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MODULAÇÃO DA VIRULÊNCIA DE CANDIDA ALBICANS POR IMUNOSSUPRESSORES ANTI-REJEIÇÃO

DOUTORADO EM
ODONTOLOGIA
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CURITIBA
2011

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Tese apresentada ao Programa de Pós graduação em Odontologia, Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Doutor em Odontologia – Área de Concentração em Estomatologia.

Orientador: Prof. Dr. Edvaldo Antonio Ribeiro Rosa

CURITIBA, 2011

Posso ter defeitos, viver ansioso

E ficar irritado algumas vezes

Mas não esqueço que a minha vida

É a maior empresa do mundo,

E posso evitar que ela vá à falência.

Ser feliz é reconhecer que vale a pena viver

Apesar de todos os desafios,

Incompreensões e períodos de crise.

Ser feliz é deixar de ser vítima dos problemas

E tornar-se um autor da própria história.

É atravessar desertos fora de si,

Mas ser capaz de encontrar um oásis

No recôndito da sua alma.

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É ter segurança para receber uma crítica,

Mesmo que injusta.

Pedras no caminho?

Guardo todas, um dia vou construir um castelo..."

Fernando Pessoa

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos parciais para a obtenção do Título de **Doutor em Odontologia**, Área de Concentração em **Estomatologia**.

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Modulação da virulência de *Candida albicans* por imunossupressores anti-rejeição

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LISTA DE ABREVIATURAS

Sap: Aspartil-proteases secretoras

YNB: *Yeast Nitrogen Base*

BSA: Albumina bovina fração V

YPD: *Yeast Peptone Dextrose*

SIB: *Sap inducer broth*

AESap: Atividade específica de Sap

RESUMO

Introdução: Apesar do aumento no número de transplantes de órgãos sólidos e no aumento da sobrevida do paciente, as infecções fúngicas, principalmente, a candidose, ainda representam uma grande ameaça aos pacientes imunocomprometidos. A modulação da virulência de *Candida albicans* por agentes imunossupressores empregados na prevenção de rejeição de transplantes de órgãos sólidos permanece desconhecida. O objetivo deste estudo foi avaliar se o tacrolimus, a prednisolona e o micofenolato, isoladamente e associados, simulando o regime triplo comumente indicado no período pós-transplante, interferem na formação de biomassa, na secreção de aspartil-proteases (Sap) e na atividade específica de Sap (AESap) de biofilmes aeróbios e anaeróbios de *C. albicans.*

Métodos: *Candida albicans* SC5314 foi cultivada em normóxia e anóxia na presença de tacrolimus 440 $pg.mL^{-1}$, prednisolona 187 $ng.mL^{-1}$, micofenolato 3 $\mu g.mL^{-1}$ isoladamente e na associação dos três fármacos nas mesmas concentrações. O controle negativo consistiu da cultura da *C. albicans* sem os fármacos. Após 72 h de crescimento sobre poliestireno, os biofilmes aeróbios/anaeróbios foram quantificados quanto às suas biomassas, secreção absoluta de Sap e AESap..

Resultados: Em biofilmes aeróbios, a associação dos fármacos reduziu a formação da biomassa ($p<0,0001$). O tacrolimus, a prednisolona e a associação dos fármacos diminuíram a secreção de Sap ($p<0,0001$). Em biofilmes anaeróbios, todos os fármacos, isoladamente e associados, promoveram redução de biomassa ($p<0,0001$), com exceção da prednisolona ($p>0,05$). Em relação à secreção de Sap, todos os fármacos, isoladamente e associados, promoveram diminuição da atividade proteolítica ($p<0,0001$).

Conclusão: Os resultados obtidos mostram que os fármacos testados promovem diminuição da virulência da *C. albicans,* mediada pelos fatores de virulência aqui avaliados, sugerindo que não existe aumento da patogenicidade.

Palavras chaves: *Candida albicans*, Imunossupressores, Aspartil-proteases, Biofilme.

INTRODUÇÃO

Dados apresentados pelo *National Organ Procurement and Transplantation Network* mostram que foram realizados 23.288 transplantes de órgãos sólidos nos Estados Unidos em 2008 (1), sendo que transplantes renais responderam por 13.743/23.288 (59,01%). No Reino Unido, foram realizados 2055 transplantes de órgãos sólidos em 2010 (2), sendo que rins isoladamente ou em associação com outros órgãos, responderam por 1977/2055 (96,20%). Equipes brasileiras realizaram 6402 transplantes de órgãos sólidos em 2010 (3), sendo que transplantes renais responderam por 4630/6402 (72,32%).

A despeito do incremento no número de transplantes e na taxa de sobrevida dos pacientes, o problema de infecções oportunistas, em especial infecções fúngicas invasivas e candidoses orais, permanece de difícil solução (4,5), estando associado à alta taxa de morbidade e mortalidade no período pós transplante (5,6). *Candida* spp. e *Aspergillus* spp. são os mais frequentemente patógenos responsáveis pelas infecções fúngicas, sendo a *Candida albicans*, o fungo mais comumente encontrado (5).

A *C. albicans* possui um número de atributos de virulência e entre os mais estudados estão a sua capacidade para formar biofilme (7) e a secreção de enzimas hidrolíticas extracelulares, as aspartil proteases (Saps), codificadas por uma família de dez genes (8).

É creditado à imunossupressão crônica, derivada da terapia pós-transplante, a responsabilidade pela morbidade advinda de infecções por *Candida albicans* e por outras *Candida* spp. (9-12). Associado a isso, nossa equipe tem mostrado que diferentes xenobióticos podem causar alterações significativas na virulência de *C. albicans* (13-17), o que ainda não foi avaliado para fármacos utilizados na terapia anti-rejeição.

O tratamento imunossupressor mais comumente adotado para a terapia de manutenção pós transplante é um regime triplo que inclui a associação do tacrolimus, um inibidor da calcineurina, o micofenolato (ácido micofenólico), um fármaco antiproliferativo que inibe a enzima inosina monofosfato desidrogenase e corticosteróides (18,19). O presente estudo buscou avaliar a possibilidade de modulação que o tacrolimus, a prednisolona e o ácido micofenólico (micofenolato), isoladamente e associados, simulando o regime triplo, exercem na formação de biomassa, secreção absoluta de aspartil-protease e AESap, em biofilmes aeróbios e anaeróbios da linhagem selvagem de *C. albicans* SC5314.

