UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS - *CAMPUS* DE BOTUCATU
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOTECNOLOGIA
(PPG-FARMATEC)

DENIZE JUSSARA RUPOLO DALL'AGNOL

EFEITOS DA TERAPIA IMUNOSSUPRESSORA NA ESTRUTURA E FUNÇÃO DO TRATO GASTRINTESTINAL DE RATOS

Tese apresentada ao Instituto de Biociências, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu para obtenção do título de Doutora em Farmacologia e Biotecnologia.

Orientadora: Profª Drª. Madileine Francely Américo

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Palavras-chave: Biosusceptometria de Corrente Alternada; Eletrogastrografia; Imunossupressores; Motilidade Gastrintestinal; Transplante de órgãos.

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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE DENIZE JUSSARA RUPOLO DALL'AGNOL, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA E BIOTECNOLOGIA, DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.

Aos 06 dias do mês de março do ano de 2018, às 08:30 horas, no(a) Sala B - Central de Aulas do IBB, reuniu-se a Comissão Examinadora da Defesa Pública, composta pelos seguintes membros: Profa. Dra. MADILEINE FRANCELY AMERICO - Orientador(a) do(a) Instituto de Ciências Biológicas e da Saúde / Universidade Federal de Mato Grosso, Profa. Dra. ROZEMEIRE GARCIA MARQUES do(a) Depto. de Cirurgia e Ortopedia / Faculdade de Medicina de Botucatu - Unesp, Prof. Dr. GUSTAVO TADEU VOLPATO do(a) Instituto de Ciências Biológicas e da Saúde / Universidade Federal de Mato Grosso, Profa. Dra. FLAVIA BONAMIN do(a) Faculdade Eduvale / Avaré, Profa. Dra. PATRÍCIA FIDELIS DE OLIVEIRA do(a) Departamento de Fisiologia / Instituto de Biociências de Botucatu - UNESP, sob a presidência do primeiro, a fim de proceder a arguição pública da TESE DE DOUTORADO de DENIZE JUSSARA RUPOLO DALL'AGNOL, intitulada **EFEITOS DA TERAPIA IMUNOSSUPRESSORA NA ESTRUTURA E FUNÇÃO DO TRATO GASTRINTESTINAL DE RATOS**. Após a exposição, a discente foi arguida oralmente pelos membros da Comissão Examinadora, tendo recebido o conceito final: APROVADA. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelos membros da Comissão Examinadora.

Profa. Dra. MADILEINE FRANCELY AMERICO

Profa. Dra. ROZEMEIRE GARCIA MARQUES

Prof. Dr. GUSTAVO TADEU VOLPATO

Profa. Dra. FLAVIA BONAMIN

Profa. Dra. PATRÍCIA FIDELIS DE OLIVEIRA

A Deus e minha família, a vós não dedico esta tese, pois dedico a minha vida.

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"Aqueles que passam por nós não vão sós. Deixam um pouco de si, levam um pouco de nós"
(Antoine de Saint-Exupery).

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"*Deus jamais tirou os olhos de você. Nem tampouco deixou de escutar suas orações". (Pe. Fábio de Melo)*

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"A primeira fase do saber, é amar os nossos professores" (Erasmo Rotterdam).

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"Todas as vitórias ocultam uma abdicação" (Simone de Beauvoir).

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"Um irmão pode ser a continuação de nós mesmos [...]" (Autor desconhecido).

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"O cientista não é o homem que fornece as verdadeiras respostas; é quem faz as verdadeiras perguntas" (Claude Lévi-Strauss).

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É grande a admiração por quem fala com abundância e sabedoria" (Cícero).

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"Coração não é tão simples quanto pensa. Nele cabe o que não cabe na despensa" (Leonardo Fressato).

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"Transportai um punhado de terra todos os dias e fareis uma montanha" (Confúcio).

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"A gentileza, é um jeito suave de colher amigos" (Dayse Sene).

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"Das grandes realizações que conquistei tive apenas um trabalho, cultivar bons amigos, depois disso todo o resto se fez" (Felipe Gallesco).

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"A fé vale mais que o tamanho,
Vale mais do que a força, é maior do que o medo
Porque no fundo você é do tamanho dos seus sonhos"
(Ivo Mozart).

RESUMO

DALL AGNOL, D.J.R. Efeitos da terapia imunossupressora na estrutura e função do trato gastrintestinal de ratos [tese]. Botucatu: Programa de pós-graduação em Farmacologia e Biotecnologia, Universidade Estadual Paulista "Júlio de Mesquita Filho – UNESP" Botucatu/São Paulo; 2018.

Os imunossupressores são utilizados após o transplante de órgãos e no tratamento de doenças autoimunes, desencadeando diversos efeitos colaterais. No esquema tríplice de imunossupressão pós-transplante, cada droga ou a combinação delas podem alterar a estrutura e/ou funcionamento do trato gastrintestinal. Apesar da importância do trato gastrintestinal e de conhecer os efeitos da ingesta oral de imunossupressores, há poucos estudos enfocando esses aspectos e vários desafios éticos para essas avaliações no homem. Desse modo, objetivou-se avaliar as alterações provocadas pela imunossupressão nos parâmetros de esvaziamento gástrico, frequência e amplitude das contrações gástricas, bem como as alterações histológicas no estômago de ratos. Para isso foram utilizados a Biosusceptometria de Corrente Alternada (BAC) e a Eletrogastrografia (EGG). No primeiro capítulo, avaliou-se individualmente sete imunossupressores de diferentes classes (Tacrolimo, Ciclosporina, Micofenolato Mofetil, Azatioprina, Sirolimo, Everolimo e Prednisona) comumente utilizados na terapia imunossupressora. Todos os imunossupressores estudados, exceto o Micofenolato Mofetil causaram alguma alteração nos parâmetros gastrintestinais analisados em ratos. Já o segundo capítulo apresenta os efeitos da terapia imunossupressora combinada, baseada em três classes de imunossupressores (inibidores da calcineurina, antimetabólitos e glicocorticoides). Um dos grupos experimentais recebeu Tacrolimo associado à Micofenolato Sódico e Prednisona, enquanto outro foi tratado com Ciclosporina associada à Azatioprina e Prednisona. Ambas as terapias alteraram o esvaziamento gástrico, a amplitude de contração gástrica e reduziram a espessura das camadas circular e longitudinal no estômago de ratos, embora a frequência tenha sido alterada apenas no grupo tratado com Ciclosporina associada à Azatioprina e Prednisona. Nossos dados apontaram que a monoterapia e a terapia combinada, alteram vários parâmetros da atividade motora ao mesmo tempo, podendo comprometer as funções básicas gastrintestinais.

Palavras-chave: Imunossupressores; Motilidade Gastrintestinal; Biosusceptometria de Corrente Alternada; Eletrogastrografia; Transplante de órgãos.

ABSTRACT

DALL AGNOL, D.J.R. Effects of immunosuppressive therapy on the structure and function of the gastrointestinal tract of rats [thesis]. Botucatu: Graduate Program in Pharmacology and Biotechnology, São Paulo State University "Júlio de Mesquita Filho - UNESP" Botucatu / São Paulo; 2018.

Immunosuppressants are used after organ transplantation and in the treatment of autoimmune diseases, triggering several side effects. In the triple regimen of post-transplant immunosuppression, each drug or combination of them may alter the structure and / or function of the gastrointestinal tract. Despite the importance of gastrointestinal tract and knowledge of the effects of oral intake of immunosuppressants, there are few studies focusing on these aspects and several ethical challenges for these evaluations in humans. The aim of this study was to evaluate the effects provoked by immunosuppression in gastric emptying parameters, frequency and amplitude of gastric contractions, as well as the histological aspects in the stomach of rats. Alternating Current Biosusceptometry (BAC) and Electrogastrography (EGG) were used. In the first chapter, seven immunosuppressants from different classes (Tacrolimus, Cyclosporine, Mycophenolate Mofetil, Azathioprine, Sirolimus, Everolimus and Prednisone) commonly used in immunosuppressive therapy were evaluated individually. All immunosuppressants studied except Mycophenolate Mofetil, caused some alteration in the gastrointestinal parameters analyzed in rats. The second chapter presents the effects of combined immunosuppressive therapy based on three classes of immunosuppressants (calcineurin inhibitors, antimetabolites and glucocorticoids). One of the experimental groups received Tacrolimus associated with Mycophenolate Sodium and Prednisone, while another was treated with Cyclosporine associated with Azathioprine and Prednisone. Both therapies altered gastric emptying, amplitude of gastric contraction, and reduced the thickness of the circular and longitudinal layers in the stomach of rats, although the frequency was disturbed only in the Cyclosporine group associated with azathioprine and prednisone. Our data showed that monotherapy and combination therapy alter several parameters of motor activity at the same time, and may compromise basic gastrointestinal functions.

Keywords: Immunosuppressants; Gastrointestinal Motility; Alternating Current Biosusceptometry; Electrogastrography; Organ transplantation.

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LISTA DE ABREVIATURAS E SIGLAS

AZA – Azatioprina

BAC – Biosusceptometria de Corrente Alternada

CD28 - Molécula co-estimulatória de linfócitos

CMM – Complexo Motor Migratório

CNI – Inibidor da Calcineurina

CSA – Ciclosporina

DNA – Ácido Desoxirribonucleico

EGG – Eletrogastrografia

EVR – Everolimo

FDA – Food and Drug Administration

GI – Gastrintestinal

HLA – Antígeno Leucocitário Humano

ICC – Célula Intersticial de Cajal

IFN-γ – Interferon Gamma

IL-2 - Interleucina-2

IL-4 – Interleucina – 4

IMPDH – Imosina Monofosfato Desidrogenase

MMF – Micofenolato Mofetila

MPA – Ácido Micofenólico

MPS – Micofenolato Sódico

mTOR – Proteína alvo da rapamicina em mamíferos

NFAT – Fator Nuclear de Células T ativadas

NF-kB – Fator Nuclear Kappa B

PRED – Prednisona

RNA – Ácido Ribonucleico

SNA – Sistema Nervoso Autônomo

SNE – Sistema Nervoso Entérico

SRL – Sirolimo

TAC – Tacrolimo

TGI – Trato Gastrintestinal

TNF-α – Fator de Necrose Tumoral Alfa

6-MP – 6- mercaptopurina

SUMÁRIO

1 INTRODUÇÃO

1 INTRODUÇÃO

O transplante de órgãos é o tratamento cirúrgico de escolha para doenças que de outra forma seriam incuráveis, aumentando a expectativa e a qualidade de vida desses pacientes a curto prazo. Todavia, a utilização da terapia imunossupressora causa diversas complicações pós-transplante que influenciam nos resultados a longo prazo, e uma das principais complicações são os efeitos colaterais gastrintestinais (TAYLOR; WATSON; BRADLEY, 2005; JOLLY; WATSON, 2011). Além disso, dependendo da gravidade de tais sintomas, é necessária redução ou descontinuação da dose, o que representa um risco significativo de perda do enxerto (LUCAN; BERARDINELLI, 2016).

Determinar precisamente as alterações nos parâmetros da motilidade gastrintestinal ocorridas após imunossupressão, amplia o conhecimento cientifico dos profissionais de saúde e facilita o manejo dessas complicações com uma significativa redução de agravos ao paciente transplantado.

Modelos experimentais adequados e uma técnica não invasiva são requisitos importantes para o estudo dos efeitos dos imunossupressores no trato gastrintestinal, já que no homem diversos procedimentos são inviáveis devido a preceitos éticos. Neste trabalho utilizou-se a Biosusceptometria de Corrente Alternada (BAC) e a Eletrogastrografia, devido as características não invasivas de ambas, além de permitirem repetições de registros (LIMA et al., 2017), para avaliar as alterações nos parâmetros gastrintestinais causadas pela monoterapia imunossupressora (Capítulo 1) e também pela terapia combinada tripla em ratos (Capítulo 2).

1.1 O trato gastrintestinal e suas funções

O trato gastrintestinal (TGI) é um sistema de grande importância para a sobrevivência de um animal, por ser o responsável pela digestão dos alimentos, absorção de água, eletrólitos e nutrientes necessários para manutenção de um funcionamento celular adequado. O TGI é formado por diversos órgãos que se comunicam entre si e nas duas extremidades com o meio externo. Os principais compartimentos do TGI são: cavidade oral, faringe, esôfago, estômago, intestinos delgado e grosso, reto e ânus (AIRES, 2012). Cada um deles possui estrutura própria, que está intimamente relacionada à sua função, no entanto a parede do TGI é constituída por várias camadas, sendo dispostas do interior para o exterior da seguinte forma: mucosa, submucosa, muscular e serosa (BREDENOORD; SMOUT; TACK, 2016).

A mucosa forma a barreira entre o conteúdo luminal e o ambiente interno, sendo constituída por epitélio e uma fina camada de músculo liso e a lâmina própria. A camada muscular externa circunda a submucosa, e é constituída por duas camadas de fibras musculares lisas, sendo a mais interna a musculatura circular e a mais externa a longitudinal (BREDENOORD; SMOUT; TACK, 2016). Nas diversas regiões do TGI, as camadas musculares da parede e sua inervação são adaptadas e organizadas para serem úteis as funções motoras daquela região (KELLOW et al, 1999). Sendo assim, a camada muscular circular, é responsável principalmente pela movimentação do conteúdo luminal ao longo do trato (BERTONI et al., 2008; AZUMA et al., 2016), enquanto o músculo longitudinal reduz a distância que o conteúdo deve percorrer no TGI (AZUMA et al., 2016). A motilidade gastrintestinal resulta de contrações coordenadas das camadas musculares (SANDERS et al., 2016), assegurando mistura e propulsão do conteúdo luminal.

Entre as duas camadas musculares, localiza-se o plexo mioentérico que está envolvido no controle da motilidade gastrintestinal, enquanto na camada submucosa encontra-se o plexo submucoso, que controla a secreção, absorção e o fluxo sanguíneo da mucosa. Essa organização de plexos ganglionares formam o sistema nervoso entérico (SNE) que é independente, embora trabalhe em conexão os outros sistemas, é composto por aproximadamente 100 milhões de neurônios (CAMILLERI, 2006). Comparável ao coração, o trato gastrintestinal possui atividade mioelétrica ao longo de sua extensão, e são as Células Intersticiais de Cajal (ICCs) que geram o sinal de marcapasso, ou seja, o ritmo elétrico basal (ondas lentas) (JONES; BRATEN, 2008; VAN HELDEN et al., 2010; YIN; CHEN, 2013). São as ondas lentas que determinam a frequência das contrações gástricas (YIN; CHEN, 2013), quando ocorre alguma alteração na frequência das ondas lentas, observa-se disfunções na motilidade gastrintestinal (SANDERS; KOH; WARD, 2006). A frequência das ondas lentas gastrintestinais é dependente da espécie, no homem por exemplo, observa-se ondas lentas com frequência de 3 ciclos por minuto (cpm) no estômago, 12 cpm no duodeno e 9 cpm no íleo terminal (BREDENOORD; SMOUT; TACK, 2016; YIN; CHEN, 2013). Em ratos foi observado por Marques et al., (2014) uma frequência de atividade elétrica gástrica em torno de 4,5 ciclos por minuto.

Além do ritmo elétrico basal (ondas lentas), há um segundo tipo de atividade elétrica, denominada potencial em ponta (BREDENOORD; SMOUT; TACK, 2016), pois as ondas lentas por si só não causam contração. Os potenciais em ponta são despolarizações rápidas da membrana celular do músculo liso, que ocorrem apenas durante a fase de despolarização da onda lenta (JOHNSON, 2014). A intensidade das contrações gastrintestinais é proporcional à amplitude das ondas lentas e à frequência dos potenciais de ação. A frequência dos potenciais

em ponta, assim como a amplitude das ondas lentas são reguladas tanto pelo SNE quanto pelo Sistema Nervoso Autônomo (SNA), sendo que a estimulação colinérgica eleva a força contrátil, e a noradrenérgica a diminui (AIRES, 2012).

