

UNIVERSIDADE ESTADUAL DO OESTE DO PARANÁ - CAMPUS DE CASCAVEL  
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE  
PROGRAMA DE PÓS-GRADUAÇÃO *STRICTO SENSU* EM BIOCÊNCIAS E SAÚDE –  
NÍVEL MESTRADO

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**EFEITO DA DERIVAÇÃO DUODENO-JEJUNAL SOBRE A  
SECREÇÃO DE INSULINA EM RATOS OBESOS PELA  
DIETA DE CAFETERIA**

CASCAVEL-PR

Fevereiro/2015

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Dissertação apresentada ao Programa De Pós-Graduação *Stricto Sensu* em Biociências e Saúde – Nível Mestrado, do Centro de Ciências Biológicas e da Saúde, da Universidade Estadual do Oeste do Paraná, como requisito parcial para a obtenção do título de Mestre em Biociências e Saúde.

Área de concentração: Fatores que influenciam a morfofisiologia orgânica

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CASCADEL-PR

Fevereiro/2015

**Dados Internacionais de Catalogação-na-Publicação (CIP)**

M492e

Mendes, Mariana Carla

Efeito da derivação duodeno-jejunal sobre a secreção de insulina em ratos obesos pela dieta de cafeteria. /Mariana Carla Mendes. Cascavel, PR: UNIOESTE, 2015.

81 p.

Orientadora: Prof<sup>a</sup>. Dr<sup>a</sup>. Sandra Lucinei Balbo

Orientadora: Prof<sup>a</sup>. Dr<sup>a</sup>. Maria Lúcia Bonfleur

Dissertação (Mestrado) – Universidade Estadual do Oeste do Paraná, Campus de Cascavel, 2015.

Programa de Pós-Graduação *Stricto Sensu* em Biociências e Saúde, Centro de Ciências Biológicas e da Saúde.

1. Derivação duodeno-jejunal. 2. Secreção de insulina. 3. Massa das células- $\beta$ . 4. Obesidade. 5. Dieta da cafeteria. I. Balbo, Sandra Lucinei. II. Bonfleur, Maria Lúcia. III. Universidade Estadual do Oeste do Paraná. IV. Título.

CDD 21.ed. 616.398  
CIP-NBR 12899

Ficha catalográfica elaborada por Helena Soterio Bejio CRB-9<sup>a</sup>/965

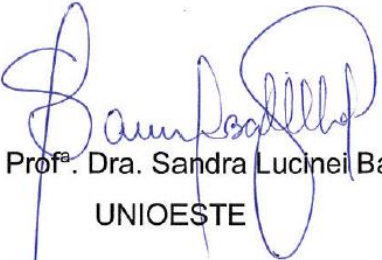
FOLHA DE APROVAÇÃO

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Esta dissertação foi julgada adequada para a obtenção do título de Mestre em Biociências e Saúde e aprovada em sua forma final pelo Orientador e pela Banca Examinadora.

  
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(fevereiro/2015)

## **DEDICATÓRIA**

Dedico esse trabalho ao meu amado avô José Florentino Mendes (em memória). Um homem que pra mim foi o maior exemplo de honestidade, sabedoria e simplicidade. E que tenho certeza que me apoiaria nessa jornada e que, onde estiver, está feliz por mais essa conquista! A ti meu avô meu amor eterno.

## AGRADECIMENTOS

A **Deus**, pelo dom da vida, pela inteligência e sabedoria dada nesse período. Principalmente pela paciência nos momentos difíceis e também por não me abandonar nos momentos que me senti totalmente sozinha. A Ti meu Deus, toda honra e glória sempre.

A minha mãe **Sonia**, por todo amor, carinho e paciência. Agradeço também pelo apoio nessa etapa desde incentivo ao apoio financeiro. Ao meu pai **Luiz**, pela paciência, compreensão, e amor que reservou pra mim. Agradeço meu pai por sonhar esse sonho comigo, e por ser esse parceiro. Obrigada aos dois por abrirem mão dos seus próprios sonhos para me ajudar a conquistar os meus.

A minha irmã **Luana** e meu cunhado **Diego** que apesar da distancia sei que sempre torceram pela minha felicidade e meu sucesso.

A minha orientadora **Sandra**, primeiramente pela confiança depositada desde a graduação, pela paciência em me ensinar. Obrigada pelas conversas, conselhos, puxões de orelha e carinho. Você foi verdadeiramente uma mãe, aliás uma jovem-mãe! A minha co-orientadora **Maria Lúcia**, pela gigantesca paciência em cada erro, por confiar em mim, por ter me ensinado cada passo dentro do laboratório e por ter aceitado minhas limitações. Vocês são minha inspiração e cada aprendizado e momento vivido serão guardados em meu coração.

Ao pessoal do **LAFEM** que durante esses anos me acompanharam no laboratório. Em especial gostaria de agradecer a **Camila**, ao **Rodrigo** e a **Iala**, por auxiliarem durante todo o período que a pesquisa foi realizada. Aos demais, meu agradecimento por todo esforço, aprendizado e palavra amiga nesse tempo.

A minha amiga **Fernanda**, primeiramente por ter me dado o empurrão para a vida científica. Você faz parte dessa conquista! Obrigada pela amizade de anos, pelas palavras amigas e pelos conselhos. A minha amiga **Cintya**, que desde sempre acreditou em mim, me dando forças para continuar, e sendo minha intercessora. A minha amiga **Ana Flávia**, que dividiu comigo a vida no laboratório, que me ajudou em cada experimento, que me ajudou com as dúvidas e principalmente que foi super companheira...a saudade de você é grande! Ao meu amigo **Dener**, que mesmo trilhando caminhos totalmente diferentes sempre se fez presente na minha vida, e sempre torceu pelo meu sucesso e minha felicidade.

As amigas que a XXX turma de enfermagem UNIOESTE me proporcionou: **Wevellen, Jolana e Giovanna...** vocês podem não ter acompanhado de perto o passo a passo disso tudo, mas sempre se fizeram presentes de alguma maneira. Obrigada por tudo!

Ao técnico do laboratório de técnicas operatórias da UNIOESTE **Assis Escher**, por toda ajuda durante o período operatório dos animais. Por ter dedicado do tempo que poderia ter passado com sua família para me ajudar a conquistar mais esse sonho.

E finalmente a **III Turma do Mestrado em Biociências e Saúde**, pelo companheirismo, carinho, brincadeiras e por tudo que dividimos nesses dois anos. Nossa turma foi demais! Sempre demonstramos um cuidado com o outro e as aulas não seriam as mesmas sem vocês! Obrigada pelo apoio, pelas risadas, churrascos, e principalmente pela mão amiga que sempre estenderam a mim!

## RESUMO

O presente estudo avaliou o efeito da derivação duodeno jejunal (DDJ) em ratos obesos pela dieta de cafeteria (CAF) sobre a morfofisiologia, com ênfase na ação de agentes potencializadores da secreção de insulina. Ratos *Wistar* com 8 semanas de vida receberam dieta padrão formando o crupo controle (CTL) ou CAF *ad libitum*. Após 10 semanas, os ratos CAF foram submetidos à falsa operação ou a DDJ, formando os grupos CAF SHAM e CAF DDJ, respectivamente. Após dois meses, foram avaliados os parâmetros de obesidade, resistência à insulina (RI), morfologia do pâncreas, secreção de insulina estimulada por glicose, carbacol (CCh), PMA, IBMX e Forscolina, e a expressão proteica de glicoquinase (GCK), receptor muscarínico M3, proteína quinase C e A (PKC e PKA) e Sintaxina-1, nas ilhotas. Os animais do grupo CAF SHAM tornaram-se obesos, hiperglicêmicos, hiperinsulinêmicos, intolerantes à glicose e resistentes à insulina. Além disso, apresentaram aumento na massa da ilhota e das células  $\beta$ . A secreção de insulina estimulada por 11.1 mM de glicose na presença de CCh, PMA, Forskolin ou IBMX foi significativamente maior no grupo CAF SHAM em relação ao grupo CTL. A DDJ não alterou o peso corporal, mas normalizou a glicemia, insulinemia, a RI e diminuiu a massa das ilhotas e das células  $\beta$ . A secreção de insulina estimulada pelos diferentes secretagogos também foi normalizada no grupo CAF DDJ. A expressão proteica da GCK, PKC, PKA e sintaxina não foi alterada no grupo CAF SHAM em relação ao CTL, todavia a expressão do receptor M3 foi reduzida. A DDJ não influenciou na expressão dessas proteínas. Em conclusão, a cirurgia de DDJ normalizou a glicemia, a insulinemia e a RI, reduziu a massa das ilhotas e das células  $\beta$  bem como, normalizou a secreção de insulina via PKC e PKA.

**Palavras-chave:** Derivação duodeno jejunal; secreção de insulina; massa das células  $\beta$ ; obesidade; dieta de cafeteria.



## ABSTRACT

This study was designed to evaluate whether DJB in western diet (WD)-obese rats could have effects on pancreatic  $\beta$ -cell morphology and islet responsiveness to potentiating agents involved with the protein kinase A (PKA) and C (PKC) pathway. Male *Wistar* rats with 8 weeks of life were fed a standard rodent chow diet (CTL group) or WD *ad libitum*. After 10 weeks, WD rats were submitted to sham operation or DJB, forming WD SHAM and WD DJB group, respectively. After two months, the obesity parameters, insulin resistance (IR), pancreas morphology, insulin secretion stimulate by different secretagogues and islet protein expression were verified. WD SHAM rats displayed obesity, hyperglycemia, hyperinsulinemia, glucose intolerance and IR. In addition, they also presented an increase in islets and  $\beta$ -cells area and mass. The glucose-induced insulin secretion stimulated by 11.1 mM glucose in the presence of carbachol (CCh), PMA, Forskolin or IBMX was higher in WD SHAM islets than CTL islets. DJB surgery did not alter body weight but normalized glucose, insulinemia, IR, and decreased islet and  $\beta$ -cells mass. The insulin release stimulated by glucose in the presence of CCh, PMA, Forskolin or IBMX was too normalized in WD DJB group. The expression of glucokinase, PKC, PKA and syntaxin was not altered in WD SHAM rats, but the M3 receptor was down-regulated. DJB did not influence these protein expressions. DJB surgery normalized glicemia, insulinemia and IR, reduced islets and  $\beta$ -cell mass and normalized insulin secretion by PKC and PKA pathway.

**Keywords:** Duodenal–jejuna bypass surgery; insulin secretion;  $\beta$ -cell mass; obesity; western diet rats

## SUMÁRIO

|   |           |
|---|-----------|
| <b>RESUMO.....</b>  | <b>7</b>  |
| <b>ABSTRACT.....</b>  | <b>9</b>  |
| <b>LISTA DE FIGURAS.....</b>  | <b>11</b> |
| <b>LISTA DE ABREVIATURAS.....</b>                                   | <b>12</b> |
| <b>INTRODUÇÃO.....</b>  | <b>14</b> |
| <b>REVISÃO DE LITERATURA.....</b>                                   | <b>18</b> |
| Obesidade e <i>diabetes mellitus</i> tipo 2.....                    | 18        |
| Secreção da insulina.....   | 19        |
| Proliferação das células $\beta$ pancreáticas.....                  | 21        |
| Cirurgias bariátricas.....  | 22        |
| Modelos experimentais de obesidade animal e dieta de cafeteria..... | 25        |
| <b>REFERÊNCIAS.....</b>   | <b>28</b> |
| <b>ARTIGO CIENTÍFICO.....</b>                                       | <b>40</b> |
| <b>APÊNDICES.....</b>   | <b>68</b> |
| <b>ANEXOS.....</b>  | <b>69</b> |

## LISTA DE FIGURAS

|  |         |
|--|---------|
| <b>Figura 1</b> Desvio ou Derivação Duodeno-jejunal (DDJ) proposta por Rubino <i>et al.</i> (2006) (A);<br>Derivação Duodeno-jejunal modificada proposta por Jurowich <i>et al.</i> (2013)<br>(B)..... | Pág. 24 |
|--|---------|

## LISTA DE ABREVIATURAS

|                  |  |
|------------------|--|
| AC               | Adenilato Ciclase                                    |
| Acetil-CoA       | Acetil-coenzima A                                    |
| Ach              | Acetilcolina   |
| AMPc             | Adenosina monofosfato cíclico                        |
| ATP              | Adenosina trifosfato                                 |
| BPD              | Derivação biliopancreática                           |
| Ca <sup>2+</sup> | Cálcio   |
| CAF              | Dieta de cafeteria                                   |
| CCh              | Carbacol   |
| CTL              | Controle   |
| DAG              | Diacilglicerol                                       |
| DDJ              | Derivação Duodeno Jejunal                            |
| DM               | Diabetes mellitus                                    |
| DM2              | Diabetes mellitus tipo 2                             |
| GCK              | Glicoquinase   |
| GIP              | Polipeptídeo inibidor gástrico                       |
| GLP-1            | Peptídeo semelhante ao glucagon 1                    |
| GLUT2            | Proteína transportadora de glicose 2                 |
| HGF              | Fator de crescimento do hepatócito                   |
| IBMX             | <i>3-isobutyl-1-methylxanthine</i>                   |
| IGF-1            | Fatores de crescimento semelhante à insulina         |
| IP3              | Inositol-1,4,5-trifosfato                            |
| KIC              | 2-ketoisocaproato                                    |
| MARCKS           | <i>Myristoylated alanine-rich C kinase substrate</i> |
| M3               | Receptor muscarínico                                 |
| PDX-1            | Fator de transcrição pancreático                     |

|      |  |
|------|--|
| PKA  | Proteína quinase A                     |
| PKC  | Proteína quinase C                     |
| PLC  | Fosfolipase C                          |
| PMA  | <i>Phorbol 12-myristate 13-acetate</i> |
| PO   | Pós-operatório                         |
| PYY  | Polipeptídeo YY                        |
| RYGB | <i>Roux-en-Y gastric bypass</i>        |
| SHAM | Falsa operação                         |

## INTRODUÇÃO

A obesidade é uma síndrome de abrangência mundial e está associada com o desenvolvimento de várias doenças, dentre elas, o *diabetes mellitus* tipo 2 (DM2). A mudança do estilo de vida, com baixa atividade física, alto consumo de alimentos hipercalóricos, níveis de estresse cotidiano elevados, não acompanhados de mudanças genéticas e fisiológicas, são apontadas como os grandes responsáveis pela promoção de doenças crônicas como o DM2 (GOTTLIEB; CRUZ; BODANESE, 2008).

