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Letícia Favara Laurino

PROPRIEDADES DO COGUMELO CULINÁRIO-MEDICINAL *Lentinus edodes* NO DESENVOLVIMENTO MATERNO-FETAL DE RATAS COM DIABETES MELITUS GESTACIONAL

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Dissertação apresentada à Banca Examinadora do Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade de Sorocaba, como exigência parcial para obtenção do título de Mestre em Ciências Farmacêuticas.

Orientadora: Profa. Dra. Marli Gerenutti

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Dissertação aprovada como requisito parcial para obtenção do grau de Mestre no Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade de Sorocaba

Aprovado em: ___/___/_____

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EPÍGRAFE

“Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer.”

(Mahatma Gandhi)

RESUMO

Introdução: A prevenção e o tratamento do GDM (diabetes mellitus gestacional) são de extrema importância, sendo a terapia nutricional uma das principais formas para o controle glicêmico adequado. A busca por produtos naturais com efeitos antidiabéticos tem aumentado na medicina preventiva, sendo o cogumelo *Lentinus edodes* um dos mais consumidos e estudados do mundo, devido ao seu valor nutricional e potenciais ações terapêuticas. A presença de β-glucanas e compostos fenólicos indica que este cogumelo pode ser usado como suplemento nutricional com alto poder antioxidante. **Objetivo:** avaliar por meio de testes pré-clínicos as propriedades da exposição diária ao *Lentinus edodes* em ratas com GDM-STZ (diabetes mellitus gestacional induzida por estreptozotocina). **Métodos:** Foram empregadas técnicas de avaliação perinatal com a exposição materna diária ao pó liofilizado de *Lentinula edodes* antes (GDM+Leb) e após (GDM+Lea) a indução do diabetes gestacional com estreptozotocina (40 mg/kg, intravenosa) no 8º dia de gestação. Foram realizados: (1) teste materno de tolerância oral à glicose; (2) avaliação da capacidade reprodutiva; (3) avaliações bioquímicas e de estresse oxidativo de sangue materno e placenta e avaliação bioquímica de líquido amniótico; (4) hemograma materno completo; (5) dosagens de insulina e (6) avaliações do desenvolvimento embriofetal. **Resultados:** *Lentinus edodes* não reduziu a hiperglicemia severa da mãe-concepto, mas promoveu melhora na tolerância materna à glicose, aumento nos níveis de insulina maternos e no líquido amniótico. Ambos os grupos tratados com o cogumelo reduziram os níveis de ALT (alanina aminotranferase) e AST (aspartato aminotranferase). Também reduziu os níveis de triglicérides das ratas GDM e de colesterol total. O *Lentinus edodes* não foi capaz de alterar a redução do ganho de peso materno, mas protegeu os animais das perdas pós-implantação e promoveu aumento das medidas fetais, o que indica possível efeito protetor do cogumelo quando administrado antes da estreptozotocina. A administração de *Lentinus edodes* no GDM-STZ melhorou alguns parâmetros de estresse oxidativo, protegendo mãe e concepto dos danos da hiperglicemia. **Conclusão:** A dose intravenosa de 40 mg/kg de STZ promoveu diabetes severo e prolongado. O *Lentinus edodes* administrado antes da STZ promoveu melhora na tolerância materna à glicose, protegeu os animais das perdas pós-implantação, apresentou redução nos níveis de colesterol total e aumento nos

níveis de insulina. Nos grupos GDM+Leb e GDM+Lea, os danos hepáticos provocados pela STZ foram revertidos. O cogumelo *Lentinus edodes* possui propriedades antioxidantes que podem minimizar os danos causados pelo diabetes mellitus gestacional.

Palavras-chave: *Lentinus edodes*. Diabetes mellitus gestacional. Estreptozotocina. Teste oral de tolerância à glicose. Estresse oxidativo.

ABSTRACT

Introduction: Prevention and treatment of GDM (gestational diabetes mellitus) are highly important, and one of the main forms of proper glycemic control is by nutritional therapy. The search for natural products with antidiabetic effects has increased in preventive medicine, and one of the most consumed mushroom, *Lentinus edodes,* is also the most studied of the world, due to its nutritional value and potential therapeutic actions. The presence of β-glucans and phenolic compounds indicates that this mushroom can be used as nutritional supplement with high antioxidant power. **Aim:** to evaluate the daily exposition to *Lentinus edodes* in GDM-STZ (gestational diabetes mellitus induced by streptozotocin) rats through preclinical trials. **Methods:** Perinatal assessment techniques were used with daily maternal exposure to the lyophilized powder of *Lentinula edodes* before (GDM+Leb) or after (GDM+Lea) the induction of gestational diabetes with streptozotocin (40 mg/kg, intravenous) on the 8th pregnancy day. Were assessed: (1) maternal oral glucose tolerance test; (2) reproductive capacity; (3) biochemical and oxidative stress of maternal blood and placenta and biochemical evaluation of amniotic fluid; (4) maternal hematological parameters; (5) insulin dosages and (6) evaluations of embryo-fetal development. **Results:** *Lentinus edodes* did not reduce the severe hyperglycemia of mother-concept, but promoted an improvement in maternal glucose tolerance, and an increase in maternal and amniotic fluid of insulin levels. Both groups treated with the mushroom reduced ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels. It also reduced triglyceride levels and total cholesterol in GDM rats. *Lentinus edodes* was not able to ameliorate the reduction in maternal body weight gain but protected the animals from post-implantation losses and promoted an increased in fetal measurements, indicating a possible protective effect of the mushroom when administered before streptozotocin. The administration of *Lentinus edodes* in GDM-STZ improved some parameters of oxidative stress, protecting the dams and concepts from hyperglycemia damage. **Conclusion:** The intravenous dose of 40 mg/kg STZ promoted severe and prolonged diabetes. *Lentinus edodes* administered before the STZ promoted improvement in maternal glucose tolerance, protected the animals from post-implantation losses, presented a reduction in total cholesterol levels and an increase in insulin levels. Liver damage caused by STZ was reversed in GDM+Leb and GDM+Lea groups. *Lentinus*

edodes mushroom has antioxidant properties that can minimize the damage caused by Gestational Diabetes Mellitus.

Key-words: *Lentinus edodes*. Gestational Diabetes Mellitus. Streptozotocin. Oral glucose tolerance test. Oxidative stress.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

ALT - Alanina aminotranferase

AST - Aspartato aminotranferase

CAT - Catalase

CEUA - Comissão de ética no uso de animais

GDM - Diabetes mellitus gestacional

GDM-STZ - Diabetes mellitus gestacional induzida por estreptozotocina

DM - Diabetes mellitus

DTNB - 5-5-ditio-bis-2-ácido nitrobenzóico

EAG - Equivalente de ácido gálico

EDTA - Ácido etilenodiamino tetra-acético

FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo

GR - Glutationa redutase

GPx - Glutationa peroxidase

GSH - Glutationa reduzida

Hb - Hemoglobina

HCl - Ácido clorídrico

HDL-col - Lipoproteína de alta densidade

H_2O_2 - Peróxido de hidrogênio

H_3PO_4 - Ácido fosfórico

HTC - Hematócrito

IV - Intravenosa

KCl - Cloreto de potássio

KOH - Hidróxido de potássio

LAPETOX - Laboratório de Pesquisa Toxicológica

LDL - Lipoproteína de baixa densidade

MDA - Malondialdeído

mM - Milimolar

NaOH - Hidróxido de sódio

nm - Nanômetro

OH - Radical hidroxila

OMS - Organização Mundial da Saúde

O_2 - Oxigênio

PLQ - Número de plaquetas

RBC - Contagem total de eritrócitos

rpm - Rotação por minuto

SDS - Dodecil sulfato de sódio

SOD - Superóxido dismutase

STZ - Estreptozotocina

TAS - Tampão acetato de sódio

TBA - Ácido tiobarbitúrico

TBARS - Substâncias reativas ao ácido tiobarbitúrico

TCA - Ácido tricloroacético

TFK - Tampão fosfato de potássio

TOTG - Teste Oral de Tolerância à Glicose

UNISO - Universidade de Sorocaba

USP - Universidade de São Paulo

WBC - Contagem total de Leucócitos

SUMÁRIO

1 JUSTIFICATIVA

O DM (diabetes mellitus) é um problema global de saúde pública que tem crescido durante as últimas décadas, refletindo maiores riscos associados tais como o sobrepeso e a obesidade. Nos últimos anos, o DM teve um aumento significante nas populações de países subdesenvolvidos comparado às de países de alta renda, trazendo perdas econômicas para sistemas de saúde e para as políticas nacionais e, consequentemente para os indivíduos portadores de DM. Esforços para melhorar o diagnóstico e o tratamento do diabetes devem ocorrer integradamente nas políticas públicas relacionadas à saúde e a educação.

Na gestação, o GDM (diabetes mellitus gestacional) aumenta o risco de morte fetal e de outras complicações, como aumento do risco no desenvolvimento de diabetes tipo 2 para a gestante e para a criança ao longo da vida. Os efeitos da exposição intrauterina ao diabetes têm implicações importantes para a saúde pública no contexto crescente do diabetes. A tendência para o desenvolvimento do DM mais precocemente pode induzir um círculo vicioso. Mudanças no hábito de vida, exercícios frequentes e dieta continuam sendo as melhores maneiras de prevenção do diabetes gestacional e seus efeitos a longo prazo.

O entendimento das informações acumuladas cientificamente sobre os tratamentos efetivos e a prevenção dessa doença são extremamente necessários. A OMS (Organização Mundial da Saúde) destaca a enorme dimensão do problema do GDM e também o potencial de reverter as tendências atuais.

As dietas desenvolvidas com alimentos funcionais têm sido consideradas uma das mais simples, importantes e não invasivas alternativas na terapia complementar no GDM.

O cogumelo culinário-medicinal *Lentinus edodes* (Shiitake) é usado medicinalmente em doenças que envolvem o sistema imune, câncer, alergias e

diabetes, sendo o segundo cogumelo mais utilizado do mundo devido à presença de alto teor de compostos antioxidantes.

A STZ (estreptozotocina) é um agente antimicrobiano, que vem sendo utilizado há algumas décadas por seu efeito diabetogênico bem caracterizado como droga β-citotóxica pancreática. A GDM-STZ (diabetes mellitus gestacional induzida por estreptozotocina) é um modelo experimental bem estabelecido para a avaliação da deficiência insulínica e dos efeitos hiperglicêmicos sobre as gestantes e seus fetos.

Os efeitos antidiabéticos do *Lentinus edodes* sobre o DM-STZ (diabetes mellitus induzido por estreptozotocina) já foram avaliados em alguns estudos *in vivo*; as β-glucanas presentes no *Lentinus edodes* parecem agir reparando as lesões em células β-pancreáticas, promovendo o aumento na síntese de insulina e a diminuição dos níveis plasmáticos de glicose. Entretanto, os estudos em gestantes diabéticas tratadas com este cogumelo são praticamente inexistentes. Sendo assim, se desconhecem os efeitos protetores da administração diária deste cogumelo, em forma de pó liofilizado, sobre ratas prenhes com Diabetes Mellitus Gestacional induzido por estreptozotocina (GDM-STZ).

Desta forma este estudo justifica-se, pois acreditamos que o cogumelo *Lentinus edodes* quando utilizado em baixas doses, pode ser uma opção eficaz como alimento funcional no tratamento do GDM-STZ.

2 OBJETIVOS

2.1 Objetivo Geral

O presente estudo busca avaliar por meio de testes pré-clínicos as propriedades da exposição diária de *Lentinus edodes* em ratas GDM-STZ, considerando suas potencialidades como alimento funcional.

2.2 Objetivos Específicos

- Avaliar a evolução gestacional e o desempenho reprodutivo de ratas GDM-STZ.

- Avaliar o desenvolvimento embriofetal por meio de análises placentária, fetal e do líquido amniótico.

- Correlacionar parâmetros de estresse oxidativo no sangue materno e placenta com dosagens de compostos fenólicos e β-glucanas.

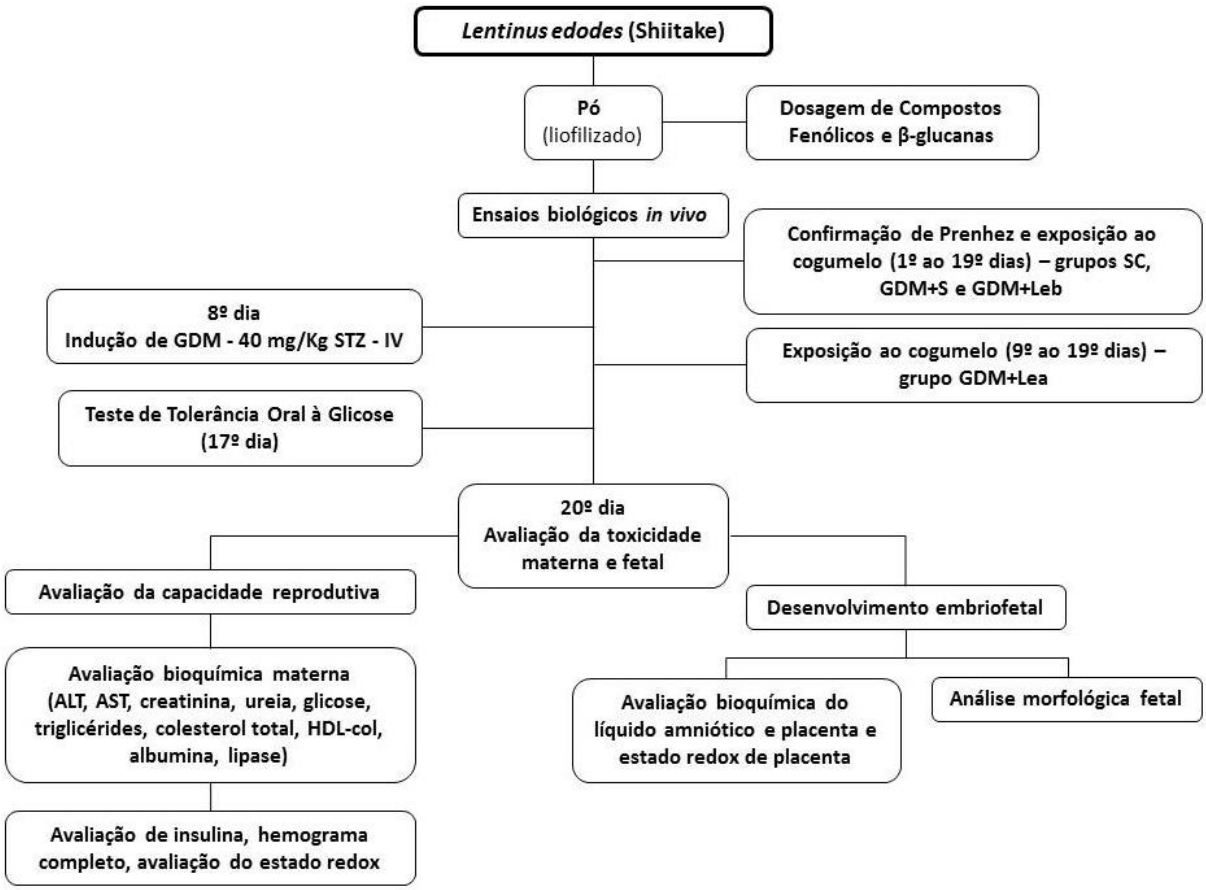
3 MATERIAL E MÉTODOS

Foi realizado um ensaio pré-clínico *in vivo* com animais GDM-STZ expostos ao cogumelo *Lentinus edodes*.

