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Avaliação da atividade imunomoduladora de nanopartículas de prata em células humanas do sangue periférico

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Avaliação da atividade imunomoduladora de nanopartículas de prata em células humanas do sangue periférico

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia, da Universidade Federal da Paraíba, como parte dos requisitos para obtenção do título de Mestre em Odontologia – Área de Concentração em Ciências Odontológicas.

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DASAIEV MONTEIRO DUTRA

AVALIAÇÃO DA ATIVIDADE IMUNOMODULATÓRIA DE NANOPARTÍCULAS DE PRATA EM CÉLULAS HUMANAS DO SANGUE PERIFÉRICO

Banca Examinadora

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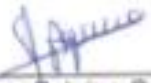

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RESUMO

Introdução: Ainda são escassos os trabalhos na literatura que avaliam a ação imunomoduladora das nanopartículas de prata (AgNPs) em células humanas do sangue total, apesar de sua crescente utilização na imunoterapia e fabricação de materiais odontológicos com atividade antimicrobiana. **Objetivo**: O objetivo deste estudo foi avaliar a atividade imunomoduladora de nanopartículas de prata em células humanas do sangue periférico. **Material e Métodos:** AgNPs foram sintetizadas seguindo o método Turkevich na concentração final de 3,72 ug/ml. A morfologia arredondada e o tamanho nanométrico das nanopartículas de prata foram verificadas por Microscopia Eletrônica de Varredura (MEV) e de Transmissão (MET) em solução estabilizada por carboximetilcelulose sódica 10 dias após a síntese. O sangue periférico foi coletado de seis voluntários saudáveis, sendo as amostras diluídas em meio RPMI1640 e incubadas por 24h/37°C/5% de CO_2, na presença de 5 µg/ml de fitohemaglutinina (PHA), 1 µg/ml de lipopolissacarídeo (LPS) e AgNP. A titulação de citocinas (TNFα, IL1β, IFN-γ, IL6, IL10, IL-4) foi realizada por ELISA. O óxido nítrico (NO) foi medido por reação de Griess. **Resultados**: Observou-se aumento da produção de IL-6 (p=0.0037) e NO (p=0.022) após a estimulação com AgNP comparada com o nível basal. A indução de IFNγ, TNF-α, IL1β, IL-4 por AgNP foi semelhante aos níveis basais. Apenas a resposta de IL-6 foi positivamente correlacionada com a produção de IL-4 (p=0.033, r=0.845). **Conclusão**: Os resultados da atividade imunomoduladora de nanopartículas de prata em células humanas sugerem o papel prefencial da IL-6 induzida por AgNP nas células do sangue periférico humano.

Palavras-chave: Nanopartículas de prata, Imunomodulação, Imunoterapia, Ensaio de sangue total.

ABSTRACT

Introduction: Is spite of the promising studies in immunotherapy and the fabrication of dental materials with antimicrobial activity, there are few studies in the literature evaluating the immunomodulatory action of silver nanoparticles (AgNP) in human cells from the whole blood. **Objective**: The aim of this study was to evaluate the immodulatory activity of silver nanoparticles in human peripheral blood cells. **Material and Methods:** Silver nanoparticles were synthesized following the Turkevich method at final concentration of 3.72 µg/ml. Nanoscale particles with rounded shape were observed by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) of the solution stabilized by carboxymethylcellulose ten days after the synthesis. Peripheral blood was collected from six healthy volunteers in containing tubes. Samples were diluted in RPMI1640 medium and incubated for 24h/37ºC/5% CO_2 in the presence 5 µg/ml phytohaemagglutinin (PHA), 1 µg/ml lipopolysaccharide (LPS) and AgNP. Cytokine titration (TNFα, IL1β, IFNγ, IL6, IL10, IL-4) was performed by ELISA. Nitric oxide (NO) was measured by Griess reaction. **Results**: Increased production of IL-6 ($p=0.0037$) e NO ($p=0.022$) was observed after AgNP stimulation. The induction of IFNγ, TNF-α, IL1β, IL-4 by AgNP was similar to basal levels. Interestingly, only the IL-6 response correlated positively with IL-4 production ($p=0.033$, $r=0.845$). **Conclusion**: Results suggest that L-6 is preferentially induced by AgNP in human peripheral blood cells.

Keywords: Silver nanoparticles, Immunomodulation, Immunotherapy, Whole blood assay.

LISTA DE ABREVIATURAS E SIGLAS

AgNP – Nanopartícula de prata

ATP – Trifosfato de Adenosina

BSA – Soro albumina bovina

cAMP – AMP cíclico

CMC – Carboximetilcelulose sódica

DNA – Ácido desoxirribonucleico

EC_{50} – Concentração do fármaco que induz metade do efeito máximo

ELISA – Ensaio imunoabsorvente ligado à enzima

FBS – Soro fetal bovino

IFNγ – Interferon gama

IgA – Imunoglobulina A

IgG – Imunoglobulina G

IgM – Imunoglobulina M

IL1β – Interleucina-1 Beta

IL-4 – Interleucina 4

IL-6 – Interleucina 6

LPS – Lipopolissacarídeo

MEV – Microscópio eletrônico de varredura

MET – Microscópio eletrônico de transmissão

NO – Óxido nítrico

PBMC – Células mononucleares de sangue periférico

PBS – Tampão fosfato-salino

PHA – Fitohemaglutinina

ROS – Espécies reativos de oxigênio

SEM – Microscópio eletrônico de varredura

TEM – Microscópio eletrônico de transmissão

THP-1 linhagem de células humanas monocíticas derivadas de pacientes com leucemia aguda monocítica

TMB - 3,3’,5,5’-Tetrametilbenzidina

SUMÁRIO

1. INTRODUÇÃO

1.1 Nanopartículas de prata

As nanopartículas de prata (AgNP) são materiais utilizados em biotecnologia básica e aplicada (LIU, 2006) com tamanho entre 1-100nm (WILLIAMS, 2008). Com forte atividade antimicrobiana, as AgNP são largamente utilizadas como principal componente de produtos de uso diário, industriais e relacionados à saúde (CHERNOUSOVA, EPPLE, 2013).