Dados mostram que o excesso de peso e a obesidade têm aumentado no Brasil. De acordo com a pesquisa “Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico” (Vigitel), realizada em 2011 pelo Ministério da Saúde, a proporção de pessoas acima do peso no Brasil aumentou de 42,7%, em 2006, para 48,5%, em 2011. Ainda de acordo com essa pesquisa, no mesmo período, o percentual de obesos subiu de 11,4% para 15,8%.

A etiologia da obesidade, por ser caracterizada como multifatorial, é de difícil interpretação, identificação e definição de estratégias de tratamento. Sabe-se que a interação entre fatores ambientais, comportamentais, culturais, genéticos, fisiológicos e psicológicos é a principal causa para o ganho de peso. Dentre estes, acredita-se que os fatores externos são mais relevantes em sua incidência do que os fatores genéticos (DÂMASO, 2003). Além disso, estudos mostram que adultos obesos desde a infância apresentam mais dificuldade no convívio social, no relacionamento amoroso e sexual, na vida profissional e nos cuidados com a saúde (MELO; SERRA; CUNHA, 2010). Devido a essa etiologia tão complexa, é necessário programas para redução do peso corporal que enfoquem as bases do comportamento alimentar e desenvolvam ações interdisciplinares que permitam obter resultados eficazes no tratamento da obesidade. Simpósios e congressos sobre o assunto devem ser desenvolvidos no intuito de discussões de diversas vertentes que envolvam essa patologia, levando em consideração métodos interdisciplinares, devendo observar aspectos genéticos e ambientais, a fisiopatologia dessa doença, os aspectos psicológicos e, também, as alternativas farmacológicas e procedimentos cirúrgicos existentes para o controle e tratamento da obesidade. Além disso, é importante disseminar a relevância e eficácia dos alimentos e das práticas alimentares para preservação da saúde e da qualidade de vida e também na prevenção e no tratamento do excesso de peso e da obesidade (BARROSO, 2012). Por isso, a obesidade e suas comorbidades não podem ser investigadas, prevenidas ou tratadas com uma visão de

mão única, é necessária a interdisciplinaridade entre diversas áreas, como a da saúde, da educação, das políticas públicas, entre outras, para que possa se realizar o melhor controle dessas doenças.

O DM é considerado uma epidemia mundial, tornando-se grande desafio para os sistemas de saúde de todo o mundo. De acordo com a Federação Internacional de Diabetes (IDF), a doença afetou 366 milhões de pessoas por todo o mundo e, acredita-se que até 2030 afete 552 milhões (IDF, 2012). O alto impacto econômico ocorre notadamente nos serviços de saúde, como consequência dos crescentes custos do tratamento da doença e, sobretudo das complicações relacionadas com o DM, sendo que os custos com o tratamento da obesidade grave atingem hoje R\$ 116 milhões (BRASIL, 2013). Além disso, a expectativa de vida é reduzida em média em 15 anos para o DM tipo 1 e em 5 a 7 anos no DM2 (BRASIL, 2006). Atualmente existem quatro milhões de mortes por ano relativas ao DM e suas complicações, o que representa 9% da mortalidade mundial total.

A obesidade está associada ao DM2 e está bem esclarecido na literatura que aproximadamente 80% dos pacientes com DM2 são ou foram obesos (FELBER, 1992). A DM2 é uma doença metabólica complexa caracterizada por alterações nos mecanismos envolvidos na regulação da secreção pancreática de insulina e no desenvolvimento da resistência à insulina nos órgãos periféricos, resultando em hiperglicemia e glicotoxicidade. A RI é inicialmente observada no tecido muscular, onde uma concentração crescente de insulina é necessária para permitir a captação de glicose pelo tecido. A resistência à insulina é influenciada tanto por fatores ambientais (obesidade, inatividade física) como por genéticos <sup>[1]</sup>

A capacidade das ilhotas pancreáticas em responder às elevações na concentração plasmática de glicose, com aumento na secreção de insulina, é o evento principal na preservação da homeostase glicêmica e inibição da instalação do quadro de DM2. Essa capacidade depende de uma regulação na maquinaria secretória das células  $\beta$ . A secreção é ajustada, continuamente, de acordo com as flutuações nas concentrações de nutrientes circulantes, em especial, a glicose (BOSCHERO, 1996). Todavia, outros fatores participam desse processo. Além dos aminoácidos, ácidos graxos, hormônios intestinais e os produzidos pelas ilhotas pancreáticas, o Sistema Nervoso Autônomo (SNA) possui um papel importante na modulação da secreção de insulina (PÉNICAUD *et al.*, 1996).

Tanto a metabolização da glicose como a ação de neurotransmissores e hormônios em receptores específicos na célula  $\beta$  pancreática, podem aumentar a formação de adenosina monofosfato cíclico (AMPc), que por sua vez, estimula a proteína quinase A (PKA) e/ou estimula a formação de inositol 1, 4, 5-trifosfato (IP3) e diacilglicerol (DAG), o qual estimula

a proteína quinase C (PKC). Todos estes eventos culminam com o aumento da concentração de cálcio ( $\text{Ca}^{2+}$ ) intracelular e exocitose dos grânulos secretórios contendo insulina (AMMALA *et al.*, 1993; BERRIDGE *et al.*, 2003; GRAPENGIESSER *et al.*, 1991; JONES; PERSAUD, 1998; RIBEIRO *et al.*, 2010; SEINO; SHIBASAKI, 2005).

As células  $\beta$ -pancreáticas, muitas vezes, conseguem compensar o estado hiperglicêmico, aumentando a secreção de insulina, por este motivo muitas pessoas obesas e com a resistência a insulina não desenvolvem DM2. Este mecanismo compensatório é possível graças à enorme plasticidade do pâncreas endócrino que é amplamente regulado pelas variações na demanda da produção de insulina. A dinamicidade dessas células tem se confirmado ao longo das últimas décadas, atualmente se sabe que a regulação da massa das células  $\beta$  e das ilhotas pancreáticas é responsável por manter a euglicemia. Alguns fatores como obesidade, gestação e pancreatômia parcial também podem levar a um aumento na massa das células  $\beta$  pancreáticas <sup>[2]</sup>; <sup>[3]</sup>. Outros fatores, como o fator de crescimento do hepatócito (HGF), lactogênio placentário, insulina, fator de crescimento semelhante à insulina (IGF-1), glicose e muitas outras moléculas têm sido relacionadas com o aumento da proliferação de células  $\beta$  em ratos e camundongos (KULKARNI *et al.*, 2012).

A obesidade e o DM2 têm sido amplamente estudados em modelos animais experimentais. Existem diversas maneiras de se induzir essa obesidade, seja por modificações genéticas, como camundongos *ob/ob*, seja pela lesão hipotalâmica através da administração do Glutamato Monossódico (MSG), ou indução da obesidade de maneira exógena, ou seja, pela oferta da dieta de cafeteria ou dieta ocidentalizada, que contém alimentos hipercalóricos. Esse tipo de obesidade é o que mais se assemelha com a obesidade humana (CEZARETTI; KOHLMANN, 2006; DIEMEN; TRINDADE, 2006).

Na tentativa de prevenir ou elaborar estratégias terapêuticas para minimizar os danos corpóreos causados pela obesidade e suas consequências, muitos tratamentos vêm sendo testados, como o incentivo a atividade física; mudança de hábitos nutricionais, tratamento farmacológico e procedimentos cirúrgicos. Estudos experimentais e clínicos sugerem que as cirurgias de derivação gastrointestinal podem alterar os mecanismos funcionais do intestino, responsáveis pelas anormalidades da homeostase glicêmica. Esta hipótese representa um novo paradigma que caracteriza o DM2 como uma doença intestinal e potencialmente tratada pela cirurgia. As intervenções no trato gastrointestinal podem levar ao controle do DM2 por mecanismos que são independentes da perda de peso (RUBINO, 2008; RUBINO, *et al.*, 2010; RUBINO, *et al.*, 2006; RUBINO, *et al.*, 2004a; RUBINO, *et al.*, 2004b). Araújo *et al.* (2012), mostraram que animais submetidos à Derivação Duodeno-Jejunal (DDJ) secretaram mais



insulina, estimulada por diferentes concentrações de glicose, quando comparados ao grupo controle. Contudo, os mecanismos envolvidos na regulação da secreção de insulina em animais obesos submetidos às cirurgias bariátricas ainda não foram esclarecidos. Desta forma, considerando que:

- a obesidade está diretamente relacionada com o DM2 e que muitos estudos recentes têm mostrado a efetividade das cirurgias bariátricas no controle dessas doenças, tanto em pacientes obesos quanto em modelos animais experimentais de obesidade;

- existem poucos estudos em relação às cirurgias bariátricas e a investigação dos mecanismos envolvidos na regulação da secreção de insulina; justifica-se a importância de estudar a secreção de insulina em ratos obesos pela dieta de cafeteria submetida à DDJ, na tentativa de responder se a cirurgia influencia a morfologia das ilhotas pancreáticas e a responsividade dessas ilhotas a diferentes secretagogos envolvidos com as vias de sinalização da PKA e da PKC.

## REVISÃO GERAL DE LITERATURA

### *Obesidade e diabetes mellitus tipo 2*

O crescimento da obesidade e as morbidades que estão associadas a essa doença é uma das maiores preocupações da comunidade médica e científica. Nas últimas décadas a obesidade alcançou proporções alarmantes, principalmente em países desenvolvidos e em desenvolvimento, atingindo indivíduos de todas as idades. Essa patologia tem sido alvo da saúde pública e vem se tornando problema de grande escala por causar complicações cardiovasculares, metabólicas, reprodutivas, neoplásicas, musculoesqueléticas e psicossociais (TADROSS; Le ROUX, 2009). De acordo com a *World Health Organization* (WHO) em 2008, cerca de 1,5 bilhões de adultos com 20 anos ou mais sofriam de sobrepeso, e mais de 200 milhões de homens e quase 300 milhões de mulheres estavam obesos no mundo todo. No Brasil, nesse mesmo período, a porcentagem de pessoas que eram alvo do sobrepeso e obesidade estava em torno de 39% (TARASTCHUK *et al.*, 2008).

Ainda de acordo com a WHO (2011), a principal causa da obesidade é o desequilíbrio energético, gerado pelo excesso de calorias ingeridas, em relação à quantidade que é gasta por um indivíduo. O Índice de Massa Corpórea (IMC) que é calculado pelo peso de uma pessoa em kilogramas dividido por sua altura em metros ao quadrado ( $\text{kg}/\text{m}^2$ ), é amplamente usado para a determinação de sobrepeso e obesidade - um resultado de  $25 \text{ kg}/\text{m}^2$  ou mais, indica sobrepeso; enquanto  $30 \text{ kg}/\text{m}^2$  ou mais, determina obesidade. Porém, é necessário que se leve em conta também o acúmulo excessivo de gordura na região abdominal, pois já existem evidências que a deposição de tecido adiposo visceral leva à obesidade central, a qual está ligada a fatores de riscos cardiovasculares (LINHARES, 2012).