MATERIAL E MÉTODOS

Fármacos e meio de cultura

Os produtos químicos usados eram de grau analítico e foram adquiridos da Merck KgaA (Darmstadt, Germany), com exceção de Yeast Nitrogen Base®Difco™ (YNB. Becton-Dickinson Co. Frankling Lakes, NJ), albumina bovinafração V (BSA), azocaseína (Sigma-Aldrich Co., St Louis, MS) e Protovit®*plus* (Bayer Healthcare AG, São Paulo, Brazil). Todas soluções e meios de cultura foram preparados com água reagente tipo II com resistência específica maior que 2 Mohm.cm^{-1} (20,21).

As concentrações finais dos fármacos, próximas daquelas plasmáticas recomendadas para a prevenção da rejeição de transplante renal estão apresentadas na tabela 1.

Tabela 1. Fármacos anti-rejeição utilizados

Fármaco*	Concentração	Procedência	Referência
Tacrolimus	440 $pg.mL^{-1}$	Zhejing Xianju Pharm Co (China)	22
Prednisolona	187 $ng.mL^{-1}$	Zhejing Xianju Pharm Co (China)	23
Micofenolato	3 $\mu g.mL^{-1}$	Sigma-Aldrich Co. (St Louis, MS)	24
Associação	Igual às anteriores		

*todos os fármacos tiveram suas purezas e potenciais certificados pelo fabricante

A cepa-selvagem SC5314 foi escolhida devido ao fato de ser uma conhecida secretora de aspartil-proteases, principalmente, quando crescida em biofilmes (14,17). Como *C. albicans* pode colonizar sítios considerados normóxicos e anóxicos, optou-se pela condução de experimentos em ambas condições atmosféricas.

A cepa foi inoculada em YPD (pH 6,5) e incubadas (37 °C, 120 rpm, normóxia). As culturas crescidas foram diluídas até OD_{600}*ca.* 0,1 em meio indutor (SIB) composto de YNB + 2% D-glucose +1% BSA, adicionado de Protovit®*plus* em uma concentração final de 0,25%. As incubações foram realizadas em normóxia ou anóxia (90% N_2, 10% CO_2) a 37 °C e 120 rpm até OD_{600}*ca.* 1,0 (~3×10^7 blastosporos.mL^{-1}).

Alíquotas de 100 µL das culturas aeróbias e anaeróbias foram inoculadas em tubos contendo 3 mL de SIB (controle) ou SIB + fármacos anti-rejeição (tabela 1), que foram incubados a 37 °C, 120 rpm, normóxia ou anóxia, por 24 h. As células foram assepticamente recuperadas por centrifugação (10000 × *g*), lavadas com NaCl 145 mM estéril e suspensas até OD_{600}*ca.* 1,0. Alíquotas de 1 mL foram transferidas para quatro poços em placas de poliestireno com 24 poços (TPP Inc., Trasadingen, Swissland). As placas foram incubadas a 37 °C e 50 rpm, em normóxia ou anóxia. Depois de 2 h, as suspensões foram drenadas e os poços foram lavados duas vezes com água destilada estéril para remover células planctônicas ou fracamente aderidas. Os

poços receberam alíquotas de 1 mL de SIB ou SIB + fármacos e as placas foram incubadas a 37 °C e 50 rpm, em normóxia ou anóxia, por 72 h. A cada 24h os meios foram renovados.

Estimativa da biomassa

Os sobrenadantes dos biofilmes foram separados por aspiração e transferidos para tubos plásticos estéreis para a determinação da atividade proteolítica. As biomassas foram cuidadosamente lavadas duas vezes com NaCl 145 mM estéril. Os poços receberam 2 mL de metanol 99% para fixação das células. Após 15 min, o metanol foi removido por aspiração e os biofilmes fixados foram secados com ar quente forçado. Os poços foram completados com cristal violeta 0,02%, por 10 min. O excesso de cristal violeta foi removido e os poços foram lavados cinco vezes com água destilada estéril. O cristal violeta impregnado foi liberado pela adição de 1 mL de ácido acético 33% e as OD_{540} foram determinadas.

Determinação da atividade proteolítica

A atividade proteolítica dos biofilmes aeróbios e anaeróbios foi determinada pela taxa de digestão de azocaseína nos sobrenadantes aspirados (14). Toda atividade medida foi creditada às aspartil-proteases secretoras (Saps), visto que outras peptidases estão presentes no sobrenadante em baixas concentrações (25,26). Foram transferidos 150 µL de sobrenadantes para tubos de vidro contendo 250 µL de azocaseina 1% (em tampão Tris-HCl 50 mM, pH 8,0). As reações foram incubadas durante 60 min, a 37 °C e foram bloqueadas com a adição de 400 µL de ácido tricloroacético 10% (TCA) e incubadas por mais 10 min em temperatura ambiente. Após

centrifugação (10000 × *g*/10 min), alíquotas de 75 µL dos sobrenadantes foram misturados com iguais volumes de NaOH 500 mM e incubados por 15 min. As OD_{440} foram medidas. Uma unidade de atividade de Sap foi arbitrariamente definida como a quantidade de enzima que aumenta em 0.001 unidades de absorbância, por minuto de digestão. Para fins de normalização, a divisão dos valores de unidades de atividade pelas respectivas absorbâncias advindas das medidas de biomassas forneceu os valores da AESap (14), que mostra exatamente a quantidade de Sap produzida por célula de *C. albicans.*

Estatística

Este estudo foi realizado em quadruplicata e em três momentos diferentes. Todos os dados foram tabulados em planilhas MSExcel® (Microsoft Co.), que foram transferidas para a interface do pacote SPSS 18.0 (SSCP, Inc.). Os grupos experimentais foram avaliados em relação às suas homogeneidades pelo teste de Levene e as diferenças entre os grupos foram acessadas através do teste de Games-Howell. Diferenças foram consideradas significativas quando o valor p foi igual ou inferior a 0,05.

RESULTADOS

Biofilmes aeróbios

A distribuição cartesiana das variáveis "atividade de Sap" [($\Delta OD_{440nm} \bullet min^{-1}$) × 1000] e "estimativa relativa de biomassa" [$\%OD_{540nm}$] para a cepa selvagem SC5314 (figura 1) mostra que a associação dos fármacos reduziu, sensivelmente, a biomassa ($p < 0,0001$), no entanto, tracolimus, prednisolona e micofenolato não alteraram a formação de biomassa, quando comparados com o controle ($p > 0,0500$). O tacrolimus, a prednisolona e a

associação dos fármacos promoveram decréscimos significativos na secreção absoluta de Sap ($p < 0,0001$).