3.1 Delineamento do Estudo

Trata-se de um ensaio pré-clínico in *vivo* (Figura 1). O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade de Sorocaba CEUA/UNISO Protocolo nº 089/2016 (ANEXO A) e recebeu apoio FAPESP (Processo nº 2015/24566-9).

Figura 1 - Desenho do estudo.



Fonte: Elaboração própria.

3.1.1 Obtenção do pó liofilizado de cogumelo

Amostras de cogumelos *Lentinus edodes* (Berk.) Pegler-cultivated strain H600 (Hokken, Shimotsuga-gun, Japan) foram adquiridas comercialmente da empresa Yuki Cogumelos Ltda *in natura,* picadas no mesmo dia da coleta em tamanhos homogêneos e armazenadas em biofreezer a -80ºC (REVCO® ULT-1386-3-D) por aproximadamente 24 h. O material congelado foi liofilizado em Liofilizador – Termo Savant, LK-40, por aproximadamente 48 h até a obtenção de 10% de massa seca. A amostra seca foi moída (moinho de facas e martelo marca Marconi®), em seguida tamisada em malha 50 e malha 60, para obtenção de partículas homogêneas. O pó tamisado foi acondicionado em embalagens plásticas herméticas mantidas em dessecador.

Figura 2 - Obtenção do pó liofilizado a partir do cogumelo *in natura*.

Nota: A: *Lentinus edodes in natura*. B: Liofilizador Termo Savant, LK-40. C: Moinho de facas e martelos. D: Pó do cogumelo seco e liofilizado.

Fonte: Elaboração própria.

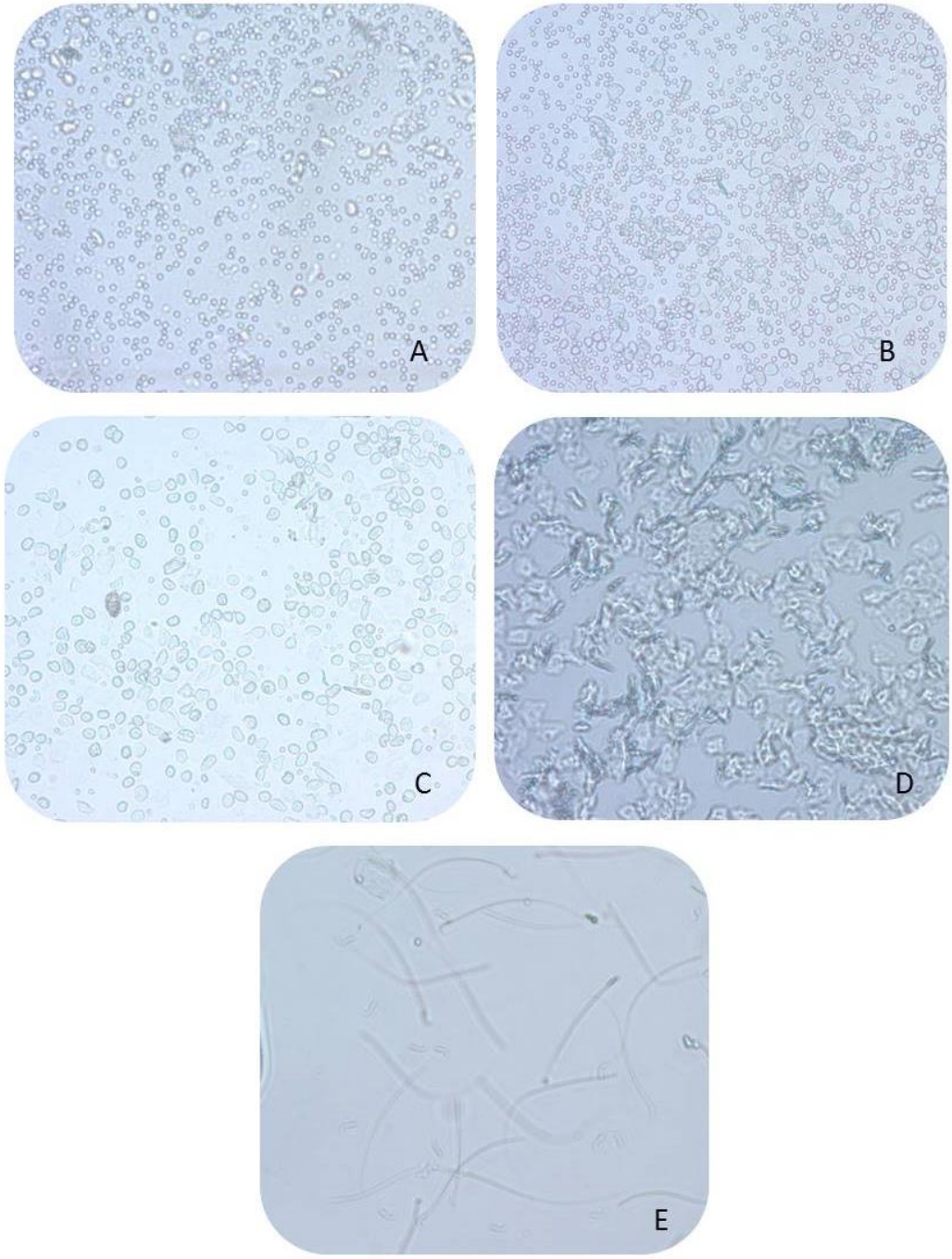
3.1.2 Evolução gestacional e desempenho reprodutivo de ratas GDM-STZ

3.1.2.1 Acasalamento, prenhez, indução do diabetes e exposição ao cogumelo

Foram utilizados Ratos Wistar, machos e fêmeas, pesando entre 180 a 200 g, adquiridos com respectivos atestados de sanidade (ANEXO B) do Biotério de Produção de Ratos da Universidade de São Paulo - USP. Os animais foram ambientados no Biotério de Experimentação Animal do LAPETOX, conforme as normas de bem-estar animal, em gaiolas ventiladas, aclimatadas e com forração de maravalha; em temperatura e ciclos claro/escuro, controlados automaticamente, a 23±3°C e 12h, respectivamente. Os animais receberam ração e água *ad libitum*. Para o acasalamento, os animais foram alojados em número de três, 01 macho com 03 fêmeas, por um período noturno de 12 horas.

Por meio de observações microscópicas (Microscópio Biológico, Modelo Axio Lab.A1, ZEISS®) (Figura 3), a indicativa do primeiro dia da prenhez foi a presença de espermatozoides no esfregaço proveniente do lavado vaginal (GERENUTTI; DEL FIOL; GROPPO, 2006).

Figura 3 - Acompanhamento do ciclo estral e da prenhez de ratas através do lavado vaginal.



Nota: A: Metaestro. B: Diestro. C: Proestro. D: Estro. E: Presença de espermatozoides (indicativa de prenhez).

Fonte: Elaboração própria.

Todas as ratas prenhes foram alojadas, também em condições especiais, considerando-se o bem-estar animal, conforme ilustra a Figura 4, em número de um animal por gaiola e divididas em quatro grupos de seis animais cada.

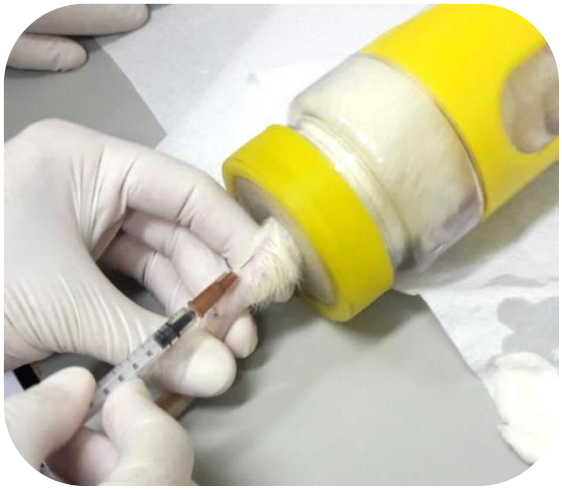
Figura 4 - Isoladores micro ambientais Alesco®.

Fonte: Elaboração própria.

Para a indução do diabetes mellitus moderado, após permanecerem em jejum de 6 horas, os animais receberam, por via intravenosa caudal, 40 mg/kg de estreptozotocina (Sigma Chemical, St. Louis, MO) diluída em 0,1 mol/L de tampão citrato, pH 4,5 (TOMA et al., 2015; VOLPATO et al., 2008), conforme ilustra a Figura 5.

A indução do diabetes foi feita via intravenosa caudal por ser mais apropriada para obtenção da dose diabetogênica esperada e devido à permanência do diabetes e à menor probabilidade de falha na indução (DA DELFINO et al., 2002).

Figura 5 - Indução do diabetes no 8º dia de gestação através da aplicação de STZ via caudal.

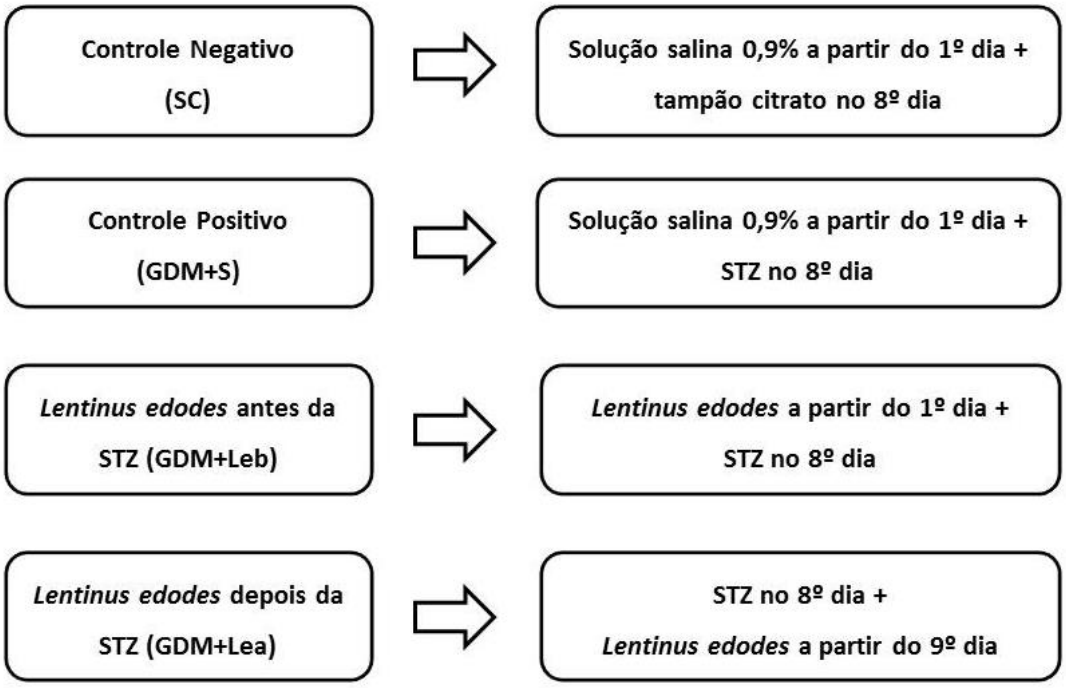


Fonte: Elaboração própria.

O estudo foi dividido em duas formas de exposição ao cogumelo: (I) antes da indução do GDM pela STZ para observar a possível proteção materna dos danos induzidos pela STZ; (II) após a indução do GDM pela STZ para se observar um possível tratamento dos danos maternos e fetais induzidos pela STZ.

As ratas prenhes foram distribuídas de forma randomizada entre os grupos, como descrito na Figura 6.

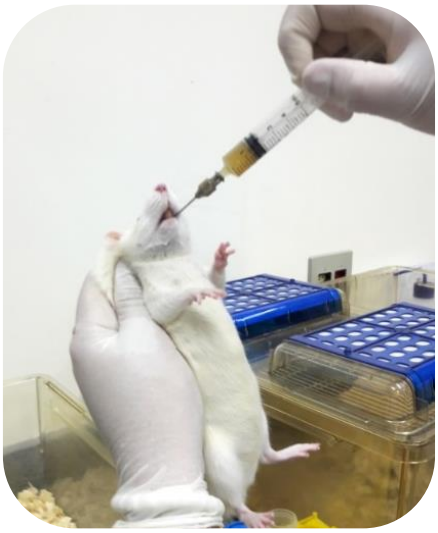
Figura 6 - Divisão dos grupos.



Fonte: Elaboração própria.

Na exposição I o *Letinus edodes* foi administrado por via oral (gavagem), conforme ilustra a Figura 7, do 1º ao 19º dia de prenhez e a STZ foi administrada no 8º dia de prenhez. Na exposição II a STZ foi administrada no 8º dia de prenhez e o cogumelo foi administrado a partir do 9º até o 19º dia.

Figura 7 - Administração do cogumelo via gavagem.



Fonte: Elaboração própria.

