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ATIVIDADE FUNCIONAL DE FAGÓCITOS DO LEITE DE MÃES HIPERGLICÊMICAS LEVE E DIABÉTICAS E SUA REGULAÇÃO NEUROIMUNOENDOCRINOLÓGICA - INTERAÇÕES COM IgA E MELATONINA

GLILCIANE MORCELI

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Sumário

Introdução

A amamentação representa integração imunológica entre a mãe e o filho. O sistema imunológico dos recém-nascidos não é totalmente desenvolvido e é, portanto, limitado na sua capacidade de responder eficazmente a vários agentes infecciosos. A imunidade materna contribui parcialmente para o desenvolvimento do sistema imunológico do recém-nascido (Takahata et al, 2003). A resistência à infecção desses recém-nascidos depende dos fatores de proteção contidos no colostro que são altamente direcionados para agentes infecciosos do ambiente materno e provavelmente, são os encontrados pela criança durante suas primeiras semanas de vida e do desenvolvimento do próprio sistema imunológico (Kelly & Coutts, 2000; Field, 2005).

As glândulas mamárias lactantes são parte integrante do sistema imune de mucosa, e os anticorpos e células presentes no leite refletem a estimulação antigênica do Sistema Imune Associado à Mucosa (MALT), tanto do intestino como das vias respiratórias. Vários trabalhos na literatura relatam que anticorpos e células do leite humano tem especificidade para uma variedade de antígenos oriundos de patógenos intestinais e respiratórios (Goldman, 1993).

Em estudos epidemiológicos, clínicos e experimentais, há evidências que o colostro e leite humanos protegem a criança de grande variedade de agentes potencialmente patogênicos, em particular os que afetam o trato gastrointestinal e respiratório (Honorio-França et al, 1997; Honorio-França et al, 2001; França-Botelho et al, 2006; Fujimori et al, 2008; França et al, 2011a). Além da proteção passiva, há na literatura relatos de que o colostro pode atuar modulando o desenvolvimento imunológico da criança (Garofalo, 2010).

Por outro lado, a composição do leite varia durante a lactação para atender a as necessidades do recém-nascido. Os benefícios do aleitamento materno para a criança são bem definidos na literatura e mais importante que o aleitamento exclusivo é a sua duração, em anos, especialmente na prevenção de algumas doenças (Bachour et al, 2012). O aleitamento materno diminui risco de síndrome de morte súbita (Bachour et al, 2012; Vennemann et al, 2009), doenças infecciosas (Bachour et al, 2012; Quigley et al, 2007) e, além disso, protege as crianças de alergias (Bachour et al, 2012; Saarinen et al, 1995), pode prevenir a asma (Bachour et al, 2012; Gdalevich et al, 2001), reduz o risco de diabete tipo 1 na infância (Bachour et al, 2012; Agostoni et al, 2009), e também pode diminuir a ocorrência do diabete tipo 2 (Bachour et al, 2012; Owen et al, 2006).

O leite contém numerosos componentes adicionais com funções nutricionais e imunológicas variadas e que ainda não foram totalmente elucidadas, bem como as interações entre estes componentes em doenças crônicas, como o diabete.

Componentes bioquímicos do colostro

O leite materno é fundamental para a saúde das crianças por ser um alimento completo, isento de contaminação, perfeitamente adaptado ao metabolismo da criança, capaz de fornecer componentes para hidratação (água) e fatores de desenvolvimento, tem efeitos sobre o organismo da criança, favorecendo o desenvolvimento emocional, cognitivo e do sistema nervoso e mantém os níveis de crescimento e desenvolvimento normais (Silva et al, 2007).

A composição química do colostro é variável com o tempo de maturação gestacional, com a hora do dia e tempo de mamada (início/fim), atende as condições particulares de digestão e é adequado ao metabolismo da criança neste período da vida (Delneri et al, 1997; Oliveira, 2005).

Entre o intervalo das mamadas são produzidos o leite anterior ou primeiro leite com menor teor de gordura e leite posterior ou último leite com a maior concentração de gorduras e calorias. Este aumento ocorre devido a estímulo hormonal da ocitocina, que induz a contração e abertura das células produtoras de gordura. Além da diferença entre a composição do leite anterior e posterior há também diferenças entre as mulheres, diferentes mamadas e no decorrer da lactação (Oliveira, 2005).

Aproximadamente por sete dias após o parto o produto da secreção lática da nutriz é chamado de colostro (Almeida, 2000). É um fluido amarelado, viscoso, rico em imunoglobulinas, proteínas, vitaminas lipossolúveis e minerais e tem mudanças nas concentrações de gorduras, calorias, lipídeos e enzimas durante a amamentação.

Em relação às quantidades de gorduras e calorias há modificações relacionadas ao horário da amamentação e a quantidade de mamadas por dia (França et al, 2010), os lipídeos, principalmente os triglicerídeos fornecem aproximadamente 50% da energia do colostro e diminuem no decorrer do primeiro ano de vida, variando de 3 a 5% no leite maduro (Woolridge et al, 1990) e a enzima superóxido dismutase tem ação antioxidante e apresenta diferenças, em concentração, relativas ao tempo e período de lactação (Kasapovic et al, 2005; França et al, 2010; França et al, 2011a).

As mães são consideradas fontes de nutrientes para o desenvolvimento adequado da criança. Deficiências nutricionais maternas podem levar a falta de nutrientes essenciais, com consequências adversas para o binômio mãe e recém-nascido. O estado nutricional materno deve ser avaliado durante a amamentação, pois pode afetar a composição do leite e é importante que durante o período de amamentação, a dieta da mãe deve ser bem equilibrada.

Componentes imunológicos do colostro

O leite materno possui inúmeros fatores imunológicos específicos e não específicos entre eles anticorpos, glicoconjugados, lipídeos, enzimas, componentes do complemento, envolvidos na ação anti-infecciosa que conferem as crianças proteção ativa e passiva (Schroten et al, 1999; Newburg, 1999; Hanson, 2007).

Há vários estudos que demostram como o colostro e leite humanos protegem a criança contra uma grande variedade de agentes potencialmente patogênicos, doenças imunológicas e também doenças tumorais (Honorio-França et al, 1997; Honorio-França et al, 2001; França-Botelho et al, 2006; Hanson, 2007; França et al, 2010; França et al, 2011a; França et al, 2011b), porém os mecanismos de atuação destes fatores ainda são parcialmente compreendidos. Estudos realizados em diferentes países em desenvolvimento têm permitido observar que quanto menor for o tempo do início do aleitamento, maior é a redução da mortalidade infantil (Edmond et al, 2006) e que os componentes solúveis e celulares presentes no colostro são importantes na prevenção deste processo e provavelmente atuem em conjunto para proteger o recém-nascido de infecções (Honorio-França et al, 1997; Honorio-França et al, 2001; França-Botelho et al, 2006; França et al, 2010, Morceli et al, 2011; França et al, 2011a; França et al, 2011b).

Entre os componentes solúveis presentes no colostro, o agente protetor em maior concentração é a imunoglobulina A do tipo secretória (SIgA). Esta proteína tem a capacidade de se ligar aos microrganismos na mucosa intestinal e, desta maneira, impedir a aderência destes à superfície da mucosa (Solórzano-Santos et al, 1993). A IgA no colostro se encontra predominantemente na sua forma dimérica (11S), é produzida por plasmócitos presentes na lâmina própria da glândula mamária, cujas subunidades estão associadas pela porção Fc à cadeia polipeptídica J ("de joining"). Ao passar pela célula epitelial liga-se a um receptor (poli IgR), parte do qual é secretado no lúmen junto com a imunoglobulina, denominado componente secretor ou peça secretória (SC).

A SIgA é relativamente resistente à degradação enzimática e por isso atua localmente no intestino do recém-nascido como primeira linha de defesa dirigida a antígenos estranhos. Esta classe de anticorpos age nos ligantes dos patógenos no trato gastrointestinal, prevenindo sua aderência às células da mucosa (Ogra &Karson, 1970; Hanson, 2007). A especificidade dos anticorpos IgA no leite humano é um reflexo dos antígenos entéricos e respiratórios da mãe, o que proporciona proteção à criança para os patógenos prevalentes no meio em que a criança está inserida (França et al, 2011a; França et al, 2011b). Recém-nascidos e lactentes são mais vulneráveis a infecções, devido à imaturidade do sistema imunológico e à maior

permeabilidade intestinal. Assim, durante esse período crítico de relativa incompetência imunológica, o leite apresenta atributos de qualidade frente às suas necessidades imunobiológicas.

Há outros isotipos de imunoglobulinas presentes no colostro como a IgG, IgM e a IgE, porém em menor concentração (Barros, 1977; Bahna et al, 1988).

As proteínas do sistema complemento, em especial C3 e C4 e os oligossacarídeos nitrogenados também estão presentes na composição do colostro. As proteínas do sistema complemento têm atividade de opsonização (Honorio-França et al, 1997) com concentrações variadas em relação a hora do dia e do estágio de maturação do leite (França et al, 2010) e os oligossacarídeos nitrogenados possibilitam a instalação da flora bífida que impede, por ação seletiva, face à sua elevada competitividade, que novas bactérias recém-chegadas à luz do intestino e os potenciais agentes patogênicos da diarréia, como a *E. coli*, dentre outras enterobactérias, colonizem o trato intestinal do recém-nascido (Novak et al, 2001).

O colostro apresenta ainda componentes bioativos como as citocinas (Kverka et al, 2007; Meki et al, 2003; Lönnerdal, 2003; Garofalo, 2010), lisozima (Lönnerdal, 2003; Marek et al, 2003), probióticos (Newburg, 2005), fatores antioxidante (Friel et al, 2008; Lindmark-Mansson & Akesson, 2000), glucanas (Newburg, 2005) como lactoaderina (Shahriar et al, 2006; Marek et al, 2003), lactoferina (Ogundele et al, 2000; Bessler et al, 2006) e transferrina (Goldman, 1993; Goldman, 2002) entre outros componentes produzidos pelo sistema imune materno. Possui também vários hormônios (Miralles et al, 2006; Lönnerdal, 2000; Abdulla et al, 2005; Lamounier et al, 2001; Fagundes et al, 2012).

Entre os hormônios, destacam-se a presença de melatonina (lllnerova et al, 1993). A melatonina é sintetizada pela glândula pineal (Claustrat et al, 2005). Possui papel importante no controle do ritmo circadiano e apresenta também outras ações protetoras (Botelho-França et al, 2011). Estudos demonstram que a melatonina pode aumentar a imunidade inata e a adquirida (Poon et al, 1994; Botelho-França et al, 2011) e estimular também células imunes inatas, principalmente leucócitos, o que representa um importante mecanismo de proteção para infecções (Botelho-França et al, 2011).

O colostro difere da maioria das secreções por conter leucócitos viáveis (10^9 cel/ml durante os primeiros dias de lactação) (Islam et al,2006), com quantidade e atividade comparáveis às leucócitos do sangue (Bhaskaram & Reddy, 1981; Honorio-França, et al, 1997).

Macrófagos do colostro têm atividade fagocitária e receptor de SIgA (Robinson et al 1991; Rivas et al 1994). São capazes de produzir radicais livres do oxigênio (Tsuda et al, 1984;Honorio-França et al, 1997; França et al, 2011a), contem IgA de superfície (França et al, 2011b) e IgA intra-citoplasmática (Brandtzaeg, 2003). Expressam marcadores de ativação como

CD11c e podem modular a função de linfócitos B e T (Rivas et al, 1994). Os neutrófilos, por sua vez, quando não estimulado apresentam menor atividade funcional (Thorpe et al, 1986; Honorio-França et al, 1997) e expressam receptor FcαR (Isoforma a.1) sem associação da subunidade γ, apresentam menor atividade microbicida, e provavelmente atuem como mediadores anti-inflamatórios (Honorio-França, 2001).Por apresentarem menor atividade funcional quando secretado no leite, acredita-se que a principal função dos neutrófilos está associada a efeitos maternos (Field, 2005).

O papel do colostro nas infecções

O sistema imune de mucosa contem numerosos fatores bioquímicos, imunológicos e células imunocompetentes, capazes de produzir radicais livres e que interagem entre si, entre as células de mucosa de ambos os tratos respiratório e gastrointestinal. A diferenciação de células imunocompetentes em membranas mucosas é mediada por antígenos em locus. Subsequentemente, as células migram para os nódulos linfáticos regionais e em seguida para os nódulos linfáticos mesentéricos, que entram na circulação pelo canal torácico para atingir outras membranas mucosas, onde permanecem prontas para serem estimuladas e sintetizar a IgA (Goldman, 2002).

Imediatamente após o nascimento há várias cepas de bactérias que passam colonizar o trato respiratório e gastrointestinal, constituindo a microbiota normal do recém-nascido. No entanto, quando há desequilíbrio na microbiota, algumas bactérias podem causar graves doenças intestinais e ou respiratórias. A diarréia aguda é a segunda causa mais comum de morte entre crianças nos países em desenvolvimento e de nível sócio-econômico baixo (Kosek et al, 2003). Entre as bactérias destacam-se a *Escherichia coli* (Meraz et al, 2006) que são classificadas de acordo com as propriedades de virulência: enterotoxicogênica (ETEC), enteropatogênica (EPEC), enterohemorragica (EHEC), enteroinvasiva (EIEC), e enteroagregativa (EAEC) (Nataro & Kaper, 1998; Robins-Browne & Hartland, 2002).

Escherichia coli enteropatogênica (EPEC) é um exemplo de micro-organismo cujo mecanismo patogênico pode levar a perda do equilíbrio da microbiota (Carneiro-Sampaio et al, 1996). A infecção por EPEC inicia-se pela simples aderência da bactéria à superfície de mucosa intestinal, sem invasão tecidual ou a liberação de toxinas. A patogênese da EPEC é uma sequência de eventos que começa com a ligação da bactéria a micro vilosidades dos enterócitos, possivelmente mediada por uma adesina bacteriana denominada *Bundle Forming Pili* (BFP), codificada por um gene plasmidial (Giron et al, 1991). Após há alterações metabólicas nos enterócitos com aumento de níveis de cálcio e há a ligação entre as bactérias e as células epiteliais, através de outra adesina bacteriana, uma proteína de 94 KDa,

denominada intimina, que determina a aderência e o desaparecimento das micro vilosidades dos enterócitos (Law, 1994).

A SIgA do colostro é capaz de inibir a aderência dos diferentes sorotipos de EPEC (Carbonare et al, 2005), e EAEC (De Araújo & Giugliano,2001), ETEC e a invasão da EIEC (Carbonare et al, 2005), enquanto IgA proporciona uma proteção para gastroenterite induzida por toxina (Cruz et al, 1988) e liga-se a antígenos de fatores de colonização impedindo a sua ação (Corrêa et al, 2006).

Oligossacarídeos, glicoproteínas e glicolípidos, também aparecem inibir a aderência de EPEC (De Araújo & Giugliano, 2001; Newburg et al, 2004; Morrow et al, 2005.) e ligam-se a fatores de colonização para impedira adesão de linhagens de ETEC a receptores da célula hospedeira (Oliveira et al, 2001). O componente secretor da IgA, em sua forma livre também atua como fator importante de defesa para agentes bacterianos patogênicos (Bessler et al, 2006).

Estudos também relatam que os fagócitos mononucleares do colostro tem atividade bactericida para EPEC equivalente aos fagócitos do sangue periférico (Honório-Franca et al, 1997; Schroten et al, 2000), e sugerem que o mecanismos de fagocitose das células do colostro é dependente da ação de opsoninas. Este mecanismo de ação dos fagócitos do colostro também protegem o recém-nascido de outros agentes patogênicos que causam diarréia em conjunto com os componentes solúveis presentes no próprio colostro (França-Botelho et al, 2006; França et al, 2011a; França et al, 2012) e a atividade funcional destas células pode ser um mecanismo adicional de proteção para as infecções por enterobactérias.

Interações entre componentes solúveis e celulares do colostro

A fagocitose é um dos principais mecanismos de defesa frente às infecções bacterianas. Durante a fagocitose ocorre processo oxidativo com a produção de derivados do oxigênio (Assad et al, 1994). Metabolitos ativos de oxigênio, tais como o ânion superóxido (O_2), peróxido de hidrogénio (H_2O_2) e radical hidroxilo (OH^-) contribuem decisivamente para a destruição de microrganismos (Kappar et al, 1995; Honorio-França et al, 2001; França-Botelho et al, 2010; França et al, 2011b). Este fenômeno biológico inicia-se com a adesão do microrganismo aos fagócitos, através de receptores não específicos ou específicos para proteínas presentes no soro ou no sobrenadante de colostro. Entre estes fatores, existem anticorpos, em particular a SIgA e proteínas do sistema do complemento (Honorio-França et al, 1997; Hanson et al, 2007).

Os fagócitos do colostro expressam receptores para a porção Fcα (CD89) (Patry et al, 1996; Honorio-França et al, 2001; Monteiro et al, 2003). Estudos sobre a natureza molecular

do receptor para a região Fc de IgA presente na superfície dos fagócitos mostraram que este receptor é constituído por uma proteína heterogênea, glicosilada, com tamanhos que variam de 55 a 100 KDa (Chevalier et al, 1989; Vidarsson et al, 1998; Honorio-França et al, 2001; Monteiro et al, 2003). O FcαR em fagócitos do colostro foi identificado com peso molecular de (55-75 KDa) semelhante aos dos fagócitos do sangue (Monteiro, 2003). A interação da IgA e seu receptor FcαR (CD89) ativa os mecanismos intracelulares resultando no aumento da fagocitose, liberação de ânion superóxido e da atividade microbicida (Honorio-França et al, 1997; França et al, 2011b).

A fagocitose tem início com adesão de bactérias previamente opsonizada pela IgA e se liga ao receptor CD89 presentes na superfície do fagócito. Com a entrada dos microrganismos ocorre ativação do sistema NADPH oxidase e a partir do oxigênio, há a geração o ânion de superóxido. Este radical superóxido é rapidamente metabolizado gerando outros metabólitos ativos do oxigênio, que potencialmente atuam na atividade citotóxica e antimicrobiana de fagócitos das mucosas (Honorio-França et al, 1997; Honorio-França et al, 2001; França-Botelho et al, 2006; França et al, 2011b; França-Botelho et al, 2011; França et al, 2012). O modelo hipotético do mecanismo bactericida via IgA-Fcα está esquematizado na Figura 1 (Anexo 1).

Há outros fatores importantes na ativação de fagócitos. Entre eles, destacam-se os fatores derivados de plantas e seus extratos (Scherer et al, 2011; Reinaque et al, 2012), bem como hormônios (Fagundes et al, 2012) em particular, o hormônio melatonina.

Na literatura há evidências da importância dos hormônios como potentes agentes imunomoduladores que melhoram a atividade funcional de células (Ianãs et al, 1991; Honorio-França et al, 2009). A melatonina é um hormônio secretado pela glândula pineal, e as suas ações benéficas foi associada à capacidade de remover radicais livres e aumentar a atividade antioxidante, porém suas funções ainda são parcialmente compreendidas. A melatonina pode ligar-se a fagócitos e determinar vários processos oxidativos no organismo (Kuhlwein et al, 2001). Dependendo da dose a melatonina pode ter efeito antioxidante (Vanecek et al, 1999; Reiter et al, 2000) ou exercer efeitos pro-oxidativo (Ianãs, 1991).

A melatonina estimula a liberação de metabolitos ativos do oxigênio pelos fagócitos do colostro e modula a atividade funcional destas células (Ianãs et al, 1991). Os mecanismos pelos quais a melatonina influencia a função imunológica envolvem a participação de outros hormônios, citocinas e receptores específicos (Rogers et al, 1997; Pandi-Perumal et al, 2008; Peschke et al, 2012).