Quando há ingestão de alimentos são simultaneamente ativados a motilidade gastrintestinal, a secreção gástrica, pancreática e a liberação de hormônios gastrintestinais que por sua vez, modulam as funções motoras, secretoras e absortivas no intestino delgado (CAMILLERI, 2006). A motilidade gastrintestinal engloba os fenômenos de atividade mioelétrica, contrátil, tônus e trânsito (KELLOW, 1999), propiciando mistura, trituração e propulsão do alimento ingerido ao longo do TGI.

A taxa de esvaziamento gástrico é um processo cuidadosamente regulado que consiste em diferentes fases bem definidas. A carga metabólica gástrica, bem como os mecanismos reguladores neurais, influências hormonais e mediadores imunológicos, cooperam para obtenção de um esvaziamento equilibrado do conteúdo do estômago para o duodeno (HELLSTRÖM; GRYBÄCK; JACOBSSON, 2006 WOUTERS; VICARIO; SANTOS, 2016). O esvaziamento de líquidos do estômago é mais rápido do que esvaziamento de sólidos, pois os mesmos distribuem-se rapidamente no estômago sem retenção nas regiões proximais (MAURER, 2012). O tempo de esvaziamento para líquidos não nutritivos em indivíduos saudáveis é de aproximadamente 20 minutos, enquanto o esvaziamento completo de sólidos ocorre aproximadamente 3-4 horas dependendo da quantidade de calorias ingeridas (CAMILLERI, 2006). Para serem esvaziadas as partículas de alimentos digeríveis precisam ter seu tamanho reduzido por trituração para aproximadamente 1-2 mm (CAMILLERI, 2006; PARKMAN; JONES, 2009; MAURER, 2016). O tempo necessário para completar a trituração de partículas sólidas deixando as mesmas pequenas o suficiente para esvaziar do estômago é referido como a *Lag phase*, ou seja, fase de atraso (MAURER, 2016). Quando as partículas atingem o diâmetro necessário, ocorre a passagem através do piloro e o esvaziamento segue uma curva quase linear (BREDENOORD; SMOUT; TACK, 2016). Camilleri et al. (1985) relatam que a duração da *Lag phase* estimada em minutos, varia de 15 a 90, com um valor médio de 60 min. em seres humanos.

O tempo de esvaziamento gástrico, assim como o transito gastrintestinal, podem ser desajustados por diversas patologias, medicamentos e plantas medicinais (ARORA, et al., 2005; LIMA et al., 2017; HAUSCHILDT et al., 2018), pois estes podem influenciar nos mecanismos responsáveis pelo controle e regulação da motilidade gastrintestinal. Dentre os medicamentos que influenciam na motilidade gastrintestinal, encontram-se os imunossupressores e como já demonstrado em nosso estudo anterior que os inibidores da

calcineurina e a prednisona estão associados a alterações das funções motoras gastrintestinais (DALL'AGNOL et al., 2014).

1.2 Regime imunossupressor e seus efeitos colaterais

O sucesso dos transplantes deve-se ao advento do uso de imunossupressores, o primeiro a relatar essa experiência foi Tom Starzl em 1963 (BARKER; MARKMANN, 2013). Em outubro de 1963, Starzl e colaboradores publicaram os resultados descrevendo o sucesso da utilização da azatioprina (AZA) associada a prednisona (PRED) para manter o enxerto renal (STARZL; MARCHIORO; WADDELL, 1963). Após décadas de utilização do protocolo proposto por Starzl, o advento da ciclosporina (CSA) na década de 1980 veio para revolucionar os transplantes, melhorando os resultados de transplantes renais e facilitando o transplante de outros órgãos sólidos (BARKER; MARKMANN, 2013). Essa droga permitiu reduzir drasticamente as taxas de rejeição e melhorar as taxas de sobrevivência do enxerto em um ano (LEE; GABARDI, 2012).

Atualmente, os regimes de imunossupressores são baseados na obtenção de concentrações sanguíneas que se acredita estarem dentro da faixa terapêutica (PENA et al., 2013). Tipicamente, os agentes imunossupressores são utilizados em doses elevadas nas primeiras semanas após o transplante, sendo as mesmas diminuídas à medida que o risco de rejeição diminui (JASIAK; PARK, 2016), sendo esta fase posterior chamada imunossupressão de manutenção. Os objetivos da imunossupressão de manutenção é prevenir episódios de rejeição aguda e otimizar a sobrevivência a longo prazo do paciente e do enxerto (LEE; GABARDI, 2012). Este tipo de imunossupressão é alcançado combinando dois ou mais medicamentos de diferentes classes para maximizar a efetividade do regime, visando componentes únicos da resposta imune.

Os regimes terapêuticos de manutenção são baseados em cinco classes de agentes imunossupressores mais comumente utilizados: (1) inibidores da calcineurina (CNIs) (ciclosporina (CSA) e tacrolimo (TAC)); (2) bloqueadores de coestimulação (Belatacept); (3) inibidores da proteína alvo da rapamicina em mamíferos (mTOR) (sirolimo (SRL) e everolimo (EVR)); (4) antiproliferativos (azatioprina (AZA); derivados do ácido micofenólico (MPA) (Micofenolato de Mofetila (MMF) e Sódico (MPS)) e (5) corticosteroides (prednisona (PRED) e prednisolona) (LEE; GABARDI, 2012).

Mundialmente os regimes imunossupressores variam entre os centros de transplantes, e com o tipo de órgão transplantado, mas geralmente incluem um CNI e um agente adjuvante,

com ou sem corticosteroides (YABU; VINCENTI, 2009; JOLLY; WATSON, 2011). O regime terapêutico adequado deve ser específico do paciente, levando em consideração as propriedades farmacológicas dos medicamentos, o perfil de eventos adversos e as potenciais interações medicamentosas, bem como as doenças preexistentes e o risco de rejeição. Além disso, o ajuste da dose deve ser cuidadoso para equilibrar o risco de rejeição com o de toxicidade (LEE; GABARDI, 2012). Na diretriz publicada pelo *Kidney Disease: Improving Global Outcomes (KDIGO) group* (2009), é sugerido que a imunossupressão no transplante renal consista em tacrolimo como inibidor da calcineurina e micofenolato como primeira escolha dentre os agentes antiproliferativos com ou sem corticosteroides.

Para pacientes transplantados renais, o Ministério da Saúde Brasileiro recomenda que seja instituído o esquema tríplice de imunossupressão para manutenção de enxertos advindos de doadores com HLA idêntico ou distinto. O esquema com prednisona, azatioprina e ciclosporina ou tacrolimo é o de primeira escolha, já o esquema com prednisona, micofenolato de mofetila ou de sódio e ciclosporina ou tacrolimo pode ser adotado em situações especificas (BRASIL, 2014).

Diante dessas recomendações faz-se necessário e importante compreender o mecanismo dos três sinais da ativação das células T e da proliferação celular, porque os esquemas tríplices atuam em mais de um alvo-chave de ativação da célula T.

A resposta imune ao aloantígeno, pode ocorrer por meio de uma sequência de três sinais de ativação da célula T (KAHAN, 2007). As células T reconhecem o aloantígeno por meio de receptores de células T (TCRs). O início da sinalização intracelular requer peptídeos adicionais conhecidos como complexo CD3, e o sinal específico do antígeno (sinal 1) é transduzido por meio do complexo TCR-CD3 (HALLORAN, 2004; KAHAN, 2007), o segundo sinal que é coestimulador depende das interações receptor-ligante entre células T e as células apresentadoras de antígenos (APCs) (KUMBALA; ZHANG, 2013). É somente quando ambos os sinais são fornecidos, que a célula T secreta as concentrações ótimas de IL-2, ativando assim, o terceiro sinal que é a interação da IL-2 com o receptor de células T, isso resulta em uma série de eventos intracelulares que conduzem à síntese de DNA, bem como a diferenciação de células T (MARION, 2003).

A combinação dos sinais 1 e 2 ativa três caminhos de transdução de sinal: a via da calcineurina, a via de proteína quinase ativada (MAPK) e a via IKK do fator nuclear κB (NF-κB). Essas três vias ativam fatores de transcrição, incluindo o fator nuclear de células T ativadas (NFAT), proteína-1 ativada (AP-1) e NF-κB, respectivamente. Várias novas moléculas e citocinas incluindo CD25, CD154, interleucina IL-2 e IL-15 são posteriormente expressas. IL-

2 e IL-15 fornecem sinais de crescimento (sinal 3) por meio da proteína alvo da rapamicina em mamíferos (m-TOR) e via fosfatidilinositol-3-quinase (PI 3K), que posteriormente desencadeiam o ciclo e a proliferação de células T (HALLORAN, 2004; DÜRRBACH et al., 2010; KUMBALA; ZHANG, 2013). As vias de ativação da célula T encontram-se ilustradas na figura abaixo.

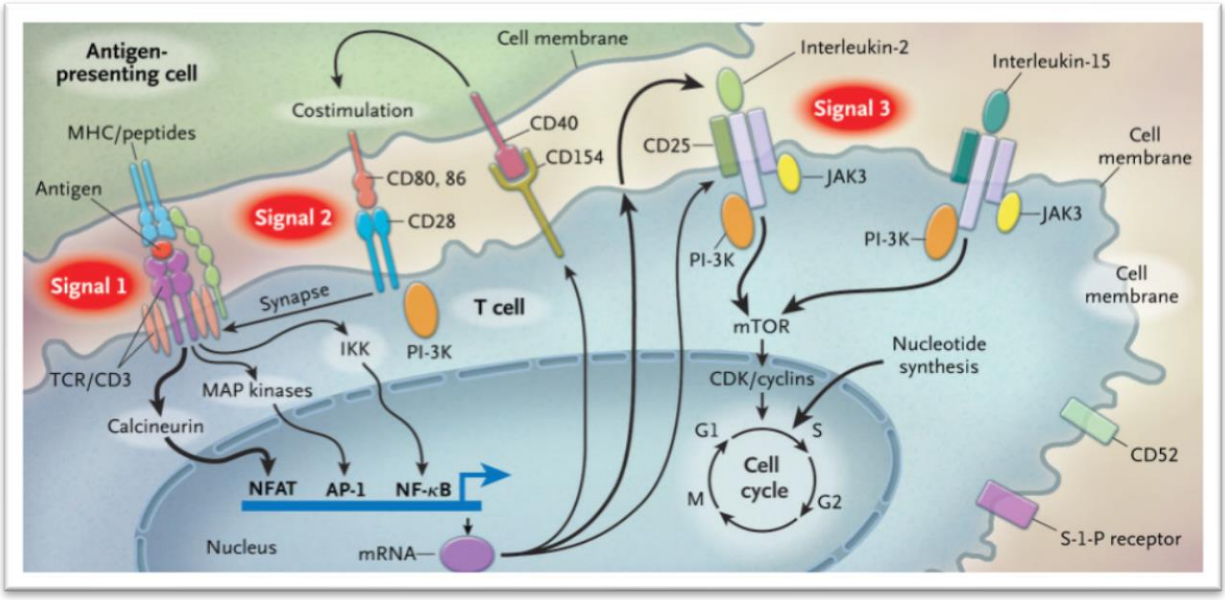

Figura 1 – Vias de ativação da célula T. Figura extraída de HALLORAN, (2004).

As células T totalmente ativadas sofrem expansão clonal e produzem um grande número de citocinas e células T efetoras (HALLORAN, 2004; SOMASUNDARAN; QUIROGA, 2011; KUMBALA; ZHANG, 2013). Para que haja o bloqueio da cascata de ativação do linfócito T, utiliza-se combinações de drogas imunossupressoras, que agem em diferentes sítios da ativação do linfócito T, essas drogas possuem um efeito final aditivo ou sinérgico, os imunossupressores comumente utilizados encontram-se descritos a seguir.

Azatioprina (AZA), além de ser utilizada após o transplante de órgãos para evitar rejeição, também pode ser empregada no tratamento de neoplasias hematológicas, doenças intestinais inflamatórias e condições autoimunes, tais como a artrite reumatoide e doença celíaca (MALTZMAN; KORETZKY, 2003; EL-BESHBISHY et al., 2011; IQBAL; CHAUDHARY; ARSALAN, 2017). Azatioprina é um pró-fármaco, metabolizada para 6-mercaptopurina (6-MP), por meio da redução por glutationa e outros compostos contendo grupo sulfidrilo, em seguida, convertido enzimáticamente em ácido tioúrico (6-TU), 6-metilmercaptopurina (6-MMP) e 6-tioguanina (6-TG) (MALTZMAN; KORETZK, 2003; JOLLY; WATSON, 2011). A 6-MP atua como um antimetabólito, após a sua incorporação no

DNA celular, altera a síntese e a função do RNA, reduzindo desta maneira a proliferação de células T. Azatioprina também interfere no sinal de coestimulação de linfócitos T por meio da molécula CD28, esta sinalização do receptor de CD28 é mediada por fosfatases, tais como a GTPase Rac1. Um dos produtos metabólicos de azatioprina, o 6-tioguanina, resulta na geração de 6-tioguanina trifosfato (6-thioGTP), que se liga ao GTPase Rac1 em lugar do Trifosfato de guanosina (GTP). O bloqueio de Rac1 converte o sinal de coestimulação de CD28 em um sinal apoptótico, suprimindo assim os linfócitos ativados (MALTZMAN; KORETZKY, 2003; TAYLOR; WATSON; BRADLEY, 2005).

Azatioprina apresenta absorção gastrintestinal, baixa biodisponibilidade e meia-vida plasmática muito curta (entre uma e duas horas) (NETO et al., 2008). Há uma interação medicamentosa importante entre azatioprina e Alopurinol, este último proporciona uma maior formação de metabólitos ativos da azatioprina que são responsáveis por significativa mielotoxicidade e imunossupressão (BRASIL, 2014). Embora bem tolerada por muitos pacientes, existem efeitos adversos significativos associados ao uso da AZA, sendo os principais náuseas, leucopenia, pancreatite e risco de linfoma. Alguns autores sugerem que sejam feitas duas contagens de células brancas ao mês a pacientes em uso de AZA (SPENCE et al., 2014), pois este fármaco é conhecido por possuir ação mielossupressora (JOLLY; WATSON, 2011).

Em muitos centros de transplante, a AZA vem sendo substituída por MPA como terapia adjuvante imunossupressora. Todavia, pacientes com intolerância gastrintestinal extrema ou pacientes que planejam a gravidez, fazem o caminho inverso convertendo o uso de MPA para AZA, já que o MPA está associado a malformação fetal (YABU; VINCENTI, 2009). Estudos demonstram que não há diferenças entre AZA e MPA quanto à sobrevida do enxerto, assim como incidência de câncer e função renal (CLAYTON et al., 2012; KWON et al., 2013). Além disso há incidência mais elevada de complicações infecciosas em pacientes que fazem uso de Micofenolato (BERNABEU-WITTEL et al., 2002).

Ainda na classe dos agentes antiproliferativos, temos os derivados do ácido micofenólico, que são Micofenolato de Mofetila (MMF) e sódico (MPS). Os mesmos sofrem efeito de primeira passagem e são rapidamente convertidos no fígado em ácido micofenólico, que é o composto ativo (TAYLOR; WATSON; BRADLEY, 2005). O alvo desses imunossupressores é a enzima Inosina Monofosfato Desidrogenase (IMPDH) na via *"de novo*", que limita a velocidade de síntese de nucleotídeos de guanosina, essenciais para a síntese de DNA (SINTCHAK et al., 1996; TANG et al., 2017). Cabe ressaltar que, a maioria dos tipos de células podem gerar nucleotídeos de guanosina por duas vias, a via de IMPDH e uma via de

salvamento, porém os linfócitos não possuem a via de salvamento e desta maneira, o bloqueio da via de IMPDH resulta em cessação relativamente seletiva da proliferação de linfócitos (TAYLOR; WATSON; BRADLEY, 2005). Nos linfócitos, o MPA possui potentes efeitos citostáticos, inibindo as respostas proliferativas de células T e B, assim como suprime a formação de anticorpos por células B (PIEDRAS et al., 2011). Em outros tipos celulares que também são parcialmente dependentes da via "*de novo"*, como por exemplo as células epiteliais gastrintestinais, a presença do MPA pode ocasionar danos consideráveis as mesmas (DAVIES et al., 2007).