Vários estudos têm demonstrado a aplicação das AgNP na área biomédica. Chen et al. (2006) avaliaram a influência da incorporação de AgNP em curativos no tratamento de queimaduras. Foram investigados 191 pacientes com queimadura de segundo grau. 65 indivíduos foram tratados com nanopartícula de prata (grupo A), 63 com sulfadiazina de prata (grupo B) e 63 com vaselina (grupo C). Os autores observaram que os grupos A e B apresentaram o mesmo efeito de redução bacteriana, contudo o tempo de cura da nanopartícula de prata foi significantemente maior.

Böswald et al. (1999) relataram o uso das AgNP nos cateteres venosos, investigando a capacidade de um cateter venoso impregnado com AgNP em reduzir infecções. Os pesquisadores realizaram inserção percutânea de 165 cateteres venosos clinicamente e compararam os resultados com um grupo controle. O estudo mostrou redução estatisticamente significante de 37,7% das infecções no grupo impregnado com AgNP.

O estudo de Cohen et al. (2007) revelou aplicação na área cirúrgica. Analisou-se a atividade preventiva de malhas cirúrgicas com AgNP contra infecções. Aplicaram-se 0,31 mg/cm^2, 0.64 mg/cm^2 e 1.13 mg/cm^2 de AgNP em malhas cirúrgicas e comparou-se o resultado no grupo sem tratamento da superfície. Os autores observaram aumento da inibição de *Staphylococcus. aureus* em todos os grupos cobertos com AgNP ($P<0,001$) comparado ao grupo controle.

1.2 Efeito antimicrobiano das AgNPs

As AgNP têm oferecido grande interesse à comunidade científica devido a seu potencial efeito antimicrobiano (PAREDES, 2014). O estudo de Gouveia (2014) objetivou sintetizar e caracterizar AgNPs e avaliar sua atividade antimicrobiana, *in vitro*, sobre *Streptococcus.mutans*, a partir de dois estabilizadores poliméricos, com Poli (Vinilpirrolidona) (PVP) e Carboximetilcelulose Sódica (CMC). Concluiu-se que a síntese utilizando CMC apresentou melhor estabilização coloidal, sendo adequada para a formação de AgNP e com melhor eficácia antimicrobiana, com CIM de 9,3 µg/mL e CBM de 33,6 µg/mL, sobre *Streptococcus mutans*.

Saeb et al. (2014) sintetizaram, caracterizaram e analisaram a atividade antimicrobiana de AgNPs obtidas pela redução de nitrato de prata por sobrenadantes de *Escherichia hermannii, Citrobacter.sedlakii e Pseudomonas. putida*. Segundo os autores, a AgNP obtida de *E. hermannii* apresentou melhor atividade antimicrobiana contra *Klebsiella pneumoniae, Staphylococcus epidermitis, S. aureus* e *Escherichia coli.* Além disso, a nanopartícula de prata obtida de *Citrobacter sedlakii* apresentou melhor atividade antimicrobiana contra *P. aeruginosa.*

As AgNPs também foram sintetizadas a partir da reação de nitrato de prata ($AgNO_3$) com a alga marinha *Sargassum cinereum*. (MOHANDASS et al. 2013). Obteve-se boa atividade antimicrobiana contra as bactérias patogênicas *Enterobactor aerogens, Proteus vulgaris* e *Salmonella typhi*.

Por outro lado, Lokina et al. (2014) descreveram o uso do extrato de maçã (*Malus domestica*) para a síntese de AgNP. As AgNP de forma esférica apresentaram excelente efeito inibitório contra *S. aureus, Pseudomonas aeruginosa, Escherichia coli, Citrobacter koseri* e *Bacillus cereus*, incluindo *Candida albicans*.

O estudo de Lu et al. (2013) demonstrou a relevância da atividade antimicrobiana das AgNP na Odontologia. O autor avaliou a atividade antimicrobiana das AgNPs de 5, 15 e 55nm contra bactérias da cavidade oral. Verificou-se que a AgNP de 5nm apresentou maior atividade antimicrobiana contra *S. mutans, Streptococcus sanguis, Streptococcus mitis*. Essas bactérias

estão presentes na dentina cariada, polpa infectada, doença periodontal avançada e abscessos dentários (MARSH et al. 2009; LOVE et al. 2002; LOESCHE, 1986). Além disso, observou-se maior atividade antimicrobiana da AgNP de 5nm contra *Aggregatibacter actinomycetemcomitans* e *Fusobacterium nucleatum*. Finalmente, a bactéria aeróbia *E. coli* foi mais susceptível à AgNP que os outros microorganismos.

Além disso, Li et al. (2006) demonstraram a utilização das AgNP na fabricação de máscaras faciais embebidas com AgNP. Verificou-se 100% de redução de *E. coli* e *S. aureus* viáveis após 48h, caracterizando um material promissor no combate à transmissão de agentes infecciosos.

A interação das AgNP com colônias de fungos foi estudada por Monteiro et al., (2014) que investigaram a susceptibilidade de biofilmes de *C. albicans* e *Candida glabrata*, em estágios intermediário e maduro, à influência das AgNPs. Os resultados mostraram que as AgNPs promoveram reduções significativas na biomassa total e UFCs de *Candida*, variando de 23% a 51,5%. Além disso, os estágios intermediário e maduro de desenvolvimento não interferiram na susceptibilidade dos biofilmes de *C. albicans* e *C. glabrata* às AgNPs, exceto para a numeração *C. albicans* 324LA/94 UFC. Portanto, esses autores demonstraram que as AgNP podem ser utilizadas no tratamento de estomatites associadas a *Candida*, microrganismos abundantes na cadidade oral (BUDTZ-JORGENSEN et al. 1970).