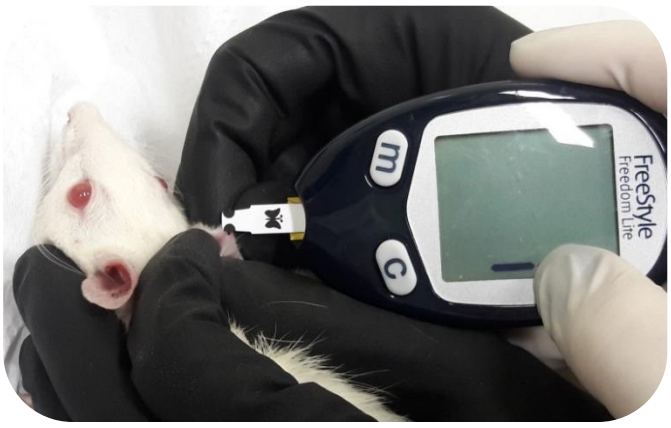
Em todos os grupos estudados, após 48 horas da administração da STZ ou do tampão citrato, os animais permaneceram em jejum por 6 horas e, após, o sangue periférico do pavilhão auricular foi colhido para dosagem de glicose (Monitor de Glicemia FreeStyle Lite, Abbott). Não houve óbito e todos os animais que apresentaram glicemia acima de 120 mg/dL foram considerados diabéticos e inclusos nos grupos.

A dose 100 mg/Kg/dia de *Lentinus edodes* foi definida com base em estudos prévios (FRIZO et al., 2014; GROTTO et al., 2016). O peso dos animais foi acompanhado diariamente.

3.1.2.2 Teste Oral de Tolerância à Glicose (TOTG)

Para o acompanhamento da GDM-STZ foi empregado o teste oral de tolerância à glicose (TOTG), que foi realizado no dia 17º de prenhez. Após jejum de 6 horas, uma solução de dextrose foi administrada por gavagem (2 g/kg de peso corporal); e as amostras de sangue colhidas do pavilhão auricular foram utilizadas para determinações de glicemia, em 0, 10, 20, 30, 60 e 120 minutos (DE MELLO et al., 2001), utilizando Monitor de Glicemia FreeStyle Lite, Abbott (Figura 8).

Figura 8 - Teste oral de tolerância à glicose.

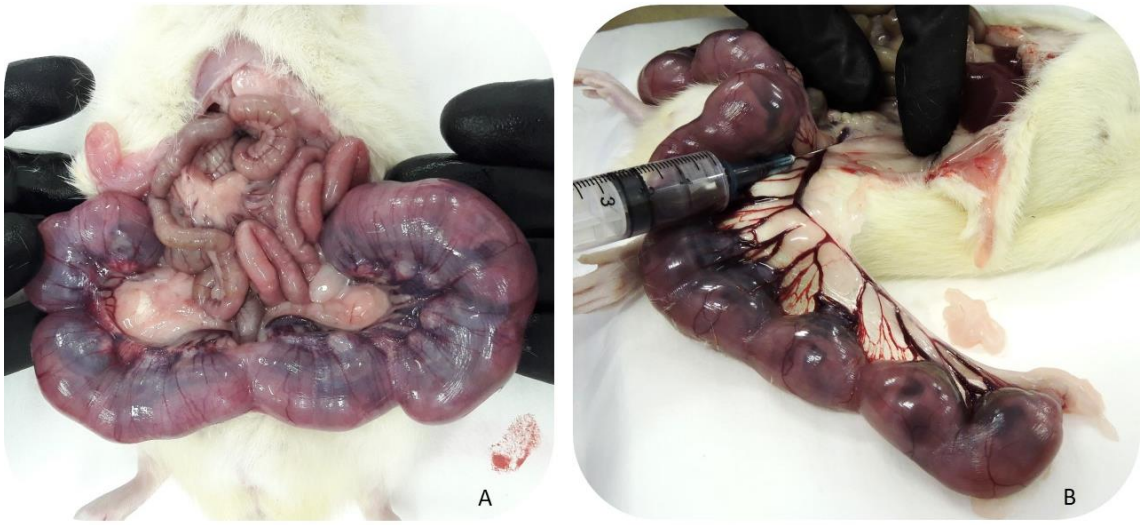


Fonte: Elaboração própria.

3.1.2.3 Procedimento de cesárea e coleta de material biológico para análises

No vigésimo dia de prenhez, as ratas não estavam em jejum e foram anestesiadas com quetamina (anestésico geral na dose de 100 mg/Kg) e cloridrato de xilazina (relaxante muscular na dose de 6 mg/Kg), via intraperitoneal. Foram realizadas as cesáreas, com uma incisão longitudinal na linha alba para exposição do útero e dos ovários. O sangue foi coletado por punção venosa hepática (Figura 9) em seringa previamente heparinizada, e transferido para dois tubos: (tubo A) contendo anticoagulante ácido etilenodiamino tetra-acético (EDTA) para avaliação dos parâmetros hematológicos e de estado redox e (tubo B) sem o anticoagulante e com gel separador para análises bioquímicas.

Figura 9 - Procedimento de cesárea e coleta de sangue.



Nota: A: Exposição do útero. B: Coleta do sangue materno por punção venosa hepática.
Fonte: Elaboração própria.

Os seguintes parâmetros hematológicos maternos foram analisados com o auxílio do equipamento automático Hematologia XS 1000i WAS, Roche®: hemoglobina, hematócrito, contagem total de leucócitos (WBC), contagem total de eritrócitos (RBC) e número de plaquetas (PLT).

Para o estado redox foram analisadas as atividades/concentrações de glutationa reduzida (GSH), glutationa peroxidase (GPx), catalase (CAT) e malondialdeído (MDA) por espectrofotometria (PerkinElmer®, Lambda 35), conforme descrito no item 3.1.4.2.

Os seguintes parâmetros bioquímicos foram analisados com o auxílio do equipamento automático Cobas C111, Roche®: alanina aminotranferase (ALT), aspartato aminotranferasee (AST), albumina, creatinina, ureia, colesterol total, lipoproteína de alta densidade (HDL-col), triglicérides, glicose e lipase. A partir dos resultados de medicação fotométrica que determinam a quantidade de absorbância do fluído, os parâmetros foram calculados pelo equipamento.

Na avaliação hepática, as determinações das atividades das aminotransferases (ALT e AST) foram realizadas por métodos enzimáticos, cinéticos e ultravioleta. O índice de Ritis foi calculado através da divisão dos

níveis de AST por ALT (RIEF et al., 2016) para avaliação da extensão da lesão hepática.

A concentração sanguínea de albumina foi determinada por método colorimétrico, não enzimático e de ponto final.

A avaliação clínica laboratorial do perfil renal foi determinada pela concentração sanguínea de creatinina e ureia, realizadas por métodos cinéticos e colorimétricos, sendo o primeiro não enzimático e o segundo, enzimático.

As medidas de colesterol total, HDL-colesterol e triglicérides foram feitas por métodos enzimáticos colorimétricos de ponto final, sendo o HDL-col obtido pela separação da fração HDL das demais lipoproteínas por meio da formação de complexos hidrossolúveis com íons de magnésio e sulfato de dextrano.

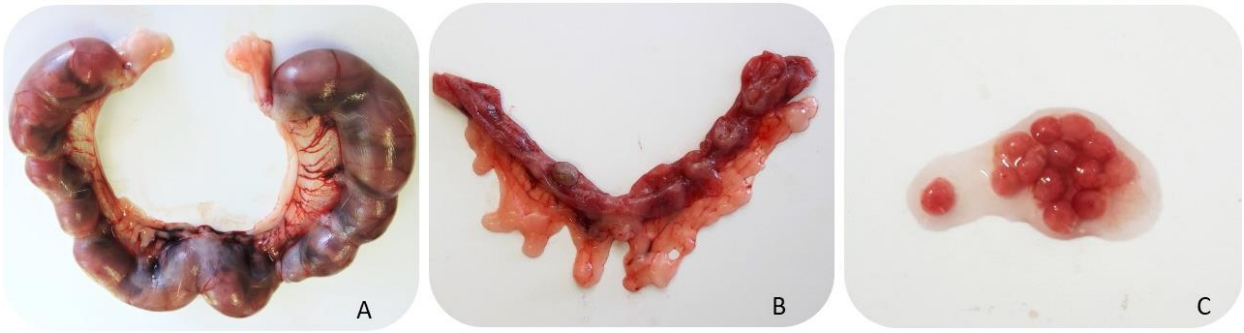
A glicose foi obtida pela utilização de métodos enzimáticos, colorimétricos e de ponto final.

A dosagem plasmática de insulina foi realizada por teste Elisa, de fase sólida, baseado no princípio Sanduíche, utilizando-se kit comercial específico para ratos, Rat/Mouse Insulin Elisa Kit - Merck®.

3.1.2.4 Avaliação da capacidade reprodutiva.

Para avaliação da capacidade reprodutiva, os cornos uterinos foram seccionados para a retirada dos sacos gestacionais e os ovários foram retirados. Em seguida o útero foi inspecionado quanto ao número de fetos, a vitalidade fetal, número de implantações e de reabsorções visíveis. O útero e os ovários foram pesados em balança (Ohaus®-AS200S) e os corpos lúteos foram contados (Figura 10). Após a realização do procedimento as ratas foram sacrificadas por aprofundamento anestésico, seguido de ruptura diafragmática.

Figura 10 - Inspeção do útero quanto ao número de fetos, de implantações e reabsorções visíveis e contagem de corpos lúteos.



Nota: A: Retirada do útero e dos ovários. B: Inspeção do útero quanto ao número de implantações e reabsorções. C: Contagem de corpos lúteos.

Fonte: Elaboração própria.

Para avaliar as perdas pós-implantação, foi utilizada a equação abaixo (GERENUTTI et al., 2014).

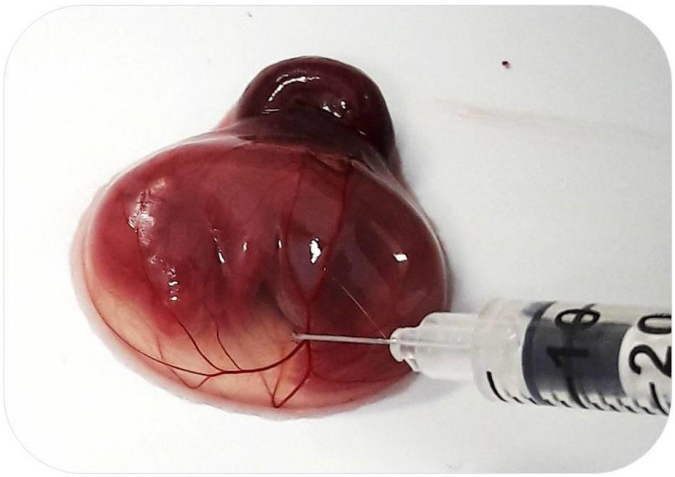
$$\%\ Perdas\ Pós\ implantação = \frac{nº\ de\ implantações - nº\ de\ fetos\ vivos}{nº\ de\ implantações}\ x\ 100$$

3.1.3 Desenvolvimento embriofetal e análise placentária

3.1.3.1 Coleta e processamento de líquido amniótico

O líquido amniótico foi coletado dos sacos gestacionais e transferido para dois tubos tipo eppendorf (Figura 11). O processamento das amostras foi feito através de uma adaptação do método utilizado por Zogno, Miglino e Oliveira (2004). No momento da análise, a amostra de líquido amniótico foi descongelada e centrifugada por 10 minutos a 2500 rpm e 22ºC, o sobrenadante foi utilizado para realização das análises de glicose e lipase (equipamento Cobas C111, Roche®). A dosagem de insulina foi realizada por teste Elisa, de fase sólida, baseado no princípio Sanduíche com kit comercial específico para ratos, Rat/Mouse Insulin Elisa Kit - Merck®.

Figura 11 - Coleta de líquido amniótico do interior do saco gestacional de fetos de ratas.

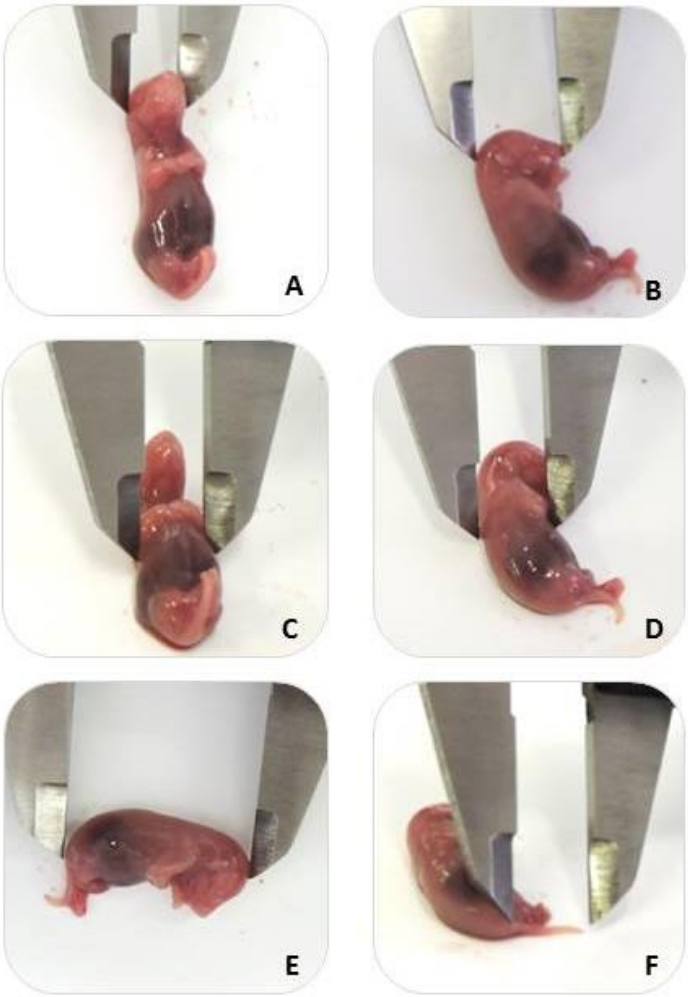


Fonte: Elaboração própria.

3.1.3.2 Avaliação morfológica fetal

Após a ruptura do saco embrionário os fetos foram separados das placentas, pesados em balança (Ohaus®-AS200S), e analisados através de medidas morfométricas externas: ântero-posterior do crânio, látero-lateral do crânio, ântero-posterior do tórax, látero-lateral do tórax, crânio-caudal e cauda (Figura 12); anestesiados e sacrificados com halotano (saturação de cuba).