Estudos sobre afinidade de vários análogos da melatonina indicam que os grupos 5-metoxi-n-acetil são necessários para interação do hormônio ao seu receptor. Com a introdução do composto (2-I125)-melatonina como marcador foi identificado três tipos de receptores,

MT-1, MT-2 e MT-3, com especificidade para a melatonina e N-acetil-Serotonina (Sugden et al, 2004). Ao utilizar o N-acetil-Serotonina (NAS) que é um precursor da melatonina com elevada afinidade para o seu receptor, observa-se que há aumento na liberação ânion superóxido e que esta interação aumentou também a atividade fagocítica e microbicida. Esta interação foi confirmada através da utilização de um antagonista do receptor da melatonina específico para o MT-3, o methoxicarbonilamina-N-acetiltriptamina (MCA-NAT) (Pickering et al, 1992; Reppert et al, 1996; Le Gouic et al; 1997; Dubocovich et al; 1998), que aumentou liberação ânion superóxido, a fagocitose e a atividade microbicida dos fagócitos do colostro e do sangue. O uso de antagonista competitivo da melatonina – Luzindol (Dubocovich et al, 1998), foi capaz de bloquear a interação melatonina-receptor e determinou menor atividade fagocitária destas células (Jockers et al, 2008).

O mecanismo pelo qual a melatonina interage com o receptor MT-3 presente na superfície de fagócitos do colostro e no sangue, aumentando a atividade funcional dos fagócitos está descrito na Figura 2 (Anexo 2).

As interações entre anticorpos e hormônios podem ser um mecanismo importante para a defesa do sistema imune de mucosa. A chave de interações entre as células e receptores sugerem um mecanismo adicional para proteção de infecções respiratórias e gastrointestinais.

Influência da hiperglicemia sobre a imunidade materna e o colostro

O aleitamento materno é recomendado por numerosas agências de saúde como alimento ideal durante o primeiro ano de vida, por causa de seus múltiplos benefícios imediatos e em longo prazo (ADA, 2009). A literatura tem reportado a relação entre amamentação e o desenvolvimento de algumas doenças, incluindo a obesidade e diabete (Taylor et al, 2005).

Diabetes mellitus é doença metabólica caracterizada por níveis elevados de glicose no sangue. É o resultado da ausência ou insuficiência de secreção de insulina do pâncreas, com ou sem comprometimento concomitante de ação da insulina (ADA, 2012) e tem sido associada a alterações no metabolismo (Balda & Pacheco-Silva 1999). Esta complicação pode preceder a gestação, como nas portadoras de Diabete Melito tipo 1 (DM1) e tipo 2 (DM2). Quando identificada na gestação, é reconhecida como DM Gestacional (ADA, 2012).

Há crescente interesse em compreender os efeitos do aleitamento materno sobre o diabete, bem como os mecanismos envolvidos. Vários estudos demostram o papel do aleitamento materno na promoção do crescimento e proteção para infecções e doenças como diabete (Taylor et al, 2005; França et al 2011b; Morceli et al, 2011; França et al, 2012). A

amamentação pode modificar a evolução natural do DM1 por proteger contra certo número de doenças virais, impedindo ou retardando o início da doença (Bognetti et al, 1992) e também é associada a melhores desfechos maternos, tais como , menor risco de câncer de mama e de ovário, e DM2 (ADA, 2009).

A curta duração na amamentação seguida pela introdução precoce de leite bovino é fator de risco para o DM1 em crianças (Gerstein et al, 1996) e a falta de amamentação também aumenta o risco de DM1 (Malcova et al, 2006), enquanto que a amamentação prolongada provavelmente reduz o risco de DM2 no futuro (Stuebe et al, 2005).

Os componentes do leite humano e as características fisiopatológicas maternas ainda é uma questão controversa. Alguns estudos sugerem que características maternas causam variações nos componentes do colostro e do leite, enquanto outros relataram que essas mudanças dependem do estado nutricional materno ou de doenças prévias (FNB, 2000; Olivares et al, 2007). Na literatura estudos buscam elucidar os efeitos do metabolismo da glicose materna e a lactação, mas pouco se sabe sobre os componentes imunológicos no colostro de nutrizes que tiveram gestações complicadas por distúrbios hiperglicêmicos. Níveis elevados de glicose podem atuar no metabolismo oxidativo celular alterando a liberação de enzimas, a produção de espécies reativas de oxigênio e a atividade funcional de células (França et al, 2011b).

O controle glicêmico inadequado durante a gestação altera as fases da lactogênese, dificultando a amamentação (Oliveira et al, 2008). Essas alterações metabólicas podem interferir na composição e nos mecanismos imunológicas do colostro e leite de nutrizes hiperglicêmicas.

A hiperglicemia materna determina alteração na composição bioquímica e imunológica do colostro (França et al, 2011b; Morceli et al, 2011; França et al, 2012). O colostro de nutrizes diabéticas apresenta maior concentração de glicose, proteína total e lipase e menor conteúdo de gordura, de anticorpos da classe IgA e IgG, de proteínas do sistema complemento e menor atividade funcional de fagócitos, Figura 3 (Anexo3) (Morceli et al, 2011; França et al, 2011b). Assim, o controle glicêmico adequado em mães diabéticas é fundamental para corrigir eventuais anormalidades na composição do leite (Vanbeusekom et al, 1993; Morceli et al, 2011).

Não foram encontrados na literatura estudos com o hormônio melatonina no colostro de nutrizes hiperglicêmicas. No entanto, sabe-se que a melatonina afeta o metabolismo da glicose (Peschke et al, 2010; Peschke et al, 2012; Korkmaz et al, 2009). Os pacientes diabéticos têm menores níveis de melatonina no soro no período diurno e maior expressão de receptores de melatonina no pâncreas (Tutuncu et al, 2005; Peschke 2008). O papel da melatonina em

prevenir ou retardar o início do diabete, no entanto, não está bem estabelecido, porque os estudos que demostram os efeitos benéficos da melatonina foram realizados depois do desenvolvimento de manifestação clínica da doença. (Hussain et al, 2006; Kadhim et al, 2006). Além disso, as ações da melatonina sobre a fisiologia endócrina do pâncreas, incluindo a provável redução na incidência do diabete não são bem descritos (Peschke, 2008).

Por outro lado, as glândulas mamárias lactantes são parte integrante do sistema imune de mucosa (Brandtzaeg, 2010). Como os componentes imunológicos que são conferidos através do aleitamento materno representam todo o repertório imunológico vivenciado pela mãe, o grau em que estes componentes imunológicos atuam depende da duração, frequência e exclusividade da amamentação. Assim, um adequado tempo de amamentação pode ser importante para mães hiperglicêmicas. Apesar das alterações em componentes bioquímicos e imunológicos, as mulheres com diabete devem ser encorajados a amamentar e prolongar o tempo de amamentação, pois o leite é uma excelente fonte de nutrientes e componentes imunológicos, e ainda as taxas de desenvolvimento de crianças amamentadas estão relacionadas com a quantidade total de leite que consomem e o tempo de amamentação.

Objetivos

OBJETIVO GERAL

Avaliar a atividade funcional de fagócitos do leite de mães hiperglicêmicas leve e diabéticas e suas interações com anticorpos IgA e o hormônio melatonina.

OBJETIVOS ESPECÍFICOS

- ✓ Analisar a concentração de proteínas totais, anticorpos (IgA, IgG e IgM) e proteínas do sistema complemento (C3 e C4) no sobrenadante de colostro e no sangue;
- ✓ Analisar a composição bioquímica de enzimas (SOD, lípase, amilase) e de gorduras e calorias no sobrenadante de colostro e no sangue;
- ✓ Analisar a ativação celular no colostro, através da liberação do ânion superóxido na presença de IgA e Melatonina;
- ✓ Avaliar a fagocitose, a atividade microbicida e o mecanismo de ativação dos fagócitos do colostro, na presença de IgA e Melatonina;
- ✓ Avaliar o mecanismo de ativação dos fagócitos do colostro via IgA-receptor CD89 e via Melatonina-Receptor.

Método

DESENHO E LOCAL DO ESTUDO

Estudo prospectivo, tipo corte transversal, realizado no Serviço Especializado de Diabete e Gravidez da Faculdade de Medicina de Botucatu/UNESP (FMB-UNESP), referência para as gestações complicadas por diabete e hiperglicemia gestacional leve.

TAMANHO AMOSTRAL

Para o cálculo do tamanho amostral considerou-se a diferença das médias de hemoglobina glicada entre os grupos hiperglicemia gestacional leve e *diabete mellitus* gestacional, pela menor distância entre os resultados e o maior tamanho da amostra. Assumindo uma perda de seguimento da ordem de 10% e corrigindo para os efeitos dos erros α (1%) e β (20%) atribuídos ao estudo, estimou-se a necessidade de avaliar, no mínimo, 30 gestantes por grupo.

VARIÁVEIS

Variáveis de Controle

a) Presença (sim) ou ausência (não) de tabagismo e de hipertensão arterial (NHBPEP, 2000);
b) Idade gestacional, em semanas completas, na entrada do protocolo de tratamento do Serviço;
c) Média glicêmica mantida na gestação, categorizada em adequada (MG <120mg/dL) e inadequada (MG ≥120mg/dL) (Rudge et al, 1995; Rudge et al, 2000);
d) Tipo de diabete e distúrbios hiperglicêmicos.

Variáveis Independentes

Grupos de gestantes:

a) ND (não diabéticas com fator de risco para diabete gestacional) (TTG100g e PG normais).
b) HGL (hiperglicemia gestacional leve) (TTG100g normal e PG alterado);
c) DM (diabete melito, gestacional, ou clínico tipo 1 e tipo 2) (TTG100g e PG alterados);

Variáveis Dependentes

a) Determinação da concentração de proteínas totais, proteínas do sistema complemento (C3 e C4)e anticorpos (IgA, IgG e IgM) no sangue e sobrenadante de colostro de mães hiperglicêmicas;
b) Determinação da composição de enzimas (SOD, lipase, amilase) e de gorduras e calorias no sangue e sobrenadante de colostro de mães hiperglicêmicas;

c) Determinação da fagocitose e atividade microbicida pelas células do colostro na presença de bactéria opsonizada pela IgA;
d) Determinação da fagocitose, atividade microbicida pelas células do colostro estimuladas por melatonina na presença de bactéria;
e) Determinação dos mecanismos de interação entre IgA-receptor e melatonina-receptor.

SELEÇÃO DOS SUJEITOS

Identificação dos sujeitos

As gestantes com potencial para a inclusão no estudo foram identificadas no Ambulatório Especializado de Diabete e Gravidez e na Maternidade do Hospital de Clínicas da FMB/UNESP. Para isso foi desenvolvido protocolo específico, considerando os critérios de inclusão, exclusão e descontinuidade pré-definidos. Diante de um caso elegível, o pesquisador principal explicava os objetivos e as técnicas de coleta de sangue e de obtenção do colostro e, se de acordo com a participação, a gestante assinava o Termo de Consentimento Livre e Esclarecido.

Os critérios de inclusão, exclusão e descontinuidade estão descritos a seguir.

Critérios de Inclusão

a. Ser classificada nos grupos definidos no delineamento do estudo;
b. Idade gestacional mínima de entrada no protocolo de tratamento de 30 semanas, para as portadoras de HGL e DMG, e de 20 semanas para as DM 1 e 2;
c. Idade gestacional no parto entre 37 a $41^{6/7}$ semanas, reações sorológicas negativas para hepatite, HIV e sífilis;
d. Realizar assistência pré-natal e parto no Serviço;
e. Assinar o Termo de Consentimento Livre e Esclarecido.

Critérios de Exclusão

a. Gravidezgemelar;
b. Malformações fetais.

Critérios de Descontinuidade

a) Abandono do pré-natal no Serviço;
b) Malformação fetal diagnosticada no momento do parto;
c) Perda de dados relativos ao parto e ao período neonatal.

TÉCNICAS DE COLETA E PROCESSAMENTO DAS AMOSTRAS

Métodos de coleta e avaliação

A coleta de sangue materno foi realizada entre 37 a 42 semanas de gestação, em tubos Vacutainer. O colostro foi coletado manualmente, com técnica adequada, sempre no período da manhã e no intervalo entre duas mamadas, no período correspondente entre 48 a 72 horas após o parto. A quantidade máxima de sangue e colostro coletada foi de 15 ml. Essas técnicas seguiram as orientações protocolares das boas práticas clínicas.

Média glicêmica da gestação

Foi calculada pela média aritmética de todas as dosagens de glicemia plasmática observadas nos perfis glicêmicos realizados durante a gestação: (i) a partir do diagnóstico realizado entre a 24ª e a 28ª semana de gestação para os grupos: HGL e DM e (ii) a partir da primeira consulta de pré-natal para gestantes com DM prévio à gestação (DM1 e DM2). Como a Média Glicêmica (MG) da gestação é marcador da qualidade do controle glicêmico materno, todos os resultados obtidos no estudo foram ajustados pelos níveis da média glicêmica obtida nos diferentes grupos, categorizados em MG < 120 mg/dL (controle adequado) e MG ≥ 120 mg/dL (Rudge et al, 2000).

Métodos de processamento e análises das amostras

Obtenção do soro

As amostras de sangue foram centrifugadas por 15 minutos a 160G a temperatura ambiente. O soro foi separado e armazenado a -80°C para análises posteriores.

Obtenção do sobrenadante do colostro

As amostras de colostro foram centrifugadas por 10 minutos a 160G sob-refrigeração a 4°C, separando-as em três fases: o "botão" celular, a porção fluida intermediária e a camada superior de gordura. A camada superior composta de gordura foi desprezada, o sobrenadante (porção fluida) foi reservado para posterior análise e, o "botão" celular, conservado para a avaliação da atividade funcional dos fagócitos.

Determinação dos níveis de glicose

Os níveis de glicose foram determinados pelo método enzimático. Para isso, utilizou-se 20µL de sobrenadante de colostro, soro e o padrão (concentração 100 mg / dL - Doles) e foram colocados em solução tampão fosfato 2,0 mL a 05 M, pH7.45, (contendo 0.03mm amino-antipiridina, 15 mM de hidroxibenzoato de sódio, 12U/L glicose e 0,8 U/L oxidase peroxidase).

As suspensões foram misturadas e incubadas por 5 min a 37°C. As reações foram lidas em espectrofotômetro na faixa de DDO a 510 nm.

Determinação da concentração de proteínas totais

As proteínas totais foram determinadas pelo método colorimétrico. Foram pipetados 20µl de amostras de colostro, soro e o padrão (concentração de 4g/dL - Labtest) e colocados em 1,0 mL de reagente Bioret (íons de cobre em meio alcalino). As suspensões foram misturadas e incubadas por 10 min a 37°C. A seguir as reações foram lidas em espectrofotômetro na faixa de DDO a 545 nm.

Determinação da concentração de amilase

Amilase foi determinada pelo método colorimétrico. Foram pipetados 500µL de substrato (amido de 0,4 g/l + tampão fosfato 100nmol/L, pH 7,0) e incubado por 2 min a 37°C. As amostras de colostro e soro (10µL) foram incubadas com o substrato por 7 min a 37°C. Após este período, 500 mL de solução de iodo foram adicionados (50mmol/L). As suspensões foram misturadas e a leitura realizada em espectrofotômetro na faixa de DDO a 660 nm. Foi realizado ensaio de controle utilizando-se apenas o substrato e iodo.

Determinação da concentração de lipase

A lipase foi determinada pelo método colorimétrico. Amostras de 1 ml de colostro e de soro foram misturadas com o tampão Tris (100 mmol/L de metano hidroximetilamina pH 8.5, fluór fenilmetil sulfonil 8 mmol/L, 3 mmol/L de ácido ditionitrobenzóico - DTNB e 100 mmol/L de acetato de sódio, pH 6,0) e incubadas por 2 min a 37°C. Após esse período, 100 ml de tributitato de propanol piridina (20 mmol/L e surfactante) foram adicionados à solução e agitados por 30 min a 37°C. Após adicionou-se 2,0 mL de acetona (PA), as suspenções foram homogeneizadas e mantidas em temperatura ambiente por 3 min. Em seguida foram centrifugadas a 400G durante 5 min e a leitura foi realizada em espectrofotômetro, na faixa de DDO a 410 nm. Foi realizado ensaio de controle sem flúor fenilmetil sulfonil (inibidor enzimático).

Determinação da concentração da superóxido dismutase (CuZn-SOD)

A análise da enzima CuZn-SOD foi realizada pelo o método de redução do "Nitro Blue Tetrazolium" (NBT – Sigma - Novelli et al, 1993). Amostras de colostro, sangue foram colocadas em tubos de vidro e tubos padrão e branco. O volume para cada amostra foi de 0,5 ml. No tubo padrão adiciou-se 0,5 ml de solução hidro alcóolica e em todos os tubos foram adicionados 0,5 ml de solução cloroformio-etanol (proporção 1:1) e 0,5 mL da mistura reativa

(NBT e EDTA). Em seguida acrescentou-se 2,0 ml de solução carbonato-hidroxilamina pH 10.2 no padrão e nas amostras.

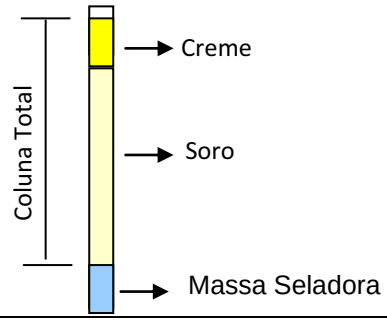
Todos os tubos permaneceram em repouso durante 15 minutos. A leitura foi realizada espectrofotômetro, na faixa de DDO a 560nm, acertando o zero com a mistura reativa. Para determinar os resultados superóxido dismutase (SOD) utilizou-se a fórmula:

CuZn SOD-= (Ab padrão - Ab amostra / Ab padrão) x 100 =% de redução de NBT / CuZn - SOD.

Os resultados foram expressos em unidades internacionais (UI) de CuZn-SOD.

Determinação do teor de gordura e da concentração de calorias

As amostras de colostro foram aquecidas a 40°C por 15 minutos em banho-maria e submetidasà agitação. Os tubos capilares (2 µl) foram preenchidos com cerca de ¾ da amostra, selados com lacre e, em seguida, centrifugado por 15 min. A centrifugação separou as amostras em forma de creme e soro.



A coluna de creme e a coluna total foram medidas, e o teor de gordura e calorias calculadas pelas seguintes fórmulas:

1. Teor de Gordura = % creme (mm) - 0.59 / 1.46,

onde a % creme = coluna de creme (mm) x 100/ coluna total (mm);

2. Kcal / L = (66.8 x % creme) + 290.

Determinação das concentrações de IgA, IgM e IgG

As concentrações de IgA, IgG e IgM no soro e no colostro foram determinadas por imunodifusão radial quantitativa (RID) de acordo com a técnica de Mancini et al (1965). Tubos contendo 10 ml de agarose 1% foram aquecidos até a fusão em banho-maria, e a seguir transferidos para banho a 56°C para estabilizar a temperatura. Os anticorpos foram adicionados à agarose e misturados por inversão do tubo. As misturas foram colocadas entre duas placas de vidro, separadas por um espaçador. Após a solidificação, as placas foram perfuradas e as amostras aplicadas.

Para a determinação de IgA, IgG e IgM do colostro e do soro utilizou-se a curva padrão Kallestad.

Determinação da concentração de C3 e C4

As concentrações de C3 e C4 presentes no colostro e sangue foram determinadas pelo método de turbidimetria. Amostras de colostro foram diluídas 1:12 (v / v) com solução salina (9 g / l), os níveis de C3 e C4 foram determinados utilizando-se soros anti-C3 e anti-C4 (Bioclin). A curva padrão de calibração foi obtida pelo Multical (Bioclin). As suspensões foram misturadas e incubadas a 37^{o}C por 15 min. As reações foram lidas em espectrofotômetro,na faixa de DDO a 340 nm.