Quando comparados ao controle, todos os tratamentos promoveram uma redução da AESap, com exceção do micofenolato. A associação dos fármacos promoveu a maior redução da AESap, comparada ao controle e aos fármacos isoladamente (tabela 2).

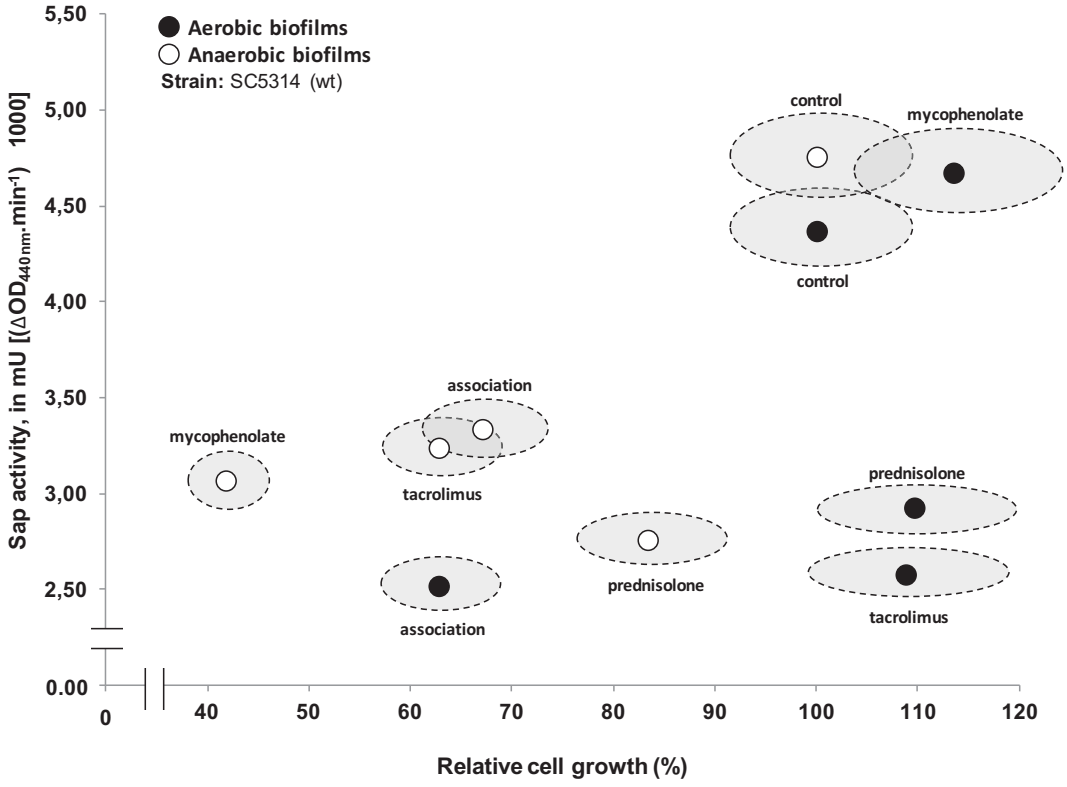

Figura 1. Influência dos fármacos anti-rejeição na atividade absoluta de Sap e na estimativa relativa de biomassa de biofilmes aeróbios/anaeróbios de *C. albicans* SC5314. Áreas elípticas ao redor dos círculos de valor médio representam intervalos de confiança 95% (atividade de Sap *vs* crescimento relativo).

Tabela 2. Influência de fármacos anti-rejeição na atividade específica de Sap (AESap) de *C. albicans* SC5314

Tratamento	Biofilme aeróbio AESap			p*	Biofilme anaeróbio AESap			p
Controle	11,555	±	1,915	A	6,487	±	0,756	a
Tacrolimus440 pg.mL^{-1}	7,028	±	0,925	B	7,030	±	1,123	a
Prednisolona187 ng.mL^{-1}	6,179	±	0,617	BC	4,528	±	0,923	b
Micofenolato3 µg.mL^{-1}	11,006	±	2,136	A	9,932	±	0,782	c
Associação	5,676	±	1,057	C	6,801	±	0,860	a

* Letras similares para tratamentos sob um mesmo fenótipo indicam não haver diferenças entre eles ($p > 0.05$). Não foram comparados os resultados em função da atmosfera de incubação.

Biofilmes anaeróbios

Todos os fármacos, isoladamente e associados, promoveram uma redução significativa ($p < 0,0001$) na biomassa dos biofilmes da cepa SC5314 (figura 1), quando comparados ao controle, com exceção da prednisolona ($p = 0,7689$). Reduções significativas ($p < 0,0001$) na secreção absoluta de Sap foram observadas para todos os tratamentos. O micofenolato foi o fármaco que promoveu a maior redução da biomassa, quando comparado ao controle e aos demais tratamentos ($p< 0,05$).

Em relação a AESap (tabela 2), o micofenolato promoveu a maior atividade proteolítica ($p < 0,0001$) e a prednisolona a menor ($p < 0,0001$), quando comparados ao controle e demais tratamentos. Não houve diferença significativa entre o tacrolimus, a associação de fármacos e o controle ($p>0,05$).

DISCUSSÃO

O tratamento imunossupressor moderno preconizado para receptores de transplantes de órgãos sólidos visa controlar episódios de rejeição, mas deve contemplar também, a minimização de efeitos colaterais indesejáveis (27). Embora o papel das *Candida* spp. nas infecções invasivas e de mucosa esteja

muito bem estabelecido (28-30), essas entidades ainda são consideradas como oportunistas que se aproveitam da incompetência imunológica do paciente para se manifestarem. Quando da concepção da idéia deste estudo, inédito, os autores hipotetizaram que além da promoção de imunosupressão, as moléculas ensaiadas também poderiam modular algum fator de virulência constitutivo, como as aspartil-proteases, já que é reportado que o tacrolimus e o micofenolato possuem atividade antifúngica (31).

Os resultados aqui obtidos revelaram que os fármacos, isoladamente ou em conjunto, promoveram alterações variáveis na formação de biomassa e na secreção de Sap, não seguindo um padrão predizível.