Os níveis de concentração máximos do ácido micofenólico ocorrem aproximadamente uma hora após a dose (PIEDRAS et al., 2011; DONG et al., 2014). A farmacocinética de MPA exibe grande variabilidade inter e intraindividual com uma diferença de mais de 10 vezes na exposição ao fármaco, em pacientes adultos após o transplante de rim, fígado e coração (DONG et al., 2014). Esses mesmos autores relatam que os principais efeitos colaterais do MPA incluem leucopenia, desconforto gastrintestinal e diarreia. Com o objetivo de reduzir os efeitos colaterais gastrintestinais (GI) frequentes causados pelo uso de MMF, desenvolveu-se o Micofenolato Sódico de revestimento entérico (EC-MPS), que previne a liberação do MPA em condições ácidas (WECLAWIAK et al., 2011). Nos estudos de Reinke et. al. (2011) e Ortega et al. (2011), a conversão de MMF para EC-MPS reduziu as complicações GI em receptores de enxerto renal, melhorando sua qualidade de vida.

Diversos autores relataram que o MPA pode causar alterações histopatológicas em diferentes segmentos do TGI. No estômago foi relatado que este medicamento causa danos nas glândulas parietais e apoptose de células epiteliais, já no intestino delgado há inflamação aguda, atrofia das vilosidades, dilatação de criptas, aumento da apoptose, assim como de linfócitos intraepiteliais (NGUYEN et al, 2009; WECLAWIAK et al., 2011; COYNE; CAMPBELL, 2012; LEE S et al., 2013;WONG et al., 2015). Os derivados do MPA são amplamente utilizados em combinação com CNIs, proporcionando uma imunossupressão ótima, favorecendo assim a redução da dose de CNIs e consequentemente, os efeitos nefrotóxicos dos últimos (KIDNEY DISEASE: IMPROVING GLOBAL OUTCOMES (KDIGO) GROUP, 2009).

A classe dos inibidores da calcineurina é representada pelos imunossupressores Ciclosporina e Tacrolimo, que ainda são considerados essenciais para o transplante de órgãos sólidos. A ciclosporina é um peptídeo lipofílico, extraído do fungo *Tolypocladium inflatum gams* (KAHAN, 1989). O fármaco foi descoberto no início da década de 1970, no laboratório da Sandoz, atual Novartis na Suíça (TEDESCO; HARAGSIM, 2012). Após sua aprovação pela *Food and Drug Administration* (FDA), nos Estados Unidos em 1983, foi capaz de revolucionar

os resultados obtidos no transplante de órgãos (GUADA et al., 2016). O tacrolimo é um macrolídeo com grande potencial imunossupressor, aproximadamente 100 vezes maior que a CSA, sendo também utilizado após transplantes de órgãos e isolado de uma estirpe de *Streptomyces tsukkubsensis* em 1984 (BARREIRO et al., 2012; CHEN et al., 2012).

O mecanismo de ação dos CNIs depende da formação de um complexo com a ciclofilina, enzima que se liga à calcineurina inibindo a sua função de fosfatase. Com a inibição da calcineurina, há também uma inibição da translocação do Fator Nuclear de Células T ativadas (NFAT) para o núcleo. Isso impede a transcrição dos genes de citocinas pró-inflamatórias como interleucina-2 (IL2), interleucina-4 (IL4), interferon-γ (IFN-γ) e o fator de necrose tumoral alfa (TNF-α), inibindo parcialmente a ativação da célula T, desta maneira os linfócitos T não respondem satisfatoriamente à estimulação do antígeno (MATSUDA; KOYASU, 2000; HALLORAN, 2002; TAYLOR; WATSON; BRADLEY, 2005; GUADA et al., 2016). A absorção intestinal, tanto da ciclosporina como do tacrolimo, é baixa e variável, podendo ser influenciada por diversos fatores como a ingestão concomitante de alimentos, diabetes, diarreia e alterações gastrintestinais em geral (NAESENS; KUYPERS; SARWAL, 2009).

O metabolismo da CSA é principalmente hepático, apresentando tempo de meia vida de 6,4 a 8,7 horas (TEDESCO; HARAGSIM, 2012). Medicamentos que inibem as enzimas do citocromo P-450 como cetoconazol, eritromicina e bloqueadores dos canais de cálcio, podem causar uma elevação nos níveis séricos de CSA, assim como os medicamentos que induzem o citocromo P-450 (valproato, fenobarbital, fenitoína entre outros) podem diminuir os níveis de CSA (KAHAN, 1989). Os principais efeitos colaterais associados à Ciclosporina incluem nefrotoxicidade e hipertensão arterial (ITALIA; BHARDWAJ; RAVI KUMAR, 2006; GUADA et al., 2016), sendo responsável por grande parte das perdas de enxertos pós transplante.

A nefrotoxicidade induzida pela ciclosporina geralmente é reversível e dose-dependente, sendo caracterizada por aumentos nos níveis séricos de ureia e creatinina, a CSA também pode causar alterações estruturais irreversíveis como fibrose intersticial durante o tratamento a longo prazo (ITALIA; BHARDWAJ; RAVI KUMAR, 2006). A monitorização dos parâmetros que avaliam a função renal deve ser obrigatória em pacientes que utilizam este fármaco. O sintoma gastrintestinal mais comum relatado com o uso da ciclosporina é a gastroparesia (MAES et al., 1999). Diversos fatores, como alterações na motilidade gastrintestinal e lesão hepática, podem comprometer a biodisponibilidade do tacrolimo. O tacrolimo, assim como a ciclosporina, também pode ter seu metabolismo alterado devido a

interações medicamentosas, com fármacos que inibem ou competem com as enzimas do citocromo P450 e com a glicoproteína-P (P-gP) (SIKMA et al., 2015). Os principais efeitos colaterais associados ao tratamento com tacrolimo são nefrotoxicidade, neurotoxicidade, distúrbios no metabolismo da glicose, distúrbios GI e hipertensão arterial, todos dose-dependentes (PIEDRAS, R et al., 2011).

O tacrolimo possui uma janela terapêutica muito estreita e altamente variável, e mesmo em baixas concentrações sanguíneas (2-6 ng/mL) pode causar nefrotoxicidade, devido a isso esse fármaco requer uma monitorização frequente a fim de manter o ajuste da dose (THÖLKING et al., 2017). As doses usualmente utilizadas em transplante de órgãos sólidos variam de acordo com o tipo de órgão transplantado e com a resposta do paciente ao regime imunossupressor. No trato gastrintestinal, o tacrolimo provoca mais diarreia quando comparado com a ciclosporina (MAES et al., 1999; LEVY et al., 2004), devido a sua conhecida estrutura de macrolídeo, que possui efeito pró-cinético nos receptores intestinais de motilina (MAES et al., 1999; TEIXEIRA et al. 2014).

No estudo de Teixeira et al. (2014), os autores observaram um esvaziamento gástrico de 47 min para pacientes que utilizavam tacrolimo associado a azatioprina e prednisona, sendo aproximadamente 4 vezes mais rápido do que em voluntários saudáveis. Estes mesmos efeitos de aceleração do esvaziamento gástrico foram observados em nosso estudo prévio em animais tratados com monoterapia imunossupressora com TAC na dose de 3mg/kg/dia. Já os animais tratados com CSA (15 mg/kg/dia) não apresentaram diferenças significativas no esvaziamento gástrico quando comparados ao grupo controle (DALL'AGNOL et al., 2014).

Além do CNIs e dos agentes antiproliferativos, os corticosteroides ainda são utilizados consistentemente no transplante de órgãos sólidos, tanto na indução como na manutenção da imunossupressão. O mecanismo de ação dos glicocorticoides na imunossupressão acontece em parte quando o receptor de glicocorticoide (GR) se liga diretamente na AP-1 (Apoproteina-1) e no NF-kB (fator nuclear kappa B), interferindo na ativação da transcrição destas duas proteínas, que assim como o NFAT, estão envolvidas transcrição do gene para IL-2, e consequentemente na diferenciação e proliferação de linfócitos T (SPIES et al., 2011; RAMAMOORTHY; CIDLOWSKI, 2016). Além disso, os glicocorticoides estão envolvidos na supressão da maturação e função das células dendríticas. Porém, esses medicamentos não apenas suprimem a atividade das células dendríticas, mas reprogramam-nas para as chamadas "células dendríticas tolerogênicas", que podem induzir um estado de hiporresponsividade nas células T e induzir a formação de células T reguladoras (T_{reg}) (BASCHANT; TUCKERMANN, 2010; COUTINHO; CHAPMAN, 2011).

Os efeitos adversos associados aos corticosteroides podem ser divididos em precoces e secundários associados ao uso prolongado destes. Como eventos precoces pode-se citar os efeitos colaterais cosméticos (como acne; fácies cushingóide e edema), intolerância à glicose, distúrbios de humor e dispepsia. Já os efeitos secundários incluem osteoporose, miopatia e suscetibilidade a infeções (LICHTENSTEIN et al., 2006; BASCHANT; TUCKERMANN, 2010).

Em um estudo recente publicado por Lima et al., (2017), em que ratos jovens foram tratados com doses baixas (0,625 mg/kg/dia) e altas (2,5 mg/kg/dia) de prednisona por 15 dias, houve uma aceleração no tempo médio de esvaziamento gástrico dos animais. Além disso, os mesmos autores também observaram que o tratamento com prednisona em ambas as doses foi capaz de diminuir a altura das vilosidades intestinais, assim como reduzir o número de mastócitos da mucosa GI. A partir desses resultados é possível inferir que em transplantes, os corticosteroides podem exacerbar os efeitos adversos dos CNIs.

Devido aos efeitos adversos associados aos corticosteroides, diversos estudos propõem regimes imunossupressores de manutenção que não os incluam, poupem ou os retirem (KIDNEY DISEASE: IMPROVING GLOBAL OUTCOMES (KDIGO) GROUP, 2009). No entanto, uma revisão da literatura publicada recentemente na *Cochrane Database of Systematic Reviews* revelou que os protocolos poupadores de esteroide ou a retirada após o transplante renal aumentam significativamente o risco de rejeição aguda (HALLER et al., 2017), fato esse que reforça a importância dos corticosteroides como adjuvantes na terapia imunossupressora.

1.3 Epidemiologia das complicações gastrintestinais em transplantes

Levando em consideração que o Brasil possui o maior sistema público de transplantes do mundo, há grande demanda por conhecimento relativo aos efeitos adversos dos medicamentos imunossupressores no TGI. A incidência de complicações gastrintestinais varia de acordo com o órgão transplantado, sendo que após transplante renal atinge em torno de 20% (HARDINGER et al. 2004). No entanto, em pacientes com transplante cardíaco, a taxa de complicações gastrintestinais ascende para aproximadamente 40%, sendo diarreia a mais frequente (58,6%), comprometendo as atividades diárias desses pacientes (DIAZ et al., 2007). Já os pacientes que receberam transplante de fígado apresentaram uma prevalência de complicações GI em torno de 50%, sendo a diarreia também a mais comum (HERRERO et al., 2007).

Em um estudo retrospectivo com mais de 40.000 receptores de transplante de rim com acompanhamento médio de três anos, os autores registraram 7.103 casos de diarreia e 8.104 perdas de enxerto, sendo que destes 4.201 pacientes foram a óbito (BUNNAPRADIST et al., 2008). Esses mesmos autores descobriram que o risco de perda de enxerto e morte do paciente é mais que o dobro em pacientes com diarreia não infecciosa. Esta observação é de particular importância porque a sobrevivência do paciente e do enxerto só pode ser mantida com terapia imunossupressora ao longo da vida.

Recentemente, em um estudo que avaliou 51 transplantados renais submetidos a colonoscopia com biopsia em um corte de tempo de 15 anos, os autores observaram que apenas 22% dos pacientes tinham colite aguda. Entretanto, 33% dos pacientes avaliados foram diagnosticados com lesão relacionada a terapia imunossupressora, sendo que a resolução dos sintomas ocorreu com a cessação das drogas, a maioria (53%) destas biópsias de cólon demonstraram apoptose das células epiteliais das criptas e/ou distorção de arquitetura intestinal (PITTMAN; JESSURUN; YANTISS, 2017).

Diante do exposto, é perceptível que os medicamentos imunossupressores utilizados no manejo dos transplantes de órgãos proporcionam a manutenção do órgão transplantado, porém o seu uso implica em inúmeros efeitos indesejados para o paciente. Convém destacar, nesse contexto, as complicações gastrintestinais que são responsáveis por consideráveis taxas de morbidade e mortalidade associadas ao transplante (GAUTAM, 2006; VEROUX, et al., 2012). Para minimizar os efeitos GI em pacientes transplantados, inclui-se diferentes abordagens gerais, sendo que a mais utilizada é o uso de medicamentos profiláticos como, por exemplo, protetores gástricos ou terapia anti-infecciosa. Além disso, a realização de procedimentos diagnósticos para identificar a causa da complicação e a modificação da imunossupressão também são utilizadas (GIL-VERNET et al., 2007). No entanto, sem conhecer os efeitos que os imunossupressores causam individualmente ou em combinação nas funções motoras gastrintestinais, permanecem as dificuldades em conduzir o tratamento das complicações GI.

1.4 Biosusceptometria de Corrente Alternada e Eletrogastrografia

Investigar alterações gastrintestinais no pós-transplante de órgãos denotam grande importância e requerem métodos precisos e bem definidos, nos quais a escolha da técnica é fundamental para garantir um diagnóstico preciso e com menor número de intervenções possíveis ao paciente. As técnicas biomagnéticas ganharam representatividade no estudo de parâmetros de motilidade do trato gastrintestinal por serem técnicas não invasivas e desprovidas de material

ionizante (CORÁ et al., 2005). Os métodos biomagnéticos são utilizados no estudo do trato gastrintestinal desde a década de 1950 (WENGER; HENDERSON; DINNING, 1957), porém a Biosusceptometria de Corrente Alternada (BAC) foi aprimorada e aplicada no Brasil na década de 1990 por Miranda, Baffa, e colaboradores para avaliar a fisiologia do trato gastrintestinal no homem (MIRANDA et al., 1992; BAFFA et al., 1995).

O sistema BAC se baseia na utilização de dois pares de bobinas alternadas de excitação e detecção em configuração que minimiza os ruídos ambientais (MIRANDA et al., 1992). A BAC utiliza como traçadores ou marcadores, materiais com alta susceptibilidade magnética, como o óxido de ferro com manganês (Ferrita - $MgZnFe_2O_3$) que ao ser aproximado do sensor magnético ocasiona um desbalanceamento do fluxo magnético, gerando uma intensidade de sinal que pode ser quantificada e analisada ao longo do tempo, relacionando-a com as funções fisiológicas do órgão estudado (CORÁ et al., 2005).

Quando aplicada para avaliar a contratilidade GI, sabe-se que as contrações mecânicas do estômago afastam o material do sensor posicionado na superfície abdominal, reduzindo a intensidade de sinal registrado, assim como, o relaxamento aproxima o marcador magnético dos sensores, o que, consequentemente, aumenta a intensidade do sinal. Quando utilizada para a avaliação do esvaziamento gástrico e transito GI, leva-se em consideração que a intensidade do sinal é proporcional a quantidade de material no interior do órgão e que no decorrer do tempo o material vai sendo deslocado pelos segmentos intestinais (CORÁ et al., 2006; AMÉRICO et al., 2010a).

A BAC é uma técnica simples, de baixo custo e fácil manuseio (AMÉRICO et al., 2010b) que permite trabalhar sem intervenção anestésica, facilitando a experimentação *in vivo*, e podendo ser utilizada tanto em animais quanto no homem. Dessa forma, propicia a obtenção de resultados mais próximos das condições fisiológicas (CORÁ et al., 2005). Cabe ressaltar que esta técnica já foi validada para a determinação da motilidade gastrintestinal em ratos por meio da avaliação de contrações da parede gástrica (AMÉRICO et al., 2010a) e do esvaziamento gástrico e trânsito GI em ratos (QUINI et al., 2012).