Kasrei et al. (2014) demonstraram que as propriedades antimicrobianas das AgNP podem ser aplicadas na adição de aditivos em resinas compostas, impedindo a formação de *S. mutans* e *Lactobacillus* na superfície dentária. O estudo avaliou a formação de biofilme em discos de resina com 0% e 1% de AgNP e concluiu que esse nanomaterial inibe significativamente o crescimento desses importantes microrganismos presentes na cavidade bucal.

Nos procarionteses, as AgNPs interagem com o peptidoglicano da parede celular (YAMANAKA et al. 2015) e membrana plasmática (JUNG et al. 2008), causando a lise da célula. Além disso, os íons de prata se ligam às bases nitrogenadas do DNA bacteriano (SHRIVASTAVA, 2007), causando sua condensação e perda da capacidade de replicação e reprodução (YAMANAKA et al. 2005). Os íons de prata podem desnaturar os ribossomos, inibindo a síntese

protéica, provocando degradação da membrana plasmática (CHALOUPKA, MALAM, SEIFALIAM, 2010).

1.3 Toxicologia das AgNPs

O efeito toxicológico das AgNPs em células de mamíferos tem sido alvo de estudos. Ashahani, Hande e Valiya veettil (2009) expuseram fibroblastos pulmonares e células de glioblastoma humanas a concentrações de 25µg/mL, 100µg/mL e 200 µg/mL. Observou-se por MEV que as células do grupo controle apresentaram morfologia normal, enquanto os fibroblastos apresentaram-se mais esféricos. Os pesquisadores também verificaram a presença de endossomos contendo AgNP na membrana plasmática, demonstrando o mecanismo de entrada da nanopartícula na célula. Por outro lado, o estudo de Houssain et al., (2005) revelou que as mitocôndrias foram o primeiro alvo de células do fígado de ratos expostas a AgNP.

Carlson et al. (2008) avaliaram as interações celulares de AgNPs medindo 15nm, 30nm e 55nm em macrófagos alveolares e observaram diminuição significativa da função mitocondrial das células expostas a doses entre 10-75µg/mL, através do ensaio de viabilidade MTT. Assim, as AgNPs interferiram na cadeira transportadora de elétrons da organela.

O estudo de Ashahani, Hande e Valiya veettil (2009) também revelou que as AgNPs aumentaram a expressão de genes relacionados ao extresse oxidativo, com aumento de HO-1 mRNA. Para Rahman et al., (2009) outros genes estão envolvidos, como superóxido dismutase 2 e glutationa redutase 1. Mroz et al. (2008) observam que o extresse oxidativo ativam p53 e proteínas ligadas ao reparo de DNA, mimetizando a carcinogênese.

De acordo com Zhang et al., (2014) o estresse oxidativo causado pelas AgNPs pode desencadear resposta inflamatória, incluindo ativação da imunidade inata e aumento da permeabilidade das células endoteliais. Em doses não-citotóxicas, as AgNPs podem induzir dano ao DNA, anormalidade cromossomal e mutagenicidade.

Barkhordari et al. (2014) avaliaram a toxicidade de AgNPs em células mononucleares sanguíneas e observaram que após exposição de 6h a citotoxidade variou conforme a concentração de AgNP. A morte celular variou entre 10,9% (1 µg/mL AgNP) e 48,4% (500 µg/mL AgNP). Após exposição de 24h, houve morte celular em 56,8% das células (1 µg/mL), variando até 86,3% (500 µg/mL). A máxima toxicidade foi observada na dose de 500µg/mL. Stoehr et al. (2011) avaliaram o efeito citotóxico das nanopartículas e nanofibras de prata em células de carcinoma de pulmão A549 e observaram que nanofibras induzem maior citotoxicidade que as AgNP. Esses dados indicam que a citotoxidade das AgNPs é dependente do tempo de exposição, dose e forma dos objetos.

1.4 Importância para Odontologia

Diversos estudos têm mostrado a utilidade da atividade antimicrobiana das AgNPs em materiais odontológicos. Cheng et al. (2012) desenvolveram uma resina composta incorporando dimetacrilato de amónio quaternário, AgNPs de prata e fosfato de cálcio amorfo. Os pesquisadores, em seguida, compararam a inibição de *Streptocuccus mutans* e a produção de ácido lático no biofilme aderido a esse material em relação a uma resina composta convencional. O estudo demonstrou que houve redução estatisticamente significante de *S. Mutans* na nova resina composta ($p<0,05$) e menor produção de ácido lático, comparado ao grupo controle ($p<0,05$). Esses resultados revelam o potencial das AgNPs no desenvolvimento de novos materiais restauradores que contribuam para a redução da cárie dentária.

As AgNPs também foram empregadas na fabricação de novos sistemas adesivos, com a vantagem de inibir o crescimento bacteriano e reduzir o índice de cárie secundária nos elementos dentários. Com essa finalidade, Melo et al. (2012) adicionaram 0,1% em masssa de AgNP em um sistema adesivo e compararam a redução de biofilme e produção de ácido lático desse material com o sistema adesivo Scotchbond. Os resultados mostraram que houve redução significativa da viabilidade do biofilme e produção de ácído lático ($p<0,05$) comparado ao grupo controle, sem comprometer a união com a dentina.

Outra aplicação consiste na utilização da AgNP no tratamento endodôntico. O estudo de Samiei et al. (2013) demonstrou que a incorporação da AgNP no MTA pode ser útil na redução do biofilme radicular. O trabalho *in vitro* avaliou as propriedades antimicrobianas do MTA Angelus e MTA com AgNP a 1% em massa contra *E. faecalis*, *P. aeruginosa*, *S. aureus*, e *C. albicans*. Observou-se que a inibição bacteriana foi significantemente maior no grupo com AgNP. Portanto, o trabalho demonstra que as AgNPs podem melhorar significantemente as propriedades do materiais atualmente disponíveis na clínica odontológica, promovendo maior eficácia ao tratamento.