Além do risco de desenvolver complicações cardiovasculares, indivíduos obesos estão propensos ao desenvolvimento de problemas psicológicos, doenças respiratórias (SAMANIC *et al.*, 2006) e doenças metabólicas como dislipidemia e DM. De acordo com Felber (1992), aproximadamente 80% dos pacientes com DM2 são ou foram obesos.

De acordo com o Ministério da Saúde (2006), o DM é um grupo de doenças metabólicas caracterizadas por hiperglicemia e pode resultar de defeitos na secreção e/ou na ação da insulina, envolvendo processos patogênicos específicos, por exemplo, destruição das células  $\beta$  do pâncreas, resistência à ação da insulina, distúrbios da secreção da insulina, entre

outros. Em 2011, cerca de 366 milhões de pessoas tinham DM, e este número está previsto para aumentar para 522 milhões até 2030 (WHITING *et al.*, 2011).

A *American Diabetes Association* (2013) classifica o diabetes em quatro tipos: a) diabetes tipo 1 – que resulta na destruição da célula  $\beta$ -pancretática, levando a deficiência absoluta de insulina; b) diabetes tipo 2 - resulta em um defeito progressivo da secreção de insulina e também na sua resistência; c) diabetes gestacional - quando é diagnosticada durante a gestação; e d) outros tipos de diabetes relacionados à outras causas, como por exemplo defeitos genéticos na função das células  $\beta$ -pancreáticas. Dentre esses tipos o DM2 é mais comum correspondendo a cerca de 90% dos casos (NARAYAN *et al.*, 2000).

Existem pelo menos dois mecanismos que contribuem para a etiopatogenia do DM2: alteração na secreção de insulina e/ou a resistência à ação desse hormônio. Acredita-se que na fase inicial há predomínio da resistência, com hiperinsulinemia compensatória, com o intuito de manter a normoglicemia. Em uma fase posterior, ocorre a falência das células  $\beta$ , com diminuição da secreção de insulina (“exaustão”), resultando em hiperglicemia <sup>[4]</sup>. A RI caracteriza-se pela redução da capacidade dos tecidos sensíveis à insulina de regular a homeostase da glicose em concentrações normais do hormônio. As principais características da resistência à insulina são o aumento da lipólise no tecido adiposo, aumento da gliconeogênese no fígado e diminuição da captação de glicose pelo músculo. O indivíduo pode também apresentar resistência à insulina e hiperinsulinemia, sem falência das células  $\beta$ , dessa forma, continuar com valores glicêmicos normais. Embora a resistência à insulina seja considerada um evento primário, vários autores acreditam que, inicialmente, ocorra uma hiperinsulinemia importante para manter a normoglicemia, levando à resistência à insulina. Uma terceira possibilidade seria um defeito comum promovendo, simultaneamente, hiperinsulinemia e resistência à insulina <sup>[5]</sup>.

### *Secreção da Insulina*

O pâncreas é um órgão misto, com porção endócrina e exócrina, situado posteriormente ao estômago, tem formato alongado e apresenta três porções: cabeça, corpo e cauda. Sua parte exócrina, formada pelos ácinos produz o suco pancreático, rico em enzimas digestivas que são lançadas no duodeno por meio dos ductos pancreáticos. Sua porção endócrina, formada pelas ilhotas pancreáticas, produz entre outros hormônios, insulina e glucagon, produzidos e secretados pelas células  $\beta$  e  $\alpha$ , respectivamente, que estão relacionados com o metabolismo da glicose (MELLO, 2011).

A insulina é um importante hormônio anabólico, necessário para transporte transmembrana de glicose e aminoácidos, formação de glicogênio no fígado e músculo esquelético, conversão de glicose em triglicérides, síntese de ácido nucléico, e síntese proteica. Sua principal função metabólica é aumentar a taxa de transporte de glicose para determinadas células do corpo (COSTA; ROSSI; GARCIA, 2003).

A glicose é o principal agente estimulador da secreção de insulina a qual responde rapidamente ao aumento da concentração desse metabólito (CARVALHEIRA; ZECCHIN; SAAD, 2002). Esse metabólito é transportado para interior da célula  $\beta$  pela proteína transportadora de glicose 2 (GLUT2), a glicose é fosforilada à glicose-6-fosfato pela enzima glicocquinase (GCK) e metabolizada, elevando, desta forma, a concentração de adenosina trifosfato (ATP) que age fechando os canais de potássio sensíveis a ATP (K-ATP). A diminuição do efluxo de potássio tem como consequência a despolarização da membrana e abertura dos canais de  $\text{Ca}^{2+}$  sensíveis à voltagem. O influxo de  $\text{Ca}^{2+}$  com o consequente aumento da concentração intracelular ativa a maquinaria de exocitose, resultando na secreção dos grânulos de insulina (MATSCHINSKY, 1996).

Um efeito secundário da estimulação das células  $\beta$  pela glicose é a ativação da fosfolipase C (PLC), que hidrolisa os fosfolipídios da membrana plasmática gerando diacilglicerol (DAG) e 1, 3, 4 -trifosfato (IP3) (ZAWALICH; ZAWALICH, 1996). O IP3 promove a ativação dos canais de  $\text{Ca}^{2+}$  localizados na membrana do retículo endoplasmático, e o DAG atua sobre os canais de  $\text{Ca}^{2+}$  sensíveis à voltagem da membrana. Além disso, DAG juntamente com  $\text{Ca}^{2+}$  também ativa a PKC, responsável por estimular a exocitose dos grânulos secretórios de insulina através da fosforilação de diversas proteínas como, por exemplo, a *myristoylated alanine-rich C kinase substrate* (MARCKS), que liga actina e calmodulina e implica no movimento celular e no transporte de vesículas (GILON; HENQUIN, 2001). Outro efeito secundário da estimulação das células  $\beta$  pela glicose é através da ativação da enzima adenilato ciclase (AC). A ativação da AC aumenta o conteúdo intracelular de AMPc, que por sua vez, ativa a PKA. A PKA pode estimular a secreção de insulina pela fosforilação do canal de  $\text{Ca}^{2+}$  sensível à voltagem, permitindo a entrada do íon na célula (FLATT, 1996). Portanto, a ativação da PKA e PKC alteram a secreção de insulina via mobilização de  $\text{Ca}^{2+}$  (NESHER *et al.*, 2002; TENGHOLM; GYLFE, 2009).

Há várias substâncias, conhecidas como moduladores da secreção de insulina, que agem via sinalização da PKC e da PKA. Dentre eles, encontra-se o carbacol (CCh), um agonista da acetilcolina (Ach), o qual é um dos principais neurotransmissores potencializadores da secreção da insulina. A Ach liberada pelas terminações parassimpáticas

se liga aos receptores muscarínicos subtipo M3 na membrana das ilhotas pancreáticas, ativa a proteína G acoplada a PLC que hidrolisa os fosfoinosítídeos da membrana em IP3 e o DAG os quais estão envolvidos na secreção da insulina. Outro secretagogo é o *phorbol 12-myristate 13-acetate* (PMA), que simula o efeito do DAG na PKC, o que pode explicar a sua ação como secretor de insulina. A amplificação da secreção da insulina pela Ach ou PMA resulta principalmente de um aumento da ação do  $Ca^{2+}$  na exocitose dos grânulos de insulina (MOURAD; NENQUIN; HENQUIN, 2013).

Outra via de sinalização da insulina é a da PKA que também possui secretagogos específicos como o *3-isobutyl-1-methylxanthine* (IBMX) e a Forscolina, ambos aumentam o AMPc, sendo que a Forscolina age via estimulação da AC e o IBMX pela inibição da fosfodiesterase, que é uma enzima que hidroliza o AMPc (DYACHOK *et al.*, 2008; RIBEIRO *et al.*, 2010).

#### *Proliferação das células $\beta$ pancreáticas*

A massa das células  $\beta$  do pâncreas possui importante papel na determinação da quantidade de insulina que é secretada, e isso pode ser regulado para manter a normoglicemia em várias condições metabólicas. O aumento da massa das células  $\beta$  tem sido constantemente observado quando há um aumento da demanda metabólica como nos estados de obesidade, gestação e pancreatômia parcial (MONTANYA *et al.*, 2000). Esse mecanismo é possível pela plasticidade que o pâncreas endócrino possui, a qual é amplamente regulada pelas variações na demanda da produção de insulina.

Os processos de reposição da massa de células  $\beta$  estão classificados em proliferação (replicação das células), neogênese (diferenciação a partir de células precursoras não  $\beta$ ), hiperplasia e hipertrofia, e a redução dessas células ocorre através de mecanismos de apoptose, necrose, autofagia, hipoplasia e hipotrofia (CERF, 2013). Existem diversas substâncias que podem estimular a proliferação das células  $\beta$ , como a glicose, que Bonner weir *et al.*, (1989) demonstraram que sua infusão por 96 horas resultou em 50% de aumento nas células  $\beta$  em ratos adultos, por meio de hiperplasia e hipertrofia das células. Além da glicose, fatores de crescimento semelhante à insulina (IGF-1), insulina, hormônio do crescimento e lactogênios placentários estimulam a replicação das células  $\beta$  (BOUWENS; ROOMAN, 2005). Estudos em animais mostram que o peptídeo semelhante ao glucagon 1 (GLP1) e seu análogo exendina-4, aumentam a massa das células  $\beta$  pela estimulação da proliferação celular e inibição da apoptose (AHRÉN, 2011).

Estudos em seres humanos obesos e com resistência à insulina, mas não diabéticos, mostraram que a primeira resposta fisiológica ao aumento da demanda de insulina é um período marcado pela expansão compensatória da massa da célula  $\beta$  (BONNER-WEIR, 1994; BUTLER *et al.*, 2003; KLOPPEL; CLEMENS, 1997) e da função das mesmas (KAHN *et al.*, 2000) que juntos permitem o aumento da produção e/ou secreção de insulina. Os seres humanos com DM2 têm ilhotas menores e mostram uma redução de 40-60% da massa de células  $\beta$  em comparação com pacientes não diabéticos hiperinsulinêmicos (WESTERMARK; WILANDER, 1978). De acordo com Ahrén (2005), o aumento na massa das células  $\beta$  causado pela resistência à insulina, é dependente de neogênese e replicação aumentada; enquanto que a redução da massa de células  $\beta$  na DM2 pode ser causado por apoptose, que pode estar aumentada devido à glicotoxicidade, lipotoxicidade e outros fatores.

Em roedores a replicação é consideravelmente maior durante o final da gestação e no período neonatal (BONNER-WEIR *et al.*, 2010). A neogênese ocorre durante determinados períodos de crescimento embrionário normal e também no pós-natal, após alguns tipos de lesões pancreáticas, e pode ser induzida por fatores de crescimento e/ou citocinas. Os mecanismos envolvidos na neogênese ainda são pouco esclarecidos. Um contribuinte fortemente importante é o fator de transcrição pancreático (Pdx1). Este fator de transcrição é imprescindível para o desenvolvimento do pâncreas e para a manutenção das células  $\beta$ , uma vez que regula diversos genes como o da insulina, transportador de glicose subtipo dois (GLUT 2) e glicoquinases, de fundamental importância no funcionamento  $\beta$  celular (HOLLAND *et al.*, 2005; GUO *et al.*, 2013).

### *Cirurgias bariátricas*

Dentre várias estratégias com objetivo de reverter os efeitos indesejados da obesidade e suas comorbidades, a cirurgia bariátrica tem sido, por muitas vezes, o tratamento de primeira escolha (BUCHWALD *et al.*, 2004; CRISTOU *et al.*, 2004).

De acordo com Rubino *et al.* (2010), os procedimentos bariátricos são classificados em três categorias em relação ao seu mecanismo de ação que objetiva o emagrecimento: 1) cirurgia mal absorptiva, na qual menor quantidade de nutrientes são absorvidos no intestino; 2) cirurgia restritiva, onde é restringida a ingestão dos alimentos, levando de forma precoce à saciedade e 3) cirurgia mista, onde ambos mecanismos fisiológicos estão associados ao final do procedimento cirúrgico, a menor ingestão de alimentos e a menor absorção de nutrientes.

