasil. Alimentação saudável para pessoa idosa: um manual para o profissional da saúde [Internet]. Brasília: Ministério da Saúde; 2009 [acesso 2010 jan 13]. Disponível em: <http://200.18.252.57/services/e-books/alimentacao_saudavel_idosa_profissionais_saude.pdf>.

Book chapters

Aciolly E. Banco de leite. In: Aciolly E. Nutrição em obstetrícia e pediatria. 2ª ed. Rio de Janeiro: Guanabara Koogan; 2009. Unidade 4.

Electronic book chapters

Emergency contraceptive pills (ECPs). In: World Health Organization. Medical eligibility criteria for contraceptive use [Internet]. 4th ed. Geneva: WHO; 2009 [cited 2010 Jan 14]. Available from: <http://whqlibdoc.who.int/publications/2009/9789241563888_eng.pdf>.

Dissertations and theses

Duran ACFL. Qualidade da dieta de adultos vivendo com HIV/AIDS e seus fatores associados [mestrado]. São Paulo: Universidade de São Paulo; 2009.

Electronic texts

Sociedade Brasileira de Nutrição Parental e Enteral [Internet]. Assuntos de interesse do farmacêutico atuante na terapia nutricional. 2008/2009 [acesso 2010 jan 14]. Disponível em: <http://www.sbnpe.com.br/ctdpg. php?pg=13&ct=A>.

Software

Software de avaliação nutricional. DietWin Professional [programa de computador]. Versão 2008. Porto Alegre: Brubins Comércio de Alimentos e Supergelados; 2008.

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