Embora o tacrolimus possua atividade antifúngica intrínseca (31) e demonstre uma interação sinergista com outros antifúngicos, podendo elevar a eficácia de antifúngicos azólicos pela inibição da síntese de calcineurina (31,32), cepas de *C. albicans* são intrinsicamente resistentes à ele (33). Nossos resultados mostram concordância com tal pressuposto quando aplicado aos biofilmes aeróbios, mas também mostram que ocorre redução de biomassa em biofilmes anaeróbios. É possível que, sob anóxia, o tacrolimus tenha inibido enzimas da via glicolítica e/ou fermentativa (34,35). Em ambiente anóxico, *C. albicans* desvia seu metabolismo para vias fermentativas imediatamente após a glicólise (36). Sob influência do tacrolimus, a inibição de enzimas fermentativas pode levar à uma redução de biomassa. Tal fenômeno implica em concomitante redução de demanda por nitrogênio, o que explicaria a menor secreção absoluta de Sap.

Era esperado que o micofenolato, um macrolídeo com conhecida atividade anti-fúngica (37), comprometesse a viabilidade dos biofilmes. De forma adversa, nos biofilmes aeróbios, a exposição ao micofenolato resultou

em um posicionamento cartesiano à direita e acima do controle, ainda que sem significância estatística. Em princípio, tal achado foi considerado como inesperado, entretanto, já foi anteriormente demonstrado que, em concentrações maiores que aquelas aqui empregadas, o micofenolato apresenta propriedade fungistática e não fungicida, causando arrestamento de células-filhas nas primeiras horas de exposição (38). Esse arrestamento, derivado de falhas na citocinese de blastosporos, não deve se aplicar às hifas verdadeiras formadas em presença de BSA.

Por outro lado, a ausência de oxigênio molecular parece ser determinante para que o micofenolato reduza a biomassa final e a secreção de Saps. Essa observação está em consonância com aquilo que era hipoteticamente esperado.

Ainda que haja uma íntima relação entre o uso de glicocorticóides e eventos de candidose invasiva, esses são amplamente empregados na prevenção da rejeição (39). Embora em altas concentrações (4 mM, para aplicações tópicas), a prednisolona possa elevar a taxa de germinação e a liberação de fosfolipase (40), uma relação direta entre prednisolona para uso sistêmico e virulência fúngica nunca foi demonstrada. Nossos resultados mostraram que apesar de não ocorrerem alterações significativas na biomassa de biofilmes crescidos em presença de prednisolona, houve um decréscimo da atividade proteolítica conferida por Sap, independentemente da atmosfera. Tal achado indica a possibilidade de redução da manifestação desse fator de virulência, o que pode ser confirmado pelas reduções nas AESap.

Sob a óptica clínica, os resultados mais importantes aqui reportados são aqueles advindos da associação dos fármacos, pois devem mimetizar a realidade terapêutica de um esquema triplo de prevenção empregando os três

fármacos. Eles levam a sugestão de que, a administração conjunta dos fármacos deve promover a simultânea redução da biomassa, da secreção absoluta de Sap e da AESap em biofilmes aeróbios. Isso equivale afirmar que ocorreu redução relativa da virulência conferida pelaSap. Por sua vez, nos biofilmes anaeróbios, o posicionamento cartesiano inferior e à esquerda sugere que ocorrem reduções importantes no potencial de virulência, ainda que sem reduções na AESap.

Em suma, dentro das limitações deste estudo, obtivemos que os fármacos utilizados no presente estudo não reduzem a biomassa de biofilmes aeróbios de *C. albicans*, mas diminuem a secreção de Sap (com exceção do micofenolato), nestes biofilmes. Clinicamente, a diminuição da secreção das Saps é um fator positivo para o paciente que se encontra imunossuprimido, pois a virulência conferida por essas enzimas se encontra diminuída, fato confirmado pela diminuição da AESap. Em biofilmes anaeróbios, todos promovem redução concomitante de biomassa e de secreção de Sap. A associação de fármacos promove reduções consistentes na biomassa e na secreção de Sap, em qualquer condição atmosférica. Esses resultados sugerem que os fármacos testados não promovem elevação da virulência da *C. albicans, c*om exceção do micofenolato*,* mediada pelos fatores de virulência aqui avaliados. No entanto, cabe ressaltar que a administração desses fármacos resulta em quadros distintos de imunocompetência, que é determinante para o estabelecimento de infecções fúngicas oportunistas.

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2. ARTIGO EM INGLÊS

Title page

Modulation of *Candida albicans* virulence by anti-rejection immunosuppressors

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Abbreviations

Sap: Secretory Aspartyl Proteases

YNB: Yeast Nitrogen Base

BSA: Bovine Serum Albumin

YPD: Yeast Peptone Dextrose

SIB: Sap Inducer Broth

AESap: Sap Specific Activity

ABSTRACT

Introduction: In spite of the growing number of solid organ transplantations and the increase in patient survival, fungal infections, especially candidiasis, still poses a significant threat to immunocompromised patients. Modulation of *Candida albicans* virulence by immunosuppressive agents remains unknown. This study aimed at evaluating whether tacrolimus, prednisolone and mycophenolate, alone and associated, simulating the triple regime usually indicated in the post transplantation period, interfere in the formation of biomass, secretion of aspartyl proteases (Sap) and the specific activity of Sap (AESap) of aerobic and anaerobic biofilm of *C. albicans*

Methods: *Candida albicans* SC5314 was grown in normoxia and anoxia in the presence of 440 $pg.mL^{-1}$ tacrolimus, 187 $ng.mL^{-1}$prednisolone, and 3 $\mu g.mL^{-1}$ mycophenolate alone and associated at the same concentration. Negative control consisted of the *C. albicans* culture without the drugs. After 72 h of growth on polystyrene, aerobic/anaerobic biofilms were assessed as to their biomasses, absolute Sap secretion and AESap.

Results: In aerobic biofilms, the drugs association was the only treatment that reduced the formation of biomass ($p < 0.0001$). The tacrolimus, prednisolone and the drugs association reduced the secretion of Sap ($p < 0.0001$) In anaerobic biofilms, all drugs, alone or associated, promoted biomass reduction ($p < 0.0001$), with the exception of prednisolone ($p > 0.05$). Regarding the secretion of Sap, all drugs, alone and associates, led to a decrease of proteolytic activity ($p < 0.0001$).