Existem vários tipos de cirurgias bariátricas, sendo que alguns autores como Mingrone *et al.* (2012), relatam que dois tipos são mais efetivos no controle glicêmico, sendo eles a

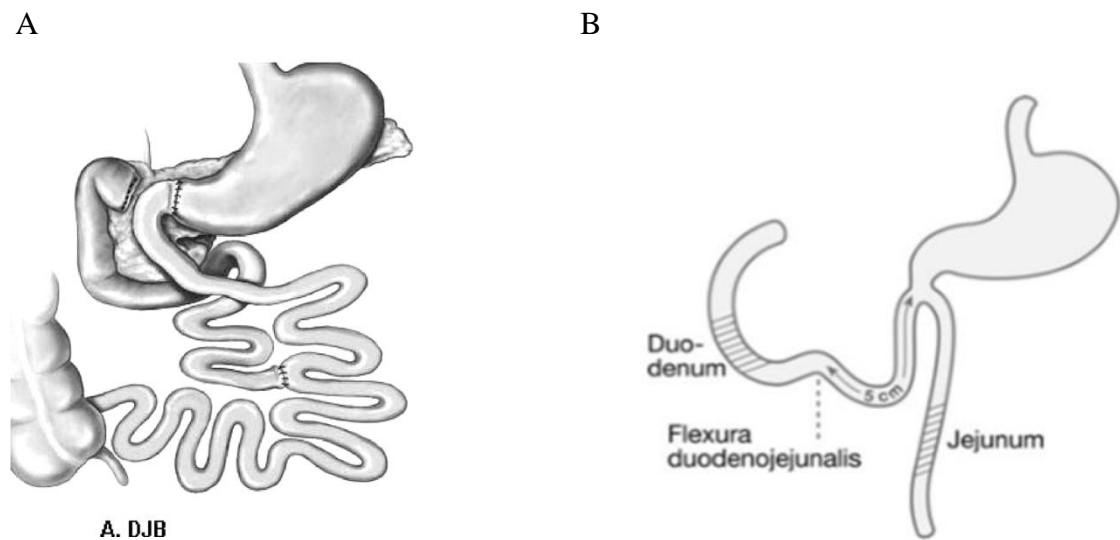
*Roux-en-Y gastric bypass* (RYGB) e a Derivação Bilio-pancreática (BPD). Buchwald *et al.* (2004) apontam que a primeira modalidade resulta em uma melhora de 84% no controle glicêmico dos pacientes com DM2 que foram submetidos a ela, enquanto a segunda pode chegar a uma melhora de 98% desses casos. Além disso, já foi comprovado que a RYGB também é efetiva para em pacientes não obesos com DM2 (COHEN *et al.*, 2006).

Para tentar elucidar melhor os efeitos da RYGB sobre o controle glicêmico e para evitar a alta taxa de mortalidade que esse procedimento causa, muitos pesquisadores tem utilizado a DDJ (Figura 2) em modelos animais de obesidade e DM2. Jurowich *et al.* (2013), mostrou que a DDJ melhorou a diabetes adquirida em um modelo animal independente da perda de peso. Já Rubino e colaboradores (2006), evidenciaram uma melhora substancial na homeostase glicêmica entre uma semana e nove meses após o procedimento cirúrgico. Em animais obesos pela dieta de cafeteria a DDJ se mostrou eficaz em restaurar a insulinemia e a sensibilidade à insulina, além de melhorar a capacidade secretória das ilhotas nesses animais (ARAÚJO *et al.*, 2012).

Existem algumas hipóteses para tentar explicar a melhora da homeostase glicêmica após cirurgias bariátricas. A *foregut hypothesis*, propõe que, após a exclusão do intestino proximal do trânsito alimentar, fatores anti-incretinas desconhecidos, os quais se opõem aos efeitos das incretinas secretadas pelo duodeno, jejuno e íleo, entre eles o GLP1, e o Polipeptídeo inibidor gástrico (GIP), como a estimulação de secreção de insulina e efeitos anti-apoptóticos sobre as células  $\beta$  pancreáticas, teriam sua produção diminuída, auxiliando na homeostase da glicemia (RUBINO; GAGNER, 2002; RUBINO *et al.*, 2006; DIXON *et al.*, 2012). Outra hipótese amplamente difundida, a *hindgut hypothesis*, sugere que a exclusão do segmento do intestino proximal faz com que haja menor absorção de nutrientes dos alimentos ingeridos, aumentando dessa forma a secreção de GLP1 e Peptídeo YY (PYY), resultando em uma melhora da homeostase da glicose (WANG *et al.*, 2008; THALER; CUMMINGS, 2009).

O efeito desse tipo de cirurgia sobre a morfologia de ilhotas pancreáticas também tem sido relatado, e geralmente está associado à secreção do hormônio GLP-1 no período de pós-operatório (PO) (SPECK *et al.*, 2011; WOODS *et al.*, 2011). Em estudo realizado em seis pacientes com hipoglicemia hiperinsulinêmica pós RYGB, Service *et al.* (2005) demonstraram que a cirurgia promoveu hiperplasia e hipertrofia das células  $\beta$ , além de aumento no tamanho das ilhotas pancreáticas, a partir de seis meses, até oito anos, de PO. Lindqvist *et al.* (2014), mostraram em um modelo de porcos não obesos e não diabéticos, que a cirurgia de RYGB foi eficaz em promover a neogênese de células  $\beta$ , o que foi demonstrado através do aumento deste tipo celular fora da ilhota pancreática. Observou-se ainda aumento

no número de ilhotas, na massa de células  $\beta$  e no número de células reativas à insulina por área de pâncreas, além de uma tendência ao aumento no tamanho da ilhota, somente vinte dias após a realização da cirurgia. Por outro lado, Meier et al. (2006), em estudo realizado com humanos que desenvolveram hipoglicemia hiperinsulinêmica oito anos após terem sido submetidos ao RYGB, demonstraram que não houve aumento da área de células  $\beta$ , tampouco aumentou proliferação celular marcada pelo antígeno Ki67, no pâncreas. Woods et al. (2011), também demonstraram que a cirurgia de DDJ não modificou a massa de células  $\beta$  e células  $\alpha$ , em camundongos C57BL/6 magros não diabéticos e obesos diabéticos, vinte e oito dias após realização da cirurgia.



**Figura 1** - (A) Desvio ou Derivação Duodeno-Jejunal (DDJ) proposta por Rubino *et al.* (2006); (B) Derivação Duodeno-Jejunal Modificada proposta por Jurowich *et al.* (2013).

#### *Modelos experimentais de obesidade animal e dieta de cafeteria*

São utilizados vários modelos experimentais animais na tentativa de compreender a fisiopatologia da obesidade e da DM2. Dentre os diferentes modelos encontram-se os oriundos de linhagens genéticas, provocadas por mutações autossômicas recessivas (ratos Zucker **fa/fa**, camundongos **ob/ob**) bem como, camundongos transgênicos e *knockouts* para



determinados genes específicos; roedores submetidos a lesões eletrolítica no hipotálamo ventromedial e lesões químicas em regiões hipotalâmicas por drogas específicas como o glutamato monossódico (BRAY; YORK, 1979).

Um modelo de dieta experimental que reflete a cultura alimentar ocidental, associada à pandemia de obesidade, é a "dieta de cafeteria" (CAF). No modelo de dieta CAF é possível analisar efeitos ambientais relacionados à obesidade, livre de possíveis efeitos genéticos (CASTELL-AUVÍ *et al.*, 2011).

Essa dieta, apesar de possuir maior valor energético, tem um baixo valor nutritivo, pois é sobrecarregada com carboidratos e gordura. Esse tipo de dieta é caracterizada por possuir a capacidade de induzir não apenas o aumento do estoque de lipídios no tecido adiposo, mas também de aumentar o estresse oxidativo por todo sistema (SCOARIS *et al.*, 2010).

A dieta de cafeteria promove hiperfagia voluntária, resulta em rápido ganho de peso, aumento da massa de gordura e gera parâmetros pré-diabéticos tais como perda da homeostase glicêmica e intolerância à insulina (BRANDT *et al.*, 2010; CASTELL-AUVÍ *et al.*, 2011; SAMPEY *et al.*, 2011). Em 15 semanas de dieta de cafeteria essas alterações podem ser evidenciadas em roedores da linhagem Wistar (DUARTE *et al.*, 2006). A resistência à insulina induzida pela dieta pode levar ao comprometimento da função de células  $\beta$  e redução da massa de células  $\beta$ , em parte, devido a um aumento em sua apoptose, e assim, levando ao diabetes (CASTELL-AUVÍ *et al.*, 2011).

Como citado anteriormente, estudos revelam que essa dieta muito se assemelha ao modelo de dieta ocidentalizada que tem culminado com o aumento nos índices de obesidade em todo o mundo. Nas últimas décadas, foi possível observar mudanças nos hábitos alimentares em diversos países, o que reflete a complexidade dos modelos de consumo e dos fatores que os determinam (PINHEIRO, 2001). Tais mudanças afetam a qualidade dos alimentos produzidos e industrializados. Na tentativa de adequar a alimentação ao ritmo acelerado do dia-a-dia, as escolhas e os hábitos de consumo passaram a apontar para alimentos mais condizentes com o novo estilo de vida, fazendo com que fossem incorporados hábitos rápidos e práticos. Estes muitas vezes são menos satisfatórios ao paladar e possuem aporte nutritivo menor do que no padrão anterior, no qual se prezava por hábitos naturais e mais saudáveis de alimentação (ABREU *et al.*, 2001; FLANDRIN & MONTANARI, 1996; OLIVEIRA, 1997).

Através da perspectiva histórica pode-se realizar a análise do consumo alimentar com base na noção de "sistemas alimentares". Estes seriam baseados em diferentes agentes sociais

(produtores, distribuidores, consumidores e Estado), estratégias e relações que se estabelecem entre eles, ao longo do tempo, de modo que se possa compreender de que formas os hábitos alimentares se constroem e evoluem (SOUZA, 2002). Além disso, é importante salientar que a formação e transformação de hábitos alimentares com o passar dos tempos tem relação estreita com a cultura e com as crenças (religiosas ou não) de um povo (PINHEIRO, 2001; ABREU *et al.*, 2001).

Atualmente, a alimentação é caracterizada pelo estilo de vida moderno, marcada pela escassez de tempo para preparo e consumo de alimentos, o que leva à emergência de alimentos do tipo *fast food*, que emprega técnicas de conservação e preparo, agregando tempo e trabalho. Estes produtos, inventados pela indústria são novos ao paladar e aos hábitos. Assim para facilitar sua aquisição, normalmente a indústria vai se valer de estratégias. De um lado a embalagem vai ser extremamente colorida e prática para chamar a atenção do consumidor. Por outro lado, esses produtos passam a ser fabricados incorporando enormes quantidades de açúcar, sal e gordura, além dos aditivos químicos, que tem se tornado cada vez mais comum (BLEIL, 1998).

A transição nutricional ocorrida neste século resultou na chamada “dieta ocidental” caracterizada pelos altos teores de gorduras, principalmente de origem animal, de açúcares e alimentos refinados e baixos teores de carboidratos complexos e fibras (MONTEIRO *et al.*, 2000). A dieta ocidental e o aumento da obesidade estão amplamente associados com a alta prevalência de doenças crônicas não transmissíveis (DCNT) e a diminuição da qualidade de vida da população (FERREIRA *et al.*, 2005). Enquanto na Europa e nos países asiáticos a mudança de hábitos ocorreu de forma gradual, o que se observa no ocidente é um acelerado ritmo de mudanças, tendo como consequência doenças como diabetes, hipertensão e cardiopatias, que atingem todas as faixas etárias e são configuradas como problemas de saúde pública (BATISTA-FILHO & RISSIN, 2003; FRANCISCHI *et al.*, 2000).

Considerando as altas taxas de obesidade associadas à esse estilo de dieta com alto teor calórico, torna-se relevante utilizar o modelo da dieta de cafeteria para demonstrar os efeitos da obesidade em animais. Da mesma forma, é importante buscar estratégias que visem combater ou minimizar os efeitos da dieta ocidentalizada sobre a obesidade e suas comorbidades.



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**DUODENAL JEJUNAL BYPASS NORMALIZES INSULIN SECRETION IN  
RESPONSE TO POTENTIATING AGENTS AND ALTERS ISLET MASSES IN  
WESTERN-DIET OBESE RATS**

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**Funding:** This study is part of the MSc Thesis of M.C. Mendes and was supported by grants  
from Fundação Araucária (223/2010); Coordenação de Aperfeiçoamento de Pessoal de Nível  
Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

**Manuscript Type:** Original article



## ABSTRACT

**Background:** This study was designed to evaluate whether duodenal jejunal bypass (DJB) in western diet (WD)-obese rats could have effects on islet responsiveness to potentiating agents involved with the protein kinase A (PKA) and C (PKC) pathway and pancreatic  $\beta$ -cell morphology.