Figura 12 - Avaliação morfológica externa de fetos (medidas realizadas com paquímetro).



Nota: Medidas - A: Látero-lateral de crânio. B: Ântero-posterior de crânio. C: Látero-lateral de tórax. D: Ântero-posterior de tórax. E: Crânio-caudal. F: Cauda.

Fonte: Elaboração própria.

3.1.3.3 Processamento da placenta

Após a ruptura do saco embrionário as placentas foram retiradas, pesadas em balança (Ohaus®-AS200S) e analisadas quanto ao estado redox.

Para avaliação de estresse oxidativo, as placentas foram armazenadas em biofreezer a -80 ºC (REVCO® ULT-1386-3-D) até o momento das análises. Um *pool* de placentas com 250 mg de cada rata foi sonicado com 5 mL de KCl 1,15% com auxílio de processador ultrassônico (Sonics® VCX 130 PB) por dois

ciclos de 30 segundos com 35% de amplificação e posteriormente processadas de acordo com as análises específicas para determinação das atividades de GSH, GPx, catalase e MDA, seguindo a metodologia descrita no item 3.1.4.2.

3.1.4 Dosagens de compostos fenólicos e β-glucanas e análise de parâmetros de estresse oxidativo

3.1.4.1 Dosagens de compostos fenólicos e glucanas

A determinação de compostos fenólicos por análise colorimétrica foi realizada em triplicata segundo o método descrito por Scalbert, Monties, Janin (1989) de acordo com as modificações realizadas por Ramirez-Anguiano et al. (2007), e os resultados foram expressos em miligramas de equivalente de ácido gálico (EAG)/grama de amostra em base seca. A determinação de glucanas foi realizada em triplicata, por hidrólises enzimáticas e químicas, realizadas por kit comercial Mushroom and yeast beta-glucan - Megazyme®.

3.1.4.2 Análise do estado redox

A determinação da concentração de GSH se deu pela quantificação dos tióis totais, que foi realizada por espectrometria UV-VIS, baseada no método de Ellman (1959). Para isso, 150 µL de sangue total sempre mantido em banho de gelo, foi hemolisado com 100 µl de Triton X-100 a 10% e precipitado com 100 µL de ácido tricloroacético (TCA) a 30%. As amostras foram centrifugadas a 4000 rpm e 4ºC por 10 minutos em centrífuga refrigerada e os sobrenadantes separados. Após, 50 µL de sobrenadante e 50 µL de 5-5-ditio-bis-2-ácido nitrobenzóico (DTNB) 10 mM foram diluídos em 900 µL de tampão fosfato (pH 7,4), formando um complexo amarelo. Para as amostras de tecido, 250 µL de sobrenadante do homogenato (precipitado com TCA 30% e centrifugado) e 100 µL de DTNB 10 mM foram adicionados em 900 µL de tampão fosfato. A leitura foi feita em seguida, em comprimento de onda de 412 nm. Uma curva de calibração com concentrações pré-definidas de GSH (0,005; 0,01; 0,025; 0,05 e

0,1 mM) foi utilizada para o cálculo da concentração. Os resultados foram expressos em µM de sangue total e de tecido.

A atividade da enzima antioxidante GPx foi determinada em sangue total e em homogenato de placenta, baseada na oxidação do NADPH a 25ºC. O sangue foi diluído 40 vezes em tampão fosfato de potássio com EDTA, pH 7,0. Após, 20 µL da amostra diluída foi acrescentado em 880 µL de uma solução contendo glutationa reduzida, glutationa redutase, NADPH, azida sódica e 100 µL de H_2O_2. Na análise da placenta, 20 µL do homogentato foi acrescentado em 880 µL de uma solução contendo glutationa reduzida, glutationa redutase, NADPH, azida sódica e 100 µL de H_2O_2. A atividade da GPx foi monitorada por dois minutos, a 340 nm, de acordo com a metodologia de Paglia e Valentine (1967). Pela medida do decaimento da absorbância do NADPH foi possível determinar a atividade da GPx, uma vez que ela é proporcional ao consumo de NADPH. Os dados foram expressos em µmol NADPH/min/g Hb, usando valores de Hb (hemoglobina) expressos em g/L.

A atividade da enzima catalase foi avaliada por espectrofotometria UV/VIS utilizando método de Aebi (1984). O método baseia-se na decomposição do H_2O_2 pela catalase, ao longo do tempo, monitorado a 240 nm. Para tanto, o sangue foi diluído 60 vezes em tampão fosfato de potássio 50 mM, pH 7,0. Uma alíquota de 20 µL de sangue diluído foi misturada a 1910 µL de mesmo tampão fosfato de potássio, e 70 µL de H_2O_2 foram adicionados, dando início à reação. As mudanças na absorbância foram monitoradas por três minutos. Uma constante de variação (κ), relacionada com a Hb, auxilia na expressão dos valores da atividade no sangue (κ/gHb). Na análise da placenta, 20 µL do homogenato foi adicionado em 1910 µL de tampão fosfato de potássio 50 mM e 70 µL de H_2O_2, dando início à reação, sendo os resultados foram expressos em κ/gHb/min de tecido.

As substâncias reativas ao ácido tiobarbitúrico (TBARS) são importantes para avaliar a peroxidação lipídica, incluindo malondialdeído (OHKAWA; OHISHI; YAGI, 1979). Alíquotas de plasma (150 µL) foram misturados com 50 µL de NaOH e 100 µL de Milli-Q Water™ (Millipore, Billerica, MA, EUA). A mistura

foi incubada durante 30 min a 60 °C com agitação. Então, 500 µL de H_3PO_4 6%, 500 µL de ácido tiobarbitúrico (TBA) 0,8% e 100 µL de dodecilsulfato de sódio a 10% foram adicionados às amostras, que ficaram em banho-maria durante 45 minutos a 90 °C. Para análise das placentas, 250 µL de homogenato foram misturadas com 1500 µL de H_3PO_4 6%, 500 µL de TBA 0,8% e 100 µL de SDS a 10%, e foram incubadas durante 45min a 90ºC. Os produtos da peroxidação lipídica reagiram com TBA em condições ácidas para formar uma substância rosa, cuja absorbância foi lida em espectrofotômetro a 532 nm. Foi utilizada uma curva de calibração (predefinida concentrações de 0,57, 1,43, 2,86 e 5,71 µM) para calcular a concentração de MDA no material.

3.2 Análise estatística

Para análise dos resultados foi utilizado o nível de significância entre 1 e 5%. O teste de Bartlet foi usado para avaliar a homocedasticidade dos dados; a análise de variância Anova para dados paramétricos, seguida do teste Tukey-Kramer para comparações múltiplas em todos os experimentos realizados. Foi utilizado também o teste de Qui Quadrado para os resultados obtidos em porcentagem. Todos os dados foram avaliados em Software GraphPad.

4 RESULTADOS

4.1 Resultados Parciais

Os resultados parciais descritos abaixo foram apresentados na “45th Annual Conference of the European Teratology Society, September 2017, Budapest, Hungary” e publicados na revista Reproductive Toxicology (Fator de impacto JCR: 2,85).

ELSEVIER

Reproductive Toxicology

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Evaluation of the reproductive capacity of diabetic pregnancy on rats treated with medicinal mushrooms

Leticia F. Laurino, Fabia J.M. Viroel, Sara R.V. Spim, Denise Grotto, Marli Gerenutti

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https://doi.org/10.1016/j.reprotox.2017.06.139

Introduction: The high rates of beta-glucans and phenolic compounds present in mushrooms have been making their medicinal and nutritional properties attractive to the scientific field in recent decades. *Agaricus blazei*, *Ganoderma lucidum*, and *Lentinula edodes* have been used for the treatment of dyslipidemias and diabetes mellitus, with very satisfactory results. The present study was carried out to investigate the protective actions of these mushrooms when administered during the gestation of rats with STZ-induced gestational diabetes mellitus (GDM-STZ), in view of the knowledge of the cytotoxic effects of this drug.

Methods: Dosages of phenolic compounds and beta-glucan were carried out on the three species of mushrooms. Pregnant rats received orally reconstituted lyophilized powder and dehydrated *Agaricus blazei*, *Ganoderma lucidum* or *Lentinula edodes* mushrooms at daily doses of 100 mg/kg from gestation day 1 to 19; the diabetic control group received saline solution. All of the animals received STZ (40 mg/kg, i.v) on the 8th pregnancy day; blood glucose above 120 mg/dl was considered as moderate GDM-STZ. The uterus was removed through a caesarean section on the 20th day. Maternal kidney and liver toxicity were assessed. The

corpora lutea, implants, number of resorptions, live and dead fetuses were counted. Placenta and fetuses were weighted.

Results: Mushrooms presented the following potential antioxidants: *Agaricus blazei* - 8.5 g beta-glucans per 100 g of dry mushroom, 1.25 mg GAE/g of phenolic compounds; *Ganoderma lucidum* - 30 g beta-glucans per 100g of dry mushroom, 1.8 mg GAE/g of phenolic compounds; *Lentinula edodes* - 39 g beta-glucans per 100 g of dry mushroom, 1.48 mg GAE/g of phenolic compounds. The exposure of mushrooms *Agaricus blazei*, *Ganoderma lucidum* or *Lentinula edodes* before STZ administration did not alter the weight of uterus and ovaries. However, it promoted an increase in the number of fetuses per mother and reduced a percentage of fetal resorptions. *Lentinula edodes* also promoted the increase in the fetal weight. They were not observed in the placental weights of the three mushrooms when compared to the diabetic control group. A reduction in the aspartate aminotransferase (AST) rates was observed in the administration of three studied species of mushrooms when compared to the diabetic control group. *Agaricus blazei* and *Lentinula edodes* promoted reduction in the alanine aminotransferase (ALT) levels. No changes were observed in the creatinine and urea levels.

Conclusion: A quantification of phenolic compounds and beta-glucans was considered within the literature standards. *Agaricus blazei*, *Ganoderma lucidum* and *Lentinula edodes* had a protective effect at a dose of 100 mg/kg/day on a GDM-STZ toxicity, reducing the deleterious effects on the reproductive capacity and the maternal hepatic toxicity promoted by STZ. Financial Support: FAPESP 2015/24566-9 process and Prosup-Capes.

Reproductive Toxicology

Volume 72, September 2017, Page 36

P-12

Functional foods in gestational diabetes: Evaluation of the oral glucose tolerance test (OGTT) in pregnant rats treated with mushrooms

Leticia F. Laurino, Fabia J.M. Viroel, Thaisa B. Pickler, Marli Gerenutti

Show more

https://doi.org/10.1016/j.reprotox.2017.06.151

Introduction: Gestational diabetes mellitus (GDM) is one of the significant metabolic changes that can affect pregnant women. On the recommendation of the International Association of Diabetes in Pregnancy Study Groups, the screening of glycemic disorders in pregnancy should be performed for all women diagnosed or not with pre-gestational diabetes. Functional foods rich in beta-glucans such as mushrooms have important factors in the control of this diabetes type and may bring potential benefits to the maternal-fetal health.

Methods: Pregnant rats received orally reconstituted lyophilized powder and dehydrated *Agaricus blazei* (Ab), *Ganoderma lucidum* (Gl) or *Lentinula edodes* (Le) mushrooms at daily doses of 100 mg/kg from gestation day 1 to 19 (Abb, Glb or Leb) or from 9 to 19 days (Aba, Gla or Lea); the control group received saline solution (Ds). All of the animals received STZ (40 mg/kg, i.v) on the 8th pregnancy day; Blood glucose above 120 mg/dl was considered as moderated GDM. At the 17th day of gestation, the animals remained fasted for 6 hours, and after receiving 2 g/kg of dextrose via gavage. For OGTT, an assessment of glucose levels was performed from peripheral arterial blood before administration of dextrose at 10 ', 20', 30 ', 60' and 120 '.

Results: In comparison to the Ds group, the Glb group presented a reduction in the fasting glycemic levels after 10, 20 ', 30' and 60 'after the administration of dextrose; the Gla group showed a decrease in the 20 ', 30 and 60' times; the Leb and Lea groups presented a reduction in the 30 'and 120' times, and it was not significantly observed for the Abb and Aba groups. Not all differences between OGTT were found between exposure to mushrooms before and after administration of STZ, for *Lentinula edodes* or *Agaricus blazei*, only Gla presented reduction at 10 'and 20' times, comparing to the Glb. There was no significant difference between the groups

that received the mushrooms for hematological parameters of leukocytes, erythrocytes, hemoglobin and hematocrit; platelet elevation was observed in the three groups that received mushrooms before STZ administration.

Conclusion: The administration of the mushrooms *Ganoderma lucidum* (100 mg/kg/day) and *Lentinula edodes* (100 mg/kg/day) before or after exposure to STZ reduced the glucose levels in the glycemic curve in response to the glucose tolerance test; this is indicative of the use of these mushrooms as functional foods, which can be utilized in the complementary therapy of gestational diabetes mellitus. Financial Support: FAPESP 2015 / 24566-9 process and Prosup-Capes.

4.2 Resultados Finais

O resultado final deste ensaio foi formatado para publicação na revista "European Journal of Nutrition" e enviado para avaliação antes da defesa da dissertação. Sendo assim, estão apresentados aqui Introdução, Material e Métodos, Resultados, Discussão e Conclusão.

Evaluation of the therapeutic effect of Shiitake Culinary-Medicinal Mushroom *Lentinus edodes* (Agaricomycetes) on materno-fetal development in rats with gestational diabetes mellitus induced by streptozotocin

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ABSTRACT

Purpose The presence of β-glucans and phenolic compounds in *Lentinus edodes* indicates that this mushroom can be used as a nutritional supplement with high antioxidant power. The aim of this study was to evaluate the properties of daily exposure to *Lentinus edodes* in GDM-STZ (gestational diabetes mellitus induced by streptozotocin) rats and their fetuses by pre-clinical tests.

Methods Twenty-four pregnant Wistar rats were divided into four groups: two controls (negative: SC and positive: GDM+S) and two groups treated with *Lentinus edodes* - Le (before (b) and after (a) GDM-STZ: GDM+Leb and GDM+Lea, respectively). The conditions of the treated groups were: SC (saline solution 0.9%), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 1 to 19) and GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). On the 20th day of pregnancy, cesarean sections were performed, blood was collected for biochemical, hematologic parameters and stress oxidative biomarkers. Placenta and amniotic fluid were collected and fetuses were analyszed through morphological evaluation.