Dosagem do hormônio melatonina pelo método imunoenzimático

Para a dosagem do hormônio melatonina foi utilizado o Kit Melatonin ELISA (IBL). A extração da melatonina foi realizada pelo método de cromatografia de afinidade utilizando colunas padronizadas. As colunas foram colocadas em tubos de vidro e centrifugadas duas vezes com 1 ml de metanol (1 min. - 200g). A seguir as colunas foram lavadas 2 vezes com água bidestilada (1 min- 200g). Após a preparação das colunas 0,5 ml dos padrões, controles e das amostras foram aplicados e centrifugados por 1 minuto a 200g. Após aplicação das amostras e padrões as colunas foram novamente lavadas com 1,0 ml de metanol a 10 % por 1 min a 500G. A seguir realizou-se a extração do eluato contendo o hormônio melatonina, pela adição de 1,0 ml de metanol a 200G. Após a obtenção do eluato, realizou-se a evaporação do metanol através de centrífuga evaporadora (speed-vac). O material foi reconstituído com 0,15ml de água bidestilada sob agitação durante 1 min. e imediatamente analisado. 50 μl de cada padrão, controle e amostras de colostro e leite foram colocados em placa de ELISA com 50 μl de melatonina-biotina em cada poço com 50 μl de anti-soro. A placa foi incubada a 4°C durante 20 horas. Após este período o sobrenadante foi descartado, a placa foi lavada 3 vezes com tampão de lavagem e colocou-se 150 μl da enzima conjugada. Após 120 min de incubação à temperatura ambiente a placa foi novamente lavada por 3 vezes e adicionou-se dentro de cada poço 200 μl do substrato p-nitrophenyl phosfatase (PNPP) e incubado por mais 40 min sob agitação. Após este período a reação foi bloqueada com 50 μl de solução "PNPP stop" e a leitura foi realizadaem espectrofotômetro para placas com filtro de 405 nm. Os resultados foram calculados através da curva padrão e expressos em pg/ml.

Obtenção do "pool" de sobrenadante de colostro humano

O "pool" de sobrenadante do colostro foi obtido por centrifugação das amostras de diferentes puérperas normoglicêmicas, conforme descrito anteriormente. Essas amostras de colostro foram misturadas e centrifugadas durante 10 min sob-refrigeração a 4°C. O "pool" formado foi utilizado para a separação e obtenção de IgA purificada. Também alíquotas foram estocadas a -80°C para posterior opsonização da bactéria.

Separação de IgA do "pool" de colostro em coluna de afinidade anti-cadeia α

Para verificar as interações entre a IgA do colostro com o receptor Fcα foi realizado a separação de SIgA a partir do "pool" de colostro humano obtido por cromatografia de afinidade em coluna de Sepharoseanti-IgA.

Utilizou-se a coluna de Sepharose 4B ligada a anti-IgA humana, para absorção de IgA do "pool" de colostro. O preparo da Sepharoseanti-IgAfoi realizado de acordo com a técnica de March et al (1974).

A concentração de IgA presente no eluato foi determinada pela técnica de Mancini et al. (1965), conforme descrito anteriormente no item determinação dos anticorpos. As alíquotas foram estocadas a -80°C para posterior opsonização da bactéria.

Obtenção do "pool" de soro humano normal

O "pool" de soro humano normal foi obtido a partir de10 amostras de sangue. As amostras foram centrifugadas para obtenção do soro. Volumes iguais dessas amostras de soro foram misturados e centrifugados durante 10 min sob temperatura ambiente. As alíquotas foram estocadas a -80^{o}C para posterior opsonização da bactéria.

Linhagem e Culturas de <u>Escherichia coli</u> Enteropatogênica (EPEC)

Utilizou-se a *<u>Escherichia coli</u>* enteropatogênica (EPEC) HMJ 0041-1-85, sorotipo 0111: H- AL-, eae+,eaf+, bfp+ e conservada a -70°C. A cultura estoque foi mantida em ágar semi-sólido em ausência de luz. A partir desta cultura de estoque, 18 horas antes do ensaio, repiques foram feitos em tubos contendo 8 ml de TSB (TrypicSoyBroth - Difco), e incubados em estufa a 37°C por 18 horas.

Após o crescimento, as bactérias foram lavadas 2 vezes em solução salina tamponada (PBS) e a concentração ajustada para 1x10^{8} bactérias por ml, medida com espectrofotômetro (540nm, DO= 0.1).

Opsonização da EPEC

EPEC foi opsonizada conforme descrito por Bellinati-Pires et al (1989). Três opsoninas foram usadas:

a) Sobrenadante de colostro. Pool de sobrenadante de colostro (concentração de imunoglobulinas (g/l): IgA=7.4; IgG=0.15 e IgM=0.37).

b) SIgA purificada do colostro. A concentração da IgA purificada foi de 4,0 g/L. Para a realização dos ensaios foi ajustada para concentração de 0,74 g/L (proporção equivalente a 10% de SIgA contida em amostra padrão de colostro- Honorio-França et al, 1997).

c) Soro humano normal. Pool de soro humano normal (concentração de imunoglobulinas (g/L): IgA=2.64; IgG=14.0 e IgM=2.0.)

Colostro, soro e IgA purificada foram descongeladas e misturadas com volumes de suspensão de bactérias (concentração final de $2x10^7$ bacteria/mL e 10% de opsoninas). Outra suspensão de bactérias foi preparada na mesma concentração em meio 199 sem opsoninas e utilizada como controle de bactérias não tratadas. Todas as suspensões de bactérias foram incubadas por 30 min a 37°C e usadas nos experimentos.

Obtenção de fagócitos do colostro

Após centrifugação prévia, as células do colostro foram ressuspendidas em meio de cultura 199 (Gibco), e separadas em gradiente de densidade com Ficoll-Paque (Pharmacia) por 40 min a 160 G sob temperatura de 4°C.

A separação com o Ficoll-Paque permite que os fagócitos polimorfonucleares sedimentem-se no tubo formando o "botão" celular e há a formação de um anel rico em células mononucleares na interface entre o meio de cultura e o Ficoll-Paque. O anel celular rico em células mononucleares e o botão celular foram retirados separadamente e lavados duas vezes em meio de cultura 199 (Gibco). As células foram contadas em câmara de Neubauer, e as concentrações celulares ajustadas para 2×10^6 células/ml.

Marcação dos fagócitos do colostro com os monoclonais anti-receptor Fcα (CD89) e anti-IgA para a análise de imunofluorescência

Para verificar a presença dos receptores Fcα, as células do colostro foram pré-incubadas com 10 μl de IgG humana na concentração de 10 mg/ml, durante 20 min a 4°C para o bloqueio dos receptores Fcγ. A seguir, as células foram incubadas com 10 μl do anticorpo monoclonal anti-FcαR (CD89- 0.1 mg/ml) durante 30 min a 4°C. Foram utilizados anticorpos monoclonais anti-receptorFcα (CD89) e monoclonal IgG1 específico para FcαRI como controle. Após o período de

incubação, as células foram lavadas 2 vezes em PBS contendo 5% de BSA (soro bovino albumina) e 0,1% de azida. Os anticorpos monoclonais biotinizados foram incubados com ficoeritrina (PE) por 30 min a 4°C. A seguir as células foram lavadas em PBS (contendo 5% de BSA e 0,1% de azida) e analisadas através de citometria de fluxo.

Para verificar a presença de IgA de superfície dos fagócitos, as células foram incubadas com anticorpo monoclonal anti-IgA humana marcado com fluoresceína (FITC - Sigma). Para analisar a interação da SIgA aos receptores Fcα dos fagócitos do colostro, as células foram incubadas com a SIgA purificada do próprio colostro (1mg/ml) durante 60 min a 4°C. A seguir foram lavadas duas vezes com PBS contendo 5% de BSA. Os fagócitos foram incubados com anticorpo monoclonal anti-IgA humana marcado com fluoresceína (FITC - Sigma) durante 30 min a 4°C. Após esse período as células foram lavadas e analisadas por citometria de fluxo. O controle foi realizado com anticorpo monoclonal anti-IgG humana marcado com fluoresceína (FITC - Sigma). A ligação da SIgA purificada do colostro aos fagócitos foi determinada pela diferença entre o índice de imunofluorescência obtido com o monoclonal anti-IgA humana (IgA de superfície) e o índice de imunofluorescência obtido quando adicionou-se a IgA purificada do colostro.

Tratamento dos fagócitos mononucleares do colostro com MoAb anti- receptor Fcα

Para verificar o efeito da interação SIgA-FcαRI sobre a liberação do ânion superóxido, a fagocitose e a atividade microbicida, fagócitos mononucleares ($2x10^6$ cel/mL) foram incubados com 50μL de MoAb reativo com receptor FcαRI humano (0,1mg/mL- Medarex, Inc., West Lebanon, NH, (Shen, 1992), anticorpo monoclonal que liga-se ao domínio EC1 do FcαRI e pode bloquear a ligação da IgA (Monteiro et al, 2003) por 2h a 37°C. A seguir foram lavados uma vez com meio 199 a 4°C e imediatamente usado nos ensaios.

Incubação dos fagócitos do colostro com o hormônio melatonina, agonista, precursores e inibidores

A incubação dos fagócitos com o hormônio melatonina, N-acetil-serotonina (NAS), Luzindol e Cloromelatonina (CMLT), para modular a atividade funcional dos fagócitos foi de 30 min. Para cada ensaio realizado, como controle dos experimentos, os fagócitos MN ($2 x10^6$cel/ml) foram incubados por tempo similar, dependendo do tipo de ensaio, em meio 199 na ausência do hormônio. A concentração utilizada para o hormônio melatonina, NAS, Luzindol e CMLT foi de 100 ng/ml, concentração determinada previamente através de curva dose-resposta (França et al, 2009).

Dosagem de ânion superóxido

Para verificar a ativação celular realizou-se o ensaio de liberação de ânion superóxido com cromógeno Ferricitocromo C, segundo o método de Pick & Mizel (1981). Na presença do ânion superóxido o ferricitocromo C sofre oxidação passando a ferrocitocromo C e a mudança colorimétrica é detectável em espectrofotômentro com filtro de 550nm.

Após a separação das células e incubação com os diferentes moduladores, volumes iguais de bactérias e células foram incubados em tubos plásticos, por 15 min a 37°C sob agitação para ocorrer à fagocitose. A seguir a suspensão de células e bactérias foi centrifugada a 160G, o sobrenadante desprezado e retirado o excesso de bactéria extracelular. A suspensão de células e bactéria foi ressuspendida em 0,5 ml de PBS glicosado contendo ferricitocromo C (concentração de 2mg/ml). O controle contendo somente células foi realizado paralelamente para a verificação da liberação espontânea do ânion superóxido pelas células. As suspensões foram colocadas em placas de cultura celular de 96 poços, com volume de 100μl por poço e deixadas em estufa a 37°C durante 1 hora. A leitura foi feita em espectrofotômetro para placa com filtro de 550 nm. A concentração do ânion superóxido foi calculada através da seguinte relação: Concentração O_2^-(nmol) =DO/6,3 x 100.

Avaliação da fagocitose e atividade bactericida dos fagócitos do colostro

A atividade microbicida dos fagócitos do colostro foi avaliada pela técnica de alaranjado de acridina (Bellinate-Pires et al, 1989). Volumes iguais de suspensão de bactérias ($2x10^7$ bactérias/ml) e de suspensão de células ($2x10^6$ cel/ml - fagócitos mononucleares) foram misturados em tubos plásticos. O volume final, da mistura fagocitária, dependeu do rendimento celular das amostras de colostro e não inferior a 0,5 ml. A suspensão de EPEC e células foram submetidas à incubação prévia por 30 min sob agitação a 37°C. Após esse período a fagocitose foi interrompida pela adição de meio de cultura (meio 199) gelado e suspensão foi centrifugada por 10 min a 160G sob refrigeração a 4°C. O "pellet" foi corado com 200 μl de alaranjado de acridina (concentração 14,4 mg/ml) por 1 min e a seguir foiressuspendida em meio 199, centrifugado e lavado mais uma vez. A seguir foram feitas lamínulas e a contagem das células foi em microscópio de fluorescência. O índice de fagocitose foi calculado pelo número de células que ingeriram pelo menos três bactérias, em um total de 100 células. O índice microbicida foi calculado pelo número de células com bactérias mortas, no total de 100 células com fagocitose positiva (França et al, 2011b).

Liberação de cálcio intracelular por citometria de fluxo

Utilizou-se a imunofluorescência com o marcador Fluo-3 para avaliar a liberação de Ca^{2+} intracelular em fagócitos do colostro. Suspensões de células foram pré-incubadas ou não com TMB8 e tratadas com melatonina, NAS, CMLT e luzindol, a 37°C por 30 minutos sob em agitação. A suspensão foi centrifugada duas vezes (160xg, 10 min, 4°C) e ressuspendida em PBS contendo BSA (5mg/ml-1) e incubadas com 5 µL de Fluo-3 (1µg .mL-1) por 30 min a 37 °C. Após a incubação, as células foram lavadas duas vezes em PBS contendo BSA (5mg. mL-1, 160 x g, 10 min, 4° C). Em todos os experimentos, as células foram analisadas por citometria de fluxo. O estudo foi realizado no FACS Calibur (BD San Jose USA). Calibração e sensibilidade foram rotineiramente verificada utilizando CaliBRITE 3 Beads (Cat BD. N°340486 USA). Fluo-3 foi detectada em filtro de 530/30 nm. A proporção de liberação intracelular de Ca^{2+} foi expressa pela média geométrica (%) de intensidade de fluorescência de Fluo-3. Os experimentos foram repetidos com células de 5 amostras de colostro de diferentes doadoras, de cada grupo experimental.

Análise estatística

Para avaliar os níveis de glicose, a concentração de anticorpos, as proteínas do sistema complemento, calorias, gordura, amilase, lipase e superóxido dismutase. Para a comparação das concentrações no sangue e colostro, utilizou-se Análise de Variância (ANOVA), considerando-se o nível glicêmico e os líquidos biológicos (colostro ou soro) [*two way ANOVA*].

Para avaliar os índices de liberação do ânion superóxido, fagocitose e atividade bactericida, na presença ou ausência da EPEC opsonizada, ou tratada por melatonina, ea liberação de cálcio, utilizou-se o teste de Análise de Variância (ANOVA). Os resultados foram considerados significativos quando $p< 0,05$.

Aspectos Éticos

O projeto de pesquisa deste estudo foi aprovado pelo Comitê de Ética em Pesquisa (CEP) da Faculdade de Medicina de Botucatu, protocolo número 2903-2008.

Resultados

RESULTADOS_PUBLICAÇÕES

Artigo 1_Morceli G, França EL, Magalhães VB, Damasceno DC, Calderon IMP, Honorio-França AC. Diabetes induced immunological and biochemical changes in humam colstum. Acta Paediatrica. 100, 550-556, 2011.

Artigo2_França EL, Morceli G, Fagundes DLG, Rugde MVC, Calderon IMP, Honorio-França AC. Secretory IgA-Fcα Receptor interaction modulating phagocytosis and microbicidal activity by phagocytes in human colostrum of diabetics. APMIS. 119, 710-719, 2011.

Artigo 3_Morceli G, Honorio-França ACH, Fagundes DLG, Calderon IMP, França EL. Antioxidant effect of melatonin on the functional activity of colostral phagocytes in diabetic women. Plos One (*submitted*).

ARTIGO 1

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REGULAR ARTICLE

Diabetes induced immunological and biochemical changes in human colostrum

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ABSTRACT

Aim: This article describes the changes and relationships between biochemical and immunological parameters in the colostrum and serum of diabetic women.

Methods: Colostrum and blood samples were collected from 30 diabetic and 15 normoglycaemic women. Glucose, total protein, antibody, complement proteins (C3 and C4), fat and calorie content, amylase, lipase and superoxide dismutase (SOD) were determined.

Results: Glucose was higher in both the colostrum and serum of diabetic mothers compared to that of their normoglycaemic counterparts. In both groups, total protein was higher in colostrum than in serum. IgA and IgG were lower in the colostrum of hyperglycaemic mothers, whereas IgM did not vary between the groups. Colostral C3 protein was significantly lower in diabetic mothers, but colostral C4 protein was similar between normoglycaemic and hyperglycaemic mothers. Fat content was lower in the colostrum of the diabetic mothers, although calorie content did not vary between the groups. Amylase was lower in colostrum than in serum in both groups. Lipase was higher in the colostrum and serum of diabetic mothers. Colostral SOD was similar between the groups.

Conclusions: Our results support the hypothesis that the colostrum of diabetic mothers suffers biochemical and immunological alterations that affect the levels of its components.

INTRODUCTION

Breastfeeding promotion is an important public health strategy to mitigate infant and child morbidity and mortality as well as maternal morbidity, thereby averting healthcare costs (1). Besides being the safest and most natural way to feed a neonate (2), breastfeeding has been proven to protect neonate against a wide range of infectious and noninfectious diseases. Therefore, efforts have been directed to identify the various immunoactive substances in human breast milk that account for the protective effects observed (2,3).

Human milk is important to nutrition and immunological defence of human infants. It has an appropriate balance of nutrients provided in easily digestible and bioavailable forms (4). Even colostrum, the first lacteal secretion produced, contains the nutrients needed for growing infants and provides adequate amounts of lipids, carbohydrates and proteins (1).

Antibodies that are specific against microorganisms such as viruses, bacteria and parasites are also found in human milk and may colonize newborn gut just after delivery. Human milk is particularly rich in secretory IgA antibodies (sIgA) (1,2,5), which play a protective role against several microorganisms (6,7). It also contains other immunoglobulin isotypes, such as IgG and IgM, which provide complementary protection in the respiratory mucosa and gastrointestinal tract of newborns (8).

Colostrum and human milk also contain complement system proteins, particularly C3 and C4. Although found at lower amounts in milk than in blood, these proteins act primarily as opsonins (9). They are thus important for phagocytosis and microbicidal activity (6). Other studies have shown that, under specific circumstances, IgA, complement factors and receptors participate in the IgA-mediated triggering of the effector function of cells (10).

Breastfeeding reduces the incidence of acute illnesses and likely decreases the risk of a number of chronic diseases. Earlier studies on the long-term effects of breastfeeding showed that it affects blood pressure, obesity/overweight and diabetes (11). Clinical, epidemiological and experimental studies suggest that the baby's diet is implicated in the

Abbreviations
ANOVA, Variance analysis; C3, Complement 3; C4, Complement 4; CuZn-SOD, CuZn-superoxide dismutase; EDTA, Ethylenediamine tetraacetic acid; GI, Glycaemic index; GTT, Glycaemia tolerance test; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; NBT, Nitroblue tetrazolium; SD, Standard derivation; sIgA, Secretory immunoglobulin A; SOD, Superoxide dismutase.

etiopathogenesis of diabetes (12). After adjusted for birth weight, parental diabetes, socioeconomic status and body size, breastfeeding is in fact associated with decreased risk of type 2 diabetes later in life (11). In contrast to breastfed infants, formula-fed infants have higher concentrations of blood glucose (12).

Diabetes mellitus is a metabolic disease characterized by elevated blood glucose levels. It results from the absence or inadequate pancreatic insulin secretion with or without concurrent impairment of insulin action (1). Despite the importance and high incidence of this disorder, the immunoreactive proteins, carbohydrate, fat and enzymes in the colostrum of diabetic women are still not understood.

In this article, we describe changes and relationships among carbohydrate, immunoreactive protein, enzyme, fat and calorie levels in the colostrum of normoglycaemic and hyperglycaemic women. Serum samples of the subjects were also studied to allow comparisons between the levels of the components studied.

MATERIALS AND METHOD

This cross-sectional study evaluated 45 diabetic and normoglycaemic women (18–35 years of age) treated at the Diabetes and Pregnancy Service of the Obstetrics Discipline of Botucatu Medical School, UNESP, Botucatu, SP. The volunteers signed an informed consent form before entering the study, which was approved by the local ethics committee. All the women were evaluated at the service, and those with hyperglycaemia were included in a treatment protocol to control maternal glycaemia (13).