Com a consolidação da técnica nos últimos anos, houve uma ampliação da abrangência de aplicações desse método. A BAC vem sendo empregada em estudos na área farmacêutica (CORÁ et al., 2010; FERRARI et al., 2014), para compreensão de doenças em modelos animais (MARQUES et al., 2014; ANJOS-RAMOS et al. 2017; HAUSCHILDT et al., 2018) e para caracterização e detecção de nanopartículas magnéticas em sistemas biológicos (QUINI et al., 2017; PRÓSPERO et al. 2017).

Outra técnica amplamente utilizada é a eletrogastrografia (EGG), um método atraente para estudar a eletrofisiologia do estômago, pois não é invasivo e não interrompe o processo contínuo do estômago (YIN; CHEN 2013). A EGG tem sido frequentemente utilizada na avaliação do efeito do estresse, disritmias gástricas e eficácia das terapias farmacológicas, mais comumente terapêutica procinética (MAURER, 2012; YIN; CHEN 2013). Esta técnica consiste em utilizar eletrodos cutâneos simples para registrar a atividade mioelétrica gástrica (MAURER, 2012).

Essa tese incorporou dois artigos que utilizaram a BAC e a EGG para avaliar *in vivo* as alterações gastrintestinais advindas da utilização de imunossupressores, tendo como objetivo:

- Investigar os efeitos da monoterapia imunossupressora na motilidade gastrintestinal de ratos utilizando a BAC.
- Investigar os efeitos induzidos no trato gastrintestinal de ratos após terapia imunossupressora tripla, utilizando a BAC e a EGG.

2 CAPÍTULO 1

Gastrointestinal disorders after immunosuppression: an experimental model to evaluate the influence of monotherapy on motility parameters

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Experimental Physiology

Research Paper

Gastrointestinal disorders after immunosuppression: an experimental model to evaluate the influence of monotherapy on motility parameters

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Edited by: James Jones

New Findings

- **What is the central question of this study?**
  The aim was to propose an animal model for investigating the effects of immunosuppressive monotherapy on gastrointestinal motility using a non-invasive biomagnetic technique.
- **What is main finding and its importance?**
  In our experimental study, immunosuppressive drugs currently in use accelerated gastric emptying whilst increasing the frequency and amplitude of gastric contractions after treatment, except for Mycophenolate and azathioprine. Alternating current biosusceptometry is a useful tool to evaluate side-effects of drugs on the gastrointestinal tract, which will help in understanding the symptoms and improving clinical management of patients.

The aim was to propose an animal model for investigating the effects of immunosuppressive monotherapy on gastrointestinal motility using a non-invasive biomagnetic technique. Male Wistar rats were randomly distributed into the following treatment groups: ciclosporin, tacrolimus, prednisone, sirolimus, mycophenolate mofetil, everolimus, azathioprine and control. Each animal was treated for 14 days by gavage with dosages ranging from 1 to 20 mg kg^{-1} day^{-1} considering the area-to-volume ratio and hepatic metabolism. Gastrointestinal transit and gastric contractility measurements were evaluated by alternating current biosusceptometry before and after treatment. Gastric emptying was faster in animals treated with tacrolimus, prednisone, sirolimus and everolimus compared with control animals (126.7 ± 12.7 min). There was a significant increase in the frequency of contractions after ciclosporin, tacrolimus, azathioprine and sirolimus treatment compared with control animals (4.6 ± 0.3 cycles min^{-1}). Increases in the amplitude of contraction were observed after treatment with tacrolimus, sirolimus and everolimus compared with control rats (34.9 ± 6.0 dB). The results showed that our animal model was suitable for demonstrating that most immunosuppressive drugs currently in use impaired at least one gastrointestinal motility parameter. As a non-invasive technique, alternating current biosusceptometry is a

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potentially useful tool for evaluation of side-effects of drugs in gastrointestinal tract, helping us to understand the symptoms to improve clinical management of patients.

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Introduction

Each year worldwide, an increasing number of transplantations are performed and more people are diagnosed with autoimmune disorders, requiring the use of immunosuppressive drugs (Yang *et al.* 2005; Meaney, 2015) to block, in part, some steps of the immune response (Halloran, 1996). Several immunosuppressive drugs combinations are used to provide adequate immunosuppression (Helderman & Goral, 2002); however, gastrointestinal (GI) complications have been reported as drug-related adverse effects (Ponticelli *et al.* 2010; Teixeira *et al.* 2012; Lucan & Berardinelli, 2016). Even minor disturbances of GI function can impair the quality of life in organ transplant recipients (Ponticelli *et al.* 2010), threatening the long-term stability of the transplant because of reduced adherence to the therapy (Chapman, 2004). Symptoms with different degrees of severity, such as reflux, diarrhoea and constipation, are often closely related to the immunosuppressive therapy (Machnicki *et al.* 2008). As immunosuppressive therapy uses at least three different drugs, it is not clear whether the GI side-effects are associated with a specific drug or with the combined therapy. Hence, an animal model could be helpful for investigating the influence of immunosuppressive monotherapy on the GI tract in order to evaluate whether the side-effects are related to disturbances in the motility parameters.

Gastrointestinal motility includes contractility, gastric emptying and gastrointestinal transit, and these parameters can be evaluated by several gold-standard techniques; however, their disadvantages comprise ionizing radiation, invasiveness and inability to analyse motility parameters simultaneously (Smout & Mundt, 2009). Biomagnetic techniques are used to study magnetic fields generated by magnetic materials (Corá *et al.* 2010), allowing an experimental approach in physiological conditions. Alternating current biosusceptometry (ACB) was recently validated and established as an effective technique for recording the frequency and amplitude of contraction, gastric emptying and GI transit in rats (Américo *et al.* 2010; Quini *et al.* 2012).

The aim of the present study was to propose an animal model for investigation of the effects of immunosuppressive monotherapy on gastrointestinal motility using a non-invasive biomagnetic technique.

Methods

Ethical approval and animals

All procedures were performed in accordance with the Guide of the Care and Use of Laboratory Animals (Brazilian College of Animal Experimentation) and approved by the Institutional Animal Care and Use Committee (protocol number 23108.049862/13-3, University of Mato Grosso/Brazil).

Sixty-four healthy male Wistar rats (270–340 g) were used, and they were handled to be conditioned to the experiments. Food and water were provided *ad libitum* during all experimental procedures, except at night before measurements (10 h fasting).

After treatment with the immunosuppressive drugs, the animals underwent an evaluation of GI transit for 6 h, being handled carefully to avoid unnecessary stress. It is important to mention that the ACB sensor does not cause any type of pain to the animal. For the GI contractility assessments, the animals were anaesthetized with 30 mg kg^{-1} pentobarbital, I.P. (Abbott Laboratories, Chicago, IL, USA), only to contain them. To assess the level of consciousness by the degree of antinociception (lack of response to noxious stimuli) after the induction of general anaesthesia, we gently pinched the hindpaw of the animals and observed the musculoskeletal response and respiratory function. At the end of the experiments, the rats were killed with an overdose of pentobarbital (100 mg kg^{-1}, I.P.).

The experiments were carried out according to the guidelines laid down by the Brazilian College of Animal Experimentation animal welfare committee and conform to the principles and regulations of the journal's ethical policy, detailed by Grundy (2015).

Alternating current biosusceptometry sensor

A single ACB sensor (Br4-Science®, São Paulo, Brazil) consists of a set-up of induction coils for monitoring of magnetic signals generated by magnetic materials in response to an externally applied magnetic field (Corá *et al.* 2010). As the signal intensity depends on the amount

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Table 1. Dose of immunosuppressive drugs as monotherapy administered to rats for 14 days consecutively

Group	Dose (mg kg^{-1} day^{-1})	*n*
Control	0.0	20
Ciclosporin (CSA)	15.0	12
Tacrolimus (TAC)	3.0	10
Prednisone (PRED)	1.0	9
Mycophenolate mofetil (MMF)	20.0	7
Sirolimus (SRL)	1.0	10
Azathioprine (AZA)	20.0	9
Everolimus (EVR)	1.5	7

of magnetic material and distance between the sensor and the magnetic sample, the sensor is placed on the abdominal surface to record in real-time intensity values for quantification of gastric emptying and contractility with good a signal-to-noise ratio (Quini *et al.* 2012). Technical details on instrumentation have been published elsewhere (Corá *et al.* 2010; Quini *et al.* 2012).

Study design and drug formulation

Initially, 20 animals were randomly selected to compose the control group. Gastrointestinal transit and gastric contractility were evaluated after gavage of 2.5 ml distilled water per animal per day, with a 16 h interval between measurements. Afterwards, the animals were randomly distributed into seven groups, according to the drug scheduled, as follows: ciclosporin (CSA), tacrolimus (TAC), prednisone (PRED), sirolimus (SRL), mycophenolate mofetil (MMF), everolimus EVR and azathioprine (AZA). The animals were treated for 14 consecutive days with variable dosage as shown in Table 1. On day 14, the animals received a solid magnetic pellet, and the measurements were performed as described below. The time line for the experimental procedures is shown in Fig. 1.

All following drugs were diluted in distilled water and administered by gavage daily for 14 consecutive days (Table 1): ciclosporin in oral microemulsion formulation (SANDIMMUN NEORAL®, Novartis Pharma Stein AG, Stein, Switzerland); Tacrolimus capsule (PROGRAF®, Janssen-Cilag, São Paulo, Brazil); Prednisone tablet (METICORTEN®, Wyeth, São Paulo, Brazil); Mycophenolate Mofetil tablet (CELLCEPT®, Roche, São Paulo, Brazil); Everolimus tablet (CERTICAN®, Novartis Pharma Stein AG, Stein, Switzerland); Sirolimus tablet (RAPAMUNE®, Wyeth Pharmaceuticals Company, Guayama, Porto Rico); Azathioprine tablet (AZA, IMUSSUPREX®, Germed Pharmaceutical LTDA, São Paulo, Brazil). Dosages were selected by focusing on clinically relevant dosages and considering that the dosage should be higher in rats than in humans because of a different body area-to-volume ratio and faster hepatic metabolism (Malinowski *et al.* 2011).

The faeces were monitored and graded for the degree of diarrhoea by the following score: 0, firm faeces; 1, malformed faeces; 2, watery faeces with perianal staining; and 3, severe perianal staining (Ball *et al.* 1996).

Gastrointestinal transit and contractility

A solid magnetic pellet was prepared using 0.5 g of ferrite powder ($MgZnFe_2O_3$; Imag, Ribeirão Pires/São Paulo, Brazil) and 1.5 g of laboratory chow. The GI tract is not able to absorb ferrite powder, which is

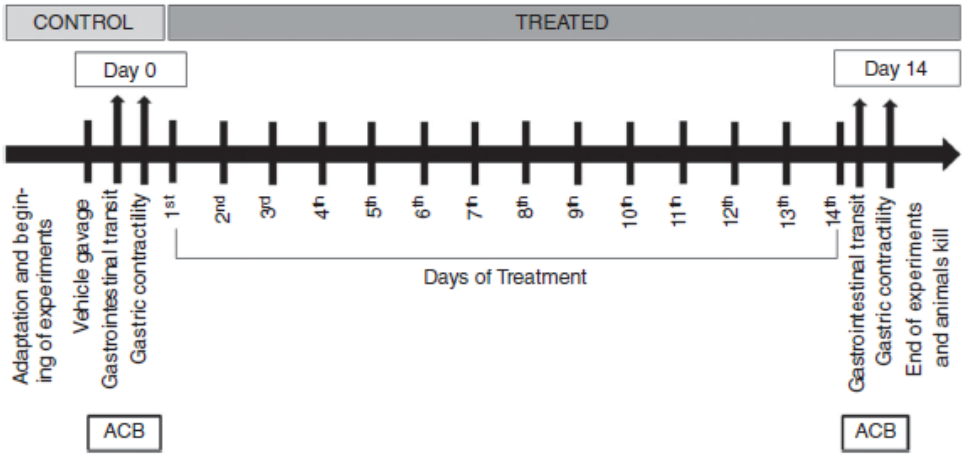

Figure 1. Schematic experimental set-up and time line of the experimental protocol used for all administered drugs
Abbreviation: ACB, alternating current biosusceptometry.

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Table 2. Gastrointestinal transit parameters obtained in rats before (control) and after treatment with immunosuppressive drugs

Transit	Control	CSA*	TAC*	PRED*	MMF	SRL	AZA	EVR
MGET	126.7 ± 12.7	122.4 ± 11.9	106.6 ± 15.3†	106.7 ± 8.0†	120.4 ± 8.1	109.7 ± 10.7†	122.8 ± 10.0	98.3 ± 6.2†
MSBTT	102.0 ± 15.9	124.7 ± 14.9‡	128.3 ± 15.6‡	124.4 ± 11.9‡	116.5 ± 16.8	127.9 ± 12.7‡	111.0 ± 9.6	139.2 ± 8.9‡
MCAT	229.0 ± 8.8	247.3 ± 24.2§	234.8 ± 11.5	231.1 ± 9.8	236.8 ± 13.2	237.7 ± 13.0	231.4 ± 9.2	237.6 ± 6.0

Groups are as in Table 1. Abbreviations: MCAT, mean caecum arrival time; MGET, mean gastric emptying time; and MSBTT, mean small intestinal transit time. Data are expressed as means ± SD. Analysis was by ANOVA with a Dunnett's *post hoc* test. *Data have already been published by Dall'Agnol *et al.* (2014). †$P < 0.01$ compared with control (MGET); ‡$P < 0.01$ compared with control (MSBTT); and §$P < 0.01$ compared with control (MCAT).

eliminated in the faeces (Quini *et al.* 2012; Matos *et al.* 2016).

On day 0, the animals (control group) were gavaged with vehicle, and 10 min after spontaneous ingestion of the meal described above, measurements of GI transit and contractility were started. Based on anatomical references, the ACB sensor was used to identify the maximal magnetic signal intensity on the points corresponding to the stomach and the caecum (Quini *et al.* 2012). Subsequent measurements were made in awake rats at those two reference points at regular 15 min intervals for at least 6 h.

Gastrointestinal contractility measurements were performed 16 h after the transit monitoring. After ingestion of the magnetic pellet, animals were anaesthetized with 30 mg kg^{-1} pentobarbital, I.P. (Abbott Laboratories). The ACB sensor was placed on the anterior abdominal surface of animals kept in the supine position, and signals were acquired continuously for 40 min. The ACB signal was acquired at a sampling rate of 20 Hz per channel with a multichannel recorder system (MP100 System; BIOPAC Inc., Santa Barbara, CA, USA) (Américo *et al.* 2010). On day 14, the treated animals received a solid magnetic pellet, and the measurements were performed as described above.

Data analysis

In order to verify the potential effect of immunosuppressive monotherapy on the GI tract, the following analyses were performed. Gastrointestinal transit was analysed using the statistical moment (Podczeck *et al.* 1995) to calculate the mean gastric emptying time (MGET) as the time t (in minutes) taken for a mean amount of magnetic material to leave the stomach; mean caecum arrival time (MCAT) as the time t (in minutes) taken for a mean amount of magnetic material to enter the caecum; and mean small bowel transit time (MSBTT) as the difference between MCAT and MGET.

To quantify gastric contractility parameters, all raw magnetic signals were initially analysed in MatLab (Mathworks, Inc., Natick, MA, USA) by visual inspection and applying bi-directional Butterworth bandpass filters by fast Fourier transform (FFT), with a cut-off frequency of 50–120 mHz. The highest frequency peak for each FFT was determined as the gastric dominant frequency, and the smallest was identified as signal noise. Amplitude of contraction (A) was calculated as the relationship between power of gastric peak (P) and power of noise peak (P'), expressed in decibels, as follows: $A = 10 \log_{10}(P/P')$ (Lu *et al.* 2005).

Statistical analysis

Normality of continuous variables was evaluated using the Kolmogorov–Smirnov test. When normally distributed, data were evaluated using ANOVA followed by Dunnett's *post hoc* test. The Pearson correlation coefficient (r) was used to analyse the relationship between variables. Only coefficients above 0.80 were considered significant. Differences were considered significant at a P value <0.05.