Conclusion: The results indicated that the drugs tested led to a reduction in the virulence of *C. albicans,* mediated by virulence factors assessed here, suggesting that there is no increased pathogenicity.

Keywords: *Candida albicans*, Immunosuppressors, Aspartyl proteases, Biofilm.

INTRODUCTION

Data presented by the National Organ Procurement and Transplantation Network show that 23,288 solid organ transplantations were made in the United States in 2008 (1), and kidney transplantations accounted for 13,743/23,288 (59.01%). In the United Kingdom, 2,055 solid organ transplantations were made in 2010 (2), and kidneys, alone or associated with other organs, accounted for 1,977/2,055 (96.20%). Brazilian medical teams made 6,402 solid organ transplantations in 2010 (3), and kidney transplantations accounted for 4,630/6,402 (72.32%).

In spite of the increment of transplantation numbers and patient survival rates, the problem of opportunistic infections, particularly invasive fungal infections and oral candidiasis, remains difficult to solve (4,5), being associated to high morbidity and mortality rates in the post-transplantation period (5,6). *Candida* spp. And *Aspergillus* spp. are the pathogens most often responsible for fungal infections, and *Candida albicans* is the fungus most commonly found (5).

The *C. albicans* has a number of virulence attributes and among the most studied are its ability to form biofilms (7) and secretion of extracellular hydrolytic enzymes, the aspartyl proteases (Saps) encoded by a family of ten genes (8).

Chronic immunosuppression, derived from post-transplant therapy, has been blamed for morbidity from infections by *Candida albicans* and other *Candida* spp. (9-12). Associated to this, our team has shown that different xenobiotics can cause significant changes to *C. albicans* (13-17) virulence, which has not been assessed by drugs used in anti-rejection therapy yet.

The immunosuppressive therapy most commonly adopted in the post renal transplant maintenance therapy is a triple regime that includes the association of tacrolimus - a calcineurin inhibitor, mycophenolate (mycophenolic acid) - an anti-proliferative drug that inhibits the enzyme inosine monophosphate dehydrogenase, and corticosteroids (18,19). The present study aimed to evaluate the possibility that tacrolimus, prednisolone and mycophenolic acid (mycophenolate), alone or associated, simulating the triple therapy, might modulate biomass formation, absolute secretion of aspartyl protease (Sap) and AESap in aerobic and anaerobic biofilms of the wild-type strain of *C. albicans* SC5314.

MATERIAL AND METHODS

Drugs and culture medium

Analytical grade chemicals were used, acquired from Merck KgaA (Darmstadt, Germany), except for Yeast Nitrogen Base®Difco™ (YNB. Becton-Dickinson Co. Frankling Lakes, NJ), bovine serum albumin fraction V (BSA), azocasein (Sigma-Aldrich Co., St Louis, MS) and Protovit®*plus* (Bayer Healthcare AG, São Paulo, Brazil). All the solutions and culture media were prepared with type II reagent water with specific resistivity greater than 2 Mohm.cm^{-1} (20,21).

Final drugs concentrations, close to the plasmatic concentrations recommended for prevention of renal transplant rejection are presented in table 1.

Table 1. Anti-rejection drugs used

Drus*	Concentration	Origin	Reference
Tacrolimus	440 $pg.mL^{-1}$	Zhejing Xianju Pharm Co (China)	22
Prednisolone	187 $ng.mL^{-1}$	Zhejing Xianju Pharm Co (China)	23
Mycofenolate	3 $\mu g.mL^{-1}$	Sigma-Aldrich Co. (St Louis, MS)	24
Association	Same as above		

*all drugs with certified purity and potency

The wild-type strain SC5314 was selected because it is a renowned secretor of aspartyl proteases, particularly when added to biofilms (14,17). As *C. albicans* can colonize sites considered normoxic and anoxic, it was decided to carry out experiments under both atmospheric conditions.

The strain was inoculated into YPD (pH 6.5) and incubated (37 °C, 120 rpm, normoxia). The grown cultures were diluted to OD_{600}*ca.* 0.1 in an induction medium (SIB) composed of YNB + 2% D-glucose +1% BSA, and Protovit®*plus* was added to a final concentration of 0.25%. Incubations were performed in normoxia or anoxia (90% N_2, 10% CO_2) at 37 °C and 120 rpm to OD_{600}*ca.* 1.0 (~3×10^7 $blastospores.mL^{-1}$).

Aliquots of 100 µL of aerobic and anaerobic cultures were inoculated into tubes containing 3 mL of SIB (control) or SIB + anti-rejection drugs (table 1), which were incubated at 37 °C, 120 rpm, in normoxia or anoxia, for 24 h. Cells were aseptically recovered by centrifugation (10000 × *g*), washed with sterile 145 mM NaCl and suspended to OD_{600}*ca.* 1,0. Aliquots of 1 mL were transferred to three wells in polystyrene 24-well plates (TPP Inc., Trasadingen, Switzerland). Plates were incubated at 37 °C and 50 rpm in normoxia or anoxia. After 2 h, suspensions were drained and wells were washed twice with sterile distilled water to remove planktonic cells or poorly adherent cells. Wells received aliquots of 1 mL of SIB or SIB + drugs and plates were incubated at 37

°C and 50 rpm, in normoxia or anoxia, for 72 h. Every 24 hours the media were renewed

Biomass estimate

Biofilm supernatants were separated by aspiration and transferred to sterile plastic tubes for determination of the proteolytic activity. Biomasses were carefully washed twice with sterile 145 mM NaCl. Wells received 2 mL of 99% methanol for cell fixation. After 15 min, methanol was removed by aspiration and fixed biofilms were dried with forced hot air. Wells were filled with 0,02% crystal violet, for 10 min. Excess crystal violet was removed and wells were washed five times with sterile distilled water. Impregnated crystal violet was released by addition of 1 mL 33% acetic acid and OD_{540} were determined.