Results Lentinus edodes did not reduce the severe hyperglycemia of the mother-concept but promoted an increase in maternal insulin levels. The mushroom reduced the levels of ALT (alanine aminotransferase), and AST (aspartate aminotransferase), triglyceride and total cholesterol, when compared with SC group. *Lentinus edodes* protected the animals from post-implantation losses and promoted an increase in the morphological parameters of fetuses, indicating a possible protective effect of the mushroom when administered before STZ. The administration of *Lentinus edodes* in GDM-STZ improved some oxidative stress parameters.

Conclusion The intravenous dose of 40 mg/kg STZ promoted severe and prolonged diabetes. *Lentinus edodes* administered before the STZ promoted improvement in maternal glucose tolerance, protected the animals from post-implantation losses, presented a reduction in total cholesterol levels and an increase in insulin levels. Liver damage induced by STZ was reversed in GDM+Leb and GDM+Lea groups. *Lentinus edodes* mushroom has antioxidant properties that can minimize the damage caused by GDM.

KEYWORDS *Lentinus edodes*; Gestational Diabetes Mellitus; Streptozotocin; Oxidative stress parameters.

Introduction

Diabetes Mellitus (DM) is a chronic disease that requests multifactorial strategies for glycemic control [1]. In diabetic patients, due to abnormal metabolism of insulin, cells and tissues do not use blood glucose, resulting in hyperglycemia, leading these patients to the risk of developing other metabolic and functional complications [2]. Hyperglycemia is manifested by insulin resistance and/or secondary insulin deficiency caused by progressive loss of mass or β-pancreatic cell function. The recommendations of the "Standards of Medical Care in Diabetes" include screening, diagnosis, and known therapeutic actions to improve the quality of life of diabetic patients [1].

Gestational Diabetes Mellitus (GDM) is one of the complications that, although temporary, can affect pregnant women. There is not an adequate treatment, effecting both dam and concept, increasing the risk of fetal loss, congenital malformations, premature birth and tendency to develop type 2 diabetes in the future [3–5]. Fetuses of diabetic mothers are developed in a hyperglycemic intrauterine environment of oxidative stress and they are adapted by altering insulin resistance and secretion, which impairs glucose tolerance in adult life and increases the risk of developing type 2 diabetes mellitus [6]. In addition, animal models have shown that diabetes can be transmitted to the fetus by intrauterine exposure to maternal hyperglycemia, which may contribute to a worldwide epidemic of diabetes, further emphasizing the need for adequate glycemic control during pregnancy [7].

The altered intrauterine environment may lead to cardiovascular complications and obesity, in addition to impairment in embryofoetal development [8]. Therefore, prevention and treatment of GDM are extremely important during the prenatal and postnatal phases. Physical activity and nutritional therapy are the first treatment choices for glycemic control, and insulin is only introduced when exercise and diet do not maintain normoglycemia [9].

The search for natural products with antidiabetic effects has increased in preventive medicine. Some species of edible mushrooms with high nutritional value and low-calorie content, are recommended for diabetic patients due to the presence of phenolic compounds, which can reduce the oxidation of macromolecules [10, 11].

Lentinus edodes, popularly known as Shiitake, is one of the most consumed and studied mushroom species in the world, not only for its nutritional value but also for its potential therapeutic actions [12]. This mushroom contains essential nutrients such as proteins, carbohydrates, fatty acids (linoleic, palmitic and oleic) and high energy value, in addition to being used in low-calorie diets. The presence of tocopherols

and phenolic compounds (p-hydroxybenzoic, p-coumaric and vanillic acid) indicates that this mushroom can be used as a supplement with high antioxidant power [13].

Regular consumption of this mushroom can increase immune function, improve metabolic profile and reduce lipid levels and oxidative stress [14, 15]. According to Spim et al. [16], in a preclinical study with hiperlipidemic animals, the exposure to *Lentinus edodes* demonstrated a hypolipidemic, hypoglycemic, hepatoprotective and renoprotective effects at a dose of 100 mg/kg/day, in addition to protecting against oxidative stress induced by high lipid intake. The authors suggest that the mushroom may be a natural source of supplementation in patients with metabolic disorders due to the presence of high concentrations of β-glucans and fibers.

β-glucans present in *Lentinus edodes* have a great impact on the *in vitro* digestion of starch, since they increase the concentration of unabsorbed starches in the small intestine, improving satiety, insulin sensitivity and balance the sugar in the body, which prevents the occurrence of diseases such as diabetes and obesity. In addition, they reduce caloric intake, being an alternative to produce foods with low glycemic index [17].

An *in vitro* study with rat insulinoma cell line (INS-1) has shown that lentinan, a purified component of *Lentinus edodes*, is a potential pharmacological agent in preventing damage associated with oxidative stress associated with diabetes, by protecting the dysfunction and apoptosis of streptozotocin-stimulated β-pancreatic cells [18].

In GDM, glycemic and oxidative stress control may help to reduce risks for pregnant women, but more studies are needed to clarify the short- and long-term effects of oxidative stress in fetuses born to diabetic mothers [19].

Just as interest in mushrooms as a potential therapy for diabetes grows, detailed studies to monitor the progress of the literature and its anti-diabetic effects are needed [2, 20].

Material and Methods

Reagents and solutions

Hydrochloric acid (HCl), potassium hydroxide (KOH), sodium acetate buffer (SAB), exo-β-1,3-glucanase, β-glycosidase, glucose oxidase/peroxidase, amiloglycosides and invertase, streptozotocin, citrate buffer, saline solution, dextrose, xylazine, ketamine, ethylenediamine tetra-acetic acid (EDTA), Rat/Mouse Insulin Elisa Kit - Merck®, potassium chloride (KCl), trichloroacetic acid (TCA), Triton X 100,

potassium phosphate buffer (TFK), 5-5-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH), sodium azide, hydrogen peroxide, sodium hydroxide (NaOH), malondialdehyde (MDA), thiobarbituric acid (TBA), phosphoric acid (H_3PO_4), sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA) or from Yeast and Mushroom, Megazyme, Bray, Eire.

Ethical approval of the study protocol

The study protocol was approved by the Commission of Ethics in the Usage of Animals of the University of Sorocaba (approval number 089/2016; São Paulo, Brazil).

Preparation, Composition of *Lentinus edodes* and Doses

Lentinus edodes (Berk.) Pegler-cultivated strain H600 (Hokken, Shimotsuga-gun, Japan) was provided by the commercial company Yuki Mushrooms (São Paulo, Brazil). The samples of fresh mushroom were trimmed, and cut into slices, on the same day of use and lyophilized (Thermo Fisher Scientific, USA®) for 48 h; the yield reached the ratio of 10% dry mass. The dried sample was milled (Marconi® - MA340, Brazil ®) after was sieved (50 mesh and 60 mesh), and packed in airtight plastic containers kept in a desiccator. For the characterization of the sample, phenolic compounds (method adapted from Scalbert, Monties, Janin [21] and β-glucans (β-Glucan Assay kit - Yeast & Mushroom; Megazyme, Bray, Eire) were dosed. *Lentinus edodes* presented the following potential antioxidants: 39 g of beta-glucans per 100 g of dry mushroom and 1.48 mg of GAE/g of phenolic compounds. Daily doses of 100 mg/kg of *Lentinus edodes* was chosen based in Grotto et al. [22] study, wich did not promote hepatic damage in rats.

Animal experiments

Wistar rats (*Rattus norvegicus* var. Albinus) which body weight varied from 250 to 300 g for the males and from 180 to 200 g for the females were purchased from USP (*Biotério de Produção de Ratos*, Universidade de São Paulo, SP, Brazil). We allowed animals to habituate to the Alesco® microenvironment isolation cages under standard environmental conditions (23 ºC, 12:12 h dark/light cycle) while providing industrialized dry food (Purina®, São Paulo, Brazil) and tap water *ad libitum*.

Assays with GDM-STZ

To allow mating, we housed up one male with four females for overnight periods. Each morning, the vaginal smear was taken under the microscope [23]. The presence of sperm (Biological Microscope, Axio Lab. A1, ZEISS®), was designated as gestational day 1. On the eighth day of pregnancy, fasting blood glucose (6 hours) was dosed in the Freedom Lite Freestyle (Abbott), diabetes was induced by administration of streptozotocin (40 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.1 mol / l citrate buffer (pH 4.5) intravenously. After 48 hours, the fasting glucose (6 hours) was performed again. Animals with glycemia above 120 mg/dL were considered diabetic [24, 25].

A total of 24 pregnant females caged in isolation from each other were randomly assigned to one of four groups: negative control (SC: saline solution 0.9%), positive control (GDM+S: Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 1 to 19) and GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). Daily at 2:00 pm, from day 1 to day 19 of the gestational period, females received orally: 0.9% saline solution or *Lentinus edodes*.

Oral Glucose Tolerance Test (OGTT)

Oral glucose tolerance tests (OGTT) were conducted at day 17 of pregnancy according to published methods [26]. Briefly, after fasting for 6h, the rats received 2g of dextrose/kg body weight, and blood was collected at 0, 10, 20, 30, 60 and 120 minutes, using Glycemic Monitor FreeStyle Lite, Abbott.

Blood collection and reproductive performance

On the 20th day of pregnancy, females were anesthetized with xylazine (6 mg/kg) and ketamine (100 mg/kg) administered by intraperitoneal injection. Cesarean sections were performed using a longitudinal incision along the linea alba. Blood samples were collected from hepatic veins and divided into two tubes. One tube 'A' for blood cell counts and oxidative stress evaluation containing ethylenediamine tetra-acetic acid (EDTA). One vacuum tube 'B' with anticoagulation gel separator for evaluation of biochemical parameters. Females were then sacrificed and their womb and ovaries removed. All sampled material were conserved at -80ºC before analysis except blood. Uterine cavities were checked for the number of fetuses, implantations, and visible resorptions. The ovaries and uterus were weighed, and corpus luteal counted.

Maternal hematologic parameters

Blood from tube A was analyzed in a Sysmex® XS-1000i equipament to determine the following parameters: RBC (red blood cell), hemoglobin, hematocrit, platelet (PLT) and white blood cells (WBC) counts. After this procedure, 500µl of blood were packed in eppendorf tube and stored at -80ºC.

Maternal biochemical profile

Biochemical parameters were measured in an automated system (Cobas 111 Roche® spectrophotometer). The following parameters were determined: alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, creatinine, urea, glucose, triglycerides, total cholesterol and high-density lipoproteins (HDL-Chol).

Embryofetal development and placental analysis

Collection and processing of amniotic fluid

Amniotic fluid was extracted from gestational sacs and the samples were frozen immediately and stored at -80˚C for future processing. The insulin was dosed with a commercial kit (Rat/Mouse Insulin Elisa Kit - Merck®).

Fetal morphological evaluation

Morphological effects due to exposure to *Lentinus edodes* mushroom in the offspring were checked comparing body measurements. Length in mm of the sections: anteroposterior and latero-lateral of the skull, anteroposterior and latero-lateral of the thorax, cranium-caudal, and tail were measured using a pachymeter.

Placenta processing

Placentas were removed, weighed, frozen immediately and stored at -80˚C for future processing. The analyses were made with 250 mg of pooled placentas. The samples were homogenized with 5 mL 1,15% KCl and then processed according to the specific analyzes for oxidative stress.

Evaluation of Oxidative Stress Biomarkers

Malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GPx), and catalase (CAT) were evaluated in both blood and placenta samples. To the placenta, a placenta homogenate was prepared to weigh approximately 0.250 g of tissue and to place in a tube with 5.0 mL of 1.15% KCl cold. The tissue was homogenized with the aid of an ultrasonic processor, always in an ice bath.

The activity of the enzyme CAT was evaluated by UV/VIS spectrophotometry using Aebi [27] method. The method is based on the decomposition of hydrogen peroxide (H_2O_2), added to the test, by tissue CAT, over time, monitored at 240 nm. Blood sample was diluted (1:60) in TFK 50 mM. An aliquot of 20 μL of diluted blood was mixed to 1910 μL of phosphate buffer , and 70 μL of H_2O_2 was added, thereby initiating a reaction that was monitored for 3 min. For placenta analysis, 20 μL of the homogenate was diluted in 1910 μL phosphate buffer and the H_2O_2 was added, initiating the reaction. To evaluate the activity of the enzyme, calculations were performed based on the fresh placentamass (0.250 g). To the blood, a constant of variation (k), related to hemoglobin (Hb), was used to obtain a value for CAT blood activity (k/g Hb).

To determine the antioxidant levels of GPx, Paglia and Valentine [28] method were followed, based on the oxidation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Blood was diluted (1:40) in phosphate buffer (pH 7.0) and 20 μL of the diluted sample was added to 880 μL of a solution containing GSH, glutathione reductase, NADPH, sodic azide and 100 μL of H_2O_2. For placenta analysis, 20 μL of the homogenate was added to 880 μL of a solution containing GSH, glutathione reductase, NADPH, sodic azide and 100 μL of H_2O_2. GPx level was monitored in a spectrophotometer at 340 nm for 2 min.

For GSH, Ellman [29] method was followed for the quantification of total reduced thiols. Briefly, 150 μL of blood maintained in an ice bath was vortex-mixed with 100 μL of 10% Triton X100 and 100 μL of 30% trichloroacetic acid and it was centrifuged at 4 °C, at 4000×g for 10 min. Then, 50 μL of the supernatant and 50 μL of 5,5′-dithiobis-(2-nitrobenzoic acid) (10 mM) and 900 μL of phosphate buffer (1 M) were pipetted and formed a yellow complex. For the tissue, 600 μL of the homogenate was added to a solution of trichloroacetic acid (30%), vortex-mixed and centrifuged. 250 μL of the supernatant was then added to a buffered solution, and a chromophore (the 5,5'-dithiobis-(2-nitrobenzoic acid)) was pipetted for reading at 412 nm. To calculate the GSH concentration, a calibration curve with predefined concentrations

of GSH (0.005, 0.01, 0.025, 0.05 and 0.1 mM) was employed. Calculations were done based on fresh placenta mass (0.250 g).