Subject evaluation

During prenatal care, 30 pregnant volunteers were diagnosed with diabetes, and 15 were confirmed to be normoglycaemic by the Glycaemia Tolerance Test (g-OGTT 100 g) and by their glycaemic profile (GP) (13). Colostrum and blood samples from these subjects were analyzed according to maternal glycaemic status: normoglycaemic (normal 100 g-OGTT and normal GP; n = 15) and diabetes clinical (abnormal pre-pregnancy 100 g-OGTT and insulin dependent; n = 30). The mean and standard deviation for gestational age were 38 ± 0.8 weeks in normoglycaemic and 37 ± 0.2 weeks in hyperglycaemic groups and newborns' birth weight were 2048.3 ± 411.8 in normoglycaemic and the 3140.1 ± 492.1 in hyperglycaemic groups. The subjects continued attending the facility, irrespective of diagnosis, and the hyperglycaemic patients followed a specific treatment for glycaemic control (13). The variables controlled in both groups during pregnancy were smoking status (yes/no), arterial hypertension (yes/no) and glycaemic index (GI), which was the mean level of plasma glucose measured over the gestation. GI was classified as adequate (GI < 120 mg/dL) and inadequate (GI ≥ 120 mg/dL) (13).

Colostrum sampling

About 48–72 h post-partum, 15-mL colostrum was collected from the volunteers. Supernatant was obtained by colostrum centrifugation at 160 G for 10 min at 4°C. The upper fat layer was discarded, and the aqueous supernatant was stored at −80°C for later biochemical and immunological analyses.

Blood sampling

We collected 15 mL of blood from each mother in tubes without anticoagulant. We centrifuged the blood samples at 160 G for 15 min, until serum separation. Serum samples were stored individually at −80°C for further glucose, enzyme and protein determination.

Glucose determination

Glucose levels were determined by the enzymatic system. Samples of 20 μL colostrum/serum, standard of 100 mg/dL (Doles), were placed in 2.0 mL phosphate buffer solution (0.05 M, pH7.45, with aminoantipyrine 0.03 mM, 15 mM sodium p-hydroxybenzoate, 12 kU/L glucose oxidase and 0.8 kU/L peroxidase). The suspensions were mixed and incubated for 5 min at 37°C. The reactions were read on a spectrophotometer at 510 nm.

Total protein determination

Total protein was determined by the colorimetric method. Samples of 20 μL of colostrum/serum, standard of 4 g/dL (laboratory test), were placed in 1.0 mL Biuret reagent (ions of copper in alkaline medium). The suspensions were mixed and incubated for 10 min at 37°C. The reactions were read on a spectrophotometer at 545 nm.

Separation of colostrum and blood leucocytes

The colostrum and blood leucocytes were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden) as described by Honorio-França et al. (6).

IgA, IgM and IgG determination

Serum/colostrum concentrations of IgA, IgG and IgM were determined by quantitative radial immunodiffusion (RID), according to Mancini et al.'s technique (14). A tube containing 10 mL of 1% agarose was heated to fusion in a double boiler and then transferred to bath at 56°C for temperature stabilization. Anti-human IgA, lamb serum (Biolab), anti-human IgM (Sigma) and anti-human IgG (Sigma) antibodies were added to the agarose and mixed by tube inversion. The mixture was placed between two glass plates separated by a spacer. After solidification, the plates were perforated and the samples applied. Antibody content in the colostrum samples was determined using the Kallestad standard curve.

C3 and C4 determination

The concentrations of C3 and C4 were determined by the turbimetric method. Colostrum and serum samples were diluted at 1:12 (v/v) with a saline solution (9 g/L). C3 and C4 levels were determined in sample supernatants using C3 and C4 antisera (Bioclin) diluted at 1:12 (v/v). A calibration curve obtained by the Multical (Bioclin) calibrator was used to determine the standard curve. Samples of 10 μL of colostrum standard and positive or negative control sera (Bioclin) were placed in 500 μL buffer solution (sodium

chloride 0:15 mol + L, Tris 50 mmol + L, 6.0000 PEG 50 g + L, sodium azide 15:38 nM). The suspensions were mixed and incubated for 15 min at 37°C. The reactions were read on a spectrophotometer at 340 nm.

Creamatocrit analysis
Colostrum samples were water-bath-heated at 40°C for 15 min and subjected to vortex mixing. Capillary tubes (2 μL) were filled to approximately three quarters with the samples, sealed with sealing wax and centrifuged for 15 min. Centrifugation separated the samples into cream and serum. The cream column and the total column were measured, and fat and Kcal content calculated using the following formulae:

$$\text{Fat content} = \%\,\text{cream} - 0.59/1.46,$$

where the % cream = cream column (mm) × 100/total column (mm);

$$\text{Kcal/L} = (66.8\% * \%\,\text{cream}) + 290.$$

Amylase determination
Amylase was determined by the colorimetric method. A volume of 500 μL substrate (amide 0.4 g/L + phosphate buffer 100 nM, pH 7.0) was incubated for 2 min at 37°C. Samples of 10 μL colostrum/serum were inoculated in the substrate and incubated for 7 min and 30 s at 37°C. After this period, 500 μL of iodine solution was added (50 mM). The suspensions were mixed and read on a spectrophotometer at 660 nm. A control assay was carried out using only the substrate and iodine.

Lipase determination
Lipase was determined by the colorimetric method. Samples of 1.0 mL colostrum/serum were mixed with Tris buffer (100 mM hydroxymethylamine methane, pH 8.5), phenylmethyl sulfonyl fluoride (8 mM) and DTNB (3 mM dithionitrobenzoic acid, 100 mM sodium acetate, pH 6.0) and incubated for 2 min at 37°C. After this period, 100 mL of tributyrate pyridine propanol (20 mM surfactant) was added to the solution and stirred for 30 min at 37°C. The suspensions were subsequently added with 2.0 mL of acetone (p.a.), homogenized and kept still at room temperature for 3 min. The suspensions were then centrifuged at 400 G for 5 min and read at 410 nm. A control assay was carried out without phenylmethyl sulfonyl fluoride (enzymatic inhibitor).

CuZn-superoxide dismutase determination (CuZn-SOD – E.C.1.15.1.1)
Analysis of the CuZn-SOD enzyme was performed using the nitroblue tetrazolium (NBT) reduction method (Sigma). Spectrophotometric reading was performed at 560 nm (15). The individual samples were placed in glass tubes, with another tube containing a standard solution. Each tube contained 0.5 mL of the sample, and the standard tube contained 0.5 mL of hydro-alcoholic solution. Next, 0.5 mL of chloroform-ethanol solution (1:1 ratio) and 0.5 mL of reactive mixture (NBT increased by EDTA) were added to the tubes. The experimental and standard solutions received 2.0 mL of buffer carbonate, and the pH was increased to 10.2 after the addition of hydroxylamine. The tubes remained still at room temperature for 15 min and were subsequently read at 560 nm. Superoxide dismutase (SOD) was calculated as follows: CuZn-SOD = (Ab standard – Ab sample/Ab standard) × 100 = % reduction of NBT/CuZn – SOD. The results were expressed in international units (IU) of CuZn-SOD.

Statistical analysis
Two-way ANOVA was used to evaluate glucose, antibody concentration, complement protein, calories, fat, amylase, lipase and SOD, considering the glycaemia level as one factor and the biological materials (colostrum or serum) as the other. Statistical significance was considered when $p < 0.05$. P-values of '$p = 0.0000$' were recorded as '$p < 0.0005$'.

RESULTS

Glucose concentration
This was higher in both the colostrum and serum of diabetic mothers than in the respective samples from normoglycaemic mothers (Table 1).

Total protein concentration
This was similar between normoglycaemic and diabetic mothers. Irrespective of glycaemia, total protein levels were higher in colostrum than in serum (Table 1).

The effect of hyperglycaemia on colostrum and blood leucocytes
Retrieval and viability of colostrum leucocytes were not affected in diabetic group compared to normoglycaemic ($p < 0.05$ – Table 1).

Immunoglobulin concentration
Diabetic mothers had lower levels of IgA and IgG in colostrum and IgG in the serum. Serum IgA did not vary between the groups. IgA was predominantly found in colostrum, whereas IgG was mostly found in serum (Table 2). IgM levels in colostrum and serum were similar between the groups. Serum IgM levels were lower in diabetic mothers than in normoglycaemic mothers (Table 2).

Complement concentration
In colostrum, the levels of C3 protein were significantly lower for diabetic mothers than for normoglycaemic mothers, but in serum samples this was similar between the groups. Only in normoglycaemic mothers were C3 levels higher in colostrum than in serum. C4 protein in colostrum and serum did not vary between normoglycaemic and diabetic mothers (Table 2).

Fat and calorie content
As shown in Figure 1, fat concentration was lower in the colostrum of diabetic mothers, but calorie level did not vary between the groups.

Table 1 Glucose level, total protein concentration and count and viability of leucocytes in colostrum and serum from normoglycaemic and hyperglycaemic mothers

Parameter		Normoglycaemic	Hyperglycaemic	Statistical
Glucose level (mg/dL)	Colostrum	69 ± 22.0	147 ± 35.8*	F = 0.52; p = 0.51 (comparing the colostrum and serum)
	Serum	76 ± 5.4	122 ± 12.7*	F = 16.7; p = 0.0008 (comparing the groups)
Total protein (g/dl)	Colostrum	15.4 ± 1.8	13.3 ± 0.9	F = 1.066; p = 0.3152 (comparing the colostrum and serum)
	Serum	6.5 ± 0.5†	5.7 ± 0.8†	F = 42.6561; p = 0.0000 (comparing the groups)
Leucocytes count	Colostrum	4.9 ± 0.5	4.8 ± 0.6	F = 0.55; p = 0.058 (comparing the colostrum and serum)
($\times 10^6$ cells/ml)	Serum	5.1 ± 0.7	5.2 ± 0.6	F = 1.718; p = 0.320 (comparing the groups)
Viability of Leucocytes (%)	Colostrum	96 ± 4.7	93 ± 3.2	F = 1.85; p = 0.71 (comparing the colostrum and serum)
	Serum	97 ± 5.4	95 ± 4.1	F = 0.8424; p = 0.9160 (comparing the groups)

Data presented as mean ± standard deviation (SD).
*Statistically significant differences between the normoglycaemic and hyperglycaemic mothers, considering the same sample (colostrum or serum).
†Comparing the colostrum and serum, considering the same group (normoglycaemic and hyperglycaemic mothers).

Table 2 Immunoglobulins and complement protein concentrations in colostrum and serum from normoglycaemic hyperglycaemic mothers

Parameter		Normoglycaemic	Hyperglycaemic	Statistical
IgA (mg/dL)	Colostrum	397.8 ± 52.0	298.6 ± 33.9*	F = 378.638; p = 0.0000 (comparing the colostrum and serum)
	Serum	55.4 ± 7.1†	46.2 ± 4.3†	F = 10.0067; p = 0.0004 (comparing the groups)
IgG (mg/dL)	Colostrum	169.8 ± 67.3	71.0 ± 20.6*	F = 168.587; p = 0.00005 (comparing the colostrum and serum)
	Serum	2014.6 ± 161.3†	1585.5 ± 213.5†	F = 6.3690; p = 0.0036 (comparing the groups)
IgM (mg/dL)	Colostrum	36.2 ± 8.2	39.1 ± 7.9	F = 9.5539; p = 0.0035 (comparing the colostrum and serum)
	Serum	34.5 ± 6.5	35.7 ± 4.6†	F = 1.6181; p = 0.2061 (comparing the groups)
C3 (mg/dL)	Colostrum	164.2 ± 11.0	142.5 ± 11.5	F = 7.8195; p = 0.0071 (comparing the colostrum and serum)
	Serum	134.5 ± 6.5†	135.7 ± 14.6	F = 0.7424; p = 0.5150 (comparing the groups)
C4 (mg/dL)	Colostrum	121.6 ± 11.9	127.3 ± 10.9	F = 23.1283; p = 0.0001 (comparing the colostrum and serum)
	Serum	130.0 ± 12.2	116.6 ± 27.0†	F = 0.6473; p = 0.5326 (comparing the groups)

Data presented as mean ± standard deviation (SD).
*Statistically significant differences between the normoglycaemic and hyperglycaemic mothers, considering the same sample (colostrum or blood).
†Statistically significant differences between colostrum and serum, considering the same group (normoglycaemic and hyperglycaemic mothers).

Amylase concentration

Both groups had similar amylase concentration, generally lower in colostrum than in serum (Table 3).

Lipase concentration

Within each group, lipase levels were similar between serum and colostrum, but it was usually higher in diabetic mothers (Table 3).

CuZn-SOD concentration

In colostrum samples, CuZn-SOD level was similar between the groups, but in serum it was higher in the diabetic group than in the normoglycaemic one. Only normoglycaemic mothers had higher CuZn-SOD levels in colostrum than in serum (Table 3).

DISCUSSION

Other studies have attempted to elucidate the effects of lactation on maternal glucose metabolism (12). Along these lines, we demonstrate the effects of mother's hyperglycaemia on the biochemical and immunological composition of colostrum. Similar to the milk of diabetic women (16), glucose concentration in the colostrum of diabetic mothers contained increased glucose levels.

Protein concentration in colostrum was not affected by diabetes, and in both groups it was at higher levels than in serum. Earlier studies on colostrum macronutrients report that in normoglycaemic women this secretion contains low protein concentration, whereas in diabetic women, the protein levels showed within the reference limits (17). In fact, protein and glucose levels in the colostrum of diabetic women are likely maintained at normal levels and at constant ratio with capillary concentrations (18). This suggests that adequate glycaemic control can correct any abnormalities in milk composition (18). The improper control of glycaemia may result in undesirable consequences such as compromised breastfeeding because of delayed lactogenesis transition from phase I to II (19).

There is an ever-increasing interest in understanding the breastfeeding effects on offspring as well as the mechanisms involved. A number of studies show the role of breastfeeding in growth promotion and against infections and diseases such as diabetes (12). Colostrum and human milk have been thoroughly studied in recent years, ever since their action against infections was confirmed. Human milk was first used clinically as a vehicle for passive immunity transfer, but its immune components are now known to be highly immunoreactive, exhibiting time-dependent alterations (20).

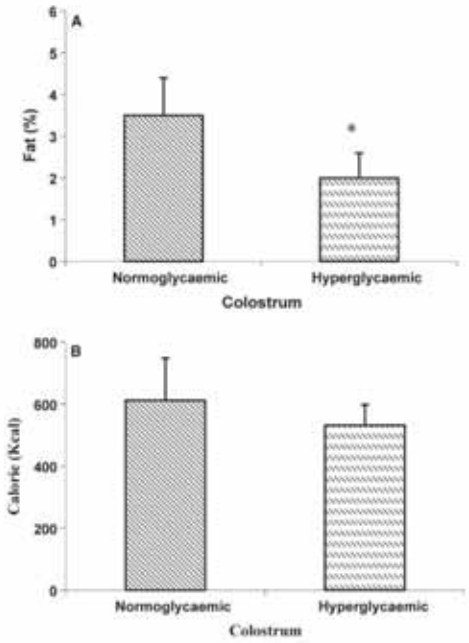

Figure 1 Fat (A) and calories (B) in the colostrum of normoglycaemic and hyperglycaemic mothers. Data presented as mean ± standard deviation (SD). *p = Statistically significant differences between control and hyperglycaemic groups. F (A) = 1.4341; p = 0.2499; F (B) = 6.1746; p = 0.0249.

The immunoreactive proteins (IgA, IgG and C3) in colostrum may be lowered by diabetes, as we observed in the study. This finding disagrees with other studies that found secretory IgA concentrations in the colostrum of insulin-dependent diabetic women comparable with those measured in the colostrum of normoglycaemic women, neither of which are distinguishable from serum IgA levels in a reference population (16).

Human milk is particularly rich in secretory IgA (5), which can block bacterial adherence to human epithelial cells (20), neutralize toxins, prevent attack and penetration by viral infections (21), in addition to avoiding tissue damage and energy loss (2). These functions are complemented by IgM and IgG antibodies (9) and, in particular, the complement proteins C3 and C4 (9). Because changes in blood antibody levels may be related to changes in B lymphocytes (22), the low IgG and IgM concentrations in the serum of diabetic mothers suggest that hyperglycaemia could affect this lymphocyte class.

The complexity of human milk makes this secretion the ideal food source for babies for at least their first 6 months of life. This early nutrition is an important environmental input that can induce lifelong effects on metabolism, growth and major disease processes, such as diabetes mellitus (1). The amount and composition of milk is probably independent of the mother's diet. Milk composition changes during lactogenesis, and these changes can be used as biochemical markers of the onset of milk secretion (22).

Normal baby development is sustained by the balanced gain of fat and calories provided by breastfeeding. Even so, fat content is lower in the colostrum of diabetic mothers, as shown in the present and in other studies (17). Conversely, calorie concentration did not vary between normoglycaemic and hyperglycaemic mothers. This is possibly a result of the higher lipase levels in the colostrum of diabetic women than in their normoglycaemic counterparts. It has been suggested that high lipase levels compromise etherification and fatty acid synthesis in the mammary gland, thereby altering milk composition (17). Our results corroborate the hypothesis that diabetes changes lipid metabolism in the mammary gland. Enzymatic action for converting fat into calories is likely accelerated, thereby ensuring energy intake for growth and proper development of newborns of diabetic mothers.

Human colostrum is also rich in biologically active molecules, which are essential for antioxidant functions. Their soluble components act in a child's gut without, however, provoking an inflammatory response (23). These compounds may be enzymes that are important as immune protectors (2) or for infant development. This is the case for amylase, which increased activity in pregnant diabetic women (24). We did not detect differences in amylase concentration in the colostrum of normoglycaemic and hyperglycaemic mothers, but these levels were lower than in human serum.

Table 3 Amylase, lipase and superoxide dismutase (CuZn-SOD) concentrations in colostrum and serum from normoglycaemic and hyperglycaemic mothers

Enzymes		Normoglycaemic	Hyperglycaemic	Statistical
Amylase (U/dL)	Colostrum	54.1 ± 9.4	55.5 ± 9.5	F = 20.0515; p = 0.0004 (comparing the colostrum and serum)
	Serum	73.1 ± 3.3*	72.4 ± 6.5*	F = 0.0152; p = 0.8987 (comparing the groups)
Lipase (UI)	Colostrum	5.8 ± 1.5	8.5 ± 1.3†	F = 0.0015; p = 0.9687 (comparing the colostrum and serum)
	Serum	4.8 ± 1.9	9.3 ± 4.3†	F = 4.2307; p = 0.04504 (comparing the groups)
CuZn-SOD (U/mg protein)	Colostrum	53.6 ± 7.1	59.1 ± 1.2	F = 11.49; p = 0.02348 (comparing the colostrum and serum)
	Serum	35.7 ± 9.1*	61.7 ± 11.4†	F = 4.8629; p = 0.01028 (comparing the groups)

Data presented as mean ± standard deviation (SD).
*Statistically significant differences between colostrum and serum, considering the same group.
†Statistically significant differences between the normoglycaemic and hyperglycaemic mothers, considering the same sample (colostrum or blood).

Amylase activity is high in colostrum and may confer to breastfed infants the ability to digest starch (24,25). This is therefore indispensable to their normal growth and development (25). In addition, some studies show that amylase associated with the SOD enzyme exerts a protective action against pancreatic lesions (15). In the present study, we demonstrated that SOD concentration in colostrum did not vary between normoglycaemic and hyperglycaemic mothers but that it was higher than in human serum. The activity of SOD in human milk appears to be 10–25 times higher than in serum (23) and may change significantly during lactation to meet the different needs of newborn development (26). Other studies show that the highest activity of this enzyme in milk occurs at 3 weeks of lactation and that it decreases after 4 months of lactation (26).

Diabetic patients usually present delayed defence factors, decreased antioxidant defence enzymes as well as failure on oxidative burst, an important biochemical and immunology pathway. Hyperglycaemia severely compromises the different endogenous antioxidant defences of diabetic patients with diabetes (27). These defences may involve enzymatic pathways (27), including those of CuZn-SOD. Changes in SOD activity have been found in chemically induced diabetic animals (28). However, the effects of diabetes on SOD synthesis are a matter of controversy. Some authors reported increased levels of SOD, whereas others observed a decrease or even normal SOD concentrations in diabetes (29). Nevertheless, the antioxidant capacity of the colostrum of diabetic mothers, which increases in mature milk, is vital for the newborn in the first days of life (30).