Results

According to the criteria adopted, no significant alterations were observed in faeces (i.e. absence of diarrhoea) after drug treatments. Table 2 shows GI transit parameters evaluated before (control) and after immunosuppressive monotherapy. The MGET for animals treated with TAC, PRED, SRL and EVR was shorter compared with control animals ($P < 0.01$). The MCAT was delayed only with CSA treatment ($P < 0.01$). The MSBTT was slower for CSA, TAC, PRED, SRL and EVR treatments ($P < 0.01$). For MMF and AZA, there were no significant difference in MSBTT compared with control animals.

Figure 2 shows typical signals of gastric contractility obtained for an animal from the control group and for a representative animal from each group after treatment. Table 3 shows GI contractility parameters evaluated before and after a course of immunosuppressive monotherapy. A significant difference in frequency of contractions after CSA, TAC, AZA and SRL therapy compared with control animals was observed ($P < 0.01$). For PRED, MMF and EVR treatments, there was no significant change in this parameter. Marked increases in the amplitude of contraction were observed after therapy with TAC, SRL

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and EVR ($P < 0.01$). For CSA, PRED, MMF and AZA therapy, no significant changes were observed.

The Pearson correlation between gastric emptying and the frequency or amplitude of contractions was determined (Fig. 3). A negative correlation ($P = -0.51$) between gastric emptying and the frequency of contractions was observed before therapy ($P < 0.05$). After treatments, it a complete absence of correlation among these parameters was observed.

Discussion

Our data showed that at least one parameter of GI transit and/or contractility was impaired for the immunosuppressive drugs used in monotherapy, except for MMF and CSA. Upper GI symptoms are often related to the use of many medications and may be attributable in part to side-effects of drugs on GI transit and contractility (Rozov-Ung *et al.* 2014). Gatrointestinal symptoms contribute remarkably to deterioration of

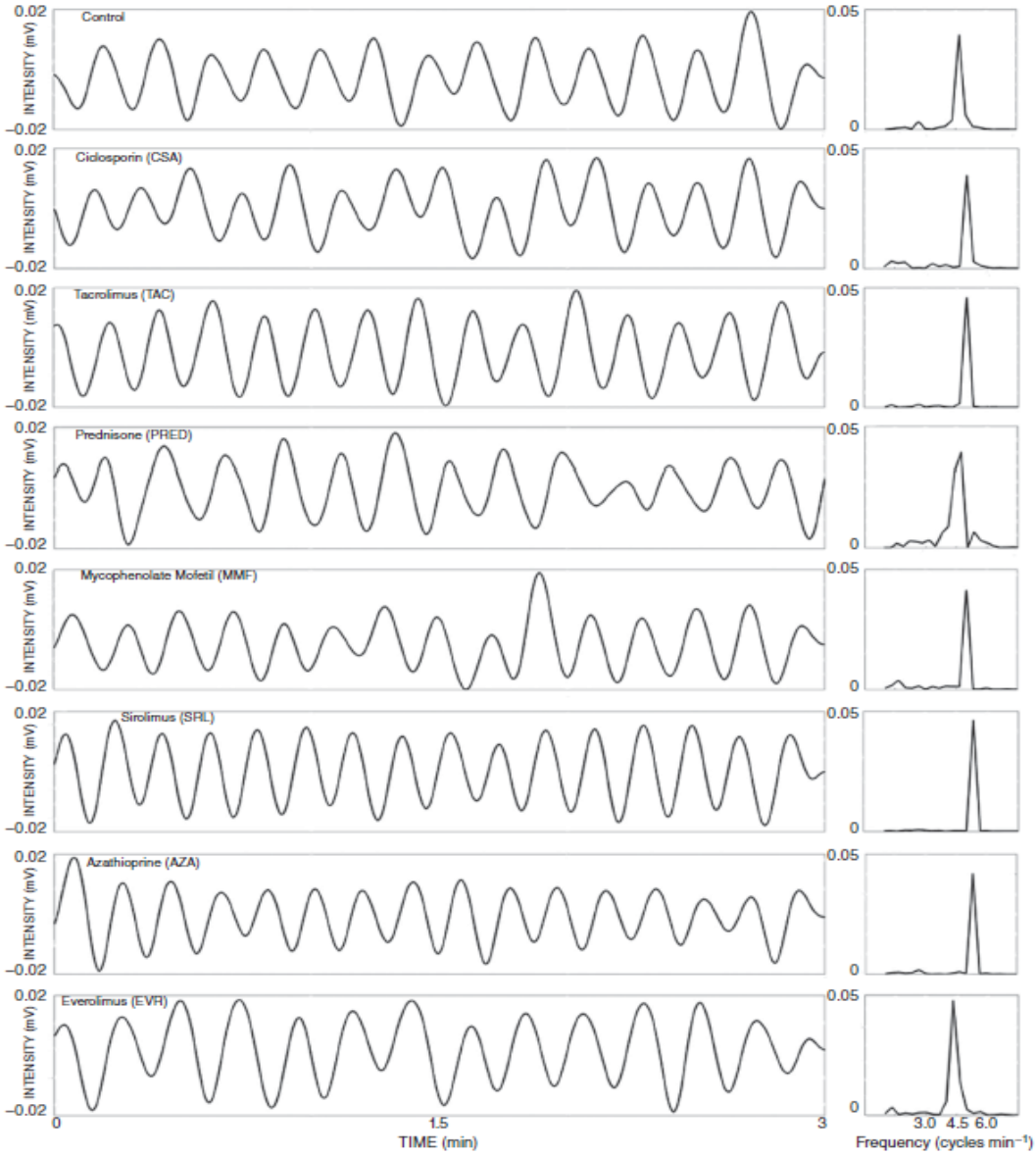

Figure 2. Profile of gastric contractility signals (left panel) and their respective Fourier transform (right panel) obtained in rats before (control) and after treatment with immunosuppressive drugs

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Table 3. Amplitude and frequency of gastric contractions obtained in rats during control conditions and after treatment with CSA, TAC, PRED, MMF, SRL, AZA and EVR

Gastric contraction	Control	CSA	TAC	PRED	MMF	SRL	AZA	EVR
Frequency (cycles min^{-1})	4.6 ± 0.3 (~76 mHz)	5.0 ± 0.3 (~84 mHz)*	5.0 ± 0.3 (~84 mHz)*	4.6 ± 0.4 (~76 mHz)	4.8 ± 0.5 (~80 mHz)	5.1 ± 0.3 (~85 mHz)*	5.2 ± 0.3 (~88 mHz)*	4.7 ± 0.2 (~78 mHz)
Amplitude (dB)	39.4 ± 6.0	38.1 ± 5.6	45.6 ± 3.1†	38.4 ± 4.7	41.6 ± 5.3	46.0 ± 7.0†	42.3 ± 5.7	46.8 ± 4.0†

Groups are as in Table 1. Frequency of gastric contractions is expressed as cycles per minute and milliherz. Amplitude (intensity of gastric contractions) is expressed as decibels. Analysis was by ANOVA with Dunnett's *post hoc* test. Data are expressed as means ± SD. *$P < 0.01$ compared with control frequency; and †$P < 0.05$ compared with control amplitude.

health-related quality of life, affecting patients' daily activities (Ponticelli *et al.* 2010). A non-invasive technique, such as ACB, can be a valuable tool to investigate the influence of long-term drug treatment on the GI tract.

Immunosuppressive drugs are used worldwide to prevent allograft rejection, and some of them are essential for the treatment of autoimmune diseases. Therefore, monitoring drug-related side-effects is crucial to the management of patients. In our study, side-effects

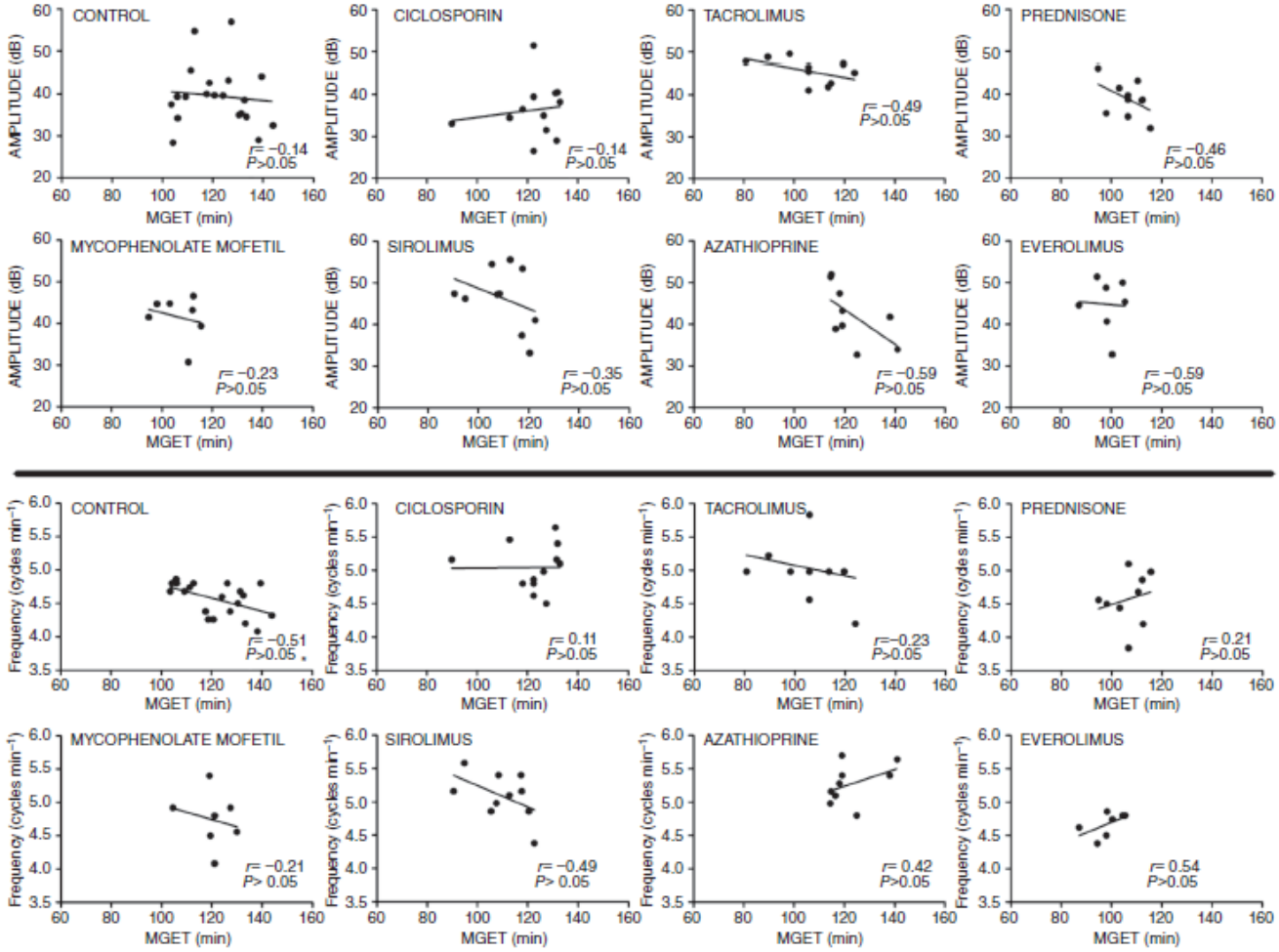

Figure 3. Pearson correlation scatter plots showing a comparison between gastric emptying and amplitude of gastric contraction (top panels) and also between the frequency of gastric contraction and gastric emptying (bottom panels) for all groups treated

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of immunosuppressive monotherapy on GI motility and contractility parameters were evaluated in rodents. Animals treated with TAC had an accelerated gastric emptying, whereas intestinal transit time was prolonged compared with control rats (Table 2; Dall'Agnol *et al.* 2014). Tacrolimus also increased the frequency and amplitude of gastric contractions. Tacrolimus is a macrolide immunosuppressant, which has a stimulatory effect on gastric emptying attributable to possible interactions with motilin receptors (Maes *et al.* 1999; Van Vlem *et al.* 2002; Lim *et al.* 2012; Teixeira *et al.* 2012). When administered in association, the prokinetic effect of TAC is able to overcome the inhibitory effects of CSA and may be beneficial in improving gastric motor function (Henry, 1999; Maes *et al.* 1999; Teixeira *et al.* 2015). Teixeira *et al.* (2015) demonstrated in humans that TAC in triple immunosuppressive therapy (TAC + AZA + PRED) was able to accelerate gastric emptying and caecum arrival compared with control and CSA + AZA + PRED treatment.

Studies focusing on the influence of sirolimus and everolimus on GI motility are scanty (Itoh *et al.* 1984). Everolimus and SRL are structural analogues of erythromycin (Maes *et al.* 2003), which interrupt the interdigestive migrating motor complex in progress and induce a burst of contractions propagating from the stomach to the ileum. This effect mimics the phase III of the migrating motor complex, through activation of motilin receptors (Itoh *et al.* 1984; Otterson & Sarna, 1990; Annese *et al.* 1992). In our study, SRL hastened gastric emptying and also increased contractility, i.e. the frequency and amplitude of contractions. Sirolimus is a macrolide extensively used in transplant clinics; however, severe diarrhoea has been related as the main adverse effect (Kahan, 2008). Sirolimus was not approved in treatments after liver transplantation by the US Food and Drug Administration because of the high incidence of hepatic artery thrombosis, graft loss and death (Ganschow *et al.* 2014).

Everolimus also accelerated gastric emptying and prolonged intestinal transit time compared with control animals (Table 2). Maes *et al.* (2003) demonstrated that gastric emptying in patients treated with EVR was faster compared with control subjects and similar to TAC treatment. Everolimus increased the amplitude of contraction, but not the frequency (Table 3). Everolimus was developed to improve the pharmacokinetic profile of sirolimus (Ganschow *et al.* 2014) and has been associated with ciclosporin to avoid acute rejection (Salvadori & Bertoni, 2011). In a randomized and double-blind study, EVR caused a greater incidence of diarrhoea than MMF treatment (Vítko *et al.* 2005).

After CSA treatment, intestinal transit and caecum arrival were delayed, although gastric emptying was similar to control values (Table 2). Systematic review indicates that patients receiving CSA have more episodes of constipation compared with TAC treatment (Webster *et al.* 2005). In contrast, CSA increases the frequency of gastric contraction, which can be associated with symptoms such as nausea (Koch, 2011).

At dosage regimens of 20 mg kg^{-1} day^{-1}, rats treated with MMF showed no significant effect on upper GI motility, corroborating the study of Maes *et al.* (2003). Gastrointestinal effects already reported were associated with impaired colonic motility (Teixeira *et al.* 2012) and other factors, such as hormones and reduction of villi, can be associated. However, such correlations were not evaluated in the present study.

Azathioprine is one of the most commonly used immunosuppressive drugs for preventing graft rejection and autoimmune disease (Estakhri *et al.* 2012). Azathioprine is generally well tolerated by the GI tract and, in our study, it increased the frequency of contraction. This drug could trigger a gastric dysrhythmia, which can be associated with unexplained nausea and vomiting in patients (Yin & Chen, 2013).

Despite the knowledge about anti-inflammatory/immunosuppressive effects of corticosteroids, very little is known about their effects on GI motility (Henry, 1999). Surprisingly, treatment with PRED hastened gastric emptying. Steroid therapy by itself has many side-effects and may worsen the side-effects of TAC (Anil Kumar *et al.* 2008). Our findings regarding prednisone treatment, which is present in the majority of post-transplant therapeutic schemes, emphasizes the importance of further studies focusing on this long-established drug.

In the control group, there was a negative correlation between MGET and the frequency of contraction, i.e. a slowed gastric emptying could be associated with a low frequency of contraction (Fig. 1). However, after treatment there was no significant correlation between MGET and contractility. Patients with delayed gastric emptying showed a significantly lower percentage of normal gastric slow waves and significantly reduced amplitude in postprandial electrogastrography (Chen *et al.* 1996; Cucchiara *et al.* 1997). However, monotherapy at the doses used in our study was not responsible for gastroparesis, a common complication after organ transplantation (Filichia & Cendan, 2008).