**Methods:** Male *Wistar* rats with 8 weeks of life were fed a standard rodent chow diet (CTL group) or WD *ad libitum*. After 10 weeks, WD rats were submitted to sham operation or DJB, forming WD SHAM and WD DJB group, respectively. After two months, the obesity parameters, insulin resistance (IR), insulin secretion stimulate by different secretagogues, islet protein expression and pancreas morphology were verified.

**Results:** WD SHAM rats displayed obesity, hyperglycemia, hyperinsulinemia, glucose intolerance and IR. The glucose-induced insulin secretion stimulated by 11.1 mM glucose in the presence of carbachol (CCh), PMA, Forskolin or IBMX was higher in WD SHAM islets than CTL islets. The expression of glucokinase, PKC, PKA and syntaxin was not altered in WD SHAM rats, but the M3 receptor was down-regulated. In addition, they also presented an increase in islets and  $\beta$ -cells area and mass. DJB surgery normalized glucose, insulinemia and IR. The insulin release stimulated by glucose in the presence of CCh, PMA, Forskolin or IBMX was also normalized in WD DJB group. DJB did not influence the islets protein expressions. In addition, a decreased in islet and  $\beta$ -cells mass was observed after DJB.

**Conclusions:** DJB surgery normalized glycemia, insulinemia and IR, normalized insulin secretion by PKC and PKA pathway and reduced islets and  $\beta$ -cell mass.

**Keywords:** Duodenal-jejunal bypass surgery; insulin secretion;  $\beta$ -cell mass; obesity; western diet rats

## INTRODUCTION

The prevalence of type 2 diabetes (T2D) in the world is rising in association with an increasing frequency of overweight and obesity <sup>[1]</sup>. The T2D is characterized by hyperglycemia due to insulin resistance in peripheral tissues together with reduction in pancreatic  $\beta$ -cell function and/or mass <sup>[2, 3]</sup>.

Glucose-induced insulin secretion by  $\beta$ -cells begins with its uptake promoted by the glucose transporter (GLUT)-2, once in the  $\beta$ -cell cytosol; this sugar is phosphorylated by glucokinase (GCK) enzyme and metabolized, increasing the ATP/ADP ratio. Subsequently, the ATP closes ATP-sensitive  $K^+$  channels, which depolarizes the  $\beta$ -cell membrane, which activates voltage-gated  $Ca^{2+}$  channels, promoting  $Ca^{2+}$  influx that activates the insulin granule exocytosis <sup>[4, 5]</sup>. Insulin secretion can be amplified in response to hormones and neurotransmitters. Gut hormones, like the incretins glucagon like peptide (GLP)-1 and glucose dependent peptide (GIP) enhances insulin secretion by interaction with G-coupled receptors that activates adenylate cyclase and increase intracellular concentration of cyclic adenosine monofosphate (cAMP) that activates protein kinase (PK)-A. In addition, acetylcholine (Ach) released by parasympathetic nerves to the  $\beta$ -cell increases hormone secretion by interaction with muscarinic type 3 (M3) receptors that activate phospholipase C which hydrolyses phosphatidylinositol biphosphate (PIP2) increasing intracellular concentration of inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 release of  $Ca^{2+}$  from internal stores and the  $Ca^{2+}$  together with DAG activates the PKC <sup>[6]</sup>.

Duodenal jejunal bypass (DJB), a malabsorptive bariatric operation, is an experimental procedure that can induce rapid and sustained amelioration of glucose homeostasis independently of weight loss in experimental pre and diabetic rodents and in obese subjects <sup>[7-9]</sup>. The mechanism of action by which DJB improves body glucose control remains unknown. Some reports indicate that the modification in intestinal transit increase incretins secretion

that improve  $\beta$ -cell function and insulin action<sup>[10, 11]</sup>. However, increased incretins levels are not observed in all cases of DJB-induced better glucose control<sup>[12]</sup>. Possibly a modification in the regulation of autonomic nervous system also occurs and contributes to DJB effects<sup>[13]</sup>. Since the incretins and parasympathetic nervous system actions are modified in  $\beta$ -cells in pre and diabetic conditions<sup>[14, 15]</sup>, here we investigate the glucose homeostasis,  $\beta$ -cell responsiveness to potentiating agents and the endocrine pancreatic morphology in western-diet (WD)-obese rats at 2 months after DJB.

## **METHODS**

### *Experimental groups*

All experiments protocols were approved by the UNIOESTE's Committee on Ethics in Animal Experimentation (license n°.: 8709). Eight-week-old male *Wistar* rats were distributed in two groups: WD and CTL group, which received cafeteria diet and soft drink and standard diet and water, respectively. The cafeteria diet consisted of standard chow (Biobase, Brazil), Italian salami (Sadia, Brazil), mini bread rolls (Nutrella, Brazil), corn chips (Cheetos, Pepsico, Brazil), marshmallow (Fini, Brazil), mixed sausage (Sadia, Brazil), chocolate cake (Renata, Selmi, Brazil), corn-based cookies (Zadimel, Brazil), mortadella (Frimesa, Brazil), bacon snacks (Trophy, Helena, Brazil), chocolate wafer biscuits (Bauduco, Brazil) and 350 ml of degassed Coca-Cola (Coca-Cola, Brazil) and Guarana (Antarctica, Brazil)<sup>[16]</sup>. Standard diet comprised of 3.8 kcal/g (70% carbohydrate, 20% protein and 10% fat; Biobase®, Águas Frias, SC, BRA). All experimental groups were housed in standard cages and maintained on a 12 h light/dark cycle (lights on 07:00–19:00 h), controlled temperature ( $22 \pm 1^\circ\text{C}$ ) and had free access to their foods, water or beverages.

### *Duodenal-jejunal bypass (DJB) and Sham operations*

At 10 weeks after cafeteria diet induced obesity, half of WD rats were submitted to duodenal-jejunal bypass (WD DJB group) as described by Jurowich et al. <sup>[17]</sup>, a less invasive procedure and with less mortality rate. Briefly, WD rats were deprived of food for 16-18 h and were anesthetized with isoflurane (Isoforine®, Cristália, SP, Brazil). After laparotomy, DJB surgery was performed by postpyloric transection of the duodenum, closure of the duodenal stump, and reconstruction of the intestinal passage by terminal lateral duodenojejunosomy 5 cm aboral of the flexura duodenojejunalis. The effectiveness of duodenal ligation was determined by injection of saline through the stomach and observing that no saline passed beyond the area of ligation. The others WD rats were submitted to Sham-operation (WD SHAM group). In the WD SHAM groups, a midline incision in the anterior abdominal wall was made; the stomach, duodenum and intestines were massaged, and the incision was closed. In the first seven days of the post-operative period WD DJB, WD SHAM and CTL groups received their respective diets in a liquid form. The animals continued to receive cafeteria diet two months postoperatively, when the euthanasia happened. Three animals died from post-surgical complications.

#### *Food intake and feces production*

At two weeks before the end of experimental period, five rats in each group were maintained in metabolic cages during 3 days to measure food intake, urine and fecal elimination over 12 h <sup>[18]</sup>.

#### *Intraperitoneal Glucose Tolerance Test (ipGTT)*

At the end of experimental period and after 8 h fasting, the rats received an intraperitoneal (ip) injection of 2g/kg body weight (BW) glucose. The blood was collected by the tail vein

before (time 0) and after 15, 30, 60, 120 and 180 min of the glucose administration to measure the glycemia using a glucose analyzer (Abbott®, Optium Xceed, Alameda, CA).

#### *Obesity parameters and plasma biochemical analysis*

All rats groups were fasted for 8h and euthanized by decapitation. Final BW and nasoanal length (NAL) were measured to obtain the Lee Index [from the ratio of BW (g)  $1/3$ /NAL (cm) x 1000]. Retroperitoneal and perigonadal fat pads were removed and weighed. Blood samples were collected to measure glycemia (as above cited) and insulinemia by radioimmunoassay (RIA). Tissue insulin sensitivity was evaluated by the previously validated homeostasis model assessment (HOMA) using the HOMA index of insulin resistance [(HOMA-IR) = fasting insulin (U/mL) x fasting glucose (mM)/22.5] <sup>[19]</sup>.

#### *Pancreatic islet isolation and static insulin secretion*

Pancreatic islets were isolated by the collagenase digestion. For static incubation, groups of four islets from each group were first incubated for 30 min in Krebs-Ringer Bicarbonate Buffer (KRB) containing: 115 mM NaCl, 5 mM KCl, 2.56 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM NaHCO<sub>3</sub>, 15 mM HEPES, supplemented with 5.6 mM glucose, 3 g of BSA/L, and equilibrated with a mixture of 95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4. This medium was then replaced with fresh buffer and the islets were incubated for further 1 h with 5.6 mM glucose in the presence of 10 mM L-Leucine, 40 mM KCl or 100 μM Tolbutamide. In another set of experiments, the islets were incubated with 11.1 mM glucose plus 100 μM carbachol (Cch), 100 nM phorbol 12-myristate 13-acetate (PMA), 10 μM forskolin or 1 mM 3-isobutyl-1-methyl-xantine (IBMX). Aliquots of the supernatant at the end of the incubation period were kept at -20°C for posterior insulin measurement by RIA.

### *Western Blotting*

For muscle insulin action evaluation, 5 rats of all groups were anesthetized *ip* with a mixture of ketamine (90 mg/kg, Vetbrands®, Paulínia, SP, BRA) and xylazine (9 mg/kg, Vetbrands®, Paulínia, SP, BRA). A laparotomy was performed and a fragment from the gastrocnemius muscle was collected. Subsequently, 1 U/kg insulin was administered into the portal vein and at 1 min and 30 s, other muscle fragment was collected. Samples were solubilized in homogenization buffer at 4°C containing 100 mM tris pH 7.5, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 1% triton-X 100. For protein expression in islets, isolated islets were solubilized in 100 µL homogenization buffer as described above. The muscle and islets extracts were then centrifuged at 12 600 *g* at 4°C for 40 min to remove insoluble material. The protein concentrations in the samples were assayed using the Bradford method. After, the samples were incubated at 100°C for 5 min with Laemmli buffer containing β-mercaptoethanol. Proteins were separated by electrophoresis on biphasic polyacrylamide gel (SDS-PAGE). Afterwards, samples were transferred to nitrocellulose membranes (BioRad®, Hercules, CA, USA) that were treated with a blocking buffer (5% non-fat dried milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20) and subsequently, were incubated with primary antibodies against phospho (p)-Akt<sub>1/2/3</sub><sup>Ser473</sup> (1:1.000; cat. sc-7985R), PKAα (1:1.000; C-20; cat. sc-903), PKCα (1:1.000; H-7; cat. sc-8393), Syntaxin -1 (1:1.000; HPC-1; cat.sc-12736), GCK (1:1.000. H-88; cat. sc- 7908) or mAChR M3 (1:1.000; C-20; cat. sc- 7474).

Visualization of specific protein bands was made by incubating the membranes for 2 h with a peroxidase-conjugated secondary antibody (1:10.000; cat.7074, Cell Signaling Technology, Boston, MA, USA), followed by exposure to X-ray film (Kodak, Manaus, AM, BRA). After assaying the target proteins, western blotting was repeated using mouse monoclonal antibody

to  $\alpha$ -tubulin (1:1.000, cat. T5168, Sigma-Aldrich Chemicals, St Louis, MO, USA) as an internal control. The band intensities were quantified by optical densitometry using Image Tool software (<http://ddsdx.uthscsa.edu/dig/itdesc.html>).

#### *Histological process and analyses of endocrine pancreas*

For morphometric analysis, pancreases from CTL, WD Sham and WD DJB rats were removed, weighed and fixed 24 h with Bouin's solution. Then, the tissue was dehydrated in ascending concentrations of alcohol, and impregnated in paraffin. From each pancreas 7  $\mu$ m serial sections were obtained (every 20<sup>th</sup> sections) and two of these sections were randomly selected and processed for the insulin immunoperoxidase reaction. All islets in each section were systematically capture using an image system coupled to a microscope (Olympus DP71; Olympus BX60; Olympus, Japan ). Islet,  $\beta$ -cell, and non  $\beta$ -cell areas were measured using the Image-Pro-Plus Media, Cybernetics Program (USA). The islet and  $\beta$ -cell masses were calculated by dividing the sum of the total islet or  $\beta$ -cell areas, respectively, per pancreas area section and multiplying per pancreas weight<sup>[20]</sup>. To identify collagen fibers in pancreatic islets, the Mallory's trichrome staining was done.

#### *Statistical analysis*

Data were expressed as means  $\pm$  SEM. Statistical analysis were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA), the groups were compared using one-way analysis of variance (ANOVA) followed by Tukey post test, except for the comparison between the non-stimulated and insulin-stimulated conditions in Figure 1D, which was analyzed using Student's *t* test. The level of significance was  $P < 0.05$ .