MDA was quantified using Ohkawa; Ohishi; Yagi [30] methods for thiobarbituric acid reactive substances with modifications. Briefly, 150 μL of plasma aliquots were mixed with 50 μL of NaOH and 100 μL of Milli-Q Water and incubated with agitation for 30min at 60ºC. Then, 500 μL 6% H_3PO_4, 500 μL 0.8% thiobarbituric acid (TBA), 100 μL of 10% sodium dodecyl sulfate (SDS) were added to samples. For placenta analysis, a volume of 250 μL of the homogenate was added to 6% H_3PO_4 and 0.8% thiobarbituric acid (TBA). The samples were incubated at 90 °C for 45 min and then read in a spectrophotometer at 532 nm. A calibration curve with different concentrations of MDA standard was used to calculate the concentration of MDA in plasma. Calculations were done based on the fresh placenta mass (0.250 g).

Statistical analyses

Data are presented as mean ± S.D. of animals (n = 6) and tested for homoscedasticity by Bartlett test. Homogeneous data were analyzed by ANOVA and Tuckey-Kramer Test in multiple posterior comparisons. Chi-square test was used evaluate differences in pre-implantation loss and p-values < 0.05 were considered significant. Results were analyzed using GraphPad Prism Software.

Results

Gestational evaluation

After 48 hours of streptozotocin administration, the GDM groups presented increased glycemic levels compared to SC (SC=60.0±5.0, GDM+S=202.34±34.65, GDM+Leb=209.34±35.98, GDM+Lea=193.0±22.7 mg/dl - $F=19.26$, $P<0.001$), showing that it is possible to reproduce GDM.

The animals of the GDM groups showed a reduction of the weight gain when compared to the SC group (SC=110.25±26.80, GDM+S=59.0±16.0, GDM+Leb=54.0±11.0, GDM+Lea=54.0±29.0 g - $F=3.919$, $P=0.0027$).

Glycemic profile on GMD

Biochemical data are reported in Fig. 1. The oral glucose tolerance test (OGTT) showed differences at all times between the GDM and SC groups ($F=19.26$, $P<0.001$) and reduction of the glycemic profile of GDM+Leb and GDM+Lea animals in relation to GDM+S, in 30 min ($F=19.26$, $P<0.001$) and 120 min ($F=19.26$, $P<0.05$). Insulin levels in GDM+S animals, both in the dams ($F=7.241$, $P=0.0059$) and in the amniotic fluid ($F=4.332$, $P=0.0205$), were reduced in relation to the SC, and in GDM+Lea group these levels remained reduced in the pregnant woman compared to SC. Regarding the lipase parameter, only the dams in GDM+Lea group presented a significant reduction in relation to the SC ($F=4.672$, $P=0.0139$). Both plasma and amniotic fluid glucose levels of GDM animals were higher than those in the SC. After 20 days, there is a sustained and severe gestational diabetes mellitus.

[insert Figure 1]

Maternal hematologic and biochemical evaluation

Regarding hematological parameters, there was an increase in platelet levels ($F=3.674$, $P=0.0491$) of GDM groups compared to SC group. There was an increase of hematocrit in the GDM+S when compared to the SC group, however the group GDM+Lea presented reduction in the hematocrit in relation to the group GDM+S ($F=3.965$, $P=0.0329$) (Figure 2).

Hepatic lesions were observed with an increase in AST ($F=9.968$, $P=0.0014$) and ALT ($F=23.23$, $P<0.0001$) in the GDM+S group, although in the GDM+Leb and GDM+groups, this alteration was not observed when compared to the SC group. In De Ritis [31] ratio, all groups presented an AST/ALT rate greater than 1. Regarding renal function, there was a decrease in the albumin parameter ($F=5.189$, $P=0.0100$) in all GDM animals in relation to the SC group. Creatinine ($F=1.349$, $P=0.2917$) and urea ($F=0.8366$, $P=0.4923$) were not altered.

All the GDM rats showed a reduction in HDL-cholesterol levels in relation to the SC group ($F=53.63$, $P<0.00001$). The GDM+S and GDM+Lea groups showed an increase in total cholesterol levels ($F=9.973$, $P=0.0018$) compared to the SC group. However, GDM+Leb showed a reduction in total cholesterol when compared to GDM+S. There was an increase in triglycerides ($F=4.417$, $P=0.0413$) in GDM+S in relation to SC group, and a reduction of this parameter in GDM+Lea in relation to the GDM+S.

[insert Figure 2]

Reproductive performance of female rats

In the evaluation of the reproductive capacity of the animals (Table 1), it can be observed that the GDM+S group presented reduced uterus weight in relation to the SC group (F=0.5187, P=0.6774), but there was no difference between treated diabetic groups and SC. The ovaries weight was decreased (F=3.336, P=0.0287) and placental weight was increased (F=10.66, <0.0001) in GDM+S and GDM+Leb groups when compared to the SC. Compared to SC, GDM+S and GDM+Lea groups showed an increase in post-implantation losses (P=0.0287), and GDM+Leb presented a reduction in the same parameter when compared to GDM+S. GDM+Lea group had a higher percentage of post-implantation loss compared to GDM+Leb. No changes were observed in the number of alive fetuses per mother between the GDM and SC groups (F=0.8614, P=0.4790).

[insert Table 1]

Embriofetal development

GDM+S presented reduction of all the morphometric measures when compared to SC. However, GDM+Leb group presented an increase in laterolateral (F=16.45, P<0.0001) and antero-posterior (F=14.87, P<0.0001) measurements of skull and tail (F=12.64, P<0.0001) in (F=17.02, P<0.0001) and antero-posterior (F=26.00, P<0.0001) of the thorax and cranium-caudal when compared to SC. GDM+Lea group presented a reduction of the skull, thorax and cranium-caudal measurements (F=16.28, P<0.0001) when compared to GDM+Leb group and also presented reduction of all parameters when compared to SC (Figure 3).

[insert Figure 3]

Oxidative stress on maternal blood and placenta

The oxidative stress results shown in Figure 4 indicate that there was a decrease in blood CAT (F=4.788, P=0.0156) in GDM+S in relation to the SC, and in GDM+Leb this parameter was increased in

relation to GDM+S. CAT activity in placenta did not present significant alterations. Regarding GPx in total blood , GDM+Leb and GDM+Lea groups presented no difference compared to SC, but GDM+S presented a reduction of this level in relation to the SC group (F=3.212, P=0.0617). The GDM groups presented increased GPx placental activity in relation to SC (F=7.811, P=0.0015). There was no differences in GSH levels in blood, an there was an increase in placental concentration of GSH in GDM+S and GDM+Lea in relation to SC (F=5.871, P=0.0056). An increase in MDA concentration in maternal plasma (F=9.791, P=0.0012) in GDM+S and GDM+Lea was observed when compared to SC and, in relation to the group GDM+S, GDM+Leb and GDM+Lea were decreased. In placenta analysis, MDA concentration was increase in GDM+Leb and decrease in GDM+S and GDM+Lea in relation to SC (F=5.150, P=0.0096).

[insert Figure 4]

Discussion

STZ is an antimicrobial agent, which has been used for decades due to its well-characterized diabetogenic effect as a pancreatic β-cytotoxic drug [32]. GDM-STZ is a well-established experimental model for the evaluation of insulin deficiency and hyperglycemic effects on fetuses [33]. However, Caluwaerts et al. [34] affirm that only doses higher than 40 mg/kg STZ are a good model of GDM-STZ studies. On the other hand, the great variability of plasma glucose levels allows defining GDM-STZ as moderate (glycemia between 120 and 200 mg/dl) or severe (glycemia superior to 200 mg/dl). In our trials, although the dose of STZ was considered low, the animals developed severe GDM-STZ.

The glucose oxidation and the glycation of non-enzymatic proteins, associated with the increase of oxygen free radicals, favor the development of diabetic complications in pregnant women. Therefore, exogenous antioxidant agents such as functional foods to prevent and treat DM-STZ have been extensively studied [35, 36]. *Lentinus edodes* mushroom, a promising functional food, has essential nutrients being used in low-calorie diets and in the prevention of diseases related to oxidative stress [13].

Studies with GDM-STZ have shown that pregnant diabetic rats present a reduction in weight gain when compared to non-diabetic animals [37, 38]; in our study, this reduction in weight gain was not reversed by exposure to *Lentinus edodes*.

The oral glucose tolerance test aims to diagnose uncontrolled glycemic levels mainly during pregnancy. In SC rats, insulin absorption in OGTT was in agreement with the expected physiological response and blood glucose levels returned to the original state at the end of the test [39]. However, in diabetic rats, there were no reduction in response time or blood glucose levels, which were increased at all times studied when compared to the control group [40, 41]. In the groups treated with *Lentinus edodes*, there were a reduction in response time or blood glucose levels. To evaluate the endocrine and exocrine functions of the pancreas, insulin and lipase levels were dosed, respectively. When evaluating the dam-concept glycemic profile, we observed that *Lentinus edodes*, although not reducing the severe hyperglycemia of the dam-concept, when administered before STZ promotes an improvement in maternal glucose tolerance, an increase in both maternal and amniotic fluid Zhang et al. [18] showed that some compounds of *Lentinus edodes* are able to prevent the inhibition of insulin synthesis in DM-STZ, similar to our results. On the other hand, pancreatic lipase dosages show that STZ at the employed dose did not promote other pancreatic injuries in GDM rats, indicating preserved exocrine function. Regarding endocrine function, it is possible to observe that, possibly due to the route and the dose used for the administration of STZ, there was a prolonged and severe DM and destruction of β-pancreatic cells. However, there was the better preservation of this organ in the group earlier exposed to the mushroom (GDM+Leb). When blood insulin levels are high, glucose should be lower, but the GDM+Leb group had higher insulin levels and blood glucose levels remained increased, indicating, possibly, insulin resistance.

DM-STZ can induce important hematological alterations in both the white and red blood cells [42, 43] and can also lead to an increase in the number of platelets, inducing an increase in platelet aggregation [44]. Our results indicate that *Lentinus edodes* maintained the high number of platelets in GDM-STZ animals. Cho, Mooney, and Cho [45] showed that hyperglycemia in DM increases hematocrit values because it renders the erythrocyte membrane more rigid with the aggregation of red cells causing an increase in viscosity and this elevation can also be explained by the increased permeability of the capillary wall. The GDM groups treated with *Lentinus edodes* presented reduction of hematocrit.

Hepatic and renal abnormalities and dyslipidemia are common in GDM-STZ [46]. Elevated ALT and AST levels in the blood represent liver damage and are observed in mice with DM-STZ [47, 48]. The membrane permeability of liver cells may be dysfunctional due to the accumulation of fat in hepatocytes caused by insulin deficiency [49]. GDM+S group had higher levels of these enzymes than the non-diabetic animals and the diabetic groups treated with *Lentinus edodes* showed a decrease in ALT and AST levels

when compared to the GDM+S group, indicating that the STZ damage was reversed by the mushroom. Akamatsu et al. [50] observed that the polyphenols of the aqueous fractions of *Lentinus edodes* have hepatoprotective effects, decreasing the activity of ALT and AST in hepatic lesions induced by dimethylnitrosamine, reducing liver inflammation and cell death by necrosis. De Ritis ratio is calculated by dividing AST levels by ALT levels and allows to observe the damage extension caused by toxic hepatitis [51]. All groups in this study had a De Ritis ratio greater than or equal to 1, which means that the liver damage was severe but less extensive.

GDM+S group presented a lipid profile similar to those found in Afiune et al. [52] and El-Sayyad et al. [53] studies, confirming the reproducibility of GDM induction by STZ, in wich GDM-STZ rats presented increased levels of triglycerides and cholesterol and reduced HDL-cholesterol. Spim et al. [16], when evaluating the ingestion of *Lentinus edodes* associated with a high-fat diet, suggests that this specie of mushroom has a hypocholesterolemic action, reducing total cholesterol and triglycerides levels. Xu et al. [54], when administering polysaccharides obtained from *Lentinus edodes*, also observed a reduction in oxidative stress induced by hypercaloric diet in rats, in addition to the reduction of total cholesterol, triglycerides, and low-density lipoprotein (LDL-col) levels. These results corroborate with Yu et al. [14] that observed that the consumption of aqueous extract of Shiitake was able to reduce the levels of triglycerides. In our study, although *Lentinus edodes* was used as food, it was also able to reduce triglyceride levels in GDM+Leb and GDM+Lea rats and, in addition, GDM+Leb group also showed a reduction in total cholesterol levels, when compared to GDM+S group.

These data may be indicative of nephrotic syndrome secondary to diabetes, since they consist of hypoalbuminemia associated with dyslipidemia, with normal levels of urea and creatinine [55] and elevated platelet levels [56].

Both moderate and severe GDM-STZ promote a reduction in the weight gain of pregnant rats, even though there is an increase in food intake [41, 57]. *Lentinus edodes* mushroom, at the dose used in this study, was not able to alter the reduction in weight gain, however, it was able to protect the animals from the post-implantation losses of the embryo when administered before the GDM-STZ induction.

In reproductive toxicity studies, special attention should be paid to the placenta, whose main function is to act as an interface between the fetus and the mother [58]. An increase in placenta weight in GDM-STZ gestation can be understood as a compensation mechanism to maximize poor maternal-fetal nutrients exchange [38]. The increased weight of placenta in GDM groups, treated or not with *Lentinus*

edodes, seems not to have been sufficient to reverse the low weight of the concepts. Calderon et al. [59] and Saito et al. [60], correlate moderate maternal hyperglycemia with fetal macrosomia, and reinforce that severe maternal hyperglycemia alters the maturation of placentas, their irrigation and characterize them as insufficiently nutritious, being responsible for small fetuses. Intrauterine exposure to moderate diabetes is associated with deficiencies in insulin secretion in adult life, while pups born from mothers with severe diabetes present depleted insulin action in adult life [7]. In our study, fetuses from GDM dams presented reduction of external morphological measures, results consistent with severe GDM-STZ. GDM+Leb promoted an increase in skull and tail parameters when compared to GDM+S, indicating possible protective effect of the mushroom when administered before STZ.

Severe hyperglycemia during pregnancy and consequent fetal hyperglycemia, responsible for fetal growth retardation, may also be related to oxidative stress and changes in the intrauterine environment [61]. The high rate of glucose in the amniotic fluid can lead to a prolonged stimulation of the fetuses β-pancreatic cells, causing a pancreatic insulin depletion and hypoinsulinemia [41]. Our results indicate that *Lentinus edodes* were able to revert fetal hypoinsulinemia in amniotic fluid, even though it did not reverse hyperglycemia.