Com relação ao uso da resina acrílica na área de Prótese, Torres et al. (2012) demonstraram a aplicação das AgNPs na prevenção de infecção por *Candida albicans* em usuários de prótese bucal. Os pesquisadores revelaram que discos de polimetilmetacrilato + AgNP reduziram significantemente a aderência de *C. Albicans* em comparação ao grupo controle, composto de resina acrílica comercial. Assim, as propriedades biocompatíves do material são uma alternativa viável à fabricação de próteses totais, com capacidade de reduzir a prevalência de estomatite protética na população.

A adição de AgNP em implantes de titânio tem sido utilizada como prevenção de infecções durante o pós-operatório. Zhao et al. (2011) inseriram amostras de implante dentário em concentrações de 0,5; 1; 1,5 e 2 M de AgNPs incubadas com *S aureus*. Os pesquisadores relataram completa redução das bactérias planctônicas durante 30 dias, período suficiente para prevenir infecção pós-operatória. Logo, a atividade antimicrobiana das AgNPs também permite a evolução dos procedimentos cirúrgicos aplicados na Odontologia, contribuindo para o tratamento e reabilitação do paciente.

Além disso, Santos Jr et al. (2014) realizaram um ensaio clínico randomizado para avaliar o efeito da aplicação de uma solução composta por nanopartícula de prata, nanopartícula de quitosana e fluoreto em 63 dentes com lesão de cárie presentes em crianças. O estudo revelou que, após 7 dias, 81% dos dentes tiveram a lesão de cárie paralisada, em oposição ao grupo controle, que não paresentou efeito. Após 5 meses, observou-se paralisação da cárie em 72,7% das lesões do grupo experimental e 27,4% no grupo controle. Aos 12 meses, 66,7% das lesões de cárie foram paralisadas no grupo experimental, enquanto 34,7% permaneceram paralisadas no grupo controle. O trabalho demononstra a eficácia

das AgNPs no tratamento da cárie dental, auxiliando o Cirurgião-Dentista no tratamento odontológico. Ainda, são necessários estudos para avaliar o efeito imunomodulador das AgNPs no organismo humano.

1.5 Imunoterapia

O uso das AgNPs como agentes imunoterápicos é visto como grande potencial de aplicação. Diante disso, torna-se necessário o entendimento dos mecanismos imunoinflamatórios que poderiam ser modulados pelos nanomateriais. Dentre algumas de grande papel inflamatório, destaca-se a interleucina-6, que possui efeito pleiotrópico no corpo humano. Assim, O estudo de Park et al. (2011) demostrou que a AgNP induz o aumento da produção de IL-8 em macrófagos, enquanto que os resultados de Yang et al. (2012) revelaram aumento de IL-1β nessas células. Por outro lado, a indução de citocinas também foi avaliada em células pulmonares de ratos no estudo de Hirn et al., (2014) no qual os pesquisadores encontraram diminuição na produção de TNF- α.

Segundo Ogata e Tanaka (2012), o bloqueio da interneucina-6 constitui uma nova estratégia terapêutica para o tratamento de doenças. Para tal, foi desenvolvido *in vivo* o tocilizumab, o anticorpo monoclonal humanizado anti-receptor de IL-6 humana (NATIONAL HORIZON SCANNING CENTRE, 2009). Conforme descrito por Ogata e Tanaka (2012), o tocilizumab se liga ao IL-6R solúvel e IL-6R transmembrana bloqueando a reação da IL-6 com esses receptores.

Itoh et al. (2004) desenvolveram um ensaio clínico randomizado piloto com 36 pacientes com doença de Crohn ativa e demonstraram que a administração de 8 mg/kg de tocilizumab durante 2 semanas reduziu a resposta de 80% desses pacientes, comparado ao grupo tratado com placebo, no qual houve redução de 39% da resposta. Essa terapia também é eficaz no tratamento de doenças como artrite reumatoide (OHSHIMA et al. 1998), doença de Castleman (NISHIMOTO et al. 2005), esclerose sistêmica (KUWAHARA et al. 2008) e doença de Behçet (HIROHATA et al. 1997).

De acordo com Hoy e Scott (2007) outro alvo da imunoterapia é o TNF. A partir do desenvolvimento do etanercept, um receptor protéico dimérico,

recombinante e solúvel, tornou-se possível inibir a ligação o TNF-α e TNF-β com receptores ligados à membrana. Calin et al. (2004) realizaram um ensaio clínico randomizado duplo cego para avaliar a eficácio do etanercept no tratamento de espondilite anquilosante em pacientes adultos comparado a um grupo placebo. Um total de 45 pacientes receberam injeções de 25 mg de etanercept 2 vezes por semana durante 12 semanas, enquanto o grupo placebo foi formado por 39 pacientes. Os resultados mostraram que os pacientes do grupo experimental obtiveram uma resposta significantemente melhor que o grupo placebo, com redução da inflamação aguda e fadiga e maior flexão da coluna, com efeitos colaterais leves ou moderados.

Conforme relatado por Robinson e Keating (2005), o infliximab é outro anticorpo monoclonal que também se liga ao TNF-α, bloqueando sua atividade biológica. O medicamento foi aprovado para pacientes com artrite reumatoide, doença de Chron e espondilite anquilosante.

Assim, o objetivo desse trabalho foi avaliar a atividade imunomoduladora de AgNPs em células humanas do sangue periférico. O estudo considerou a escassez de trabalhos *in vitro* com células humanas, que estão usualmente em contato com as AgNPs presentes em produtos industrializados e biomédicos. A avaliação desse efeito imunomodulador é importante em vista do uso das AgNPs em ensaios clínicos.

2. CAPÍTULO 1

O manuscrito a seguir foi submetido para publicação no periódico “Mediators of Inflammation” seguido do “International Journal of Biomaterials”.

EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF SILVER NANOPARTICLES IN HUMAN PERIPHERAL BLOOD CELLS

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ABSTRACT

Silver nanoparticles (AgNP) are widely used in industrial life, surface coatings of machines and medical products. However, their ability to modulate the immunological responses of human cells remains unclear. The aim of this study was to evaluate the immunomodulatory activity of silver nanoparticles in human peripheral blood cells. Nanoparticles were synthesized following the Turkevich method at final concentration of 3.72 µg/ml. Heparinized peripheral blood was collected from six healthy volunteers and samples were diluted 1:4 in RPMI1640 medium and incubated 24h/37ºC/5% CO_2 in the presence of 5 µg/ml PHA, 1 µg/ml LPS and AgNP. Nano-sized particles with rounded shape were observed by Scanning Electron Microscopy and Transmission Electron Microscopy. Cytokine titration (TNF-α, IL-1β, IFN-γ, IL-6, IL-10, IL-4) was performed by ELISA. Nitric oxide was measured by Griess reaction. It was observed an increased production of IL-6 an NO after AgNP stimulation. The induction of IFN-γ, TNF-α, IL-1β, IL-4 by AgNP was similar to basal levels. Interestingly, only the IL-4 response correlated positively with IL-6 production. Results suggest that a pro-inflammatory pattern via IL-6- and NO-dependent pathways is preferentially induced by AgNP in human peripheral blood cells.

Keywords: Silver nanoparticles; Immunomodulation; Interleukin-6; Nitric Oxide

1 INTRODUCTION

Basic and applied nanobiotechnology has performed different studies in the last decades aiming to develop new nanometer-scale materials for a wide range of applications [1]. Silver nanoparticles (AgNP) represent one these materials, which has considerable antimicrobial activity against a broad spectrum of bacteria and fungi [2, 3]. Furthermore, it has been reported that the antimicrobial property of the silver nanoparticles is achieved even from living organisms as a raw material [4-6]. For these reasons, such nanoparticles became widely used as major ingredients in industrial life, as surface coatings of machines, being incorporated in cosmetics and medical products [3, 7].

One important effect of the AgNP on mammalian cells is the induction of reactive oxygen species (ROS) production, which in turns potentiates the oxidative stress in the membrane of the cell and its organelles – such as lysosomes and mitochondria – and the nucleus. Subsequently, apoptosis or necrosis may occur as a result of the inflammatory response, DNA damage, chromosomal abnormality and mutagenicity [8].

Studies regarding the immunological effect of silver nanoparticles reported that the high reactivity of such nanoparticles and easy transportability in the cell induce high

toxicity, ROS production and increased IL-8 secretion on human macrophage immune cells [9, 10]. Moreover, it was demonstrated an increased release of IL-1β in human monocytes exposed to AgNP [11]. Conversely, other group showed no significant increase in the secretion of IL-8 in a macrophage cell line exposed to AgNP [12], whereas decreased release of TNF-α in precision-cut lung slices of rats exposed to AgNP was demonstrated [13]. The final concept about the pro- or anti-inflammatory role of AgNP is still unclear. Thus, the aim of this study was to evaluate the immune modulatory effects of silver nanoparticles in human peripheral blood cells.

2 METHODS

2.1 Synthesis of silver nanoparticles

The synthesis of the AgNP was performed by chemical reduction of silver salts. According to the method proposed by Turkevich (1951), the synthesis process was performed with the action of biocompatible reagents only. The chemical reduction of the silver salts was performed with 1% sodium citrate (Sigma-Aldrich), which consist of a stabilizing and reducing agent for silver ions. The test included 50 mL of previously sterilized deionized water, which was placed in a becker heated in a constant temperature of 89 ºC. Subsequently, 0.009 g of silver nitrate ($AgNO_3$) (Platlab) and 0.02 g of polymeric stabilizer were added in constant magnetic stirring. The synthesis route was performed with the addition of 0.02 g of sodium carboxymethylcellulose - CMC (Denver / Emfal) as a stabilizer. Then, 1 mL of 1% sodium citrate solution was added to the solution via a peristaltic pump injection (HARVARD Apparatus® - Pump 11), with drip rate of one drop per second. Stirring was monitored for 20 minutes after the addition of 1% sodium citrate. After the pre-set time 0.6 g of Pluronic ™ F68 (Sigma-Aldrich) copolymer was added in the becker, and the solution was cooled at 52°C. Stirring continued for 20 minutes. The whole synthesis of AgNP was conducted at a pH between 6.0-7.0.

2.2 Caracterization of silver nanoparticles by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

Images from SEM and TEM were obtained from the solution containing silver particles stabilized by sodium carboxymethylcellulose - CMC (Emfal, USA), as shown in Figure 1. Spherical particles dispersed in the medium were observed with considerable variation in size. Size determination was performed in the software Image J, in which the diameter of each particle was measured five times from different starting points, consisting of 55 measures. Transmission electron microscopy (TEM) micrographs indicated a polydispersity of spherical nanoparticles in the colloidal array, measuring 0.776 ± 8.41 nm in diameter. Small aggregation areas of silver nanoparticles could be observed.

2.3 Peripheral whole blood cell culture

Peripheral blood samples were obtained from 06 healthy volunteers by venopuction using heparinized vacuum tubes (BD Vaccutainer®, Brazil). Samples were diluted 1:4 in RPMI1640 medium supplemented with 10 U/ml Streptomycin/Penicillin (GIBCO, USA) in 24-well microplates (Falcon, USA) in the presence of medium alone, 1 µg /ml LPS (Sigma, USA), 5 µg/ml PHA (Sigma, USA) or 3.72 µg/ml AgNP previous diluted in the

same medium. Plates were incubated at 37 ^{o}C in a 5% CO_2 atmosphere. Supernatants were collected after 24 h, centrifuged, and stored at -20 ^{o}C until use. This work was approved by the Ethical Committee on Human Research (protocol 0123/11 CEP/CCS/UFPB).