## **RESULTS**

### *Obesity evaluation*

The final BW, NAL and Lee Index were 28%, 4% and 5% higher, respectively, in WD SHAM rats when compared with CTL ( $P < 0.001$ ,  $P < 0.01$ ,  $P < 0.001$ , respectively; Tab. 1). Also, WD SHAM rats presented a 63% and 50% higher retroperitoneal and perigonadal fat stores, respectively in comparison with CTL rats ( $P < 0.001$  and  $P < 0.0001$ ; Tab. 1). These obesity features in WD SHAM rats were accompanied by an increased in food intake ( $24.5 \pm 1.0$  g/day;  $P < 0.05$ ) but reduction in feces elimination ( $0.7 \pm 0.2$  g/day;  $P < 0.01$ ) as compared to CTL animals ( $19.0 \pm 1.0$  and  $3.6 \pm 0.3$  g/day, respectively). At 2 months after DJB operation, WD DJB rats did not alter BW, NAL, obesity parameters (Tab. 1) or the amount of food ingested and feces eliminated when compared with WD SHAM rats ( $26.8 \pm 1.5$  and  $1.2 \pm 0.4$  g/day, respectively).

### *Glucose homeostasis*

Fasted WD SHAM rats presented enhanced glycemia and insulinemia when compared with CTL ( $P < 0.01$  and  $P < 0.001$ ; Fig. 1A and 1B). During the ipGTT, WD SHAM rats presented an increased in blood glucose levels at basal (Fig. 1C,  $P < 0.01$ ) and after 15, 30, 60 and 120 min of the glucose load compared to CTL group ( $P < 0.05$ ,  $P < 0.001$ ,  $P < 0.001$  and  $P < 0.01$ , respectively). Also, the total glycemia, expressed as the area under glucose curve (AUC) during the ipGTT, was 47% higher in WD SHAM group than CTL rats ( $P < 0.001$ ; Fig. 1D). The glucose intolerance in WD SHAM rats was associated with lower insulin sensitivity in peripheral tissues, since WD SHAM group presented a 4.4-fold increase in HOMA-IR when compared with CTL ( $P < 0.001$ ; Fig. 1E) and did not enhance the expression of the pAKT<sub>1/2/3</sub> in gastrocnemius muscle in response to insulin (Fig. 1F). WD DJB rats normalized the fasting glycemia and reduced insulinemia compared to WD SHAM rats ( $P < 0.05$ ; Fig. 1B). Although the DJB operation did not modify the glucose intolerance (Fig. 1C and D), WD DJB



rats presented a reduction in HOMA-IR (Fig. 1E) and a better insulin action in gastrocnemius muscle (Fig. 1F) than WD SHAM rats.

#### *Islet insulin secretory function*

To determine whether the insulin secretory response is altered by cafeteria diet or DJB surgery, the islets was incubated at 5.6 mM Glucose in the absence or presence L-Leucine, KCl or Tolbutamide. The basal insulin release (5.6 mM glucose) was similar in both groups.

Also, when islets were exposed to the medium containing either

L-leucine, KCl or Tolbutamide no differences were observed (Fig. 2A).

In another set of experiments, the islets were incubated at 11.1 mM Glucose in the absence or presence CCh (which stimulates the formation of inositol-1,4,5-triphosphate plus

diacylglycerol through activation of PLC), PMA (which stimulated directly PKC), Forskolin or IBMX (which increase cAMP by adenylate cyclase stimulation or by phosphodiesterase inhibition, respectively). No alteration in insulin release in response to 11.1 mM glucose was

observed between the groups (Fig. 2B and C). However, isolated islets from WD SHAM rats secreted more insulin in response to CCh and PMA ( $P < 0.01$ ; Fig. 2B), as well as, in the presence of Forskolin and IBMX ( $P < 0.01$ ; Fig. 2C). These changes in islets secretory

capacity were not due to alteration in GCK, PKC, PKA or Syntaxin-1 protein content (Fig. 3A, C, D and E, respectively). A reduction of 56% in mAChR M3 protein expression was evidenced in WD SHAM islets when compared with CTL islets ( $P < 0.05$ ; Fig. 3B).

The insulin secretion in response to potentiating agents that activates the cholinergic/PKC or cAMP/PKA pathways (Fig. 2B and C), was normalized in WD DJB islets compared to WD

SHAM islets. No alterations in GCK, mAChR M3, PKC, PKA or Syntaxin-1 islets protein expression were observed between WD DJB and WD SHAM rats (Fig. 3).

### *Endocrine pancreatic morphology*

Figure 4A, B and C shows histological pancreatic sections stained for insulin from CTL, WD SHAM and WD DJB rats. The pancreatic islet architecture was not altered between the groups. Also, the pancreas weight was similar in all experimental groups (Fig. 4G). However, in pancreatic sections from WD SHAM rats the islet,  $\beta$  and non  $\beta$ -cells areas were higher than that observed in the CTL pancreas ( $P < 0.01$ ,  $P < 0.01$  and  $P < 0.01$ ; Fig. 4H, I and J, respectively). Furthermore, the islet and  $\beta$  masses were 6 and 3 fold higher, respectively, in pancreas of WD SHAM rats compared with CTL rats ( $P < 0.001$ ; Fig. 4K and L, respectively). DJB operation normalized the islet,  $\beta$  and non  $\beta$ -cell areas in WD DJB rats than WD SHAM animals ( $P < 0.05$ , Fig. 4H, I and J). In addition, an reduction of approximately 54% was observed in the islet and  $\beta$ -cell pancreatic masses from WD DJB rats compared with WD SHAM group ( $P < 0.05$ ; Fig. 4K and L). A qualitative histological analysis of the pancreases showed that WD SHAM rats had more collagen fibers (Fig. 4E) in endocrine tissue than CTL group (Fig. 4D). The islets of WD DJB animals, displayed qualitatively less collagen fibers (Fig. 4F) what was observed in islets from WD SHAM rats (Fig. 4E).

### **Discussion**

Currently, bariatric operation is the most effective tool for the treatment of obesity<sup>[21]</sup> and comorbidities, as T2DM<sup>[7]</sup>. DJB surgery improved glucose homeostasis before weight loss<sup>[9, 17, 22, 23]</sup>, however the mechanisms involved in this improvement are unknown. In this way, herein, using obese rats by western diet, we showed that DJB improved glycemia, insulinemia and IR without changes in BW and fat content. Furthermore, WD DJB rats normalized glucose-induced insulin secretion in the presence of CCh, PMA, forskolin and IBMX and presented a reduction in islets and  $\beta$ -cell area and mass.

As previously reported <sup>[9, 24]</sup> the consume of western diet leads an increased in BW, excessive fat content, hyperphagia, reduction in stools production (Tab. 1), glucose intolerance, higher glucose levels and hiperinsulinemia (Fig. 1B). Two months after DJB surgery, the glycemia was normalized and the insulinemia decreased in WD DJB group (Fig. 1B). The others parameters have not changed. Decreased food intake <sup>[25]</sup> and reduced in the absorption of nutrients from the gut <sup>[26]</sup> are some possible explanations to improves in the glucose homeostasis after bariatric operation, independent of weight loss. However, our results indicate that other factors may be involved in this process.

Obesity is frequently associated with IR, which reduces glucose uptake in peripheral tissues <sup>[27]</sup>. In the present work, WD SHAM animals showed an increase in HOMA-IR and a reduction in AKT phosphorylation in the gastrocnemius muscle. AKT is a serine/threonine kinase that when phosphorylated by PI3 kinase, provokes the targeting of glucose transporter (GLUT4) to the cell membrane in adipose tissue and muscle <sup>[27]</sup>. Few reports study insulin signaling after bariatric operation and the data are controversial <sup>[23, 25, 28-30]</sup>. Herein, although the glucose intolerance not changes after DJB, the IR was modified, since the HOMA-IR was reduced (Fig. 1E) as well as, WD DJB animals improved insulin signaling in the gastrocnemius muscle due an increased in AKT phosphorylation (Fig. 1F). Possibly, the normalization of glycemia and the reduction in insulinemia observed in WD DJB animals is associated with improvement in IR.

To compensate IR, the endocrine pancreas has the ability to promote functional and morphological changes in pancreatic islets leading to hyperinsulinemia <sup>[31]</sup>. WD female rats showed a reduction in insulin secretion induced by depolarizing agents <sup>[32]</sup>. Insulin secretion was increased in islets from WD male rats when stimulated by 11.1 mM glucose <sup>[9]</sup>. Also, insulin secretion stimulated by different glucose concentrations and the total insulin content was greater in WD Swiss mice <sup>[33]</sup>. In relation to effects of DJB on static insulin secretion,

one study showed an increase in insulin release stimulates by 11.1 mM glucose in isolated islets from WD DJB rats was related<sup>[9]</sup>. On the other hand, DJB performed in hypothalamic obese rats, decreased glucose-stimulated insulin secretion<sup>[29]</sup>. In our study, the static insulin secretion in the presence of 5.6 mM and 11.1 mM glucose (Fig. 2A and 2B, respectively) and 5.6 mM plus Leu, KCl and tolbutamide (Fig. 2A) was not altered by WD or DJB surgery.

Also, no alteration in GCK protein expression was observed between all groups.

Others metabolic substrates are able to induce insulin release. The importance of the PKC and cAMP/PKA pathway for insulin secretion process has long been demonstrated<sup>[34, 35]</sup>. When islets were stimulated by 11.1 mM glucose in the presence of CCh or PMA (Fig. 2B), insulin secretion was higher in WD SHAM group than CTL group. The acetylcholine muscarinic (M)-3 receptor protein expression in islets was decreased in WD SHAM animals (Fig. 3B), but PKC expression was not altered in this rats (Fig. 3C). Altered autonomic activity contributes to enhance  $\beta$  cell insulin secretory through increased parasympathetic and decreased sympathetic activity in rats high – carbohydrate<sup>[36, 37]</sup>. Since the PNS potentiates insulin secretion by M3 receptor, which activates phospholipase (PL)-C/PKC pathway<sup>[38]</sup>, we postulate that reduction in M3 receptor in islets from obese animals in the present study, may be due to  $\beta$ -cell physiological adaptation in function of increased in parasympathetic activity. However, the increase in insulin release by Cch and PMA persists yet, and we suggest that probably is due to alterations in calcium influx. DJB surgery normalizes insulin secretion in response of CCh and PMA. Recent studies have been shown that sensitivity of vagal innervation is increased after bariatric operation<sup>[39]</sup>. One recent report using Zucker *fa/fa* rats, showed that improve on glycemia after DJB is reliant on central and peripheral vagus inputs<sup>[25]</sup>. In according with these results, we hypothesized that DJB may have altered the vagal inputs to the central nervous system, normalizing insulin secretion in WD DJB animals. Also, in the present work, islets from WD SHAM animals when stimulated by 11.1 mM glucose

plus forskolin or IBMX, released more insulin than islets from CTL animals (Fig. 3B), suggesting an increased in [cAMP]<sub>i</sub>. PKA phosphorylates several proteins, resulting in increased [Ca<sup>++</sup>]<sub>i</sub>, insulin granules exocytosis and maintenance of  $\beta$ -cell mass<sup>[40]</sup>. Herein, PKA (Fig. 3D) and syntaxin (Fig. 3E) proteins expression was not changed by WD. For the first time, we observed that DJB surgery normalize glucose-stimulated insulin secretion in the presence of forskolin and IBMX. Nevertheless, the mechanism involved remains unknown. In addition to alterations in the function of pancreatic beta cells, IR may lead to changes in the morphology of islets. Studies in rodents<sup>[41]</sup> and humans<sup>[42]</sup> exhibit a dramatic increase in the mass of  $\beta$ -cells in obesity. Herein, WD SHAM rats presented an increased in islets and  $\beta$ -cell area (Fig. 4H and I, respectively), as well as, an enhanced in islets and  $\beta$ -cell mass compared to CTL group (Fig. 4J, K and L, respectively), which may explain in part the hyperinsulinemia observed in these animals. Many factors are involved with  $\beta$ -cell proliferation<sup>[43]</sup>. Parasympathetic nervous system (PNS) has been found to be involved in  $\beta$ -cell mass regulation under normal and pathological conditions<sup>[44-47]</sup>. There are few reports that investigated the effects of DJB on the islets morphometry. Woods et al.<sup>[48]</sup>, using C57BL/6J mice treated with HFD, not observed alterations in  $\beta$ -cell mass after DJB procedure, but found an increased in  $\alpha$ -cell/  $\beta$ -cell ratio. On the other hand, GK rats submitted to DJB operation, presented an increased in  $\beta$ -cell area compared to sham-operated rats<sup>[49]</sup>. In our study, DJB normalized islets and  $\beta$ -cell area and reduced islets and  $\beta$ -cell mass. Also, qualitative analysis of pancreas showed that WD SHAM rats exhibit increased fibrous tissue in islets compared to the CTL (Fig. 4E and D, respectively) and DJB attenuates fibrosis in the islets of obese animals, corroborating to study by Speck et al<sup>[49]</sup>. In this way, we suggest that DJB have a remedial effect on morphologic islets in obese rats by cafeteria diet, which prevents the evolution to total decompensation state, leading to development of T2DM. In summary, we have shown that obese animals by WD display obesity, hyperphagia, alterations

in glucose homeostasis, increase islet mass and  $\beta$ -cells, as well as, enhances glucose-stimulates insulin secretion in the presence of different secretagogues involved in PKC and PKA pathway. DJB surgery, decreased glycemia and insulinemia and improves insulin resistance in WD DJB animals, without changed body weight. In addition, for the first time we reported that DJB reduced islets and  $\beta$ -cell mass and normalized insulin secretion by PKC and PKA pathway. We postulate that the DJB surgery may be altering ANS activity and, consequently, leading to recovery  $\beta$ -cell mass and normalizing insulin secretion. It is important to emphasize that all these alterations occur before weight loss. More experiments are needed to prove our hypothesis.