Studies focused on monotherapy are able to optimize the efficacy and limit the toxicity (Song *et al.* 2011). Some reports have discussed the synergistic and nephrotoxic effect among drugs (Nielsen *et al.* 2003) and, in all cases, toxicity depends on the exposure time and dose. Doses mimicking therapeutic effects of immunosuppressive drugs have been proposed for a rat model (Nielsen *et al.* 2003; Böhmer *et al.* 2011; Malinowski *et al.* 2011; Bohra *et al.* 2012; Dall'Agnol *et al.* 2014), and they were within the therapeutic limit and were also

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able to induce immunosuppression without causing nephrotoxicity (Böhmer *et al.* 2011). Oral bioavailability of drugs in rats is markedly lower than in humans, ~12% in mice (O'Reilly *et al.* 2010). The dosages used in rats are higher than those administered in humans because of the different body area-to-volume ratio and faster hepatic metabolism (Malinowski *et al.* 2011). Therefore, clinically relevant doses of immunosuppressants were selected in our study based on previous data and metabolic differences between rodents and humans (Malinowski *et al.* 2011). In addition, the solubility of ionizable drugs or pH-responsive formulations is significantly influenced by the differences in pH along the GI tract and interspecies differences (Merchant *et al.* 2015).

Several drugs *per se* can modify GI motor activity, and it is possible that compromised transit could affect absorption of drugs. The ACB technique can be very helpful for evaluating the severity of GI motor abnormalities *in vivo*, because stimulatory effects on the isolated GI tract do not always produce propulsion of the luminal contents (Tsubouchi *et al.* 2003). Diagnosis of GI motility disorders in immunosuppressed patients is essential, because symptoms can be prevented and complications avoided (Ponticelli *et al.* 2010). Strategies to manage GI disorders without increasing the risk of graft loss or patient mortality are mandatory because these complications can lead to changes in immunosuppression in 25% of patients (Díaz *et al.* 2007; Herrero *et al.* 2007). However, in clinical settings, it is difficult to distinguish a direct effect of immunosuppressive drug treatment from several variables.

Conclusions

Our animal model was suitable for demonstrating that most immunosuppressive drugs currently in use impaired at least one GI motility parameter. As a non-invasive technique, ACB is a valuable tool to evaluate side-effects of drugs in the GI tract and help us to understand the symptoms to improve clinical management of patients.

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Additional information

Competing interests

None declared.

Author contributions

Conception or design of the work: D.J.R.D., L.A.C. and M.F.A. Acquisition, analysis, or interpretation of data for the work: D.J.R.D., M.C.B.T., M.B.L., L.A.G., J.R.A.M. and M.F.A. Drafting the work or revising it critically for important intellectual content: D.J.R.D., L.A.C., M.C.B.T., M.B.L., L.A.G., J.R.A.M. and M.F.A. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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3 CAPÍTULO 2

Calcineurin inhibitors, antimetabolites and glucocorticoids: effects of immunotherapy in the gastric structure and function evaluated in rats

Neurogastroenterology & Motility

CALCINEURIN INHIBITORS, ANTIMETABOLITES AND GLUCOCORTICOIDS: EFFECTS OF IMMUNOTHERAPY IN THE GASTRIC STRUCTURE AND FUNCTION EVALUATED IN RATS

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Running Title: Immunosuppressive effects on the GI tract

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Abstract

Background: Triple immunosuppressive therapy is associated with several gastrointestinal disorders, such as vomiting, gastric discomfort, constipation or diarrhea. The aim of this study was to investigate the effects induced on the gastrointestinal tract of rats by triple immunosuppressive therapy. **Methods:** Male Wistar rats were randomly assigned into three experimental groups: Control: filtered water; TAC+MPS+PRED: treated with Tacrolimus plus Mycophenolate Sodium plus Prednisone; and CSA+AZA+PRED: treated with Cyclosporine plus Azathioprine plus Prednisone. The treatment was done during 14 days by gavage. Gastric emptying and contractility were evaluated by the Alternating Current Biosusceptometry (ACB) and Electrogastrography (EGG). Histological analysis, biochemistry and hematology were also performed. **Key Results**: Gastric emptying time was slower in the group treated with CSA + AZA + PRED in comparison with control group ($p<0.01$). Animals treated with TAC + MPS + PRED showed accelerated gastric emptying ($p<0.05$) compared with control. The amplitude of gastric contractions in both treated groups was higher than in the control. The frequency of gastric contractions in the CSA+AZA+PRED group was also increased ($p<0.01$). Results obtained by EGG are similar to those recorded with the ACB. The circular layer from stomach muscle had his thickness decreased in both treated groups. Longitudinal layer was only reduced by CSA+AZA+PRED treatment. **Conclusions:** Triple immunosuppressive therapy alters stomach motility and morphology in rats. Specific gastrointestinal side effects resulting from different immunosuppressive therapies still need to be elucidating in order to provide more effective and personalized therapy, to prevent and also to avoid complications in the management of patients.

Key-words: immunosuppressive drugs; Alternating Current Biosusceptometry (ACB); Electrogastrography; gastrointestinal motility; gastric emptying;

Key Points

- Triple immunosuppressive therapy in rats impaired at least one parameter of gastric motility and modified the morphological structure of the stomach. Our data also pointed out that the combined therapy triggered more intense consequences in several parameters simultaneously, especially regarding the motor activity aspects.
- Animal studies focusing on clinically relevant immunosuppressive doses are essential to understand and to avoid gastrointestinal complications, especially considering their association in clinical settings.
- ACB and EGG are efficient noninvasive techniques to measure parameters of gastrointestinal motility in an experimental model for drug evaluation.

1 Introduction

Solid organ transplantation provides a short-term impact in the recovery of health and also in the quality of life of patients with chronic diseases that would, otherwise be incurable (1); however, it requires long-term use of immunosuppressive drugs. Maintenance immunosuppressive therapy is usually achieved by combining two or more different classes of drugs to maximize the effectiveness of the regimen, since each drug has as a target specific component of the immune response (2). Worldwide, immunosuppressive regimens generally include a calcineurin inhibitor (CNI) and an adjuvant agent, with or without corticosteroids (3,4).

Despite successful transplantation, a large part of non-allograft complications includes gastrointestinal (GI) disturbances (5) which are related with considerable morbidity and mortality rates (6). Nausea, vomiting, abdominal pain, gastric discomfort, constipation and especially diarrhea are often described as adverse effects of immunosuppressive drugs (7,8,9). These symptoms are associated to the daily therapy (10) and can impact negatively on transplant outcome. Besides, depending on the severity of such symptoms, either dose reduction or discontinuation are required which significantly risk of graft loss (11).

As side-effects of immunosuppressive therapy are common, several clinical trials have been proposed in order to investigate the impact of therapy on GI tract. However, human studies may be limited not only for ethical reasons but also due to many uncontrollable variables, such as diet, nutrition, genetic conditions, socioeconomic and absence of suitable techniques as well (12). Experimental models play an important role to elucidate the side effects of drugs on the GI tract. According to experimental apparatus and study design, animal models allow correlating such effects with changes in biochemical and histological parameters. Although side effects of immunosuppressive regimens on GI tract are known, there are no consensus concerning how these effects occur and if motility parameters are affected. A plethora of techniques have been developed in order to investigate the GI tract (13, 14, 15 16); however, radiation exposure, costs and invasiveness are their main limitations. Alternating Current Biosusceptometry (ACB) is a low cost, radiation-free and easy handling biomagnetic technique (15) which allows to perform several noninvasive measurements of gastrointestinal motility in the same animal at different protocols, favoring *in vivo* experimentation (17, 18, 19). ACB can also be used in humans with safety (20, 21) providing results that are closer to the physiological conditions since it allows recording gastrointestinal motility in real time (22).

In a recent study, the assessment of each immunosuppressive drug individually administered to rats indicated that GI motility were altered (23); however, this parameter were not evaluated after triple drug therapy, a common approach used in clinical practice. The selection of an appropriate and effective immunosuppressive regimen should take into account the side effects on the GI tract. As these effects are still unclear or neglected by physicians and the studies focused on the GI motility are scanty, our aim was to investigate the effects induced on the GI tract of rats after triple immunosuppressive therapy.

2 Materials and methods

2.1 Ethical approval

The experimental procedures were approved by the Ethics Committee on Animal Research from Federal University of Mato Grosso/Brazil (protocol number 23108.049862/13-3) and followed the guidelines from the Brazilian College of Animal Experimentation animal welfare committee.

2.2 Animals and experimental groups

Twenty-three healthy male Wistar rats initially weighing 250 – 320g were housed in controlled conditions of temperature (22±3°C), humidity (60±5%) and 12-hour light/dark cycle with access to filtered water and commercial chow (Purina®) *ad libitum*. The consumption of chow was controlled only in the night before the motility measurements.

Initially, the animals were conditioned in individual cages during 7 days for adaptation and vermifugation, after starting the experimental procedures. Experimental protocol includes sterilization in a UV camera for 15 min of all material in contact with animals such as cage, chow, water and others were, this procedure had as objective the prevention of opportunistic infections coming from the immunosuppression.

The animals were randomly assigned into three experimental groups: Control group (n=9): rats received only vehicle; TAC+MPS+PRED (n=7): rats received Tacrolimus (TAC) *plus* Mycophenolate Sodium (MPS) *plus* Prednisone (PRED); CSA+AZA+PRED (n=7): animals were treated with Cyclosporine (CSA) *plus* Azathioprine (AZA) *plus* Prednisone (PRED) (Table 1).

All the drugs were diluted in filtered water and were stored in the refrigerator at 8°C. Vehicle was composed only by 2.5 ml filtered water. Each animal was treated for 14 days by

gavage with dosages ranging from 1 to 20 mg kg^{-1} day^{-1} considering the area-to-volume ratio and hepatic metabolism of each drug (23, 24, 25, 26) (Table 1).

2.3 Alternating Current Biosusceptometry and electrogastrography

Gastric emptying and gastric contractility were evaluated by the Alternating Current Biosusceptometry (ACB) technique (17, 18, 21). ACB sensor (Br4-Science®, São Paulo, Brazil) is a setup of two pairs of excitation and detection coils separated by a fixed distance. The sensor registers the difference in the magnetic flux between the pairs of coils caused by a magnetic tracer susceptible to the magnetic field induced. The intensity of the magnetic signal registered depends on the amount of magnetic material as well as the distance between the sensor and the magnetic sample (27). Technical details of the ACB have been previously described (17, 28, 29). Electrogastrography (EGG) was employed to record the electrical activity generated by the gastric muscle through cutaneous electrodes (30, 31).

2.4 Measurement of gastric emptying, contractility and gastric electrical activity

In the 14th day, after fasting overnight, animals were fed with 1.60 g commercial chow for rats blended with 0.40g magnetic tracer (ferrite powder, $MgZnFe_2O_3$; $90 \leq Ø \leq 125$ µm; Imag, Ribeirão Pires/São Paulo, Brazil). Ferrite powder is inert, non-toxic and not absorbed by the GI tract (32). Ten minutes after spontaneous ingestion of the meal described above, gastric emptying measurements were started. The animals were gently handled by the neck and the region corresponding to the stomach was delimited. Afterwards, ACB sensor was place on the abdominal surface every 15 min. for at least 4 hours towards recording the magnetic signal intensity (Figure1). Measures of gastric emptying measurements were taken without any sedation or analgesics.

All magnetic signals from the gastric emptying records were mathematically extrapolated to 6 hours and analyzed in a numerical matrix form in Origin®. Statistical moments (33) were used to calculate the Mean Gastric Emptying Time (MGET) as the time t (min) it takes for a mean amount of magnetic material to leave the stomach.

Measurements of the gastric contractility were performed immediately after the gastric emptying recordings. Rats were anesthetized with 75 mg/kg ketamine (Cetamin®, Syntec, Brazil) plus 2.5 mg/kg acepromazine (Acepran® Vetnil, Brazil) administered intraperitoneally. Animals were then laid in supine and the ACB sensor was placed on the anterior surface of the abdomen. For electrogastrography (EGG), the bipolar configuration was applied using the surface electrodes – near to ACB sensor in the abdomen – connected to a BIOPAC system

(EGG100C) (Figure 1). Simultaneous signals (ACB and EGG) were acquired during 30 minutes at a 20 Hz/channel rate digitized using a multi-channel recorder (MP100 System; BIOPAC Inc., Santa Barbara, CA, USA) and stored for further analyzes.

At the end of the GI motility measurements, the animals were killed by anesthetic overdose with 240 mg/kg ketamine plus 45 mg/kg xylazine chlorhydrate solution (Xilazin®, Syntec, Brazil) administered intraperitoneally. When cardiac arrest was diagnosed, they were decapitated for collection of blood and tissues samples. Blood was centrifuged at 3500 rpm, and plasma was stored at -80ºC.

To quantify the gastric contractility parameters, all raw magnetic signals were initially analyzed in MatLab (Mathworks, Inc., Natick, MA, USA) by visual inspection and applying bi-directional Butterworth bandpass filters with a cut-off frequency of 0.02–0.15 Hz (1.2-9.0 cpm – cycles per minute). The highest frequency peak for each FFT was determined as the gastric dominant frequency, and the smallest was identified as signal noise. Frequencies were expressed in Hertz (Hz) and then converted to cycles per minute (cpm). Amplitude of contraction (A) was calculated as the relationship between power of gastric peak (P) and power of noise peak (P'), expressed in decibel (dB), as follows: $A = 10 \log10(P/P')$ (34, 23).

2.5 Biochemical analysis

The biochemical indicators were studied to evaluate renal and hepatic function of rats submitted to treatment with triple immunosuppressive therapy. On the 12^{th} day, animals were housed individually in metabolic cages and for 24-h water and food intake was quantified. The 24-hour urine volume was also measured and stored at -80ºC.

Biochemical assays were carried out in order to quantify plasma creatinine, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and urine creatinine. Creatinine clearance (CrCl) was expressed in ml min^{-1} and was calculated as follows: (U/ S) x VM, where the clearance was equal to the V = concentration of the urine creatinine (mg dL^{-1}) divided by the S = concentration of the plasma creatinine (mg dL^{-1}) and multiplied by the VM = urinary volume per minute (ml) according to the manufacturer's specifications.

All the analyses were performed by means of semi-automatic biochemical analyzer BIO-2000 (Bioplus Products Laboratories Ltda, Barueri/SP, Brazil) and colorimetric kits (In Vitro Diagnostic Ltda, Itabira/MG Brazil).

2.5 Blood Cell Count

Blood samples were collected in EDTA-coated tube and diluted (1:20) in Turk's solution (Acetic Acid 3% - Contemporary Chemistry Dynamics LTDA, Diadema/SP). In a Neubauer camera using an optical microscope, the total leukocytes blood cell was counted. For differential counting of leukocytes, the blood smear technique was used, the blades were stained using the fast-Panoptic kit (Laborclin® Ltda, Pinhais, Paraná, Brazil). The smears were subjected to light microscopy (100x), counting 100 cells per blade. Cell populations were differentially counted based on the morphological features and the results are presented in absolute values ($10^3/mm^3$) (35).

2.6 Histological analysis

Gastric samples from antrum were fixed at Metacarn (60% methanol 30% chloroform, and 10% Glacial Acetic Acid), dehydrated in the alcohol series, diaphanized in xylol and included in paraffin. The sections were obtained with a thickness of 4.0 micrometers employing a microtome (Microm HM 355S AutomaticMicrotomes-ThermoScientific). Hematoxylin and eosin (H&E) staining was used in analysis of thickness of circular and longitudinal muscle layers (36). Images were captured on an optical microscope (Zeiss, Germany) coupled to a high-resolution camera (AxioCam ERc5s – Zeiss, Germany) and analyzed using the ZEN Blue Software 2011 (Zeiss, Germany). Values are expressed in micrometers.