Increased blood glucose is the main cause of oxidative stress in diabetes since STZ stimulates the generation of H_2O_2 in β-pancreatic cells that are destroyed by the production of nitrogen monoxide (NO) [62].

Oxidative stress occurs even in normal pregnancies due to the generation of reactive oxygen species (ROS) caused by the high metabolic activity of the placenta and maternal metabolism [63]. In GDM-STZ, ROS overproduction leads to the molecular and structural cell damage, whether in membranes, DNA, lipids or proteins, characterized by the production of free radicals greater than the defense capacity of the antioxidant system [19, 64].

Hyperglycaemia can increase in H_2O_2 production and deregulate the expression of catalase [65]. There is a decrease in its activity in different tissues and organs in DM [66], and the same happened in our study. However, administration of *Lentinus edodes* from the first day of gestation (GDM+Leb) increased plasma catalase activity compared to GDM+S.

The antioxidant system of glutathione enzymes plays an important role in cell defense against reactive free radicals and other oxidant species [67] and, if their levels are altered, the cells are susceptible to oxidative stress and cellular injury [68]. GDM+S group presented a reduction in GPx activity compared

to SC; the groups treated with mushroom, although there was no significant difference, showed a tendency to approach the SC group. Our results are in accordance with Yurkiv et al. [69], who studied the influence of *Agaricus blazei* and *Ganoderma lucidum* mushrooms on the oxidative stress in rats with DM, indicating the protective effect of the antioxidant compounds of these mushrooms and also of *Lentinus edodes* and the reduction of toxic substances (such as H_2O_2). However, in the placenta, GDM groups presented higher GSH level and GPx activity.

STZ induced diabetes mellitus intensifies the production of free radicals and increases TBARS. To prevent the formation of hydroxyl radicals can be a way of reducing damage [35], and *Lentinus edodes* mushroom presents an antioxidant potential since both treated diabetic groups presented a reduction of plasma MDA concentration in relation to GDM+S.

Conclusions

The dose of 40 mg/kg of intravenously STZ causes severe and prolonged gestational diabetes. Although *Lentinus edodes* did not reduce severe dam-fetus hyperglycemia, it promoted an improvement in maternal glucose tolerance and an increase in insulin levels. *Lentinus edodes* was not able to alter the reduction of maternal weight gain, however, it protected the animals from the post-implantation losses of the embryo when administered before GDM-STZ. The mushroom reversed the liver damage caused by STZ and reduced the triglycerides and cholesterol levels. *Lentinus edodes* mushroom has antioxidant properties that can protect against the damage caused by hyperglycemia in GDM. The best protective effects both in the dam and in fetuses were observed when administered from the first day of gestation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figure Captions

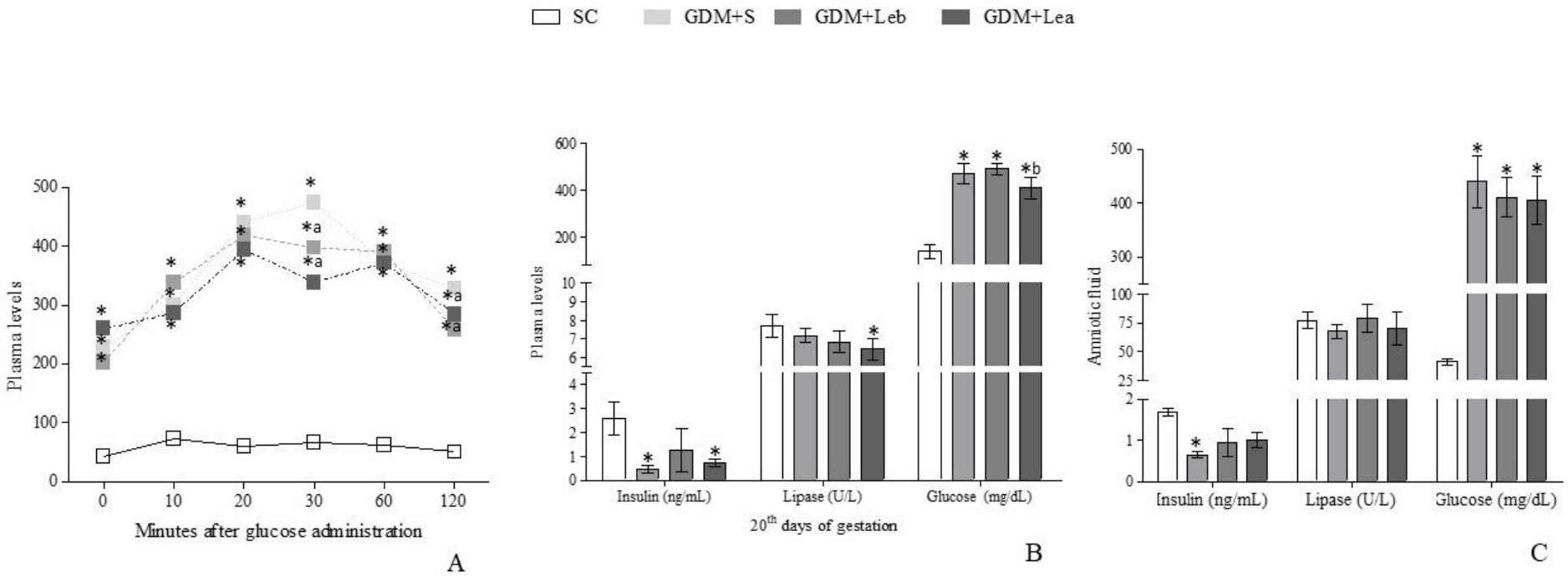
Fig. 1 – Biochemical parameters of pregnant rats.

Fig. 2 - Hematological parameters and serum biochemical outcomes of pregnant rats.

Fig. 3 - Mean length (mm) of sections of the head and body of fetuses.

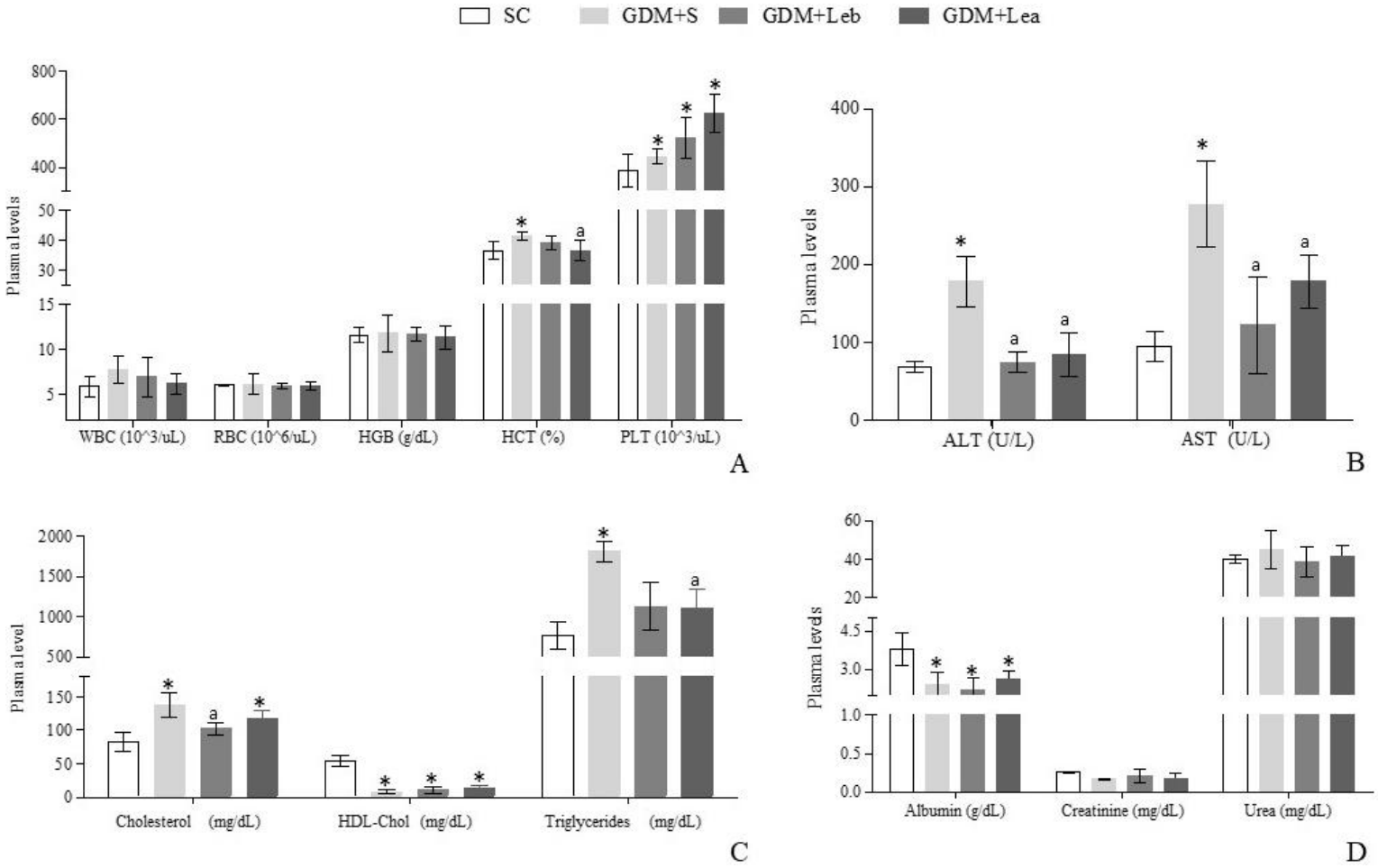
Fig. 4 - Activity level of catalase, reduced glutathione (GSH), glutathione peroxidase reduction (GPx) and MDA (malondialdehyde) on plasma and placenta of pregnant rats.

Fig. 1 - Biochemical parameters of pregnant rats.



Note: A: Oral Glucose Tolerance Test (OGTT) on 17th day of gestation. B: Serum concentration of insulin, lipase and glucose. C: Amniotic fluid concentration of insulin, lipase and glucose.
SC (0.9% saline solution), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 1 to 19), GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). Data are presented as mean ± SD (n = 5). *p<0.05 in comparison to SC group, [a] in comparison to GDM+S group, one-way ANOVA, followed by Tukey-Kramer's.

Fig. 2 - Hematological parameters and serum biochemical outcomes of pregnant rats.



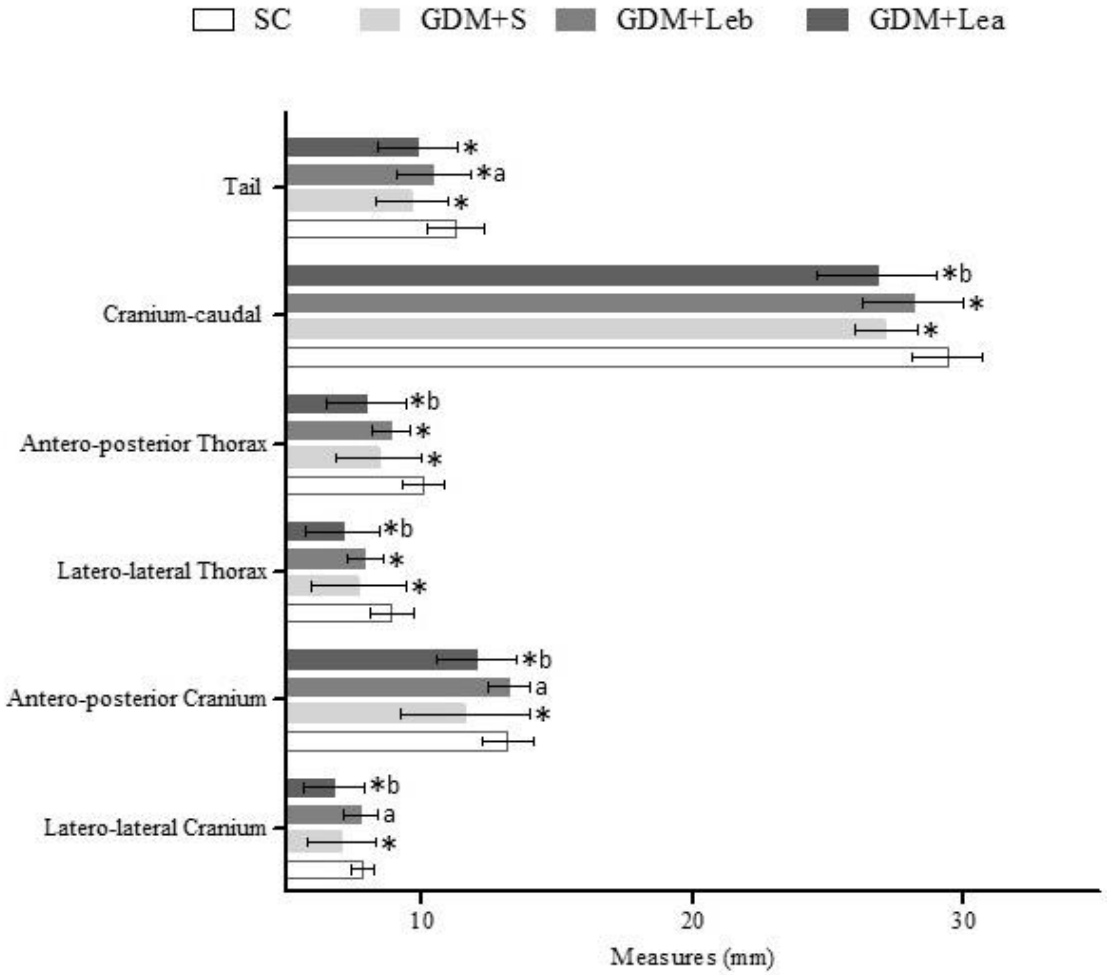
Note: A: Hematological parameters. B: Biochemical serum outcomes for hepatic analysis. C: Lipidic profile. D: Biochemical serum outcomes for renal analysis.
SC (0.9% saline solution), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 1 to 19), GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). WBC (Leukocyte), RBC (Red Blood Cells), HGB (Hemoglobin), HCT (Hematocrit), PLT (Platelet). ALT (Alanine Aminotransferase), AST (Aspartate Aminotransferase). Data are presented as mean ± SD (n = 6) or percentage. *p<0.05 in comparison to SC group, [a] in comparison to GDM+S group, one-way ANOVA, followed by Tukey-Kramer's.