### **Conflicts of interest**

All contributing authors declare that they have no conflicts of interest.

### **Acknowledgments**

We are grateful to Assis Roberto Escher Teixeira for animal care and Nicola Conran for editing English.

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**Table 1** – General features in CTL, WD SHAM and WD DJB rats.

|                                | CTL                     | WD SHAM                 | WB DJB                  |
|--------------------------------|-------------------------|-------------------------|-------------------------|
| Final Body Weight (g)          | 460 ± 10.2 <sup>a</sup> | 589 ± 13.8 <sup>b</sup> | 592 ± 17.1 <sup>b</sup> |
| Naso-anal length (cm)          | 24.3 ± 0.3 <sup>a</sup> | 25.2 ± 0.2 <sup>b</sup> | 25.7 ± 0.2 <sup>b</sup> |
| Lee Index                      | 318 ± 2.1 <sup>a</sup>  | 332 ± 2.2 <sup>b</sup>  | 326 ± 2.3 <sup>b</sup>  |
| Retroperitoneal fat pad (% BW) | 1.9 ± 0.2 <sup>a</sup>  | 3.1 ± 0.2 <sup>b</sup>  | 2.8 ± 0.1 <sup>b</sup>  |
| Perigonadal fat pad (% BW)     | 1.8 ± 0.1 <sup>a</sup>  | 2.7 ± 0.1 <sup>b</sup>  | 2.6 ± 0.1 <sup>b</sup>  |

Data are mean ± SEM (n =17-21). Different letters indicate significant difference. One-way

ANOVA with Tukey post hoc test (P < 0.05).

### Figure legends

Figure 1 - (A) Fasted serum levels of glucose and (B) insulin. (C) Changes in plasma glucose levels during ipGTT test, (D) AUC of ipGTT, (E) HOMA-IR and (F) pAkt<sub>1/2/3</sub><sup>Ser473</sup> protein expression in gastrocnemius muscle in CTL, WD SHAM and WD DJB rats. Different letters indicate significant differences. One-way ANOVA with Tukey post hoc test.  $P < 0.05$ ,  $n = 9-16$ . In D, the pAKT was determined in the absence (-) or presence (+) of insulin. \* indicate significant difference Student's *t* test;  $P < 0.05$   $n = 4-5$ .

Figure 2 - (A) Insulin secretion induced by 5.6 mM glucose in absence or presence of 40 mM KCl or 10 mM Leu in isolated islets from CTL, WD SHAM and WD DJB rats. (B) Insulin secretion induced by 11.1 mM glucose in absence or presence of 100  $\mu$ M Cch, 100 nM of PMA, 10  $\mu$ M Forskolin or 1 mM of IBMX in isolated islets from CTL, WD SHAM and WD DJB rats. Each bar represents mean  $\pm$  SEM of 7-20 groups of islets isolated from 12 rats in 2 independent experiments. Different letters indicate significant differences. One-way ANOVA with Tukey post hoc test.  $P < 0.05$ .

Figure 3 – GCK (A), mAChR M3 (B), PKC $\alpha$  (C), PKA $\alpha$  (D), and Sintaxin-1 (E) expression in isolated islets from CTL, WD SHAM and WD DJB rats. Protein extracts were processed for Western Blotting. The bars represent the mean values  $\pm$  SEM of the percentage from the ratio of densitometric values of GCK, mAChR M3, PKC $\alpha$ , PKA $\alpha$ , and Sintaxin-1 divided by  $\alpha$ -tubulin. Different letters indicate significant difference. One-way ANOVA with Tukey post hoc test.  $P < 0.05$ ,  $n = 4-6$ .

Figure 4 - Representative photomicrographs of insulin immunoperoxidase and trichrome of Mallory sections of pancreas from CTL (A, D), WD SHAM (B, E) and WD DJB (C, F)

animals. Pancreas weight (G), islet (H), beta cell (I), and non-beta cell area (J). Islet (K) and beta cell mass (L) from CTL, WD SHAM and WD DJB animals. Different letters indicate significant differences. One-way ANOVA with Tukey post hoc test.  $P < 0.05$ ,  $n = 4$ .