2.7 Statistical analysis

Normality of continuous variables was evaluated using the Kolmogorov–Smirnov test. When normally distributed, data were evaluated using One-Way ANOVA followed by Dunnett's post hoc test. The Pearson correlation coefficient (r) was used to analyses the relationship between variables and techniques. Only coefficients above 0.70 were considered significant. Differences were considered significant at a P value<0.05 and all results are expressed as mean ± standard deviation (SD).

3 Results

The treatment with TAC+MPS+PRED and CSA+AZA+PRED reduced the total leukocytes number and differential count compared with control group, confirming the immunosuppression in both treated groups, as expected (Table 2). Serum creatinine was considerably increased in the group treated with CSA+AZA+PRED ($p<0.01$) compared with

control, whereas clearance was reduced in both treated groups ($p<0.05$). Aspartate aminotransferase was decreased for both treated groups compared with control ($p<0.05$ and $p<0.01$ respectively) (Table 2).

Animals treated with triple immunosuppressive drugs had at least one gastric motility parameter altered. Gastric emptying time was slower in the group treated with CSA + AZA + PRED (131.0 ± 3.7 min.) in comparison with control group (123.0 ± 4.0 min.) ($p<0.01$). Contrariwise, animals treated with TAC + MPS + PRED (117.5 ± 3.3 min.) had gastric emptying accelerated compared with control group ($p<0.05$) (Figure 2). The amplitude of gastric contractions registered in the both treated groups (54.9 ± 3.3 dB for TAC+MPS+PRED and 50.4 ± 3.4 dB for CSA+AZA+PRED) was higher than the amplitude registered in the control group (43.0 ± 4.1) ($p<0.01$). The frequency of gastric contractions for animals treated with CSA + AZA + PRED (5.3 ± 0.3 cpm) was also higher in comparison with control (4.7 ± 0.2 cpm) ($p<0.01$) The frequency (Control = 4.8 ± 0.2 cpm; TAC+MPS+PRED = 5.0 ± 0.15 cpm; CSA+AZA+PRED = 5.2 ± 0.4 cpm) and amplitude (Control = 45.0 ± 3.8 dB; TAC+MPS+PRED = 52.7 ± 3.2 dB; CSA+AZA+PRED = 51.4 ± 3.8 cpm) results obtained by EGG are similar to those recorded with the ACB (Figure 2).

A negative correlation ($R = -0.71$) between gastric emptying and frequency of gastric contractions recorded by ACB was observed only in the control group. After treatments, there was a total absence of correlation (Figure 3).

There was a strong positive correlation between EGG and ACB recordings regarding frequency in the control and CSA + AZA + PRED groups ($R= 0.77$ and $R=0.89$, respectively). In the group treated with TAC + MPS + PRED there was no frequencies values relationship between techniques (Figure 4).

The circular layer from stomach muscle had his thickness decreased in both treated groups (168.7±8.8 µm for TAC+MPS+PRED and 104.7±5.9 µm for CSA+AZA+PRED compared with control 248.6±8.8 µm). Longitudinal layer was only reduced by CSA+AZA+PRED (32.0 ± 5.5 µm) treatment compared with control (46.0 ± 2.9 µm) (Figure 5).

4 Discussion

Our study showed that the triple immunosuppressive therapy disturbs both function and the structure of the GI tract. Our data also pointed out that the combined therapy triggered more intense consequences in several parameters simultaneously, especially regarding the motor

activity aspects. In our previous study (23), have already shown that immunosuppressive drugs administered individually affects several parameters concerning GI motility in rats. They also showed that only mycophenolate mofetil at a dose of 20 mg/kg/day did not was able to disturb gastric emptying or contractility in rats.

In the present study, gastric emptying time was faster for animal treated with TAC+MPS+PRED than for the control group. Similar results were found by Teixeira et al. (21) in a study that evaluated gastrointestinal transit parameters in renal transplant patients treated with a triple immunotherapy with TAC + AZA + PRED. Rats in monotherapy with TAC at a dose of 3 mg/kg/day had increased contractility in comparison with controls and as a consequence, gastric emptying was accelerated (23). Tacrolimus is a macrolide, which stimulates gastric emptying via motilin receptors in human stomach; however, in rats motilin and its receptors are non-existent or are non-functional (37, 38). On the other hand, Bielefeldtl et al. (39) reports that tacrolimus increases intracellular calcium concentration in intestinal smooth muscle cells and, secondarily alter intestinal contractility *in vitro*. Although the mechanisms underlying the effects of immunosuppressive drugs on GI motility are still unclear, the evidences point out for involvement of gut receptors and mucosa immune system (40).

In the group treated with CSA + AZA + PRED the gastric emptying time was slower in comparison with control group. This finding corroborates previous studies performed in renal transplant patients and healthy volunteers (41, 42, 21). Transplanted patients taking Cyclosporine show a greater tendency to develop gastroparesis compared to patients using tacrolimus (43, 44). Webster et al. (45) reported that patients who received CSA also had more episodes of constipation compared with TAC. Ekberg et al. (7) demonstrated that patients taking azathioprine had a reduced risk of suffering from diarrhea, indigestion, and GI symptoms in general.

Gastric emptying is the most important GI motor parameter in clinical practice, which is carefully regulated by neurohumoral mechanisms and metabolic load (46). Several diseases, medications, medicinal plants, biological factors, among others, can impair the gastric emptying, resulting in decreased drug absorption and bioavailability failures (47).

Our data also shown that muscle thickness from gastric antrum measured in the animals treated with TAC + MPS + PRED and CSA + AZA + PRED were decrease in the circular muscular layer which is responsible for moving the content along the GI tract (48, 49). The thickness of longitudinal muscle layer was also reduced in CSA + AZA + PRED group. Decreases in the both muscular layers thickness can affect the propulsion of the contents and compromise gastric emptying. As a consequence, even the relatively high gastric frequency and

amplitude of contractions not reflected necessarily in faster gastric emptying, as it was observed for the group treated with CSA + AZA + PRED. On the other hand, in the group treated with TAC + MPS + PRED it was observed a hypermotility, reduction only in the circular layer and accelerated gastric emptying compared with control. This finding can be partially explained by the addiction effects resulting from drugs combination and their mechanisms of action.

A negative correlation between gastric emptying and frequency of contractions was found for the control group. However, after the treatment with the triple regimen there was found a complete absence of correlation. In our previous study (23), showed the same results in animals treated with immunosuppressive monotherapy, suggesting that those drugs affect the synchronization of gastrointestinal motor functions.

In our study there was also a strong positive correlation between frequency of electrical activity measured by EGG and frequency of mechanical activity by the ACB in the control group and treated with CSA + AZA + PRED, demonstrating that both increased or decreased together. There is a one-to-one correlation between gastric contractions and slow waves under normal conditions which disappear during gastric dysrhythmia (50). In the group treated with TAC + MPS + PRED an absence of correlation between techniques was observed, suggesting that this combination of immunosuppressants caused more damage to the synchronization of these GI parameters, that combination CSA + AZA + PRED.

The experimental model in rodents proposed in this present study was acceptable to evaluate the effects of triple immunosuppressive therapy. Since immunosuppression was evidenced by the reduced number of total leukocytes in both treated groups. This effect was more pronounced in the group treated with CSA + AZA + PRED, since AZA has a myelosuppressive action (4).

Renal function was evaluated because a relation between dose and nephrotoxicity has not been well established yet in healthy rats, though seems to generally dependent on the route of administration, dose and the time of use (51, 52). In our study, there was a decrease in creatinine clearance in the groups treated with CSA + AZA + PRED and TAC + MPS + PRED compared to the control group, indicating nephrotoxicity This effect can be attributed to calcineurin inhibitors and also to a possible synergistic effect among them. Nephrotoxicity occurs because cyclosporine provoked vasoconstriction of the renal afferent arterioles which decrease renal blood flow and glomerular filtration characterizing an acute effect (53). Tacrolimus has a nephrotoxic effect similar to cyclosporine; however, these effects are dose-dependent and reversible with dose reduction or discontinuation of therapy, whereas chronic nephrotoxicity is linked to structural alterations that are not reversible (54).

The serum ALT activity was low (ranging 20-30 U L^{-1}) in both treated groups, which indicate that no clinically significant hepatoxicity was induced. However, there was a decrease in aspartate aminotransferase, this result was also found by Sereno et al. (55) in rats treated with 5 mg/kg/day CSA for 6 weeks, but new histopathological studies are needed to thoroughly investigate this change.

Alterations in the GI motility resulting from triple immunosuppressive therapy can be worsened by the drug interaction. Corticosteroids for example can contribute to the increase of adverse effects, since even low doses can modify GI motility, immune response and morphological structure in the intestine of rats (35). Gastric emptying evaluation is very important because can be associated with inadequate absorption of several drugs, including immunosuppressants, which provokes low bioavailability of the drug and rejection of the transplanted organ. However, new clinical trials are required to fully understand the gastrointestinal complications in patients treated with triple immunosuppressive drugs, considering our data and that immunosuppressive drugs compromise gastric motility when used individually. There are still gaps regarding side effects that still need to be elucidating in order to provide more effective and personalized therapy, to prevent and also to avoid complications in the management of these patients.

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Authorship

DJRD: performed the research; designed the research study; analyzed the data and wrote the paper. LAC: designed the research study; contributed with essential reagents and revised the paper. LAG: performed the research and wrote the paper. RSC: performed the research and analyzed the data. JRAM: contributed with essential reagents or tools and revised the paper. MFA: designed the research study; analyzed the data and wrote the paper.

No competing interests declared

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TABLES

Table 1 - Therapeutic doses administered to the animals during 14 consecutive days.

DRUG	**GROUP 1**	**GROUP 2**	**SOURCE**
Tacrolimus (TAC)	1.0 mg Kg^{-1} day	-	PROGRAF®, Janssen-Cilag, São Paulo, Brazil
Cyclosporine (CSA)	-	10.0 mg Kg^{-1} day	SANDIMMUN NEORAL®, Novartis Pharma Stein AG, Stein, Switzerl
Mycophenolate Sodium (MPS)	20.0 mg Kg^{-1} day	-	Myfortic®, Novartis Pharma Stein AG, Stein, Switzerl
Azathioprine (AZA)	-	3.0 mg Kg^{-1} day	IMURAN®, Aspen Pharma LTDA, Espirito Santo, Brazil
Prednisone (PRED)	1.0 mg Kg^{-1} day	1.0 mg Kg^{-1} day	METICORTEN®, Wyeth, São Paulo, Brazil

Table 2- Biochemical and Hematologic data after 14 days after vehicle (control) or treatment with triple immunosuppressive therapy

	CONTROL	TAC+MPS+PRED	CSA+AZA+PRED
Biochemical data			
ALT (U L^{-1})	22.2 ± 4.4	21.7 ± 3.2	23.5 ± 3.7
AST (U L^{-1})	111.2 ± 13.8	85.3 ± 9.1**	93.9 ± 11.2*
Creatinine (mg dl^{-1})	0.54 ± 0.09	0.49 ± 0.04	0.67 ± 0.08**
Creatinine Clearance (ml min^{-1})	0.88 ± 0.45	0.51 ± 0.03*	0.53 ± 0.16*
Blood cells data			
Total leukocytes ($10^3/mm^3$)	4.71 ± 0.85	3.57 ± 0.74*****	1.97 ± 0.85**
Neutrophils ($10^3/mm^3$)	1.56± 0.27	1.17 ± 0.36*	0.78 ± 0.23**
Eosinophils ($10^3/mm^3$)	0.03 ± 0.03	0.06 ± 0.01	0.03 ± 0.02
Monocytes ($10^3/mm^3$)	0.23 ± 0.11	0.17 ± 0.04	0.06 ± 0.01**
Lymphocytes ($10^3/mm^3$)	2.89 ± 0.68	2.17 ± 0.63*	1.10 ± 0.40**

Data are expressed as means ± SD. Analysis was by ANOVA with a Dunnett's post hoc test. Cell counts expressed in $10^3/mm^3$;

* $p < 0.05$ versus control and ** $p<0.01$ versus control.

FIGURE LEGENDS

Figure 1- Diagram showing the positioning of the ACB single-sensor (open circles) on the rat abdominal surface. **A** corresponds to gastric emptying and **B** corresponds to gastrointestinal contractility. The numbers 1, 2 and 3 correspond to the sites insertion of the electrodes for signal acquisition through the EGG.

Figure 2 - Gastric emptying (**A**) frequency and amplitude gastric obtained by ACB (**B**) frequency and amplitude gastric obtained by EGG (**C**) obtained for all groups. Abbreviations: MGET - mean gastric emptying time; cpm - cycles per minute; dB – decibels. TAC: Tacrolimus; MPS: Mycophenolate Sodium; PRED: prednisone; CSA: Cyclosporine; AZA: Azathioprine.
Data are expressed as means ± SD. Analysis was by ANOVA with a Dunnett's post hoc test.
* $p < 0.05$ compared with control and ** $p < 0.01$ compared with control.

Figure 3 - Pearson correlation scatter plots showing the comparison between gastric emptying and amplitude of gastric contraction (**A**) and the comparison between the frequency of gastric contraction and gastric emptying (**B**) for all groups evaluated. Pearson correlation coefficient (r) and * $p < 0.05$ compared with control.
Abbreviations: TAC: Tacrolimus; MPS: Mycophenolate Sodium; PRED: prednisone; CSA: Cyclosporine; AZA: Azathioprine.

Figure 4 - Pearson correlation scatter plots showing the comparison between Frequency obtained by ACB and EGG for all groups evaluated.
Pearson correlation coefficient (r) and * $p < 0.05$ compared with control.
Abbreviations: TAC: Tacrolimus; MPS: Mycophenolate Sodium; PRED: prednisone; CSA: Cyclosporine; AZA: Azathioprine.

Figure 5 - Morphometry of gastric muscle of rats control (**A**), treated with TAC+MPS+PRED (**B**) and CSA+AZA+PRED (**C**). The representative photomicrographs with H&E illustrates the graphs. Bar = 50 μm
Data are expressed as means ± SD. Analysis was by ANOVA with a Dunnett's post hoc test and* $p < 0.01$ compared with control. **Abbreviations**: CM **–** muscle circular; LM **–** muscle longitudinal**.**