Determination of protease activity

Protease activity of aerobic and anaerobic biofilms was determined by the rate of digestion of azocasein in aspired supernatants (14). The secretory aspartyl proteases (Sap) accounted for all the activity measured considering that other peptidases are present in the supernatant at low concentrations (25, 26). 150 µL of supernatants were transferred to glass tubes containing 250 µL of 1%azocasein (buffer 50 mM Tris-HCl, pH 8.0). Reactions were incubated during 60 min at 37 °C and were blocked with the addition of 400 µL of 10% trichloroacetic acid (TCA) and incubated for 10 min at room temperature. After centrifugation (10000 × *g*/10 min), aliquots of 75 µL of supernatants were mixed with equal volumes of 500 mM NaOH and incubated for 15 min. The OD_{440} were measured. A unit of Sap activity was randomly defined as the amount of enzyme that increases in 0.001 units of absorbance, by minute of digestion. For

standardization purposes, the division of values in units of activity by the respective absorbance from biomass measures provided the values of specific protease activity (AESap) (14), showing exactly the amount of sap produced by cell *C. albicans*.

Statistics

This study was performed in quadruplicate and in three different moments. All the data were tabulated in MSExcel® (Microsoft Co.) spreadsheets, which were transferred to the interface of package SPSS 18.0 (SSCP, Inc.). The experimental groups were assessed with regard to their homogeneities through the Levene`s test and differences between groups were assessed through the Games-Howell`s test. Differences were considered significant when the p-value was equal or lower than 0.05.

RESULTS

Aerobic biofilms

The distribution of Cartesian variables “Sap activity” [($\Delta OD_{440nm} \bullet min^{-1}$) × 1000] and “relative biomass estimate” [$\%OD_{540nm}$] for the wild-type strain SC5314 (figure 1) shows that the drug association significantly reduced biomass ($p < 0.0001$); however, tracolimus, prednisolone and mycophenolate did not alter biomass formation when compared to control ($p > 0.0500$). The tacrolimus, prednisolone and the drugs association promoted a significant decrease in the absolute Sap secretion ($p <0.0001$). When compared to control, all the therapies had an AESap reduction, except for mycophenolate. The drugs association led to a greater AESap reduction, compared to control and compared to drugs alone (table 2).

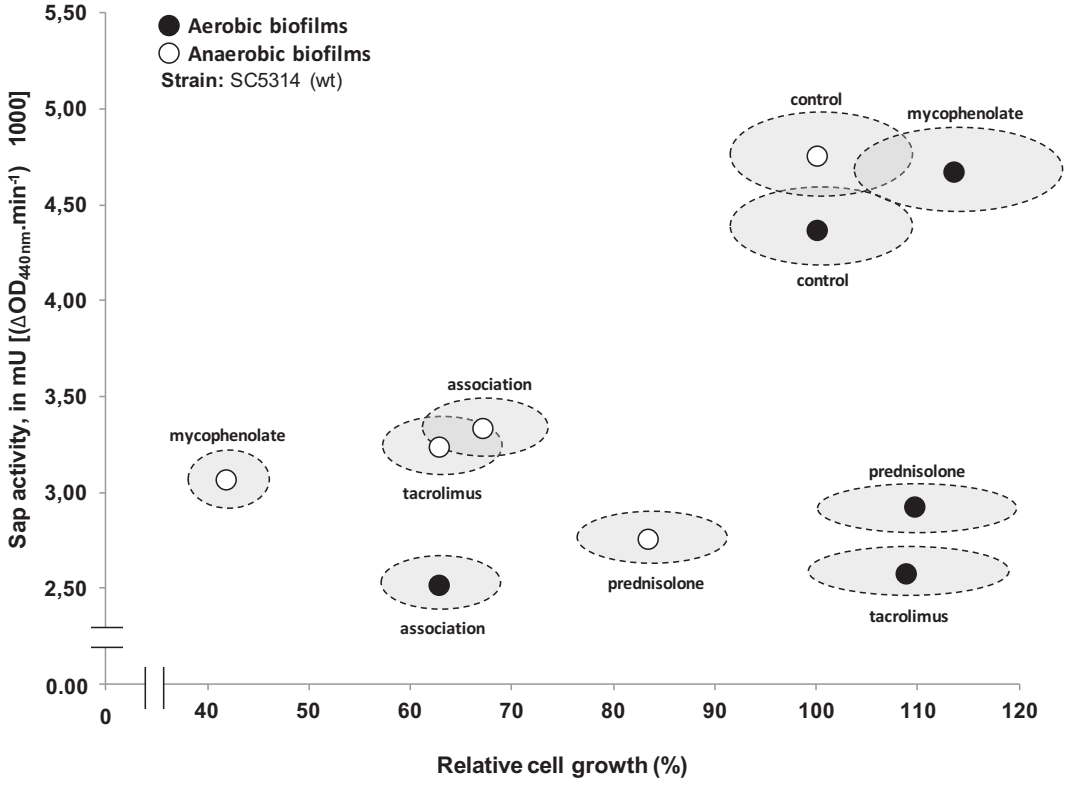

Figure 1. Influence of anti-rejection drugs in the absolute Sap activity and the amount of biomass estimate of aerobic/anaerobic biofilms of *C. albicans* SC5314. Elliptical areas around the circles of average value represent confidence intervals of 95% (Sap activity *vs.* relative growth).

Table 2. Influence of anti-rejection drugs in the specific activity of Sap (AESap) of *C. albicans* SC5314

Therapy	Aerobic biofilm AESap	p*	Anaerobic biofilm AESap	p
Control	11.555 ± 1.915	A	6.487 ± 0.756	a
440 pg.mL^{-1}Tacrolimus	7.028 ± 0.925	B	7.030 ± 1.123	a
187 ng.mL^{-1}Prednisolone	6.179 ± 0.617	BC	4.528 ± 0.923	b
3 µg.mL^{-1}Mycophenolate	11.006 ± 2.136	A	9.932 ± 0.782	c
Association	5.676 ± 1.057	C	6.801 ± 0.860	a

* Similar letters for therapies under the same phenotype indicate no difference between them ($p > 0.05$). Results were not compared in view of the incubation atmosphere.

Anaerobic biofilms

All the drugs analyzed, alone and associated, led to a significant reduction ($p < 0.0001$) in biofilm biomass of strain SC5314 (figure 1), when compared to control, except for prednisolone ($p = 0.7689$). Significant reductions ($p < 0.0001$) in absolut Sap secretions were observed in all the therapies. The drug mycophenolate led to the greatest biomass reduction when compared to control and to other therapies ($p<0,05$).