Our findings support the hypothesis that the production of milk components changes in diabetic mothers because of alterations in glucose metabolism. Therefore, adequate maternal glycaemic control of diabetic mothers is crucial to ensure that the nutritional needs of newborn babies are met and that the immunity components are properly provided. Despite the abnormalities in biochemical and immunological components, women with diabetes should be strongly encouraged to breastfeed their children. Besides being an excellent food source for newborns, breast milk decreases the high rates of maternal and infant complications. In addition, the rate of growth of breastfed infants is related to the total amount of milk they consume rather than the concentration of fat, proteins or carbohydrates in the milk.

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ARTIGO 2

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Secretory IgA–Fcα receptor interaction modulating phagocytosis and microbicidal activity by phagocytes in human colostrum of diabetics

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The effects of secretory immunoglobulin A (SIgA) interaction with its specific Fcα receptors on colostral phagocytes needs further investigation, especially with respect to diabetic women. Accordingly, we studied the colostrum of hyperglycemic women to assess SIgA interactions with Fcα receptors of macrophages as well as the functional activity of these cells. The women were divided for colostrum sampling according to their glycemic status: normoglycemia (N = 51), mild hyperglycemia (N = 23), and diabetes (N = 25) groups. We determined the FcαR expression, the IgA on the surface and the surface-bound IgA in colostrum macrophages. We also evaluated the superoxide release and bactericidal killing of these cells. Colostral phagocytes expressed FcαR, contained IgA on the surface and are able to bind to purified SIgA. The bactericidal activity of colostral phagocytes from the hyperglycemic women was similar to that of normoglycemic only when SIgA was used as opsonin. Addition of a MoAb anti-human Fcα receptor resulted in a significant decrease of superoxide release and bacterial killing by macrophages when bacteria were opsonized with purified SIgA, suggesting an interaction between SIgA and FcαR. The stimulatory effects of SIgA on the functional activity of phagocytes therefore protect infants, especially of diabetic women, against intestinal infections.

Key words: FcαRI; colostrum; phagocytes; diabetes; EPEC; SIgA.

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Breastfeeding is recommended by numerous health agencies as the preferred method of feeding during the infant's first year because of its multiple immediate and long-term benefits for both mother and child (1). The relationship of breastfeeding to the development of a number of chronic diseases, including obesity and diabetes has been extensively studied (2).

Short-term breastfeeding followed by early introduction of bovine milk is a known risk factor for type 1 diabetes in infants (3). The lack of breastfeeding also increases the risk of type 1 diabetes (4), whereas prolonged breastfeeding

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duration likely reduces the risk of future type 2 diabetes (5).

Clinical and epidemiological evidence and experimental studies suggest that diet plays an important role in the etiopathogenesis of diabetes (2, 6). Therefore, diabetic women should be strongly encouraged to breastfeed (2), to decrease the risk of diabetes for their babies (1), infant morbidity, and mortality as well as to prevent gastrointestinal and respiratory infections (7, 8).

In developing countries the enteropathogenic *Escherichia coli* is the main etiological agent of diarrhea in infants during their first year of life (9, 10). This disease is possibly prevented by colostrum and human milk, which are known to play an anti-infection, protective role against pathogens (7, 10–12). Human milk is particularly rich in secretory IgA (SIgA), which blocks bacterial adherence to human epithelial cells, neutralizes toxins, and prevents viral infections (12) and acts as an opsonin by increasing free radicals, phagocytosis, and the microbicidal activity of colostral cells (7, 8, 11). Colostral IgA and human milk have proven to be effective against a number of microorganisms (7).

In addition to antibodies, soluble bioactive components and anti-infectious factors, human colostrum contains large amounts of viable leukocytes (1×10^9 cells/mL in the first days of lactation), especially neutrophils and macrophages (13). Most of the cells present in colostrum are macrophages. They have fat globules, casein, and other particulate materials. They differ from blood mononuclear phagocytes by presenting increased motility, the amount of lysosomes, and present SIgA in surface and endocytoplasmatic (12, 13). Circulating leukocytes have phagocytic and bactericidal activity and produce free radicals (7,10,14). In bacterial infections, phagocytes are known to be the main cell lineage in host defense (14).

Human colostrum phagocytes may be activated by stimulatory signals generated by milk antibodies, especially during SIgA interaction with its Fc receptor (15). The IgA receptor family comprises a number of surface receptors, including the myeloid specific IgA Fc receptor [FcαRI or CD89 – (15)]. FcαR are expressed on the surface of blood neutrophils, eosinophils, and monocytes/macrophages (7, 15). In addition to phagocytosis stimulation, the combined action of SIgA and its Fc receptor may trigger degranulation and the production of oxygen radicals (16).

Colostral macrophages exhibit phagocytic activity and produce oxygen-free radicals. The bactericidal activity of colostral mononuclear phagocytes after opsonization with SIgA is equivalent to that of mononuclear and polymorphonuclear phagocytes from peripheral blood (10, 11).

The significance of the biological activity of SIgA and its interactions is of great importance because colostrum is the secretion that contains the highest amount of this antibody and may represent a complete micro-environment, in which both soluble and cellular components act together (12).

Some studies have reported that diabetic patients have reduced phagocytic activity, low microbicidal activity, and reactive oxygen species production due to changes in the antioxidant systems (16, 17). The reduction in phagocytic and microbicidal activity of leukocytes is likely related to an increase in blood glucose levels (17). Nevertheless, there are no studies that have investigated the functional activity of mononuclear phagocytes in the colostrum of mildly hyperglycemic and diabetic mothers, as well as the action of these defense cells on the newborn's gut and their interactions with colostral IgA. Colostrum possibly plays an important protective role against pathogens in the newborn gut, especially infants of diabetic mothers because the former are more vulnerable to infection.

This study investigated the interactions of Fcα receptor and SIgA as well as the functional activity of mononuclear phagocytes in the colostrum of diabetic women.

MATERIALS AND METHODS

The functional activity of colostrum phagocytes in hyperglycemic women, of any etiology, was evaluated in a cross-sectional study. The subjects attended the Diabetes and Pregnancy Facility, School of Medicine Obstetrics Course, UNESP, Botucatu, SP. This study was approved by the institutional Research Ethics Committee, and all the subjects gave informed written consent before entering the experimental protocol.

Subjects

We evaluated 99 women (18–45 years old) tested for hyperglycemia between the 24th and 28th weeks of pregnancy. Hyperglycemia was diagnosed by the 100 g – oral glycemia tolerance test (OGTT test), according to ADA criteria (18), and the glucose profile (GP) test, according to Gillmer's threshold values (19). After delivery, colostrum samples from these subjects were analyzed according to maternal glycemic status: normoglycemic group (normal 100 g-OGTT and normal GP; N = 51), mild hyperglycemia (normal 100 g-OGTT and abnormal GP or abnormal 100 g-OGTT and abnormal GP during pregnancy; N = 23) and diabetes group (abnormal pre-pregnancy 100 g-OGTT and insulin-dependent; N = 25). The mean and standard deviation for gestational age were 38 ± 0.8 weeks in normoglycemic, 38.3 ± 0.9 weeks in mild hyperglycemic and 37.2 ± 0.4 weeks in diabetes groups and for newborns at birth were 2048.3 ± 411.8 in normoglycemic, 3357 ± 323.2 in mild hyperglycemic groups, and the 3140.1 ± 492.1 in diabetes groups). The subjects continued attending the facility, irrespective of diagnosis, and the hyperglycemic patients followed a specific treatment for glycemic control (19).

The variables controlled were, smoking status (yes/no), arterial hypertension (yes/no), and glycemic index (GI), whose mean plasma glucose level was measured in the glycemic profiles taken during the gestation. GI was classified as adequate (GI < 120 mg/dL) or inadequate [GI≥120 mg/dL – (19)].

Separation of colostral cells About 15 mL of colostrum from each woman was collected in sterile plastic tubes between 48 and 72 h postpartum. The samples were centrifuged (160×*g*, 4 °C) for 10 min, which separated colostrum into three different phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant as reported by Honorio-França et al. (10). Cells were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden), producing preparations with 98% of pure mononuclear cells, and analyzed by light microscopy. Purified macrophages were resuspended independently in serum-free medium 199 at a final concentration of 2×10^6 cells/mL.

Purified colostral SIgA Human SIgA was purified from a defatted colostrum pool by affinity chromatography on Cyanogen Bromide-Activated Sepharose-4B (CNBr-Sepharose-4B; Sigma, ST Louis, MO, USA) bound with sheep anti-human α chain as proposed by March et al. (20). To ensure SIgA depletion, fractions eluted from the affinity chromatography column were pooled and passed five times through the same column. Bound SIgA was eluted from the column with 6N glycine – HCL buffer, pH 2.8. The purified preparations were restored to the initial volume. The concentration of SIgA was determined by simple radial immunodiffusion (RID) with a sheep anti-human α chain serum on agarose plates (10). Protein total concentration was available by Lowry Method. The purified SIgA preparation was also tested by immunoelectrophoresis with goat anti-human γ and μ chain antisera. Both IgG and IgM were undetectable in the preparation.

IgA determination by quantitative RID Secretory immunoglobulin A concentrations in colostrum from normoglycemic, mild hyperglycemic, and diabetic groups were determined as proposed by Mancini et al. (21). Antibody levels in human colostrum was determined with an anti-human IgA, lamb serum (Biolab), and the Kallestad standard curve. All the experiments were performed in duplicate and triplicate.

Immunofluorescence and flow cytometry We performed double immunofluorescence staining with MoAb anti-CD89 with anti-CD14 or MoAb anti-CD89 with anti-IgA to assess FcαR expression and detection of IgA on the surface in colostral mononuclear cells. Cells were pre-incubated with 10 μL of human IgG (Sigma, ST Loius, USA;10 mg/mL) for 20 min on ice to mask FcγR (22) before incubation with 10 μL of PE-labeled anti-CD89 (IgG1k – 0.1 mg/mL) mAb specific for FcαRI (22) and 10 μL of fluorescein isothiocyanate (FITC) conjugated, anti-CD14 mAb for 30 min at 4 °C in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA; Sigma; 5 mg/mL) and sodium azide (0.1 mg/mL). PE-labeled, irrelevant IgG1 mAb (Sigma;10 μl – 0.1 mg/mL) was used as negative control. After incubation, the cells were washed twice in PBS containing BSA (Sigma; 5 mg/mL) and sodium azide (ver marca 0.1mg/mL). For detection of IgA on the surface of MN phagocytes, colostrum cells were washed extensively and then stained with a $F(ab')_2$ fragment of anti-human IgA conjugated to FITC (Sigma) for 30 min at 4 °C. In all experiments, the cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson, Franklin Lakes, NJ, USA).

Immunofluorescence of SIgA interaction with FcαRI on colostral phagocyte surface This assay verified whether SIgA was specifically bound to the FcαRI. The colostral cells were incubated with purified colostral SIgA (1mg/mL) for 60 min at 4 °C. After incubation, the cells were washed extensively in PBS containing BSA (Sigma; –5mg/mL) and sodium azide (0.1mg/mL). The mononuclear phagocytes of colostrum were stained with a $F(ab')_2$ fragment of anti-human IgA conjugated to FITC (Sigma) for 30 min at 4 °C. A monoclonal antibody anti-human IgG-labeled and conjugated to FITC (Sigma) was used as negative control. In all experiments, the cells were analyzed by flow cytometry (FACScalibur, Becton Dickinson).

Treatment of colostral mononuclear phagocytes with MoAb anti-human Fcα receptor To assess the effect of SIgA–FcαRI interaction on superoxide anion release, as well as phagocytic and microbicidal activity, mononuclear phagocytes (2×10^6 cells/mL) were incubated with 50 μL of a MoAb reactive with human FcαRI [0.1mg/mL; Medarex, Inc., West Lebanon, NH, USA (23)], monoclonal Ab that bind in the EC1 domain of FcαRI can block IgA binding (15) for 2 h at 37 °C. They were then washed once with medium 199 at 4 °C and immediately used in the assays.

E. coli strain The enterophatogenic *E. coli* (EPEC) used was isolated from stools of an infant with acute diarrhea (serotype 0111:H2, LA1, eae1, EAF1, bfp1). This material was prepared and adjusted to 10^8 bacteria/mL, as previously described (10).

Bacterial opsonization Enterophatogenic *E. coli* was opsonized as described by Bellinati-Pires et al. (24). Three opsonins were used:

1. *Colostrum supernatant*. A pool of 10 colostral samples (immunoglobulin concentration (g/L): IgA = 7.4; IgG = 0.15; and IgM = 0.37) was defatted by repeated centrifugation at $160 \times g$ for 10 min at 4 °C. The aliquots were stocked at −80 °C and subsequently used for EPEC opsonization.
2. *Purified colostral SIgA*. Purified colostral IgA as described above. The purified SIgA was 4.0 g/L adjusted to a concentration of 0.74 g/L (to be used in a proportion equivalent to 10% of the SIgA contained in the standard colostrum sample). The aliquots were stocked at −80 °C and subsequently used for EPEC opsonization.
3. *Sera*. A pool of normal human serum samples from 10 volunteer donors [immunoglobulin concentration (g/L): IgA = 2.64; IgG = 14.0 and IgM = 2.0] was prepared, the aliquots stocked at −80 °C for subsequent EPEC opsonization.

Colostrum supernatant, serum and purified IgA were thawed and mixed with appropriate volumes of bacterial suspension to a final concentration of 2×10^7 bacteria/mL in 10% of the opsonin sources. Other bacterial suspension prepared at the same concentration in medium 199 without opsonin was used as an untreated bacterial control. Both bacterial suspensions were incubated for 30 min at 37 °C and used in the superoxide release and bactericidal assays.

Release of superoxide anion Superoxide release was determined by cytochrome C (Sigma) reduction (10, 25). Briefly, mononuclear phagocytes and bacteria, opsonized or not with the aforementioned opsonins, were mixed and incubated for 30 min for phagocytosis. Cells were then resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C (Sigma; 2 mg/mL). The suspensions (100 μL) were incubated for 60 min at 37 °C on culture plates. The reaction rates were measured by absorbance at 550 nm and the results were expressed as nmol/O_2^-. All the experiments were performed in duplicate or triplicate.

Bactericidal assay Phagocytosis and microbicidal activity were evaluated by the acridine orange method described by Bellinati-Pires et al. (24). Equal volumes of bacteria and cell suspensions were mixed and incubated at 37 °C for 30 min under continuous shaking. Phagocytosis was stopped by incubation in ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice ($160 \times g$, 10 min, 4 °C). Cells were resuspended in serum-free medium 199 and centrifuged. The supernatant was discarded and the sediment dyed with 200μL of acridine orange (Sigma; 14.4g/L) for 1 min. The sediment was resuspended in cold culture 199, washed twice and observed under immunofluorescence microscope at 400× and 1000× magnification. The phagocytosis index was calculated by counting the number of cells ingesting at least three bacteria in a pool of 100 cells. To determine the bactericidal index, we stained the slides with acridine orange and counted 100 cells with phagocytized bacteria. The bactericidal index is calculated as the ratio between orange- stained (dead) and green- stained (alive) bacteria × 100 (24). All the experiments were performed in duplicate or triplicate.

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate superoxide anion release, phagocytosis and bactericidal index in the presence or absence of opsonized EPEC. Statistical significance was considered for a p-value lower than 0.05 ($p < 0.05$).

RESULTS

The effect of hyperglycemia on colostral mononuclear phagocytes

Retrieval and viability of colostral phagocytes were not affected in hyperglycemic groups compared to normoglycemic group ($p < 0.05$). However, colostral SIgA concentration was lower ($p < 0.05$) in group diabetic group (Table 1).

Table 1. General characteristics of the experimental groups

Parameter	Group		
	Normoglycemic (N = 51)	Mild hyperglycemia (N = 23)	Diabetes (N = 25)
Glucose level (mg/dL)	81 ± 9.24	108 ± 7.8	142 ± 12.7[1]
Mononuclear phagocyte count ($\times 10^5$ cells/mL)	4.1 ± 0.5	3.9 ± 0.4	3.8 ± 0.6
Viability of mononuclear phagocytes (%)	96 ± 4.7	95 ± 5.6	93 ± 3.2
Colostral IgA (g/L)	4.2 ± 0.7	4.3 ± 0.5	3.0 ± 0.5[1]

The results represent the mean and SD of 10 experiments.
[1]intergroup differences (ANOVA, $p < 0.05$)

Colostral mononuclear phagocytes express FcαRI and carry SIgA in their membranes

The colostral mononuclear cells analyzed were CD14-bright and therefore identified as macrophages (Fig. 1). The incubation of mononuclear cells with colostral purified SIgA revealed that the macrophages had FcαRs with high levels of SIgA (Fig. 1). Table 2 shows the rate of positive bindings by fluorescence intensity of SIgA on the surfaces of colostral phagocyte and of colostrum-purified SIgA bound to the FcαR. Colostrum phagocytes from normoglycemic and of the hyperglycemic groups had similar FcαRI expression, surface SIgA, and surface-bound SIgA.

MoAb anti-FcαRI and EPEC opsonization on superoxide release

Hyperglycemic and normoglycemic groups had similar spontaneous superoxide release by colostrum mononuclear phagocytes. When exposed to EPEC (opsonized or not), superoxide release increased ($p < 0.05$) in all the groups and was higher in normoglycemic group ($p < 0.05$). Phagocytes incubated with EPEC opsonized with colostrum supernatant or SIgA had higher superoxide release than phagocytes exposed to non-opsonized bacteria ($p < 0.05$), irrespective of glycemic status. In the presence of EPEC opsonized by colostrum supernatant or SIgA, mononuclear phagocytes of all the groups had lower superoxide release when pretreated with MoAb anti-FcαRI (Table 3).

MoAb anti-FcαR and EPEC opsonization on the phagocytosis activity of colostral mononuclear cells.

Colostral mononuclear phagocytes of the hyperglycemic groups had some phagocytic activity in response to EPEC. Phagocytosis increased significantly in the presence of opsonins (colostrum

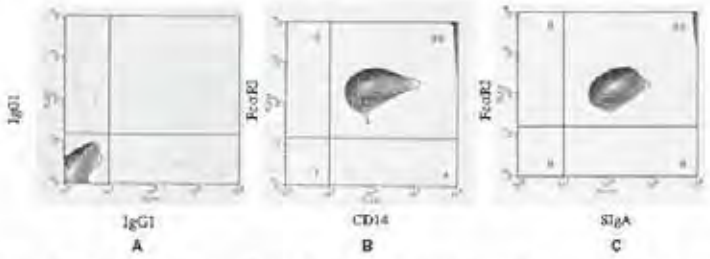

Fig. 1. Expression of FcαR bearing purified SIgA on colostral mononuclear cells. Viable cells pre-incubated with excess human IgG to block FcγR were stained directly with PE-labeled IgG1 control (A) or with a PE-labeled anti-CD89 (anti-FcαR MoAb B) or PE-labeled anti-CD89 (anti-FcαR MoAb plus colostral SIgA C). After being washed, the cells were stained with FITC labeled anti-CD14 (B) and anti-human IgA conjugated with FITC (C), as described in Materials and Methods. Two-color immunofluorescence analysis was then carried out by flow cytometry.

Table 2 Positive bindings indicated by fluorescence intensity

Type of interaction mononuclear phagocytes	Intensity (%; mean ± SD)		
	Normoglycemic (N = 12)	Mild hyperglycemia (N = 12)	Diabetes (N = 12)
IgG (control)	8.2 ± 3.8	10.2 ± 3.7	9.2 ± 1.7[1]
Surface SIgA	36.5 ± 5.8[1]	33.9 ± 4.4[1]	35.8 ± 4.6[1]
Purified colostral SIgA + Anti-IgA-FITC	85.2 ± 5.8[1]	85 ± 5.6[1]	83 ± 3.2[1]

The results represent the mean and SD of 10 experiments.
[1]contrast with the control (IgG) group (ANOVA, $p < 0.05$).