Figure 1

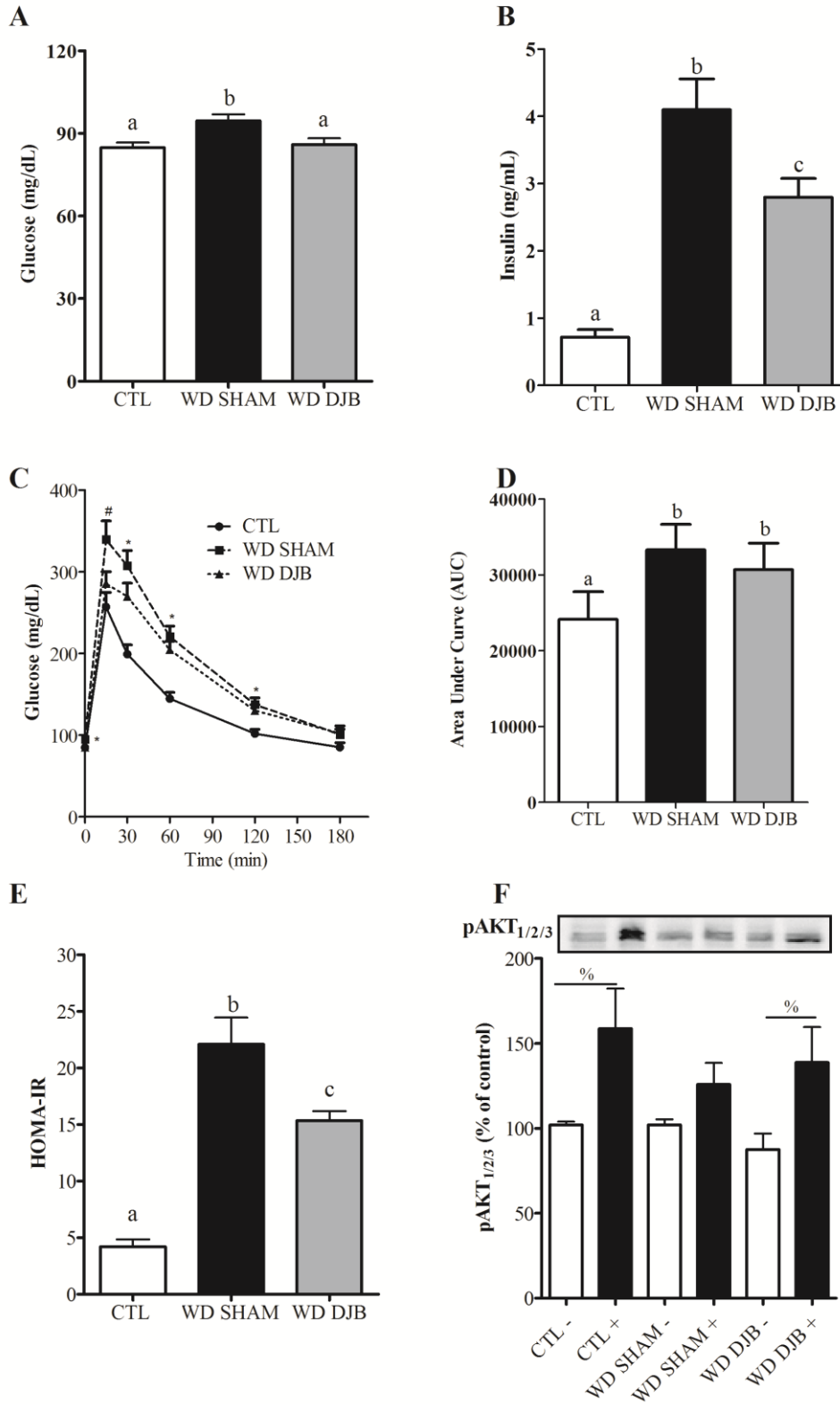






Figure 2

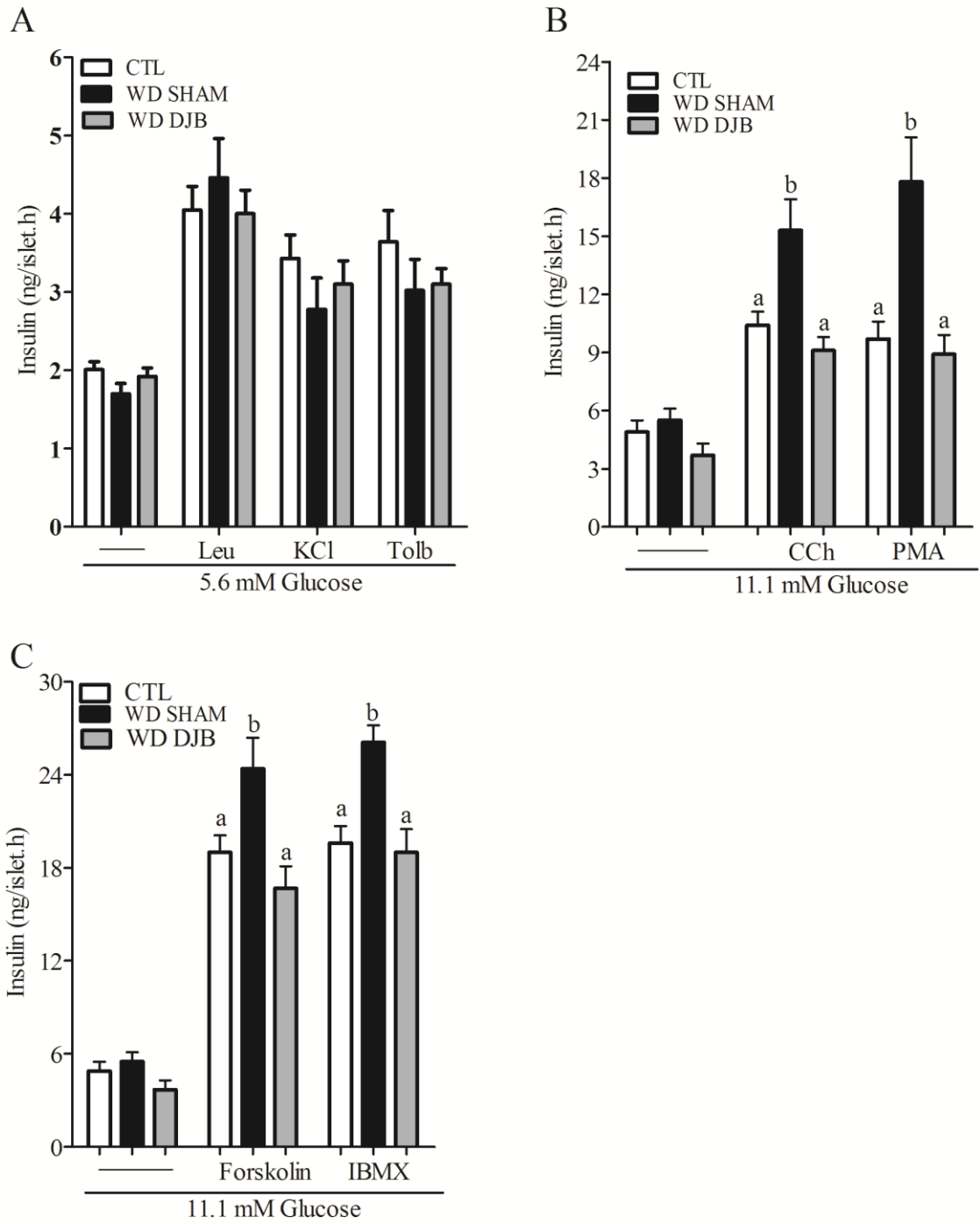


Figure 3

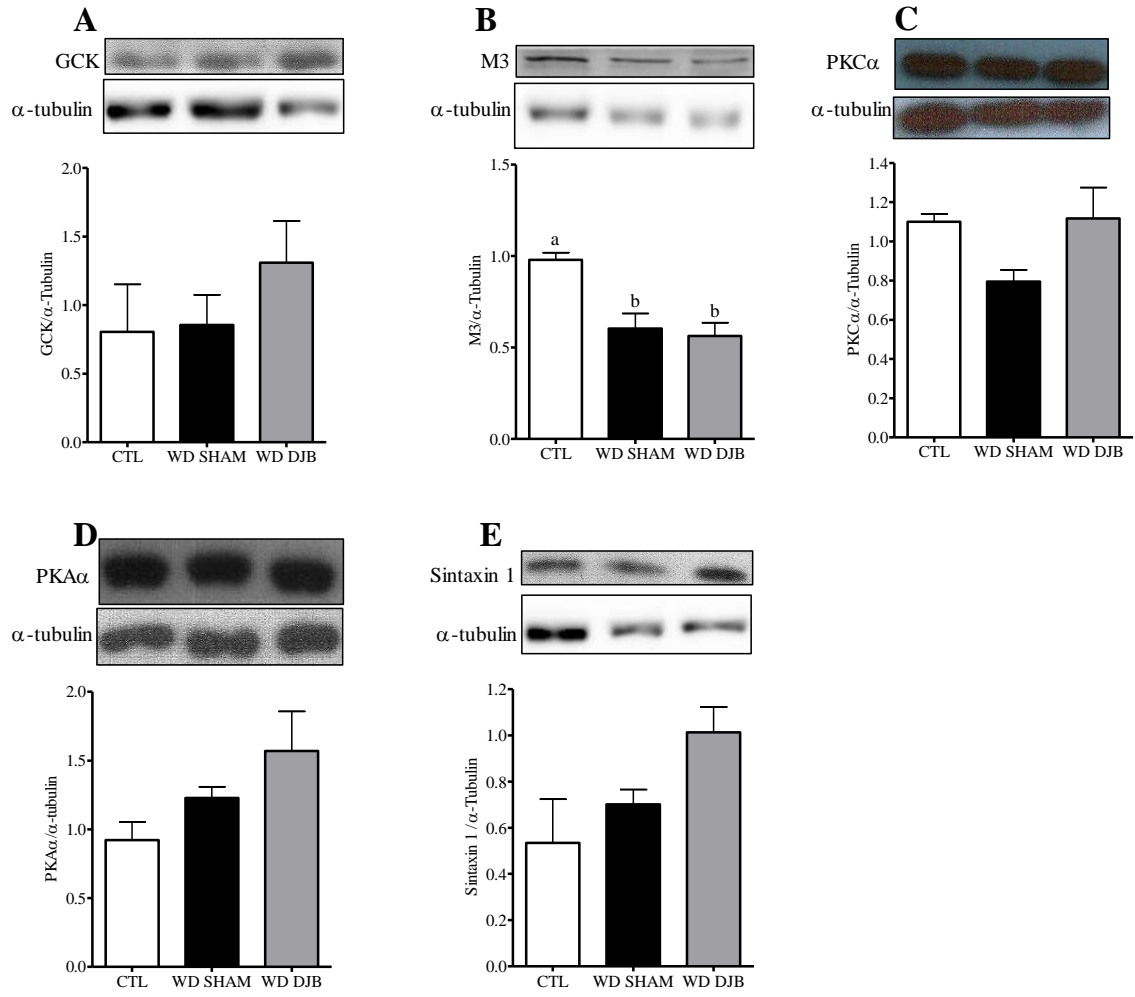
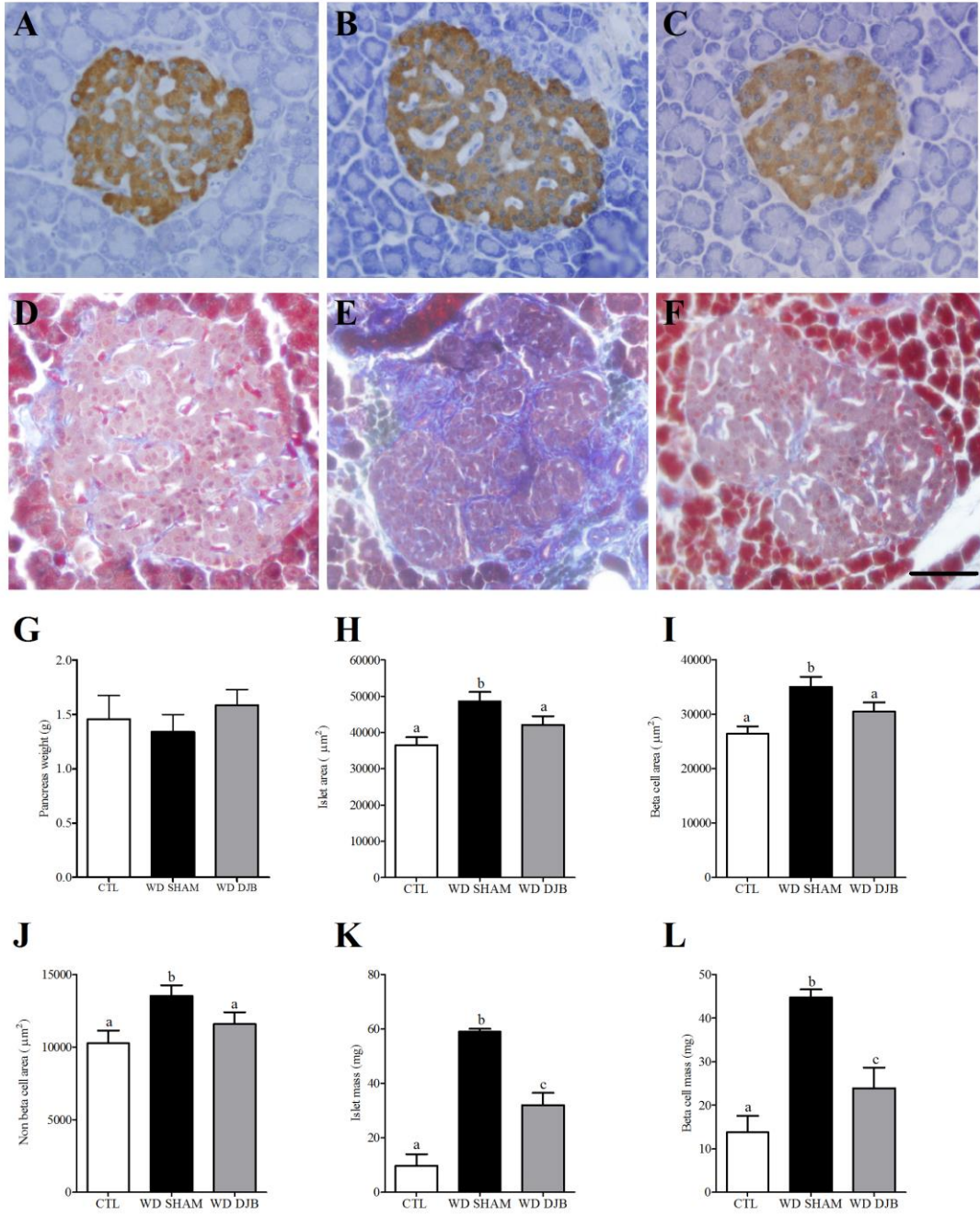


Figure 4



## APÊNDICE

|   | Valor<br>energético<br>kj/100g | Carboidrato<br>s<br>g/100g | Proteína<br>s<br>g/100g | Gord.<br>g/100g | Sódio<br>mg/100<br>g |
|---|--------------------------------|----------------------------|-------------------------|-----------------|----------------------|
| <b>Cheetos (Pepsico, Brasil)</b>                          | 1948                           | 72                         | 6,4                     | 17,2            | 676                  |
| <b>Salgadinho de Bacon Troféu ( Santa Helena, Brasil)</b> | 2200                           | 56                         | 8,8                     | 30              | 1040                 |
| <b>Biscoito de maizena (Zadimel, Brasil)</b>              | 1793                           | 73                         | 8                       | 10,7            | 300                  |
| <b>Bolo de chocolate (nutrella, Brasil)</b>               | 1798                           | 55                         | 5                       | 21,7            | 141,7                |
| <b>Coca-cola (Pepsico, Brasil)</b>                        | 178                            | 11                         | 0                       | 0               | 5                    |
| <b>Guaraná (Antartica, Brasil)</b>                        | 168                            | 10                         | 0                       | 0               | 5,5                  |
| <b>Salame tipo italiano (Sadia, Brasil)</b>               | 1822                           | 2                          | 22                      | 38              | 1140                 |
| <b>Linguiça (Sadia, Brasil)</b>                           | 1554                           | 1,4                        | 16                      | 34              | 1342                 |
| <b>Bisnaguinha (Nutrella, Brasil)</b>                     | 1328                           | 54                         | 11,2                    | 6,2             | 300                  |
| <b>Waffer de chocolate (Bauducco, Brasil)</b>             | 2176                           | 63                         | 5                       | 27              | 113                  |
| <b>Mortadela (Frimesa, Brasil)</b>                        | 845                            | 2                          | 12                      | 16              | 1545                 |
| <b>Marshmallow (Fini, Brasil)</b>                         | 1423                           | 80                         | 5                       | 0               | 46                   |

**Tabela 1** – Composição da dieta de cafeteria

## ANEXO A – CERTIFICADO DE APROVAÇÃO DO PROJETO PELO CEEAAP



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Estado do Paraná

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## CERTIFICADO

Certificamos que o Protocolo nº 8709, sob o título : “*Regulacao da secrecao de insulina em ratos obesos submetidos a derivação duodeno-jejunal*”, sob a responsabilidade de *Maria Lúcia Bonfleur*, tendo como período de realização início em 03/2009 e término 02/2012, está de acordo com os Princípios Éticos na Experimentação Animal, adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pelo Comitê de Ética na Experimentação Animal a Aulas Práticas (CEEAAP/Unioeste) em reunião realizada em 03 de dezembro de 2009, conforme Ata nº 112009.

Marechal Cândido Rondon, PR., 18/12/2009

Coordenadora do CEEAAP/Unioeste  
PORTARIA Nº 2618/2008 - GRE

**DRª PATRICIA BARCELLOS COSTA**  
Médica Veterinária  
CRMV/PR 07950

**ANEXO B – CERTIFICADO DE PRORROGAÇÃO DO PROJETO PELO CEEAAP**

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**Comitê de ética na experimentação animal e aulas práticas – CEEAAP/UNIOESTE**

Concedemos a prorrogação do término do projeto do Protocolo nº 87/09 sob o título “**Regulação da secreção de insulina em ratos obesos submetidos à derivação duodeno-jejunal**”, sob a responsabilidade da professora Dra. **Maria Lúcia Bonfleur**, para 31/12/2012.

**Cascavel, 18 de maio de 2011.**

A handwritten signature in blue ink, which appears to read 'Maria Lúcia Bonfleur'.

**Dra. Maria Lúcia Bonfleur**  
**Coordenadora do CEEAAP/UNIOESTE**  
**Portaria Nº0903/2011-GRE**

**ANEXO C – CERTIFICADO DE PRORROGAÇÃO DO PROJETO PELO CEEAAP****PARECER DE PROTOCOLO**

A solicitação de prorrogação de prazo do protocolo intitulado "Regulação da secreção de insulina em ratos obesos submetidos à derivação duodeno-jejunal", sob vossa coordenação, foi avaliado pelo CEUA como **APROVADO**.

**ATENÇÃO!**

O Certificado Experimental deste Protocolo, somente será emitido após o encerramento das atividades previstas e após o encaminhamento do Relatório Final ao CEUA. Este Parecer **NÃO** tem valor como Certificado Experimental.

Cascavel, 09/04/2013

Profa. Dra. Luciana Oliveira do Fariña  
Coordenadora do CEUA  
Portaria nº 2861/2012-GRE



## ANEXO D

### Normas da revista científica - Life Sciences

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### Acknowledgements

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, *J. Sci. Commun.* 163 (2010) 51–59.

Reference to a book:

[2] W. Strunk Jr., E.B. White, *The Elements of Style*, fourth ed., Longman, New York, 2000.

Reference to a chapter in an edited book:

[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), *Introduction to the Electronic Age*, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[4] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2003 (accessed 13.03.03).

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