With regard to AESap (table 2), mycophenolate led to a greater enzymatic activity ($p < 0.0001$) and prednisolone led to the lowest ($p < 0.0001$), when compared to control and to other therapies. There was no significant difference between tacrolimus, drug association and control ($p>0.05$).

DISCUSSION

The modern immunosuppressive therapy recommended to solid organ transplantation receptors aims to control rejection episodes but should also consider minimizing undesirable side effects (27). Even though the role of *Candida* spp. in invasive infections is well established (28-30), these entities are still considered opportunistic, which take advantage of the patient's incompetent immune system to manifest. When the idea of this study was first conceived, the authors hypothesized that, besides promoting immunosuppression, the assayed molecules could also modulate some constitutive virulence factor such as aspartyl proteases, as tacrolimus and mycophenolate are reported to have a powerful antifungal activity (31).

The results achieved here reveal that drugs, alone or associated, promote variable alterations in biomass formation and Sap secretion, and do not follow a predictable pattern.

Although tacrolimus has an intrinsic antifungal activity (29) and shows a synergist interaction with other antifungal agents, capable of enhancing efficacy of azole antifungal agents by inhibiting the calcineurin synthesis (31,32), strains of *C. albicans* are intrinsically resistant to it (33). Our results agree with this assumption when it applies to aerobic biofilms; however, they also show that a biomass reduction occurs in anaerobic biofilms. It is possible that, under anoxia, tacrolimus might have inhibited enzymes of the glycolytic and/or fermentative pathways (34,35). In an anoxic environment, *C. albicans* switches its metabolism to fermentative pathways immediately after glycolysis (36). Under influence of tacrolimus, fermentative enzyme inhibition could lead to biomass reduction. Such phenomenon implies a concomitant demand for nitrogen, which would explain lower absolut Sap secretion.

It was expected that mycophenolate, a macrolide with renowned antifungal activity (37), would compromise biofilm feasibility. Adversely, in aerobic biofilms, exposure to mycophenolate led to a cartesian position on the right and above control, although not statistically significant. In principle, such finding was considered unexpected; however, it has been previously shown that, in concentrations higher than the ones employed here, mycophenolate has fungistatic properties but not fungicidal properties, causing daughter cells to arrest in the first hours of exposure (38). This arrest, deriving from failures in blastospores cytokinesis, should not apply to real hyphae formed in the presence of BSA.

On the other hand, the absence of molecular oxygen seems to be determinant when mycophenolate reduces final biomass and Saps secretion. This observation is compliant with what was hypothetically expected.

While there might be a close relation between the use of glucocorticoids and events of invasive candidiasis, glucocorticoids are widely used to prevent rejection (39). Although at high concentrations (4 mM, for topic applications), prednisolone can increase germination rates and release of phospholipase (40), a direct relationship between prednisolone for systemic use and fungal virulence has never been demonstrated. Our results showed that, despite no significant alterations occurred in the biomass of films grown in the presence of prednisolone, there was a decrease in the proteolytic activity conferred by Sap, regardless of atmosphere. Such finding points out the possibility of reducing manifestation of this virulence factor, which can be confirmed by reductions in AESap.

From the clinical viewpoint, the most important results reported here are those arising from the association of drugs as they should mimic the therapeutic reality of a triple prevention scheme using the three drugs. They suggest that the joint administration of drugs should promote concomitant reduction in biomass, absolute Sap secretion and AESap in aerobic biofilms. That is to say that there was reduction of the virulence conferred by Sap. In turn, in anaerobic films, the Cartesian position in the lower left quadrant suggests that important reductions occur in pathogenic potential, even without reductions in AESap.

In short, within the limitations of this study, we found that the drugs used in the present study do not reduce biomass of aerobic films of *C. albicans*, but interfere negatively with Sap secretion (except for mycophenolate) in these biofilms. Clinically, the decreased secretion of Saps is a positive factor for the patient who is immunosuppressed, since the virulence conferred by these enzymes is reduced, a fact confirmed by the decrease in AESap. In anaerobic biofilms, all the drugs promote concomitant reduction of biomass and Sap

secretion. Drug association promotes consistent reduction in biomass and Sap secretion under any atmospheric condition. These results suggest that the above mentioned drugs do not raise the pathogenicity of *C. albicans* mediated by the virulence factors evaluated here. However, it should be noted that the administration of these drugs alone or associated results in distinct immunocompetence frames, which is a determinant of the establishment of opportunistic fungal infections.

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Color images must be saved as "CMYK". Images saved as "RGB" are not acceptable for printing. For further explanation of these requirements, please visit Cadmus Digital Art General Guidelines.

● Testing figures with Rapid Inspector

Prior to upload, all figures should be tested using the free pre-flight software program provided by Cadmus Rapid Inspector in order to see whether they meet journal production requirements. Prepare all figures and then go to the Cadmus Rapid Inspector site to register for an account (confirmation of the account will be sent to the email address you enter). Log in and enter the Manuscript ID number to launch an inspection session for your manuscript. Select *Transplantation* from the list of Publication Titles. The Rapid Inspector Program dialogue box will open; just click on open and the session will begin.

Follow the instructions provided to test each figure file. Drag files to the box, or select files to test using the File menu. After completing inspection, access the File menu to create and save a submission report (can be saved as an html file).

Upload the report as part of your final submission. If the figure files fail to pass Rapid Inspector, in depth help and instruction for correcting errors in the files are available at the Rapid Inspector Online Help System.

● Cost of figures

Color figures submitted in correct digital format will be charged $100 per figure (including multi-panel figures). Figures not submitted in correct digital format may incur additional charges. Once the figures have passed inspection, log on to the Transplantation manuscript submission site at **http://mc.manuscriptcentral.com/transplant**, and enter your Author Center, where you will find your manuscript listed in the "Manuscripts Accepted for First Look" queue. Click this link to view the list of accepted manuscripts pending action in a table below. Under "Actions," click "submit updated manuscript" to upload the files of the final production-ready version. These files should include the Main Body text as a document (tables can be included within this document or uploaded as separate documents), figures (loaded individually), and Rapid Inspector report. The Editorial Office will send confirmation by email when your production ready files have been reviewed and accepted for forwarding to the publisher. If the production-ready version requires modification, the Editorial Office will notify the corresponding author and the manuscript files will be returned to the First Look queue in the Author Center for further update.