Table 3 Superoxide release by colostrum mononuclear (mean ± SD, N = 10 in each treatment)

Bacteria treatment	MoAb anti-FcαRI	Superoxide release (nmol)		
		Normoglycemic	Mild hyperglycemia	Diabetes
Control (without bacteria)	No	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.2
	Yes	1.1 ± 0.3[3]	1.6 ± 0.2	1.5 ± 0.8
Bacteria + PBS	No	3.1 ± 0.6[1]	2.5 ± 0.1 [2,1]	2.3 ± 0.2 [2,1]
	Yes	2.9 ± 0.5[1]	1.9 ± 0.8[2]	1.7 ± 0.5[2]
Bacteria + serum	No	4.1 ± 0.7[1]	3.2 ± 0.5 [2,1]	3,1 ± 0.4 [2,1]
	Yes	3.5 ± 0.3[1]	3.0 ± 0.7[1]	3.2 ± 0.8[1]
Bacteria + colostrum	No	3.9 ± 1.4[1]	3.1 ± 0.4[1]	3.1 ± 1.0[1]
	Yes	1.3 ± 0.6[3]	1.9 ± 0.3[3]	1.8 ± 0.4[3]
Bacteria + purified SIgA	No	3.6 ± 0.7[1]	3.1 ± 0.5[1]	3.8 ± 0.5[1]
	Yes	0.5 ± 0.2[1,3]	0.9 ± 0.3[13]	0.6 ± 0.2[1,3]

Bacteria were opsonized with a colostrum supernatant pool, purified SIgA or a normal serum pool. In controls assays, mononuclear cells were pre-incubated with PBS.
[1]differences between each bacteria treatment and the control treatment within each group (ANOVA, $p < 0.05$).
[2]intergroup differences within each treatment (ANOVA, $p < 0.05$).
[3]differences between MoAb anti-FcαR use within each treatment and group.

supernatant or normal serum). The highest phagocytosis rates were found when EPEC was opsonized by colostral SIgA. A comparison of the groups showed that EPEC phagocytosis was in general lower in the mild hyperglycemic and diabetic groups than in normoglycemic group, except when EPEC was opsonized with SIgA (Table 4). Pretreatment of mononuclear phagocytes with MoAb anti-FcαR decreased their phagocytic activity when EPEC was opsonized by colostrum supernatant or SIgA in all the groups (Table 4).

MoAb anti-FcαRI and EPEC opsonization on the bacterial activity of mononuclear phagocytes

In general, colostral mononuclear phagocytes had low bactericidal activity against non-opsonized EPEC. With bacteria opsonized with serum or colostrum supernatant, the bactericidal activity was lower in hyperglycemic groups than in normoglycemic group. The groups showed equivalent rates of SIgA-induced EPEC elimination by mononuclear phagocytes (Table 5). Compared to untreated cells, mononuclear phagocytes pretreated with AbMo anti-FcαRI had lower phagocytic activity in response to EPEC opsonized by colostrum supernatant or SIgA (Table 5). Colostral mononuclear phagocytes were able to internalize bacteria, irrespective of the use of SIgA as opsonin. SIgA-mediated bacterial killing by colostral mononuclear phagocytes is shown in Fig. 2.

DISCUSSION

In the present study, we identified FcαRI expression by colostral macrophages in hyperglycemic, diabetic, and normal women. In every case macrophage receptors interacted with SIgA, and the FcαRI–SIgA complex formed

Table 4 Bacterial phagocytosis by mononuclear cells (mean ± sd, N = 10 in each treatment)

Opsonin source	MoAb anti-FcαRI	Phagocytosis (%)		
		Normoglycemic	Mild hyperglycemia	Diabetes
Control -Medium 199	No	49.1 ± 8.8	38.8 ± 9.4	32.8 ± 3.3+
	Yes	59.0 ± 8.0	32.3 ± 6.1+	34.6 ± 3.7+
Colostrum pool	No	62.0 ± 7.2[1]	50.6 ± 6.1 [2,1]	48.0 ± 3.5 [2,1]
	Yes	48.0 ± 8.4[3]	33.2 ± 3.4 [2,3]	44 ± 4.8
Serum pool	No	60.0 ± 9.4[1]	47.6 ± 8.6 [2,1]	49.1 ± 8.0 [2,1]
	Yes	58.6 ± 3.7	50.5 ± 5.5	57.4 ± 6.3
Purified SIgA	No	61.0 ± 6.1[1]	55.6 ± 5.6[1]	53.5 ± 6.0[1]
	Yes	48.2 ± 3.7[3]	42.0 ± 1.5[3]	40.4 ± 3.1[3]

Bacterial phagocytosis by mononuclear cells from colostrum was determined with the acridine orange method.
[1]differences between opsonin source and PBS medium (ANOVA, $p < 0.05$).
[2]intergroup differences for each opsonin treatment (ANOVA, $p < 0.05$).
[3]differences between MoAb anti-Fcα use within each opsonin treatment and group.

Table 5 Bacterial elimination index (mean ± SD, N = 10 in each treatment)

Opsonin source	MoAb anti-FcαRI	Bactericidal index (%)		
		Normoglycemic	Mild hyperglycemia	Diabetes
Control–medium 199	No	25.7 ± 5.2	19.4 ± 8.0	18.8 ± 4.6
	Yes	24.0 ± 6.0	25.1 ± 3.8	22.6 ± 3.7
Colostrum pool	No	64.9 ± 8.0[1]	40.2 ± 5.0 [2,1]	38.7 ± 2.5 [2,1]
	Yes	28.4 ± 6.1[3]	23.0 ± 6.0[3]	26.8 ± 6.2[3]
Serum pool	No	63.7 ± 6.3[1]	42.4 ± 6.1 [2,1]	41.9 ± 4.0 [2,1]
	Yes	56.2 ± 4.2	44.7 ± 2.4	47 ± 4.2
Purified SIgA	No	63.6 ± 11.4[1]	61.6 ± 2.9[1]	63.0 ± 5.1[1]
	Yes	9.2 ± 2.5[3]	16.5 ± 8.5[3]	14 ± 1.2[3]

Bacterial elimination by mononuclear cells from colostrum was determined with the acridine orange method.
[1]differences between opsonin source and PBS medium (ANOVA, $p < 0.05$).
[2]intergroup differences for each opsonin treatment (ANOVA, $p < 0.05$).
[3]differences between MoAb antiFcα use within each opsonin treatment and group.

displayed efficient bacterial-killing activity along with superoxide release. This process was more efficient when bacteria were previously opsonized with SIgA.

FcαR participates in many aspects of host defense through engagement with antibodies complexed to antigens. FcαR binding by immunoglobulins can initiate biological processes, including phagocytosis, antigen presentation, superoxide generation, and the release of cytokines and inflammatory mediators (7, 15). FcαR binding can also modulate the activation status of cells, and consequently their immune responses. For instance, the FcαRI-mediated engulfment of SIgA-opsonized bacteria and yeast particles involves neutrophil and monocyte action (15).

Nearly 70% of the colostral phagocytes have surface-bound SIgA, a proportion that is much higher than that of blood phagocytes (7). With regard to colostral mononuclear phagocytes, we found that, independent of glycemia, in a medium, 35% have surface-bound SIgA and 85% of the purified SIgA can bind a specific receptor. This binding is likely promoted by the Fcα receptors in the macrophages, which reinforces the important role they play for the protective properties of colostrum.

Colostral mononuclear phagocytes obtained in colostrum of normoglycemic women are known to kill SIgA-opsonized enteropathogenic *E. coli* via the FcαRI (10). This response is less effective in other defense cells such as the colostral neutrophils, which express of FcαR (a.1 isoform) without γ subunit association and present low bacterial-killing activity. This receptor may mediate noninflammatory effects of SIgA and the FcαR expression is therefore likely enrolled

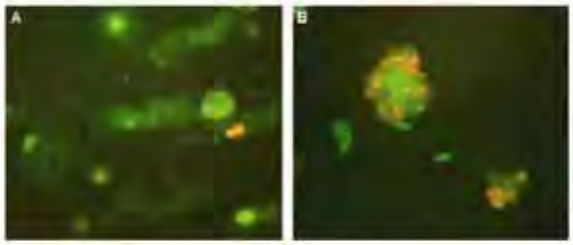

Fig. 2. Bacterial killing by colostral mononuclear phagocytes. The mononuclear phagocytes were incubated with bacteria not opsonized (A) or opsonized with purified SIgA (B) on a shaker for 30 min at 37 °C. After washing at 4 °C, cells were stained with acridine orange and analyzed by fluorescent microscopy. Orange- stained bacteria (dead) and green- stained bacteria (alive). Experiments were repeated five times and the results were comparable.

in a programed anti-inflammatory system by polimorphonuclear phagocytes (7).

The high glucose levels of diabetic patients can impair their antioxidant systems by decreasing important responses such as lysozyme release, ROS production, as well as phagocytic and microbicidal activity (26). Corroborating this, the SIgA concentration that we found in the colostrum of diabetic mothers was lower than that of the control group (27). However, SIgA levels were not low enough to compromise the SIgA–FcαRI binding rate.

Despite the low SIgA levels in colostrum from diabetic group, we observed that mononuclear phagocyte count and viability were preserved in all the groups. The activation ability of these cells, indicated by spontaneous superoxide release, was kept at the same levels in both hyperglycemic and control groups. Similar results with respect to the functional activity of leukocytes were observed in colostrum samples from patients with diabetes (17) and from animals with induced diabetes (28). Mononuclear phagocytes play an important role in host defense. They produce phagocytic NADPH oxidase, which forms superoxide, imperative for bacterial killing (14) and crucial for the success of immune responses and inflammatory reactions (29).

Superoxide release by colostral mononuclear cells in fact increased in response to EPEC exposure. The use of human serum such as opsonin was more efficient in the normoglycemic group than in hyperglycemic groups. However, the use of SIgA or colostrum supernatant to opsonize EPEC provided satisfactory superoxide release in all the groups studied. SIgA-opsonization in particular increased superoxide release along with the phagocytic bactericidal activity of colostral mononuclear phagocytes. Other studies have reported the elimination of opsonized EPEC by mononuclear phagocytes, and like in the present study, microbicidal activity was stimulated only in the presence of SIgA or other immunomodulatory agents (8, 10). Complementing these findings on normoglycemic patients, we show that colostral SIgA is an equally important opsonin in colostrum from hyperglycemic women. Although high glucose levels impair a series of physiological process, SIgA activity in colostrum seems to be unaffected. This finding is of great importance because, besides the fundamental biological activity of SIgA, this is the main antibody class in colostrum (10). The SIgA during feeding serves as an optimally antigen-targeted passive immunization of the breast-fed infant's gut and in developing countries breast-feeding is the best defense against mucosal infection (30).

The effectiveness of colostral SIgA depends on its binding to FcαRI We indeed found that macrophages pretreated with MoAb anti-FcαRI did not perform significant phagocytosis and bacterial killing, not even after bacteria opsonization with SIgA. Therefore, the SIgA–FcαR binding may activate the intracellular mechanisms of phagocytosis by macrophages. After binding FcαRI, IgA is internalized by macrophages and probably participates in the specific immune responses against mucosa-derived antigens (31). Studies have suggested that colostrum macrophages remain viable in the intestinal

mucosa for a period of 4 h (32) and are able to induce microbicidal activity in newborns (33). FcαR expression in macrophages may therefore play an important role either as a second line of defense against bacterial infection or by arming the immune system to respond to external antigens (15, 31).

The rich nutrition provided by breastfeeding in early nutrition can induce lifetime beneficial effects on metabolism, growth, immune system, neurodevelopment, and on major disease processes (6, 34). As shown in the present study, breastfeeding also provides additional protection against intestinal infections in infants of diabetes women, seemingly through colostral SIgA interactions with the Fcα receptors. This binding stimulates the functional activity of mononuclear phagocytes against EPEC. Diabetic mothers should therefore be encouraged to breastfeed their babies because this is an important strategy for combating newborn infections.

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ARTIGO 3

Title: Antioxidant effect of melatonin on the functional activity of colostral phagocytes in diabetic women

Short Title: Melatonin activity onto diabetic women colostrum

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ABSTRACT

Melatonin is involved in a number of physiological and oxidative processes, including functional regulation in human milk. The present study investigated the mechanisms of action of melatonin and its effects on the functional activity of colostral phagocytes in diabetic women. Colostrum samples were collected from normoglycemic (N=38) and diabetic (N=38) women. We determined superoxide release, bactericidal activity and intracellular Ca^{2+} release by colostral phagocytes treated or not with 8-(Diethylamino) octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) and incubated with melatonin and its precursor (N-acetyl-serotonin-NAS), antagonist (luzindole) and agonist (chloromelatonin-CMLT). Melatonin stimulated superoxide release by colostralphagocytes from normoglycemic but not hyperglycemic women. NAS increased superoxide, irrespective of glycemic status, whereas CMTL increased superoxide only in cells from the normoglycemic group. Phagocytic activity in colostrum increased significantly in the presence of melatonin, NAS and CMLT, irrespective of glycemic status. The bactericidal activity of colostral phagocytes against enterophatogenic *Escherichia coli* (EPEC) increased in the presence of melatonin or NAS in the normoglycemic group, but not in the hyperglycemic group. Luzindole blocked melatonin action on colostrum phagocytes. Phagocytes from the normoglycemic group treated with melatonin exhibited an increase in intracellular Ca^{2+} release. Phagocytes treated with TMB-8 (intracellular Ca^{2+} inhibitor) decreased superoxide, bactericidal activity and intracellular Ca^{2+} release in both groups. The results suggest that melatonin action is associated to glucose metabolism control in colostral phagocytes. In mononuclear cells of normoglycemic women, melatonin probably increases the ability of colostrum to protect against EPEC, whereas in cells of diabetic mothers it likely mediates anti-inflammatory processes.

Key words: melatonin, colostrum, phagocytes, diabetes.

Introduction

Diabetes is prevalent in young women and is increasingly related to maternal and child health issues, such as breastfeeding. Several studies have investigated the association of breastfeeding with a variety of chronic diseases, including obesity and diabetes [1,2]. Infants born to diabetic women are at higher risk of hypoglycemia because maternal hyperglycemia causes child hyperinsulinism [3]. However, the impact of breastfeeding on glucose metabolism is only partially understood.

Breastfeeding decreases the risk of diabetes development [4], infant morbidity and mortality and prevents gastrointestinal and respiratory infections [5,6,7,8,9]. Human milk contains soluble and cellular components such as lipids, carbohydrates, proteins, cells and hormones, which are important for the nutrition and immunological defense of infants [10]. Melatonin, one of the hormones contained in milk, is produced by the pineal gland and plays an important for infants [11].

Many of the benefits of melatonin and its metabolites are related to their antioxidant and anti-inflammatory properties [12,13] and prooxidant effects [14]. Melatonin also affects glucose regulation in humans [15,16]. Diabetic patients have lower diurnal serum melatonin levels and more pancreatic melatonin-receptors [17,18]. The role of melatonin in preventing or delaying diabetes onset, however, is not well established, because studies showing the beneficial effects of melatonin have been conducted after the development of the clinical manifestation of diabetes [19,20]. In addition, the actions of melatonin on endocrine pancreas physiology, including the probable reduction in diabetes incidence, have not been well described [18].

Melatonin is not a conventional hormone because it displays both receptor-mediated and receptor-independent actions. Therefore, regardless if they possess indolamine receptors, all cells in the body are a target for melatonin. Melatonin interacts with membrane (MT1 and MT2) and nuclear receptors (NMRs). Membrane receptors mediate functions such as seasonal reproduction, sleep and bone growth [16]. Using receptor-mediated and receptor independent mechanisms, melatonin seems to be involved in a number of physiological and metabolic processes. Melatonin can affect the levels of cAMP and intracellular $Ca^{2+,}$ and its action seems to be transmitted through the Gq-coupled MT1-receptor action on PLC and IP3 [21].

Human colostrum also contains large amounts of viable leukocytes, most of them macrophages. Both circulating and colostral leukocytes have phagocytic and bactericidal activity and produce free radicals [5,8,22,23]. Macrophages in diabetic patients were shown to have lower phagocytic and microbicidal activity and lower production of reactive oxygen species [8,24,25]. This suggests that the antioxidant systems of diabetic individuals are likely

compromised by high glucose levels [25]. Despite this evidence, studies on the functional activity of phagocytes in the colostrum of diabetic mothers, as well as the action of these defense cells on the gut of newborns and their interactions with melatonin are scarce. It is known that colostrum components possibly play an important protective role against pathogens in newborn guts, and colostral melatonin [11] may directly affect many gastrointestinal tissues. In addition to stimulating the immune system, melatonin prevents ulcerations of the gastrointestinal mucosa by antioxidant action [26].

The biological activity of colostral melatonin and its interactions with milk components is important because colostrum consists of a complete micro-environment, where the combined action of soluble and cellular components possibly plays a significant protective role against pathogens in newborn guts, especially considering infants of diabetic mothers who are highly susceptible to infections. Given the lack of information on this issue, the present study investigated the mechanisms of action of melatonin and its effects on the functional activity of phagocytes in the colostrum of diabetic women.

Materials and Methods

The effect of melatonin on the functional activity of colostrum phagocytes in hyperglycemic women, of any etiology, was evaluated in a cross-sectional study. The subjects attended the Diabetes and Pregnancy Facility, School of Medicine Obstetrics Course, UNESP, Botucatu, SP. This study was approved by the institutional Research Ethics Committee of Botucatu Medical School, and all the subjects gave informed written consent before entering the experimental protocol.

Subjects

We evaluated 76 women (18-45 years old) tested for hyperglycemia between the 24th and 28th weeks of pregnancy. Hyperglycemia was diagnosed by the 100g – oral glycemia tolerance test (OGTT test), according to ADA criteria [27], and the glucose profile (GP) test, according to Gillmer's threshold values [28]. After delivery, colostrum samples from these subjects were analyzed according to maternal glycemic status: normoglycemic group (normal 100 g-OGTT and normal GP; n = 38) and diabetes group (abnormal pre-pregnancy 100 g-OGTT and insulin-dependent; n = 38). The mean and standard deviation for gestacional age were 38.9±1.1 weeks in normoglycemic and the 37.3±0.9 weeks in diabetes groups and newborns'birth were 3127.3±339.4 in normoglycemic and the 3266.3±469.8 in diabetes groups. The subjects continued attending the facility, irrespective of diagnosis, and the hyperglycemic patients followed a specific treatment for glycemic control [28].

The variables controlled were smoking status (yes/no), arterial hypertension (yes/no) and glycemic index (GI), whose mean plasma glucose level was measured in the glycemic profiles

taken during the gestation. GI was classified as adequate (GI<120mg/dL) or inadequate (GI≥120mg/dL) [28].

Colostrum sampling and separation of colostral cells

About 8 mL of colostrum from each woman was collected in sterile plastic tubes between 48 and 72 hours postpartum. The samples were centrifuged (160xg, 4°C) for 10 min, which separated colostrum into three different phases: cell pellet, an intermediate aqueous phase, and a lipid-containing supernatant by Honorio-França et al [22]. The upper fat layer was discarded and the aqueous supernatant stored at -80^{o}C for later analyses. Cells were separated by a Ficoll-Paque gradient (Pharmacia, Upsala, Sweden), producing preparations with 98% of pure mononuclear cells, analyzed by light microscopy. Purified macrophages were resuspended independently in serum-free medium 199 at a final concentration of 2x10^{6} cells/mL.

Melatonin determination by the immunoenzymatic method

Melatonin was extracted by affinity chromatography, concentrated by speed-vacuum and determined using the ELISA kit (Immuno-Biological Laboratories, Hamburg, Germany). Reaction rates were measured by absorbance plate-reading spectrophotometer with a 405 nm filter. The results were calculated according to the standard curve and shown in pg/mL.