2.4 Cytokine titration (ELISA)

Cytokine (TNF-α, IL-1β, IFN-γ, IL-6, IL-10, IL-4) titration was performed by ELISA, using specific monoclonal antibodies provided in the BD OptEIA™ ELISA Set (BD Biosciences, USA). Nonspecific binding was prevented by incubation with 20% fetal bovine serum (FBS; Gibco, USA) in PBS. Culture supernatants were diluted 1:2 in 2% BSA-PBS. Enzymatic activity was developed by incubating plates with TMB (Sigma) for 1 hour and stopped using 2 N H_2SO_4 solution. Absorbance was read at 450 nm and subtracted from 560 nm filter in a microplate reader (GloMax Multi, Promega, USA). Cytokine concentrations expressed in pg/ml were determined against recombinant human cytokine standards provided in the kits.

2.5 Nitrite determination

Nitric oxide (NO) production was measured in supernatants (50 µL) after reaction with 50 µL nitrate reduction solution (5 mg/ml NADPH, 0.5 M KH2PO4, 1 U / 100 µl nitrate reductase enzyme) (SIGMA) for 18 h at 37 °C. After this step, 100 µl of Griess reagent was added (Promega, USA) and the nitrite concentration was obtained against a $NaNO_3$ standard curve, provided in the kit. Absorbance was read at 560 nm filter in a microplate reader (GloMax Multi, Promega, USA).

2.6 Statistical analysis

Nonparametric Kruskal-Wallis test was applied to compare levels among three groups according to stimuli used in culture, followed by the post hoc Dunn procedure for two-group comparisons, when indicated. Spearman correlation test was applied to evaluate any correlation among cytokines and nitric oxide production. GraphPad Prism v5.0 software was used, with a p value <0.05 being considered significant.

3 RESULTS

3.1 Cytokine and nitrite production by AgNP-stimulated cells

As shown in Figure 2, a strong production of IL-6 and NO was observed in AgNP-stimulated samples. The induction of IFN-γ, TNF-α, IL1-β, IL-4 by AgNP was similar to basal levels. Interestingly, only the IL-4 production correlated positively with IL-6 levels in AgNP-stimulated samples ($p=0.033$, $r=0.845$; Spearman correlation test).

4 DISCUSSION

Nanobiotechnology is defined as being the design, characterization and application of structures, devices and systems with nanometer scale, considering their shape and size [14]. Silver nanoparticles have been widely used in healthcare-related products, industry and daily life [3], including the surface coatings of washing machines, water purifiers, toys, and packaging materials. Besides, these particles have been applied to textiles and some cosmetics, such as sunscreens. Furthermore, they have been incorporated inside medical products, such as wound dressings, urinary catheters, surgical instruments, and bone prostheses [7]. Although widely used, the capacity of AgNP to modulate human immunological responses is still unclear.

In the present study, final concentration of 3.72µg/ml AgNP induced similar amounts of IFNγ, TNF-α, IL1β and IL4 than basal production by medium alone. However, this same concentration of AgNP was able to induce the release of IL-6 and NO to comparable levels of those induced by LPS or PHA. This result suggests that AgNP alone might induce strong pro-inflammatory profile in human blood cells. Human immunological responses to AgNP have been studied but in different aspects. Previously, it was observed that commercial colloidal solution of AgNP was able to decrease the production of IL-5, IFNγ and TNF-α by PHA-pre-activated human peripheral blood mononuclear cells (PBMC) [15]. On the other hand, it was observed an increased production of IL-1β, IL-6, IL-8 and TNF-α by HEK cells exposed to 0.34 µg/mL AgNP [16]. Studies on other animal cells observed that mouse macrophage cell lines RAW264.7 and J774.1 decreased the production of TNF-α induced by LPS only when cells were co-incubated with 100µM AgNP [17]. In contrast, it was reported an increase in TNF-α, CXCL2 and IL-1β production by rat macrophages exposed to 0, 5, 10 and 25 µg/ml AgNP [18]. In the present study, only one concentration of AgNP was selected to stimulate the samples. The selection criteria was an observation in a parallel study that 3.72µg/ml AgNP presented interesting antimicrobial results to some human pathogens (Bonan et al., unpublished work). Considering the antimicrobial potential of this concentration, it was decided to evaluate the immunological response induced by AgNP in human cells.

Interleukin-6 is a pleiotropic cytokine produced by diverse cell types such as T cells, B cells, monocytes and others [19]. It is well known that IL-6 is essential for T-cell activation and proliferation [20]. Its production seems to be induced by IL-1 and TNF-α [21]. Further investigations demonstrated that IL-6 can induce a diversity of acute phase proteins, leading to an acute phase response [22, 23]. Many human pathological conditions have been implicated to the dysregulated production of IL-6. Initial studies pointed to a key role of IL-6, together with TNF-α and IL-1 as biomarkers for strong inflammatory processes [24]. Additionally, it was established that IL-6 would play a dual role in the T cell compartment of the immune system. It was demonstrated that this cytokine was able to induce the development of Th17 cell subsets, while inhibiting T regulatory (Treg) cell differentiation. Interleukin-17-producing T cells present important activity in controlling pathogen infections but also in inducing strong autoimune diseases. Meanwhile, Treg cells control the excessive T cell responses in many pathological manifestations [25]. For these reasons, an overproduction of IL-6 is not desirable. Further work should be addressed to test whether AgNP is capable of inducing Th17 cell differentiation in a IL-6-dependent manner. This might be taken into consideration before exposing humans to a massive use of any nanostructured material, such as AgNP.