Page Proofs

The publisher's Journal Production Editor, Kerri Landis, will contact you when page proofs are ready for your review. The figures included on author's proofs are high resolution. Please inform the Journal Production Editor immediately if you have any questions concerning the quality of the figures on the proofs.

For information regarding proofs, or the status of publication of your accepted manuscript, please contact Kerri Landis at kerri.landis@wolterskluwer.com or 1-410-528-4323.

Changes in Corresponding Author's Contact Information

Please give all new information, including e-mail address, to the editorial office and to the publisher. Authors may send this information to Kerri Landis at kerri.landis@wolterskluwer.com or by fax to 1-443-817- 0912. If the journal is unsuccessful in contacting the corresponding author, the author will not receive proofs for approval, and the manuscript may not be published.

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Changes at Proofs

It is expected that the final manuscript sent to the Editor is indeed the final version, so few changes should be required at proof stage. The Journal will pay for the first 15 edits made to a manuscript at proof stage. Additional author edits may be charged to the author at $4 per change.

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COLLOQUIA: REVIEW ANDSUBMISSION POLICIES

Effective January 1, 2011, the Journal will utilize the Colloquium format for the publication of thematically related topics of high interest, and this format will replace the previously used Supplement format.

Colloquium Format: The colloquium will be treated as a single, multi-authored article which can be referenced as a single citation. It will be published as a separately bound volume with pagination usings numbers. The Special Features Editor(s) in collaboration with the guest editor will edit and organize the acceptable individual contributions based on topic(s) and content into appropriate sub-sections which will make up the text of the colloquium. The colloquium will have a single broad title and the individual sections will have short focused titles or topic headings. The Guest Editor will be the first author of the multi-authored colloquium with subsequent co-authors listed in the sequence in which their contributions appear in the sections. The names of individual authors will be listed at the beginning of each section containing their specific contribution. All references will be cited numerically in sequence throughout the sections with a single list of references at the end of the text. Tables and figures will also be numbered sequentially and be placed within the text of the specific section to which they apply. Institutional addresses, titles, position and email addresses will be listed for each author contributor. The sponsorship of each colloquium shall be indicated on the front page of the separately bound colloquium volume. Logos of companies and other institutions will not be allowed .Advertisements will not be included in the colloquium but subscription information for *Transplantation* may be included.

Each colloquium will also include an introduction/statement from the Editors outlining the principles of the specific colloquium and indicating among other things various articles recently published in *Transplantation* on related topics as well as emphasizing that the content of the colloquium has not been subjected to the Journal's standard external peer review process.

Submission and Review: All proposals for a colloquium to *Transplantation* must be submitted to either the North American Editorial Office or the European Editorial Office. The Editorial Office will forward the proposals to the Special Features Editor, designated by the Editors of *Transplantation*.

1. The Special Features Editor, in consultation with the Editors, will decide upon the suitability of the submitted proposal. The initial decision to publish a colloquium is based on the significance

and timeliness of the proposed topic and the qualifications of the Guest Editor. Final acceptance is based on review of the submitted manuscripts to ensure a balanced presentation.
2. Each colloquium must have a Guest Editor who is an expert in the designated topic. The Guest Editor is responsible for:
a) assuring the Editors of *Transplantation* that all articles in the colloquium will be subjected to internal peer review;
b) providing information regarding the nature of the peer review process;
c) compiling of subsections for inclusion in the colloquium; and
d) assisting with editing of the publication if necessary.
3. The Special Features Editor and the Editors of *Transplantation* retain the right to determine whether any individual subsection in a colloquium submitted for publication requires additional peer review. For disputed manuscripts the Editors retain authority to determine whether the final manuscript will be published. Individual authors are responsible for the content of their own contributions and for editing those contributions. The Guest Editor of the colloquium accepts responsibility for the overall quality and integrity of the content.
4. The topics for the colloquium must be of importance to *Transplantation* subscribers and related to the academic and educational mission of *Transplantation*. Priority will be given to colloquium that does not focus on a single product but rather on a field of inquiry.
5. Colloquium will be published only if there is scientific or educational logic for combining the subsections in one publication rather than publishing them separately. The number and quality of the subsections in the colloquium must be sufficient to constitute a body of important information that is current and of interest to the clinical and scientific community.
6. A colloquium based on a conference or symposium should be planned well in advance of the meeting so that manuscripts will be available either at the time of the meeting or shortly thereafter. Timely publication of such symposia is essential so that the colloquium is not out of date by the time of publication. To ensure timeliness, colloquia may not be published if the date of publication will be more than 9 months after the symposium or conference.
7. *Transplantation* will only consider publishing proceedings from symposia that are organized by an independent body of professionals in which the funding organization does not have a controlling voice. It is preferable that the Guest Editor and a majority of the members of the independent body be members of the Transplantation Society. All colloquia must have a statement indicating the source of funding, together with any restrictions. Furthermore, the Guest Editor and all contributors must clearly indicate whether there is any conflict of interest and, if so, the extent and nature of the potential conflict.
8. At submission, the contents will be reviewed to assure that there is no bias in the interest of any sponsor. *Transplantation* will not permit presentations within the scientific and educational portion of the colloquium that extol a commercial product. Publication as a colloquium does not constitute product or sponsor endorsement by *Transplantation*, and the following Disclaimer will be printed at the beginning of each colloquium: The contents of this issue represent a supplement to *Transplantation*, prepared and paid for by the sponsoring organization.
Transplantation endeavors to assure that the material presented is not biased in the interest of the sponsoring organization. However, it should be understood by the reader that the Editorial Board of *Transplantation* has generally not subjected the articles included to peer review, but rather to an internal peer review organized by the sponsoring organization. Publication of this colloquium does not constitute product or sponsor endorsement by *Transplantation*.
9. The contents (subsections) in a colloquium are subject to the same copyright regulations that apply to articles published in regular issues of *Transplantation*.

Quotes

Colloquia must be sponsored for publication. The price should be negotiated with Jim Mulligan, Publisher for Lippincott Williams & Wilkins (PH: 702-407-6614;E-mail: jim.mulligan@wolterskluwer.com). The sponsor must submit a letter of intentor purchase order to the publisher at or before the time colloquia manuscripts are delivered to the publisher. Colloquia are routinely mailed to the Journal subscribers. Information needed includes whether

a raw manuscript or camera ready copy is being submitted; estimated number of printed pages; whether color figures are included; the number of overruns (if any); and any special instructions for packaging/ shipping the overruns.