Treatment of colostral mononuclear phagocytes with melatonin and its precursor, antagonist and agonist

To assess the effects of melatonin on superoxide anion release, as well as on phagocytic and microbicidal activity, mononuclear phagocytes (2x10^{6}cells/mL) were incubated with 50 µL melatonin (MLT - Sigma, at a final concentration of 10-7M), N-acetyl-serotinin (NAS), luzindole and chloromelatonin (CMLT) for 1h at 37^{o}C. This final concentration of 10^{-7}M modulator was used in the tests [29].

To investigate the effects of intracellular Ca^{2+} on melatonin action, phagocytes (2x10^{6} cells/mL) were incubated with 10µL of 8-(Diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8 intracellular calcium inhibitor) at a final concentration of 0.1 mM-Sigma) for 1 h at 37^{o}C. The phagocytes were then washed once with medium 199 at 4°C and immediately used in the assays developed to measure superoxide release, phagocytosis and bactericidal activity.

E. coli strain

The enterophatogenic Escherichia coli (EPEC) used was isolated from stools of an infant with acute diarrhea (serotype 0111:H2, LA1, eae1, EAF1, bfp1). This material was prepared and adjusted to 10^{8} bacteria/ml, as previously described [22].

Release of superoxide anion

Superoxide release was determined by cytochrome C (Sigma, ST Loius, USA) reduction [22,30]. Briefly, mononuclear phagocytes and bacteria, opsonized or not with the aforementioned opsonins, were mixed and incubated for 30 min for phagocytosis. Cells were then resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C (Sigma, ST Loius, USA;2 mg/mL). The suspensions (100 µL) were incubated for 60 min at 37°C on culture plates. The reaction rates were measured by absorbance at 550 nm and the results were expressed as nmol/O^{2-}. All the experiments were performed in duplicate or triplicate.

Bactericidal assay

Phagocytosis and Microbicidal activity were evaluated by the acridine orange method described by Bellinati-Pires et al. [31]. Equal volumes of bacteria and cell suspensions were mixed and incubated at 37°C for 30 min under continuous shaking. Phagocytosis was stopped by incubation in ice. To eliminate extracellular bacteria, the suspensions were centrifuged twice (160xg, 10 min, 4°C). Cells were resuspended in serum-free medium 199 and centrifuged. The supernatant was discarded and the sediment dyed with 200µL of acridine orange (Sigma, ST Loius, USA; 14.4g/L) for 1 min. The sediment was resuspended in cold culture 199, washed twice and observed under immunofluorescence microscope at 400x and 1000x magnification. The phagocytosis index was calculated by counting the number of cells ingesting at least 3 bacteria in a pool of 100 cells. To determine the bactericidal index, we stained the slides with acridine orange and counted 100 cells with phagocytized bacteria. The bactericidal index is calculated as the ratio between orange-stained (dead) and green-stained (alive) bacteria x 100 [8]. All the experiments were performed in duplicate or triplicate.

Immunofluorescence and flow cytometry

We performed immunofluorescence staining at the FACS Calibur (BD San Jose USA) to assess intracellular Ca^{2+} release in colostrum phagocytes. Cells were loaded with the fluorescent radiometric calcium indicator Fluo-3. Cell suspensions, pre-treated or not with melatonin and TMB-8, mixed and incubated at 37°C for 30 min under continuous stirring. Suspensions were centrifuged twice (160xg, 10 min, 4°C) and resuspended in PBS containing BSA (5mg/mL). This suspension was incubated with 5µL of Fluo-3 (1µg/mL) for 30 min at 37°C. After incubation, cells were washed twice in PBS containing BSA (5mg/mL; 16 xg, 10 min, 4°C) and then analyzed by flow cytometry. Calibration and sensitivity were routinely checked using CaliBRITE 3 Beads (BD Cat. No 340486 USA). Fluo-3 was detected at 530/30 nm filter for intracellular $Ca^{2+.}$ The rate of intracellular Ca^{2+} release was expressed in geometric mean fluorescence intensity of

Fluo-3. Data shown in the figures correspond to one of several trials performed.

Statistical analysis

Analysis of variance (ANOVA) was used to evaluate superoxide anion release, phagocytosis, bactericidal index and calcium release. Statistical significance was considered for a p-value of less than 0.05.

Results

Melatonin concentration in colostrum

Melatonin concentration was higher in colostrum samples from hyperglycemic than normoglycemic mothers ($P < 0.05$; Figure 1).

Effects of melatonin (MLT) and TMB-8 on superoxide release by colostrum phagocytes

Hyperglycemic and normoglycemic groups exhibited similar spontaneous superoxide release by colostral mononuclear phagocytes, which did not increase with phagocyte exposure to EPEC. In the normoglycemic group, phagocytes stimulated with MLT and incubated with EPEC had higher superoxide release than those exposed to the bacteria alone ($p<0.05$), but MLT stimulation was not observed in the hyperglycemic group. Superoxide release increased when colostral phagocytes were incubated with NAS, irrespective of the group's glycemic status, whereas when incubated with CMTL this anion increased only in the normoglycemic group. Luzidole decreased superoxide release in both groups (Table 1). Phagocytes treated with TMB-8 (inhibitor of intracellular Ca^{2+}) displayed a reduction in superoxide release in all the groups studied (Table 1).

Effects of melatonin and TMB-8 on the phagocytic activity of colostral mononuclear cells

Colostral mononuclear phagocytes from the hyperglycemic groups had some phagocytic activity in response to EPEC. Phagocytosis increased significantly in the presence of MLT. The highest phagocytosis rates for EPEC were also exhibited by colostral phagocytes incubated with NAS and CMLT, independently of glycemic status (Table 2). Luzindole blocked MLT action on colostral phagocytes. The phagocytosis indexes of cells incubated with luzindole or MLT were similar to those exhibited by phagocytes incubated with bacteria alone (Table 2).

Pretreatment of mononuclear phagocytes with TMB-8 did not alter their phagocytic activity for EPEC in all the groups (Table 2).

Effect of melatonin and TMB-8 on the bactericidal activity of mononuclear phagocytes

In general, colostral mononuclear phagocytes had low bactericidal activity against non-opsonized EPEC. In the normoglycemic group, the bactericidal activity was higher when the phagocytes were incubated with MLT or NAS, but this effect was not observed in the hyperglycemic group. Incubation with luzindole decreased the effects of MLT on the bactericidal activity of colostral phagocytes. The bactericidal index of phagocytes incubated

with luzindole or MLT was similar to that of phagocytes incubated with bacteria alone (Table 3).

After incubation with MLT, NAS and CMLT, mononuclear phagocytes pretreated with TMB-8 had lower bactericidal activity than untreated cells in the normoglycemic groups (Table 3).

Intracellular Ca^{2+} release by colostral phagocytes in the presence of melatonin

Mononuclear phagocytes had low spontaneous intracellular Ca^{2+} release. In response to MLT, NAS and CMLT, colostral phagocytes displayed increased intracellular Ca^{2+} release in the normoglycemic groups (Table 4). Colostral from diabetes mothers decrease increased intracellular Ca^{2+} release (Figure 2). Incubation with luzindole decreased the effects of MLT on the intracellular Ca^{2+} release (Table 4). Pretreatment of mononuclear phagocytes with TMB-8 decreased intracellular Ca^{2+} release in both groups (Table 4 – Figure 2).

Discussion

The major findings of the present study are that melatonin was shown to increase superoxide production and bactericidal activity of colostral phagocytes from normoglycemic women, but not colostral phagocytes from hyperglycemic women. The bactericidal activity of colostral cells incubated with NAS, a melatonin precursor, was potentiated in both normoglycemic and hyperglycemic groups. Similar results were obtained when phagocytes were exposed to melatonin agonists, suggesting that melatonin and its agonist have the same mechanism of action. Finally, the mechanism of action of melatonin was shown to be dependent on intracellular Ca^{2+} release.

Superoxide production is known to increase in response to bacterial infection. In the present study, melatonin potentiated the production of these free radicals, irrespective of bacterial presence. Therefore, colostral melatonin probably enhances the protective role of colostrum against EPEC and other infections.

The beneficial actions of melatonin are associated to its ability to scavenge free radicals and increase antioxidant enzyme activity [32,33,34]. Moreover, melatonin exhibits immunomodulatotory effects [35] and stimulates immune cells [36,37]. This hormone stimulated the functional activity of phagocytes in the normoglycemic group, increasing superoxide release and EPEC killing capacity. However, an important finding in the present study was that colostral phagocytes from diabetic women were not stimulated by melatonin, showing low phagocytic and bacteria killing capacity. The insufficient stimulation of the functional activity of colostral macrophages, e.g., a failure of the prooxidant effect, indicates a diabetes-related antioxidant effect [13]. Similar results were observed in alloxan-induced diabetes models [29,38,39,40,41].

Immune cells produce a high amount of superoxide radical anion during oxidative stress [42,43,44], an important protective mechanism during infectious processes, particularly intestinal infections [5,6,7,8,9,14,22,45,46]. In the present study, colostral phagocytes from the diabetic group released superoxide, but unlike the control group, this response was not triggered by melatonin. Therefore, the uncontrolled glucose metabolism produced by diabetes may also stimulate macrophages [15,16,29].

NAS effects on superoxide release, phagocytic activity and the bactericidal capacity of colostral phagocytes was also evaluated in order to investigate the mechanisms of action underlying melatonin effects. Interestingly, binding to both MT1 and MT2 receptors was able to potentiate the activity of colostral phagocytes from normoglycemic mothers, but not those from hyperglycemic mothers. On the other hand, the agonist CMLT was able to stimulate the functional activity of colostrum phagocytes, irrespective of glycemic status, whereas the antagonist luzindole inhibited the effects of melatonin.

Melatonin plays a crucial role in a number of metabolic functions such as antioxidant and anti-inflammatory responses, and possibly as an epigenetic regulator [15]. As a multitasking indolamine, melatonin seems to be involved in several physiological and metabolic processes via receptor-mediated and receptor-independent mechanisms [47].

Melatonin-binding sites have been identified in several human immune cells such as T lymphocytes [48], bone marrow cells and Th2 lymphocytes [49], platelets [50], neutrophils, granulocytes [51] and monocytes [52]. In mammals, melatonin activates at least three distinct high-affinity receptors (MT1, MT2 and MT3). MT1 and MT2-melatonin receptors show 60% homology at the amino acid level and are encoded by separate genes [53]. The putative melatonin MT3 receptor binds to 2-[125I]-iodomelatonin and exhibits a different pharmacological profile (2-iodomelatonin > prazosin > N-acetylserotonin > melatonin) from that of the high-affinity sites (MT1 and MT2), where melatonin has significantly higher affinity than NAS [54,55,56]. All three melatonin receptors are blocked by luzindole [55]. This inhibitory effect was observed in the present study, where the luzindole concentration used blocked MT receptors, thereby decreasing superoxide release and EPEC killing by colostrum phagocytes.

When melatonin interacts with its receptors, the latter signal to second messenger 3’-5’-cyclic adenosine monophosphate (cAMP) or inositol triphosphate (IP3)/calcium(Ca2þ), changing intracellular concentrations of either cAMP or calcium. In the present study, pretreatment of colostral phagocytes with TMB-8 (intracellular calcium inhibitor) showed that the mediation of phagocytic activity by melatonin-receptor interaction is calcium dependent. On the other hand, melatonin induced intracellular calcium release by colostrum phagocytes. Some authors

have shown that TMB-8 effects include inhibition of calcium influx but not of calcium release [57], whereas others reported that TMB-8 inhibits calcium release [58].

The actions and effects of melatonin were shown to be transmitted via G-protein-coupled receptors (GPCRs), a class of membrane receptors expressed at low levels in numerous organs and cell types [59]. They are in general dependent on intracellular Ca^{2+}[21].

At physiological concentration, the pineal hormone melatonin can stimulate the natural immunity of animals because it activates colostrum phagocytes, an important defense with anti-infectious and microbicidal actions [7,8,9,30,14]. Colostral phagocytes from diabetic mothers, however, were not stimulated by melatonin. Therefore, the results obtained suggest an interactive effect of glucose metabolism and melatonin on colostral phagocytes. In colostral phagocytes from normoglycemic mothers, melatonin likely increases the ability of colostrum to protect against EPEC and other infections. In diabetic mothers, because maternal hyperglycemia modifies the functional activity of colostrum phagocytes, melatonin effects are likely limited to noninflammatory processes, with low superoxide release and bactericidal activity.

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Author Contributions

G. Morceli carried out the assay and participated in the sequence alignment and drafted the manuscript.

AC Honorio-França carried out the assay, conceived of the study, carried out the assays and participated in its design and coordination and help to draft the manuscript.

DLG Fagundes participated in the collect of samples, carried out the assays and help to draft the manuscript.

IMP Calderon participated in the design of the study and helped to draft the manuscript.

EL França participated in the design of the study and coordination and helped to draft the manuscript.

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Legends

Figure 1. Mean (±SD; N=10) melatonin levels (pg/mL) in the supernatant of colostrum from diabetic mothers *indicates difference between normoglycemic and hyperglycemic groups.

Figure 2. Intracellular Ca^{2+} release by mononuclear (MN) colostral phagocytes from diabetic mothers treated or not with TMB-8 and stimulated with melatonin. Cells were stained with Fluo-3, and immunofluorescence analyses carried out by flow cytometry (FACScalibur, Becton Dickinson, USA).

Table 1 – Superoxide release by colostrum macrophages (mean ± SD, N=8 per treatment).

Phagocytes incubated with:	TMB8	Superoxide release (nmol)	
		Normoglycemic	Diabetes
Control	No	1.2±0.4	1.2±0.1
(without bacteria)	Yes	0.5±0.2#	0.4±0.2#
Bacteria + PBS	No	1.7±0.6	1.4±0.1
	Yes	0.6±0.3#	0.3±0.1#
Bacteria + MLT	No	2.8±0.6*	1.6±0.5+*
	Yes	0.2±0.1#	0.2±0.1#
Bacteria + NAS	No	2.9±0.4*	2.3±0.4*
	Yes	0.15±0.1#	0.3±0.12#
Bacteria + Luzindole+MLT	No	0.5±0.13	0.40±0.05
	Yes	0.1±0.02#	0.45±0.03
Bacteria + CMLT	No	1,95±0.4*	1.43±0.5
	Yes	0.68±0.2#	1.0±0.3

Phagocytes were incubated with bacteria in the presence of MLT, NAS, luzindole or CMLT. In controls assays, phagocytes were pre-incubated with PBS. * indicates difference from the control treatment (ANOVA, P<0.05); + indicates intergroup differences within each treatment (ANOVA, P<0.05); # indicates differences between TMB-8 use within each treatment and group.

Table 2. Bacterial phagocytosis by colostral macrophages (mean ± SD, N=7 in each treatment), determined by the acridine orange method.

Phagocytes incubated with:	TMB-8	Phagocytosis (%)	
		Normoglycemic	Diabetes
Bacteria - Medium	No	49.1±8.8	44.2 ±2.5
199	Yes	48.8 ±3.3	42.±2.8
Bacteria + MLT	No	62.0±7.2*	75.2±3.5*
	Yes	68.4±7.9*	59.5±5.7*#
Bacteria + NAS	No	62.0±7.2*	72.5 ± 1.4*
	Yes	69.2 ±9.1*	70.5 ± 4.5*
Bacteria +	No	52.0±7.2	53.7 ± 5.9
Luzindole+MLT	Yes	54.8 ±5.7	52 ± 5.9
Bacteria + CMLT	No	76± 3.2*	79 ± 8.9*
	Yes		62 ± 2.6*#
		67.0±7.2*	

Phagocytes were incubated with bacteria in the presence of MLT, NAS, luzindole or CMLT.

* indicates differences from the 199 medium (ANOVA, P<0.05); + indicates intergroup differences within each treatment (ANOVA, P<0.05); and # indicates differences between TMB-8 use within each treatment and group.

Table 3. Bactericidal index (mean ± SD, N=7 in each treatment). Bactericidal activity by colostral phagocytes was determined with the acridine orange method.

EPEC	TMB-8	Bactericidal Index (%)	
		Normoglycemic	Diabetes
Control - Medium	No	25.7±5.2	32.3 ±5.9
199	Yes	23.5 ± 3,7	34.6±5.8+
MLT	No	64.9±8.0*	37.4 ±2.2+
	Yes	35.6 ± 11.5#	39 ± 4.4
NAS	No	63.7±6.3*	43.6± 8.8+
	Yes	38.9 ±4.6*#	36.6± 6.7
Luzindole + MLT	No	39.7 ± 5.7	42.4 ± 6.8
	Yes	32.1 ± 12.1	34.6 ± 6.5#
CMLT	No	50.7 ±16.2*	53.3 ±7.2*
	Yes	35.7±16.2#	37.1±6.8#

Phagocytes were incubated with bacteria in the presence of MLT, NAS, luzindole or CMLT. * indicates difference from the 199 medium (ANOVA, $P<0.05$); + indicates intergroup differences within each treatment (ANOVA, $P<0.05$); # indicates differences between TMB-8 use within each treatment and group.

Table 4. . Intracellular Ca^{2+} release by mononuclear (MN) colostrum phagocytes from diabetic mothers indicated by fluorescence intensity.

Phagocytes	TMB-8	Intensity (%)	
		Normoglycemic (N= 5)	Diabetes (N= 5)
FLUO3	No	35.7±10.2	29.4±8.0
	Yes	4.0±0.6#	5.1±0.8#
MLT + FLUO3	No	64.9±8.0	30.2±5.0*
	Yes	18.4±6.1#	13.0±6.0#
NAS + FLUO3	No	61.4 ± 9.3	38,5 ± 9.7+
	Yes	14,5 ± 7.9#	9.6 ± 3.9#
Luzindole + MLT +	No	13.5 ± 2.9*	4.5 ± 1.3*+
FLUO 3	Yes	10.2 ± 5.2	3.9 ± 1.1+
CMLT + FLUO3	No	58.9 ± 12.5	12.3 ± 2.5+
	Yes	7.5 ± 4.7#	7.7 ± 3.2

Phagocytes were incubated with melatonin and pre-treated or not with TMB-8. Results are expressed as mean and SD for 5 experiments with cells from different individuals. * indicates difference from the FLUO3 (ANOVA, $P<0.05$); + indicates intergroup differences within each treatment (ANOVA, $P<0.05$); # indicates differences between TMB-8 use within each treatment and group.