Another important molecule that presents strong immunological activity, the nitric oxide, had its production induced by AgNP in this study. Nitric oxide is a member of the reactive oxygen and nitrogen species (ROS and RNS), which is related to many

inflammatory responses [26]. This molecule has been associated with many antimicrobial mechanisms in macrophages. Induction of endogenous NO production as well as improvements in exogenous NO availability might protect the host from many pathogens, whereas its inhibition or blockade might favours microbial persistence and infection chronicity [27]. Noteworthy, even in restricted site-specific inflammatory processes, circulating NO-producing cells might interact with local cells improving the total NO content locally and also systemically. This overproduction of NO and other ROS/RNS would happens as an amplification signal of the immune response [28]. It has been postulated that the cell uptake of silver nanoparticles might disrupt the flux of ions and electrons across the mitochondrial membrane, resulting in the production of radical oxygen species (ROS), leading to oxidative stress and consequent apoptosis or necrosis of the cells [29]. A strong induction of both IL-6 and NO might be also important when considering that these two molecules are involved in endothelial commitment as observed in shock events of sepsis and other disseminated inflammatory conditions [30, 31]. Although not addressed here, it would be speculated from the results that AgNP might act directly inducing the STAT3/NF-κB-dependent signaling pathway. This would be a plausible explanation for preferential increase observed in both IL-6 and NO production, mimicking that observed in other stimulating conditions [32-34]. Further studies are needed to address which cells are stimulated by AgNP as well as the receptors and the signaling pathways involved in their recognition by diverse human cells.

4 CONCLUSION

The immunomodulatory activity of silver nanoparticles in human cells suggests that the IL-6-dependent pathway and NO formation are preferentially induced by AgNP in human peripheral blood cells. This observation suggests the potential pro-inflammatory activity of these nanoparticles in humans.

5 ACKNOWLEDGMENTS

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6 DISCLOSURE POLICY

The authors declare that there is no conflict of interest regarding the publication of this paper.

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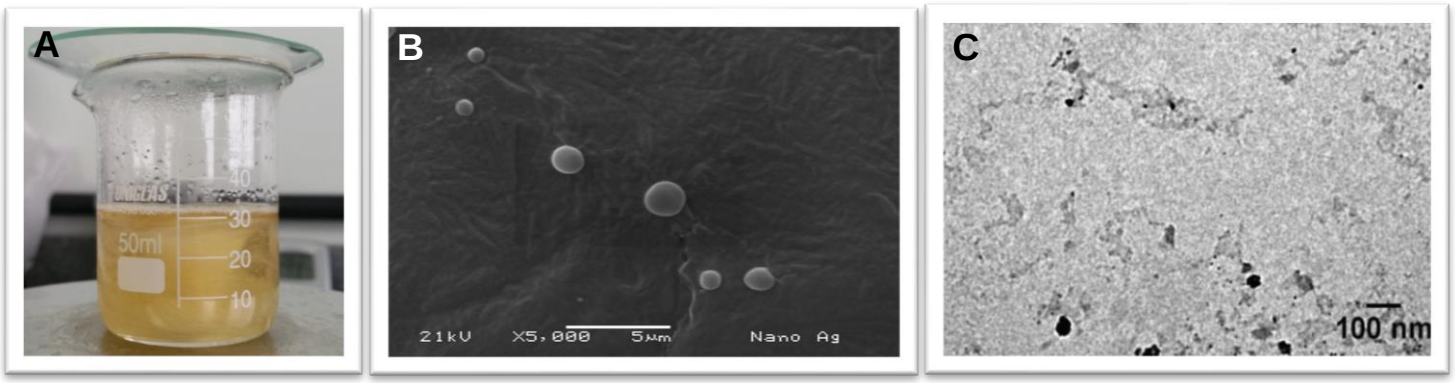

Figure 1 – Silver nanoparticle characterization. (A) Colloidal solution containing AgNP during agitation after the addition of sodium citrate. (B) SEM micrograph showing the presence of particles with micrometer and nanometer size. 5.000x magnification. (C) TEM micrographs of the nanoparticles in a colloidal array.