FIGURES

Figure 1

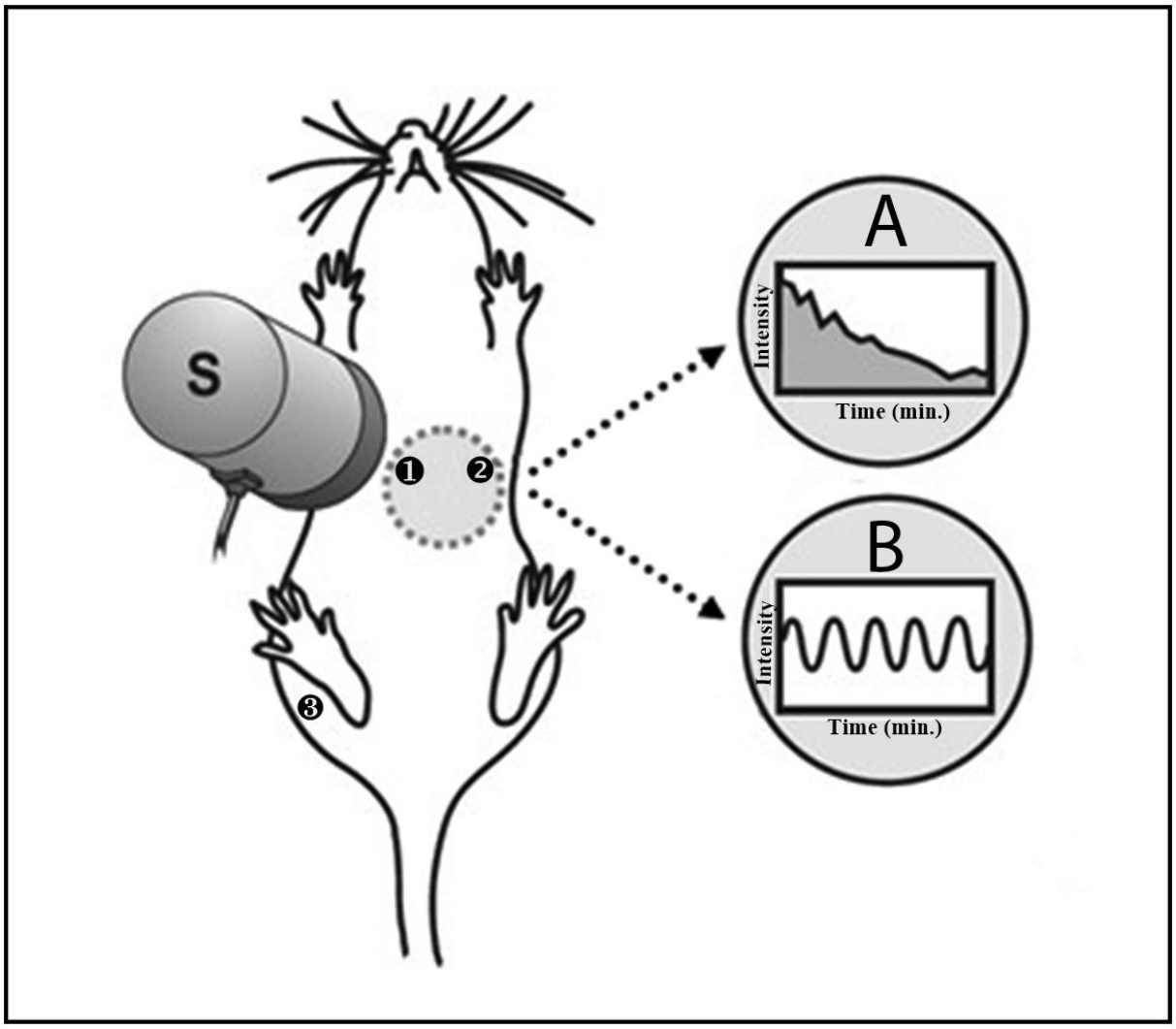
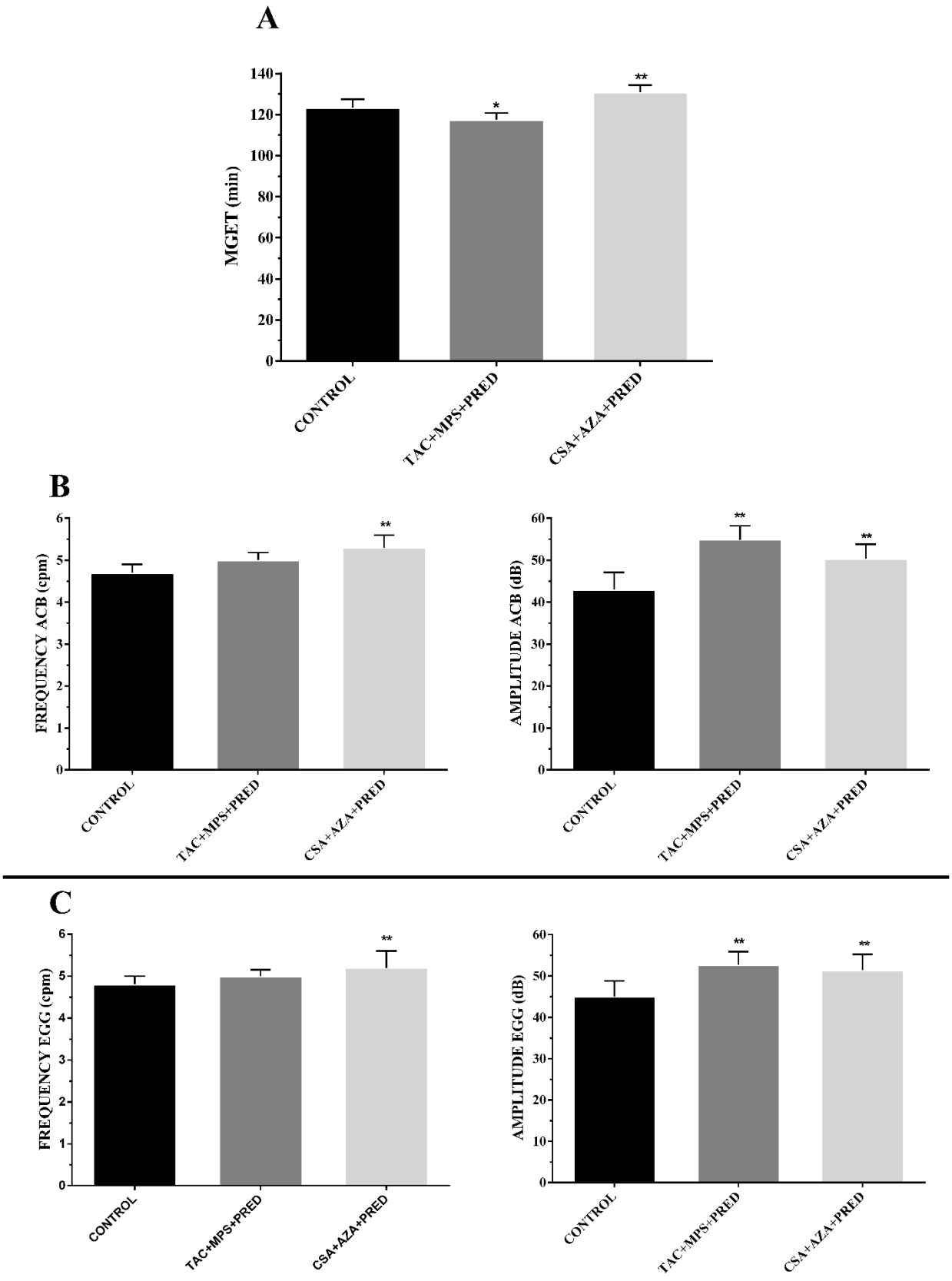


Figure 2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100
120
140
*
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CONTROL
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CSA+AZA+PRED
B
FREQUENCY ACB (cpm)
0
1
2
3
4
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CONTROL
TAC+MPS+PRED
CSA+AZA+PRED
AMPLITUDE ACB (dB)
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TAC+MPS+PRED
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CONTROL
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AMPLITUDE EGG (dB)
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CONTROL
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Figure 3

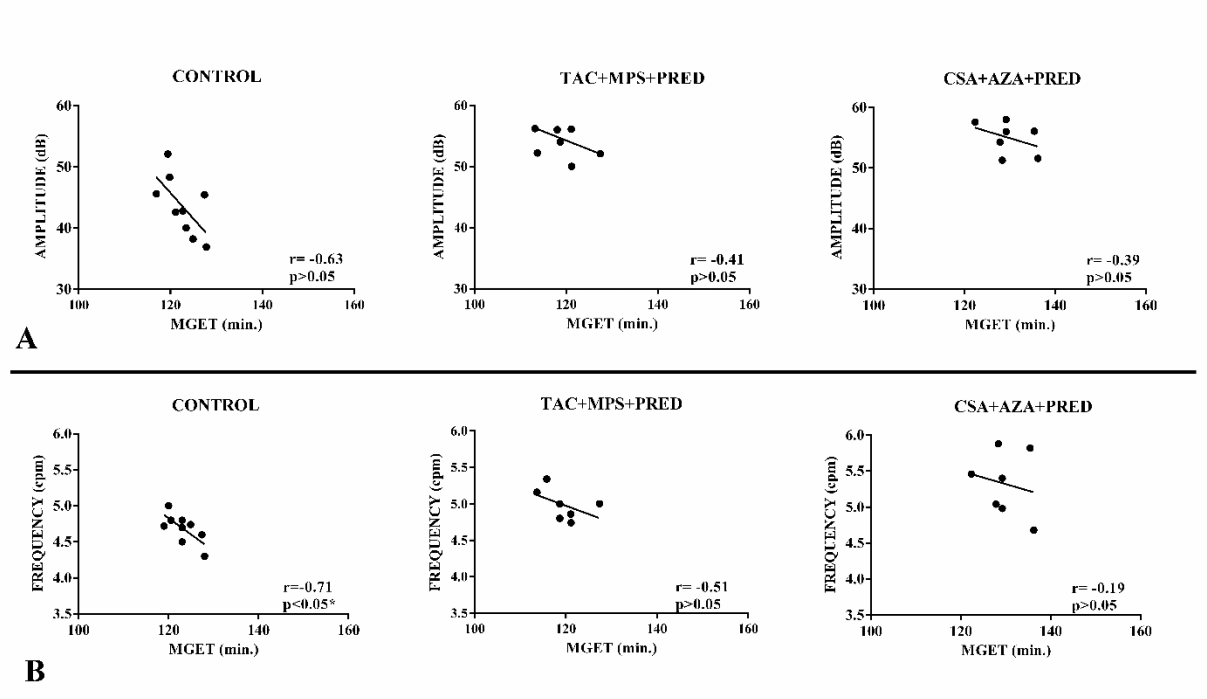


Figure 4

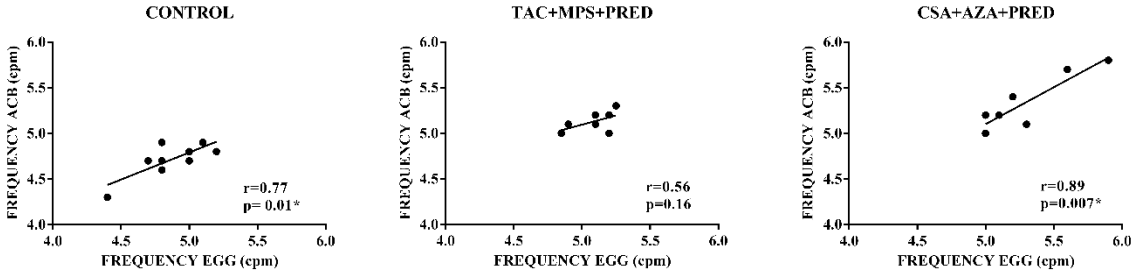
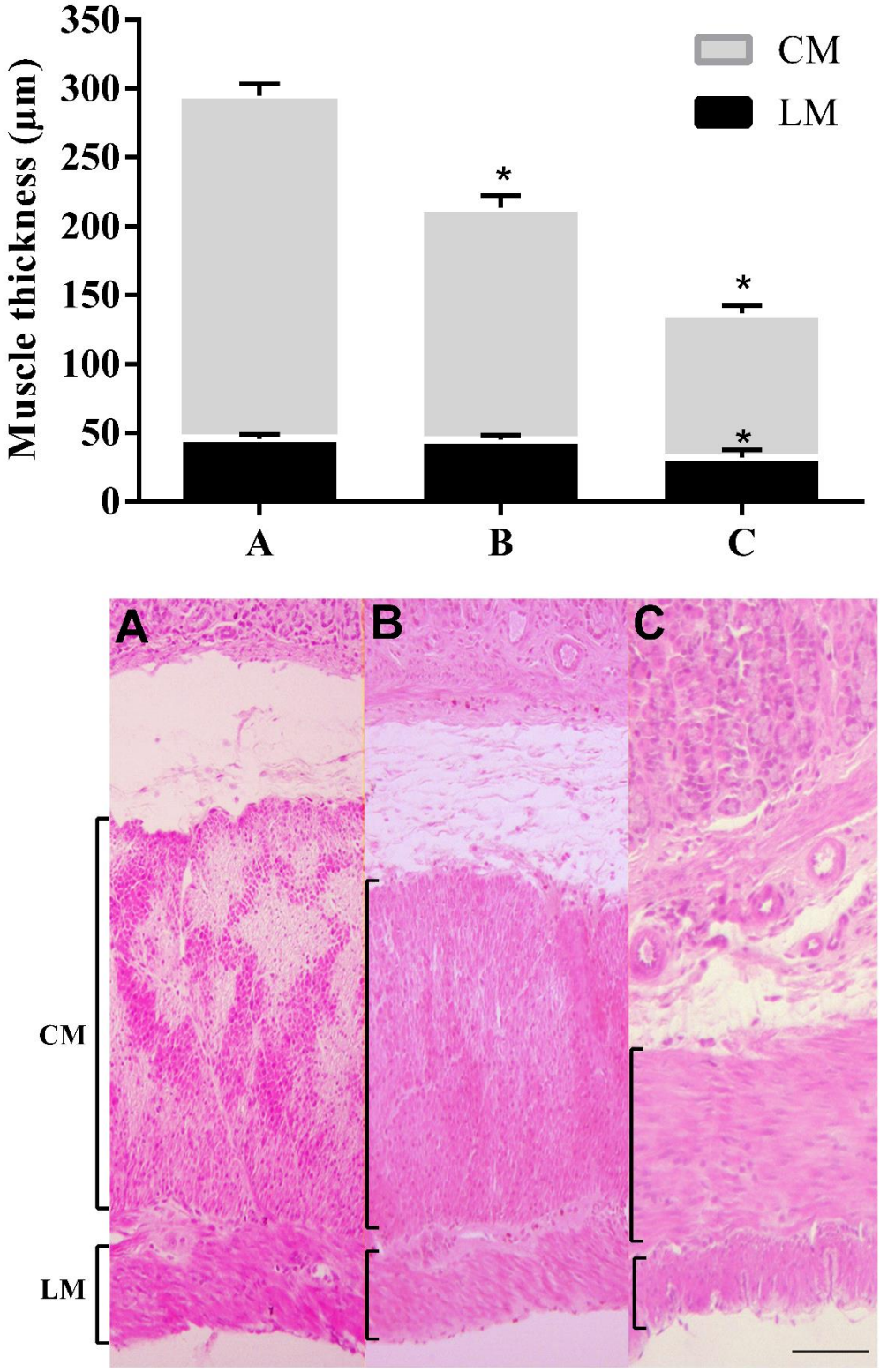


Figure 5


Muscle thickness (µm)
350
300
250
200
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100
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A
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A
B
C
CM
LM

4 CONCLUSÃO

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Neste trabalho foi possível avaliar os efeitos dos imunossupressores sob a motilidade gastrintestinal de ratos e demonstrar quais parâmetros foram afetados por estes fármacos.

Quando avaliados em monoterapia, todos os imunossupressores modificaram ao menos um parâmetro da motilidade gástrica, exceto o MMF. Isso demonstra que estes fármacos causam alterações significativas nos padrões motores do estômago, além de induzirem alterações na sincronicidade entre esvaziamento e frequência das contrações gástricas.

Quando avaliados em combinações triplas, os imunossupressores alteraram ao menos um parâmetro de motilidade gástrica e também a estrutura morfológica do estômago dos animais. Além disso, a terapia combinada desencadeou consequências mais intensas em vários parâmetros ao mesmo tempo, especialmente em relação aos aspectos da atividade motora.

A Biosusceptometria de Corrente Alternada e a Eletrogastrografia são técnicas não invasivas e eficientes para determinar a contratilidade gástrica, e no que concerne a BAC também capaz de quantificar o esvaziamento gástrico e o trânsito intestinal, em um modelo experimental de imunossupressão.

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ANEXOS

Anexo A - Certificado de aprovação no Comitê de ética em pesquisa

UNIVERSIDADE FEDERAL DE MATO GROSSO
COMITÊ DE ÉTICA NO USO DE ANIMAIS

CEUA
COMITÊ DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o Protocolo Nº 23108.049862/13-3, sobre "Caracterização dos efeitos de imunossupressores sobre a motilidade gastrintestinal de ratos" sob a responsabilidade de **Profª Drª MADILEINE FRANCELY AMERICO & Col.**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), tendo sido aprovado pelo Comitê de Ética no Uso de Animais (CEUA)-UFMT em reunião ordinária de **13/03/2014**.

CERTIFICATE

We certify that the protocol Nº 23108.049862/13-3, entitled "Characterization of immunosuppressive effects on gastrointestinal motility of rats", is in agreement with the Ethical Principles for Animal Research established by the National Council for Control of Animal Experimentation (CONCEA). This project was approved by the institutional Committee for Ethics in the Use of Animals (Federal University of Mato Grosso – UFMT) on **Mar 13, 2014**.

Cuiabá-MT, 14 de março de 2014.

Prof. Dr. Roberto Vilela Veloso
Presidente

Profª Drª Nair Honda Kawashita
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