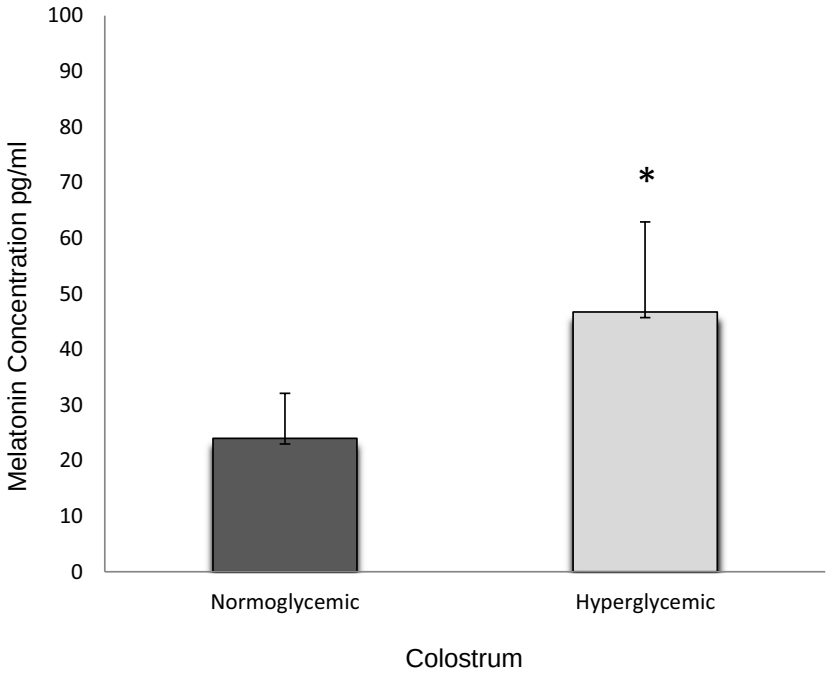
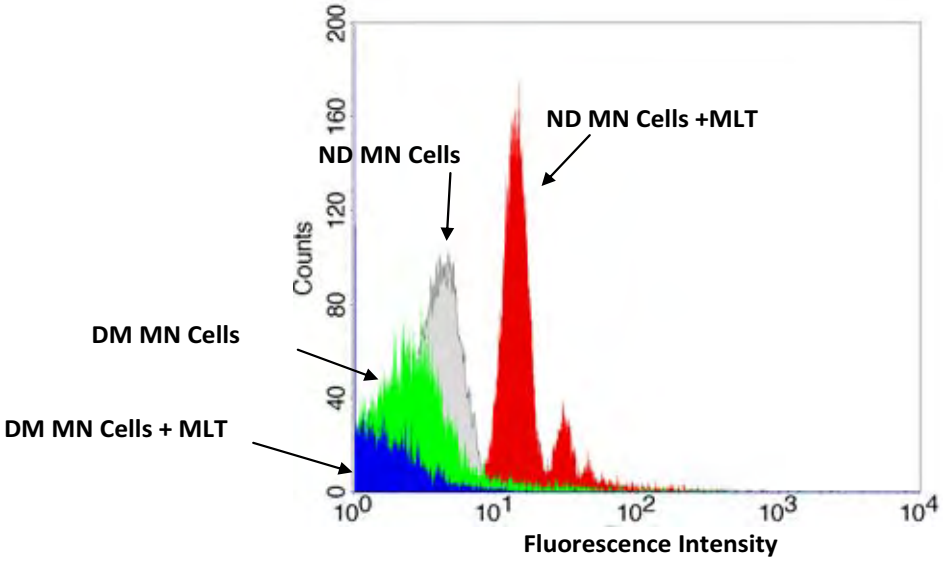

100
90
80
70
60
50
40
30
20
10
0
Melatonin Concentration pg/ml
*
Normoglycemic
Hyperglycemic
Colostrum

Figure 1.


ND MN Cells +MLT
ND MN Cells
DM MN Cells
DM MN Cells + MLT
Counts
Fluorescence Intensity
TMB-8 -

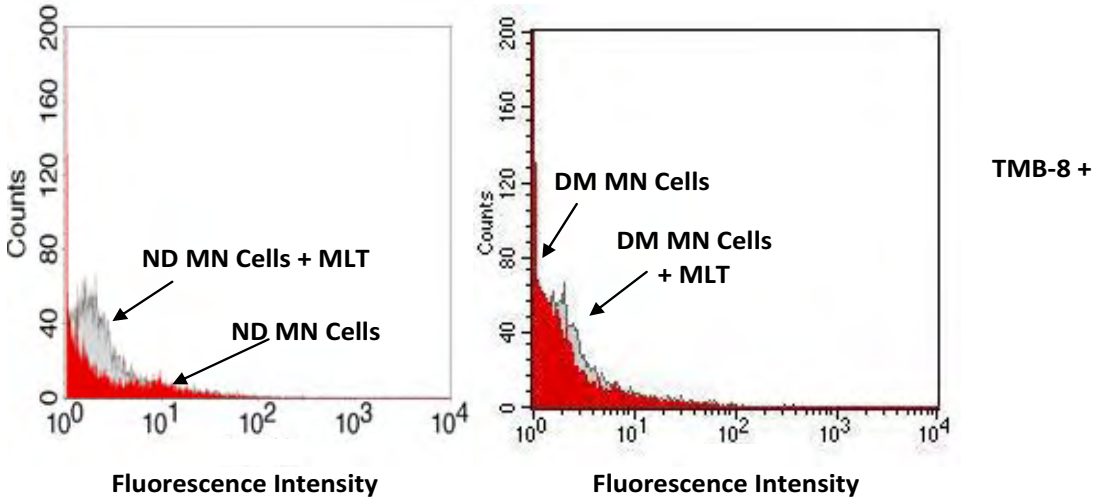

ND MN Cells + MLT
ND MN Cells
DM MN Cells
DM MN Cells
+ MLT
Counts
Fluorescence Intensity
Fluorescence Intensity
TMB-8 +

Figure 2.

Conclusões

CONCLUSÕES / principais resultados

- ✓ O colostro de mães diabéticas, apresenta menor concentração de IgA e IgG e diminuição de proteína C3 , menor teor de gordura e aumento dos níveis de glicose, proteínas totais e lipase.
- ✓ Fagócitos do colostro expressam FcαR, possuem IgA na superfície, são capazes de se ligar a SIgA purificada do próprio colostro e tem menor atividade microbicida. Autilização de IgA como opsonina restaura a atividade bactericida dos fagócitos do colostro de mães hiperglicêmicas. A adição do MoAb anti-receptor Fcα resultou em significativa diminuição da liberação do ânion superóxido e morte bacteriana pelos macrófagos quando as bactérias foram opsonizados com IgA purificada, sugerindo a interação entre SIgA e o receptor FcαR.
- ✓ A concentração de melatonina foi maior no colostro de mães diabéticas. A melatonina aumentou atividade funcional dos fagócitos do colostro de mães normoglicêmicas, mas não nas mães diabéticas. O luzindol foi capaz de bloquear a ação da melatonina sobre a atividade destas células, sugerindo interação deste hormônio a receptores específicos. O tratamento dos fagócitos do colostro com TMB-8 diminuiu a liberação de cálcio intracelular determinando menor atividade funcional destas células, em ambos os grupos estudados.

Em fagócitos do colostro de mães normoglicêmicas, a melatonina provavelmente aumenta a capacidade destas células em proteger o recém-nascido de infecções respiratórias e gastrointestinais. Em mães diabéticas, a hiperglicemia materna modifica a atividade funcional dos fagócitos do colostro, e os efeitos da melatonina são provavelmente limitados a processos não inflamatórios, com menor liberação do superóxido, cálcio intracelular e atividade bactericida.

Os resultados deste estudo suportam a hipótese de que a produção de componentes de leite em mães diabéticas apresentam alterações devido aos distúrbios no metabolismo da glicose. Portanto, o controle adequado da glicemia materna de mães diabéticas é fundamental para garantir as necessidades nutricionais e imunológicas para o recém-nascido.

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Anexos

ANEXOS

ANEXO 1_Figura 1

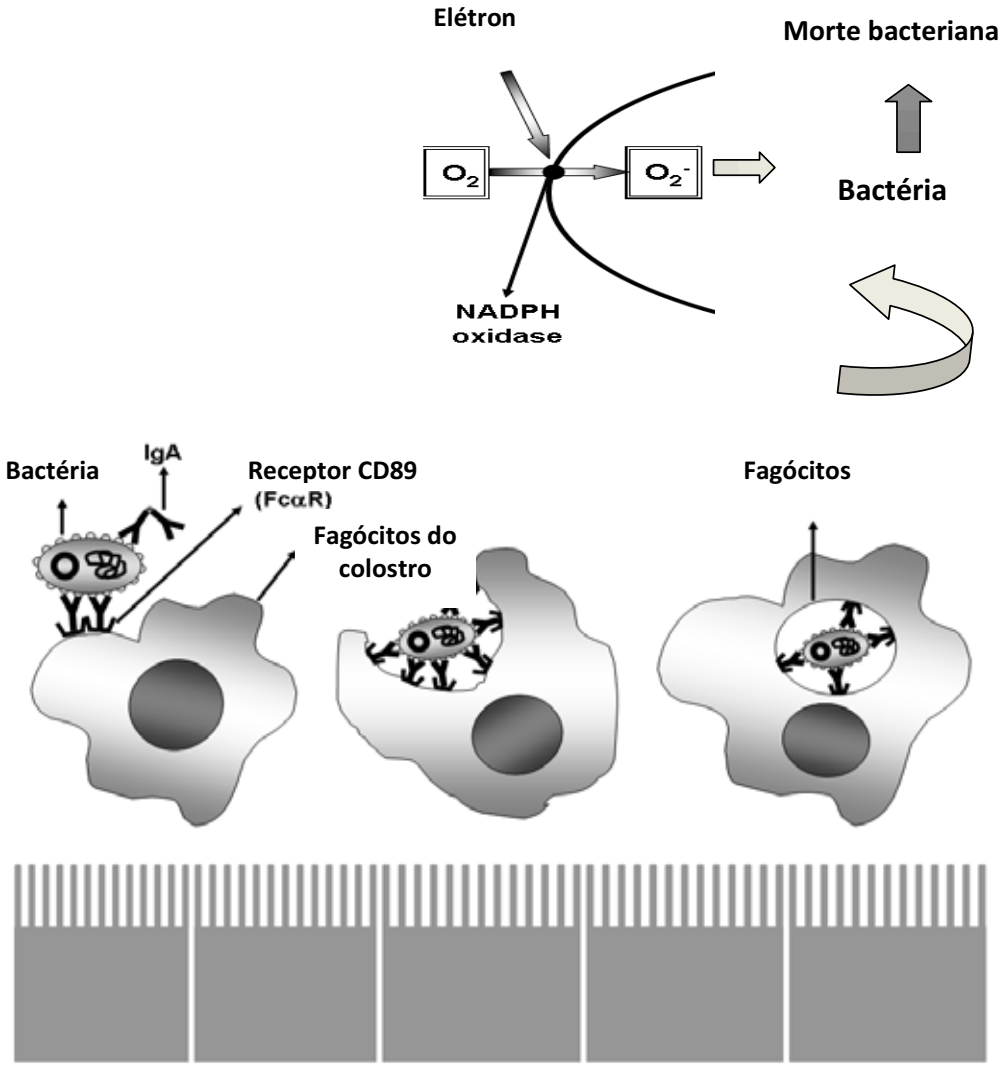

Figura 1. Modelo hipotético da atividade funcional dos fagócitos do colostro para EPEC, via interação IgA e receptor CD89(IgA-Fcα).

ANEXO 2_Figura 2

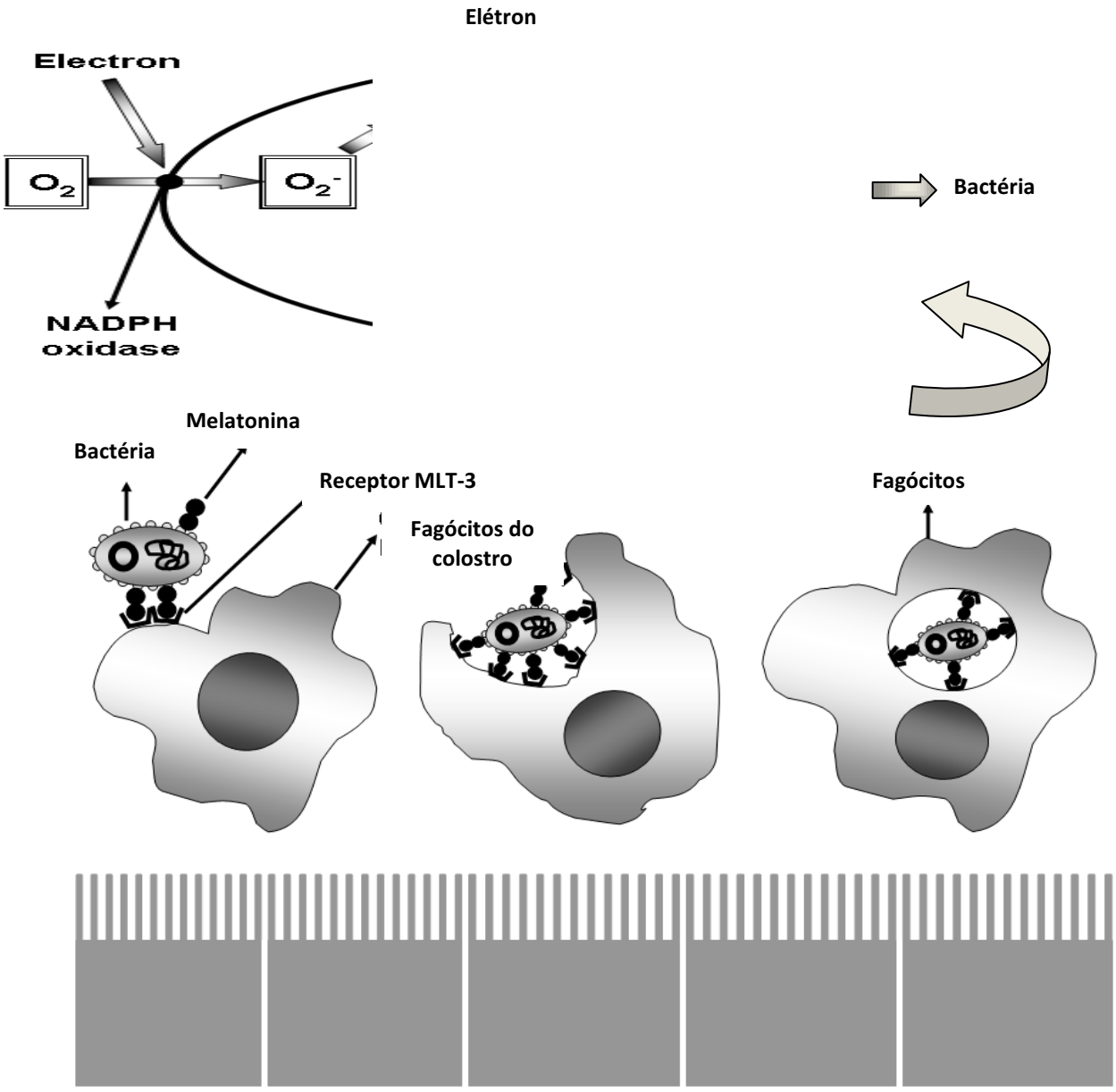

Figura 2. Modelo hipotético da atividade funcional dos fagócitos do colostro para EPEC via interação melatonina e receptor.

ANEXO 3_Figura 3

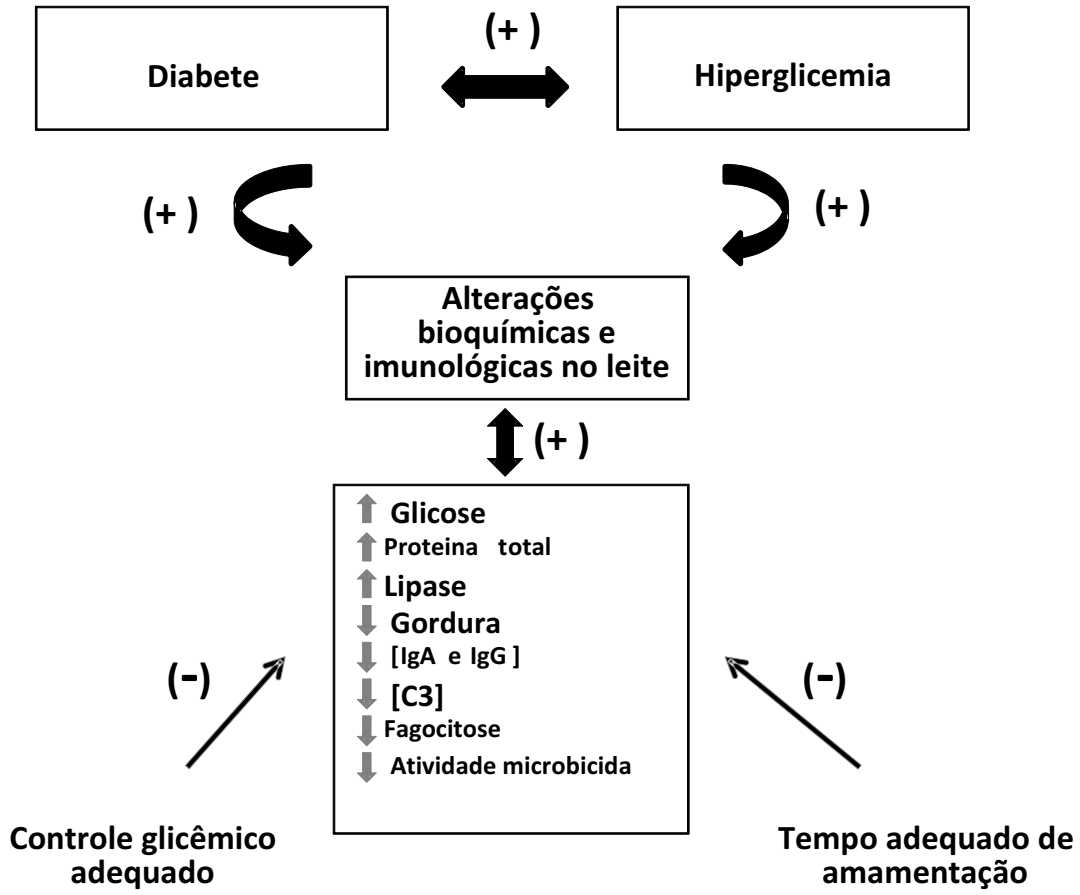

Figura 3. Relação entre o diabete e o aleitamento materno

ANEXO 4_Carta de aprovação do projeto no CEP

unesp Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

Distrito Rubião Junior, s/nº - Botucatu – S.P.
CEP: 18.618-970
Fone/Fax: (0xx14) 3811-6143
e-mail secretaria: capellup@fmb.unesp.br
e-mail coordenadoria: tsarden@fmb.unesp.br

Ética

Registrado no Ministério da Saúde em 30 de abril de 1997

Botucatu, 06 de fevereiro de 2012 Of. 34/2012

Ilustríssima Senhora
Profª. Drª. Iracema de Mattos Paranhos Calderon
Departamento de Ginecologia e Obstetrícia da
Faculdade de Medicina de Botucatu

Prezada Drª. Iracema,

De ordem do Senhor Coordenador deste CEP, informo que o Projeto de Pesquisa: **(Protocolo CEP 2903-2008)** "**Atividade funcional de fagócitos do leite de mães hiperglicêmicas e diabéticas e sua regulação neuroimunoendocrinológicas**",coordenado por Vossa Senhoria e conduzido por Adenilda Cristina Honório França, aprovado por este CEP em 01/09/2008 conta com um **sub-projeto** à saber:

Sub-Projeto: "**Atividade funcional de fagócitos do leite de mães hiperglicêmicas e diabéticas e sua relação neuroimunoendocrinológica - Interações com IgA e melatonina**", de autoria de Glilciane Morceli, orientada por Vossa Senhoria.

Situação do Projeto: **APROVADO**. Os pesquisadores deverão apresentar ao CEP ao final da execução dos Projetos o "**Relatório Final de Atividades**".

Atenciosamente,

Alberto Santos Capelluppi
Secretário do CEP

unesp Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

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Registrado no Ministério da Saúde em 30 de abril de 1997

PARECER CONSUBSTANCIADO

Projeto de Pesquisa: Atividade funcional de fagócitos do leite de mães hiperglicêmicas e diabéticas e sua regulação neuroimunoendocrinológica.

Pesquisadores: Adenilda Cristina Honório França - Orientadora: Profª Adjunta Iracema de Mattos Paranhos Calderon.

Departamento: Ginecologia e Obstetrícia.

Data da Aprovação pelo CEP: 01/09/2009.

Assunto: Relatório de Atividades do Projeto, bem como solicitação de prorrogação do Projeto por mais 12 meses.

Parecer do Relator: A pesquisadora responsável solicitou prorrogação por mais 12 meses do projeto supra citado, justificando a necessidade de completar o estudo realizando as mesmas análises no leite no sangue materno. Apresentou relatório dos dados obtidos até o presente momento (manuscrito em preparação) e modificação proposta para novo TCLE. O novo TCLE está claro e apenas acrescenta ao anterior que será feita a coleta de 8ml de sangue periférico além do sangue materno.

Somos de Parecer Favorável ao atendimento da prorrogação do prazo.

Parecer do Coordenador do CEP:

DE **ACORDO: AUTORIZO** a prorrogação por mais 12 meses do estudo supracitado, esclarecendo que ao final, deverá ser apresentado Relatório Complementar de Atividades.

Prof. Dr. Trajano Sardenberg
Coordenador do CEP