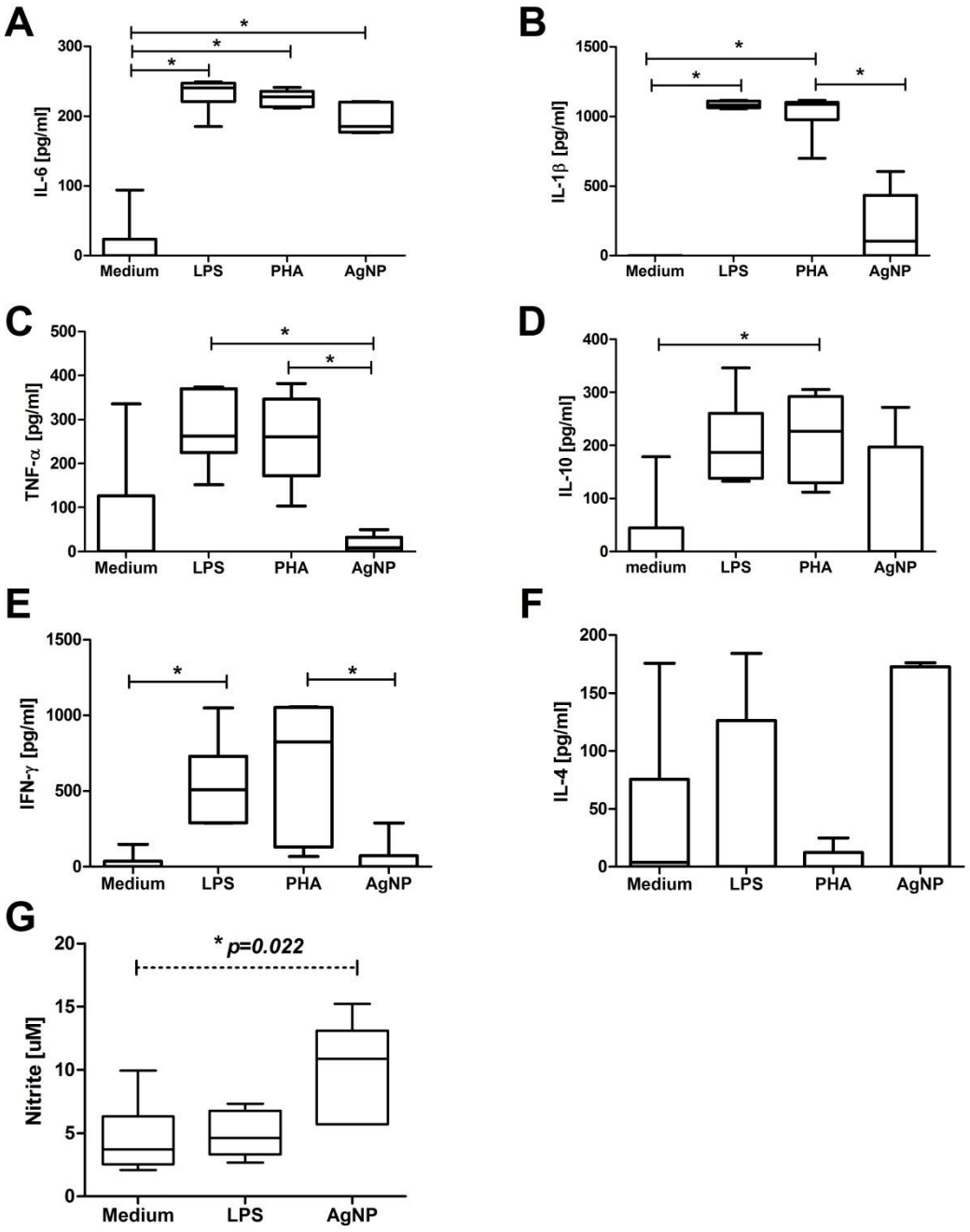

Figure 2 – Cytokine and nitric oxide production by human whole blood cells in response to Silver nanoparticles. (A) IL6, (B) IL-1b, (C) TNF-a, (D) IL-10, (E) IFN-g, (F) IL-4 and (G) nitrite levels detected on the supernatants of Peripheral blood samples cultured in presence of medium alone, LPS or AgNP. Horizontal line represents median, bars the 25th – 75th percentiles and the vertical lines 1th – 99th percentiles. Equal symbols present statistical difference on Kruskall Wallis test followed by Dunn's post hoc test, $p<0.05$.

3 CONSIDERAÇÕES GERAIS

Neste estudo, não houve padronização das amostras conforme a produção de óxido nítrico, representando uma limitação da pesquisa, visto que alguns pacientes apresentaram deficiência na produção dessa citocina. Outrossim, estudos *in vitro* não permitem representar fielmente a ação imunomoduladora das AgNPs clinicamente. Por isso, sugere-se o desenvolvimento de estudos *in vivo* para avaliar a ação imunomoduladora das AgNP em tecidos bucais, como a mucosa oral e polpa.

3. CONCLUSÃO

Os resultados da atividade imunomoduladora de AgNPs em células humanas sugerem o papel prefencial da IL-6 induzida por AgNP nas células do sangue periférico humano.

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* De acordo com as normas do PPGO/UFPB, baseadas na norma do International Committee of Medical Journal Editors - Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

ANEXO A – Normas para submissão do artigo ao periódico International Journal of Biomaterials

Author Guidelines

Submission

Manuscripts should be submitted by one of the authors of the manuscript through the online Manuscript Tracking System. Regardless of the source of the word-processing tool, only electronic PDF (.pdf) or Word (.doc, .docx, .rtf) files can be submitted through the MTS. There is no page limit. Only online submissions are accepted to facilitate rapid publication and minimize administrative costs. Submissions by anyone other than one of the authors will not be accepted. The submitting author takes responsibility for the paper during submission and peer review. If for some technical reason submission through the MTS is not possible, the author can contact ijbm@hindawi.com for support.

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Title and Authorship Information

The following information should be included

- Paper title
- Full author names
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Abstract

The manuscript should contain an abstract. The abstract should be self-contained and citation-free and should not exceed 200 words.

Introduction

This section should be succinct, with no subheadings.

Materials and Methods

This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described.

Results and Discussion

This section may each be divided by subheadings or may be combined.

Conclusions

This should clearly explain the main conclusions of the work highlighting its importance and relevance.

Acknowledgments

All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

References

Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., “as discussed by Smith [9]”; “as discussed elsewhere [9, 10]”). All references should be cited within the text; otherwise, these references will be automatically removed.

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Upon submission of an article, authors are supposed to include all figures and tables in the PDF file of the manuscript. Figures and tables should not be submitted in separate files. If the article is accepted, authors will be asked to provide the source files of the figures. Each figure should be supplied in a separate electronic file. All figures should be cited in the paper in a consecutive order. Figures should be supplied in either vector art formats (Illustrator, EPS, WMF, FreeHand, CorelDraw, PowerPoint, Excel, etc.) or bitmap formats (Photoshop, TIFF, GIF, JPEG, etc.). Bitmap images should be of 300 dpi resolution at least unless the resolution is intentionally set to a lower level for scientific reasons. If a bitmap image has labels, the image and labels should be embedded in separate layers.

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ANEXO B – Certidão do Comité de Ética

CERTIDÃO

Certifico que o Comitê de Ética em Pesquisa, do Centro de Ciências da Saúde da Universidade Federal da Paraíba – CEP/CCS aprovou por unanimidade na 4ª Reunião realizada no dia 12/07/2011, o projeto de pesquisa intitulado "IDENTIFICAÇÃO DOS FATORES SOCIOECONÔMICOS, AMBIENTAIS E IMUNOLÓGICOS RELACIONADOS COM A DINÂMICA DA TRANSMISSÃO E O ESTABELECIMENTO DA LEISHMANIOSE TEGUMENTAR AMERICANO EM HABITANTES DE ÁREA ENDÊMICA NO ESTADO DA PARAÍBA, BRASIL", do Pesquisador Lúcio Roberto Cançado Castellano. Protocolo nº. 0123/11.

Outrossim, informo que a autorização para posterior publicação fica condicionado à apresentação do resumo do estudo proposto à apresentação do Comitê.