Table 1 - Reproductive capacity of female rats.

GROUPS	Uterus weight (g)	Ovary weight (g)	Placenta weight (g)	Number of alive fetus per mother	Post-implantation loss (%)
SC	44.48 ± 13.83	0.142 ± 0.015	0.415 ± 0.09	9.75 ± 1.83	4.6
GDM+S	35.40 ± 12.49*	0.112 ± 0.019*	0.494 ± 0.143*	8.16 ± 1.53	16*
GDM+Le$_b$	41.64 ± 8.36	0.105 ± 0.020*	0.485 ± 0.102*	10.83 ± 2.03	6.9^a
GDM+Le$_a$	42.79 ± 11.79	0.123 ± 0.019	0.443 ± 0.081	11.33 ± 2.92	13.8*^{b}
	(F=0.5187, P=0.6774)	(F=3.336, P=0.0287)	(F=10.66, P<0.0001)	(F=0.8614, P=0.4790)	(P=0.0287)

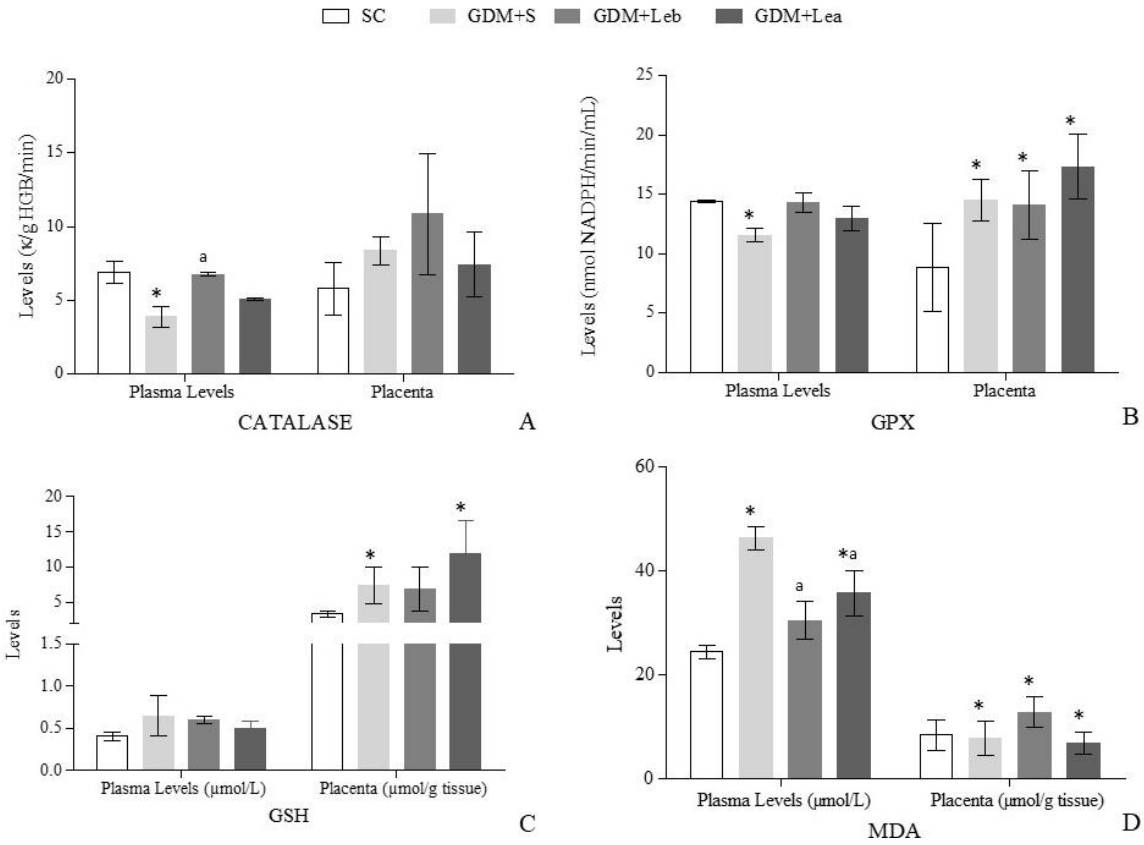
SC (0.9% saline solution), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 1 to 19), GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). Data are presented as mean ± SD or percentage. *p<0.05 in comparison to SC group, ^{a} in comparison to GDM+S group, ^{b} comparison between groups exposed to the same mushroom, one-way ANOVA, followed by Tukey-Kramer's or Chi-Square Test.

Fig. 3 - Mean length (mm) of sections of the head and body of fetuses.



SC (0.9% saline solution), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of Lentinus edodes from gestation day 1 to 19), GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). Data are presented as mean ± SD or percentage. *p<0.05 in comparison to SC group, [a] in comparison to GDM+S group, one-way ANOVA, [b] comparison between groups exposed to the same mushroom, followed by Tukey-Kramer's

Fig. 4 - Activity level of catalase, reduced glutathione (GSH), glutathione peroxidase (GPx) and MDA (malondialdehyde) on plasma and placenta of pregnant rats.



Note: A: Catalase activity on plasma and placenta. B: Glutathione peroxidase activity on plasma ad placenta. C: Reduced glutathione concentration on plasma and placenta. D: Malondialdehyde concentration on plasma and placenta.
SC (0.9% saline solution), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 1 to 19), GDM+Lea (Diabetic+100 mg/kg/day of *Lentinus edodes* from gestation day 9 to 19). Data are presented as mean ± SD or percentage. *p<0.05 in comparison to SC group, [a] in comparison to GDM+S group, one-way ANOVA, followed by Tukey-Kramer's

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ANEXO A - CERTIFICADO DE APROVAÇÃO EM CEUA-UNISO

UNIVERSIDADE DE SOROCABA

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CEUA–UNISO

PARECER

Protocolo nº 089/2016
Interessado (a): Marli Gerenutti
Orientador (a): Marli Gerenutti
Título do Projeto: Ensaios Pré-Clínicos com os Cogumelos *Agaricus blazei, Lentinula edodes* e *Ganoderma lucidum,* visando a redução e danos maternos e fetais causados pelo diabetes gestacional
Título do Experimento: o mesmo

Apresentado à Comissão de Ética no Uso de Animais - CEUA para análise, segundo a Lei No. 11.794, de 8 de outubro de 2008, que regulamenta o inciso VII do parágrafo 1º do artigo 225 da Constituição Federal, foi considerado:

[X] **APROVADO**.

[ ] **APROVADO com RECOMENDAÇÃO**, devendo o proponente encaminhar as modificações sugeridas em anexo para complementação do protocolo;

[ ] **COM PENDÊNCIA**, devendo o proponente readequar os itens do protocolo;

[ ] **REPROVADO**

Manifestação do Parecerista:

Nome: Renata de Lima

Coordenador da CEUA-Uniso

Assinatura: Renata de Lima

Data: 11/08/2016

* Encaminhar cópia deste parecer para o e-mail ceua@uniso.br e original assinado para a Seção Técnica Acadêmica

Biotério de Produção de Ratos

Universidade de São Paulo
Instituto de Ciências Biomédicas – ICB
Biotério de Produção de Ratos
Av Prof Lineu Prestes, 2048 CEP: 05508-000.
Cidade Universitária - São Paulo – SP
Tel: 3091-8491 3091 0889
E-mail: renaider@usp.br

ORIGEM: Biotério de Produção de Ratos	**SEQUÊNCIAS:** 50364
ESPÉCIE: *Rattus norvegicus*	**LINHAGEM:** Wistar / SHR / WKY
NÚMERO DE ANIMAIS/SEXO: 10 Machos	**IDADE:** Adultos

PROTOCOLO DE MONITORAMENTO DE SAÚDE – BACTERIOLÓGICO

Foram coletadas amostras de fezes do jejuno, ceco e intestino grosso, bem como amostras da orofaringe, semeadas em meios seletivos, incubadas por 24-48 horas a 37° C, em aerobiose e anaerobiose.

MICRORGANISMOS PESQUISADOS	AMOSTRA	RESULTADO	MÉTODO
Bordetella bronchiseptica	Traqueia/orofaringe	Negativo	PCR
Citrobacter rodentium	Traqueia/orofaringe/ trato	Negativo	Cultura
Corynebacterium kutscheri	Traqueia/orofaringe	Negativo	Cultura
Klebsiella oxytoca	Traqueia/orofaringe/ trato	Negativo	Cultura
Klebsiella pneumoniae	Traqueia/orofaringe/ trato	Negativo	Cultura
Mycoplasma pulmonis	Traqueia/orofaringe	Negativo	PCR
Pasteurella multocida	Traqueia/orofaringe	Negativo	Cultura
Pasteurella pneumotropica	Traqueia/orofaringe	Negativo	PCR
Pseudomonas aeruginosa	Traqueia/orofaringe/ Trato	Negativo	Cultura
Staphylococcus aureus	Traqueia/orofaringe	Negativo	Cultura
Streptococcus β hemolítico	Traqueia/orofaringe	Negativo	Cultura
Streptococcus pneumoniae	Traqueia/orofaringe	Negativo	Cultura
Salmonella spp.	Trato gastrointestinal	Negativo	Cultura
Helicobacter spp.	Trato gastrointestinal	Negativo	PCR
Car bacillus	Soro	Negativo	ELISA
Mycoplasma pulmonis	Soro	Negativo	ELISA
Clostridium piliforme	Soro	Negativo	ELISA

Outros microrganismos isolados: *Aerococcus viridans, Proteus mirabilis* e *Pseudomonas fluorescens.*

PROTOCOLO DE MONITORAMENTO DE SAÚDE – VIROLÓGICO

Foram coletadas amostras de sangue via punção cardíaca para obtenção de soro a ser testado para presença de anticorpos.

VÍRUS	RESULTADOS	LABORATÓRIO	MÉTODO
Kilham rat virus (KRV)	Negativo	LCSA – ICB	ELISA
Pneumonia virus of rat	Negativo	LCSA – ICB	ELISA
Reovirus (REO)	Negativo	LCSA – ICB	ELISA

PROTOCOLO DE MONITORAMENTO DE SAÚDE – PARASITOLÓGICO

MICRORGANISMOS PESQUISADOS	AMOSTRA	RESULTADO	MÉTODO
Aspiculuris tetráptera	Fezes	Negativo	Microscopia
Chilomastix bettencourti	Fezes	Negativo	Microscopia
Criptosporidium sp	Fezes	Negativo	Microscopia
Eimeria sp	Fezes	Negativo	Microscopia
Entamoeba sp	Fezes	Positivo	Microscopia
Giárdia muris	Fezes	Negativo	Microscopia
Hymenolepis diminuta	Fezes	Negativo	Microscopia
Hymenolepis nana	Fezes	Negativo	Microscopia
Spironucleus muris	Fezes	Negativo	Microscopia
Syphacia sp	Fezes	Negativo	Microscopia
Tritrichomonas muris	Fezes	Negativo	Microscopia
Demodex musculi	Pêlo	Negativo	Microscopia
Myobia musculi	Pêlo	Negativo	Microscopia
Myocoptes musculinus	Pêlo	Negativo	Microscopia
Notoedres muris	Pêlo	Negativo	Microscopia
Ornitonyssus bacoti	Pêlo	Negativo	Microscopia
Polyplax sp	Pêlo	Negativo	Microscopia
Psorergates simplex	Pêlo	Negativo	Microscopia
Trichoecius rombotsi	Pêlo	Negativo	Microscopia

Responsável Bacteriológico - Cyntia M. S. C. de Godoy - CRBio – 31131/01
Responsável Parasitológico - Robison José da Cruz - CRBio - 33762/01
Responsável Sorologia – Agtha de Alencar Muniz Chaves – CRBio 054385-8

Renaide Rodrigues Ferreira Gacek
Bióloga Responsável – Biotério de Produção de Ratos
CRBio 43739/01D

ANEXO C - SUBMISSÃO DO ARTIGO À REVISTA EUROPEAN JOURNAL OF NUTRITION

European Journal of Nutrition

Therapeutic effect of the Shiitake Culinary-Medicinal Mushroom Lentinus edodes (Agaricomycetes) an on development materno-fetal on gestational diabetes mellitus rats induced by streptozotocin.

--Manuscript Draft--

Manuscript Number:	
Full Title:	Therapeutic effect of the Shiitake Culinary-Medicinal Mushroom Lentinus edodes (Agaricomycetes) an on development materno-fetal on gestational diabetes mellitus rats induced by streptozotocin.
Article Type:	Original Contribution
Keywords:	Lentinus edodes; Gestational Diabetes Mellitus; Streptozotocin; Oxidative stress parameters
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Abstract:	Purpose: The presence of β-glucans and phenolic compounds in Lentinus edodes indicates that this mushroom can be used as a nutritional supplement with high antioxidant power. The aim of this study was to evaluate the properties of daily exposure to Lentinus edodes in GDM-STZ rats and their fetuses by pre-clinical tests. Methods: Twenty-four pregnant rats were divided into four groups: SC (saline solution 0.9%), GDM+S (Diabetic+saline solution 0.9%), GDM+Leb (Diabetic+100 mg/kg/day of Lentinus edodes from gestation day 1 to 19) and GDM+Lea (Diabetic+100 mg/kg/day of Lentinus edodes from gestation day 9 to 19). On the 20th day of pregnancy, cesarean sections were performed, blood was collected for biochemical, hematologic parameters and stress oxidative biomarkers. Placenta and amniotic fluid were collected and fetus was analyszed through morphological evaluation. Results: Lentinus edodes did not reduce the severe hyperglycemia of the mother-

	concept but promoted an increase in maternal insulin levels and amniotic fluid. The groups treated with the mushroom reduced the levels of ALT and AST, triglyceride and total cholesterol in GDM rats. Lentinus edodes protected the animals from post-implantation losses and promoted an increase in the morphological parameters of fetuses, indicating a possible protective effect of the mushroom when administered before STZ. The administration of Lentinus edodes in GDM-STZ improved some oxidative stress parameters. Conclusion: Lentinus edodes mushroom has antioxidant properties that can minimize the damage caused by Gestational Diabetes Mellitus.
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