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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

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PABLO EDUARDO OCAMPO ORTIZ

Botucatu, SP
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Tese apresentada junto ao Programa de Pós-Graduação em Biotecnologia Animal para obtenção do título de Doutor

Orientador: Profª. Titular. Fernanda da Cruz Landim e Alvarenga

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TÍTULO: CÉLULAS TRONCO MESENQUIMAIS CANINAS CULTIVADAS COM DESFERROXAMINE (DFO) E INTERFERON GAMMA (IFN-GAMMA), MIMETIZANDO CONDIÇÕES DE HIPÓXIA E INFLAMAÇÃO

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Dedico este triunfo a minha mãe, sem ela não seria quem sou
Minha família, apoio incondicional
Minha esposa e filho, meu todo

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LISTA DE ABREVIATURAS

Bcl2	Linfoma de células B antiapoptótico 2
Casp3	Caspase 3
Casp8	Caspase 8
Casp9	Caspase 9
cAT-MSCs	Células tronco mesenquimais derivadas de tecido adiposo canino
Cyclin D1	Ciclina D1
Cyclin D2	Ciclina D2
DFO	Desferroxiamine
DKC1	Disquerina 1
DNMT1	ADN-metiltransferase 1
EGF	Fator de crescimento epidérmico
EPO	Eritropoyetina
FGF-2	Fator de crescimento fibroblástico 2
GAPDH	Gliceraldehído-3-fosfato desidrogenase
HDAC1	histona deacetilase 1
HGF	Fator de crescimento de hepatócitos
Hif1α	Fator induzível por hipoxia 1 alpha
HRE	Elemento responsivo a hipóxia atuando em *CIS*
IDO	Indoleamina-pirrol 2,3-dioxigenase
IL-10	Interleucina 10
IL-6	Interleucina 6
IFN-γ	Interferon gamma
iNOS	Oxido nítrico sintase
MM	Meio de manutenção
MMP2	Metaloproteinase de matriz 2
MSCs	Células tronco mesenquimais
NO	Oxido nítrico
P53	Supressor tumoral 53
RT- qPCR	Análise Quantitativa PCR em Tempo Real
PGE-2	Prostaglandina E2
VEGFA	Fator de crescimento endotelial vascular

TERT	Telomerase transcriptase inversa
TNF-α	Fator de necrose tumoral alpha

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RESUMO

As células tronco mesenquimais (MSC) tem se destacado como candidatas ao uso em terapias regenerativas. Porém, foi demonstrado que nem todas as condições clínicas podem ser favorecidas pela presença das MSCs, já que suas propriedades são influenciadas pelas condições do microambiente onde serão aplicadas, que normalmente estão acompanhadas da presença de fatores inflamatórios e isquêmicos. Devido a isto, este estudo teve por objetivo avaliar os efeitos *in vitro* da adição de DFO e/ou ao IFN-γ no meio de cultivo de células tronco mesenquimais (MSCs do inglês *Mesenchymal Stem Cells)* de tecido adiposo canino (cAT-MSCs). Foram utilizadas MSCs originadas de 6 cadelas e pertencentes a um banco celular em terceira passagem. As amostras foram divididas em 4 grupos, controle (MSCs em condições normais de cultivo em meio de manutenção), grupo DFO (50 µmol de DFO no meio de manutenção), grupo IFN-γ (50 ng/mL de IFN-γ no meio de manutenção) e grupo IFN-γ/DFO (50 µmol de DFO e 50 ng/mL IFN-γ no meio de manutenção). O teste de proliferação foi feito por 144 horas de cultivo e, viabilidade, apoptose e expressão gênica foram avaliadas após 48 horas de cultivo. Na expressão genica foram analisados transcritos relacionados com apoptose (*BCL2, CASP8* e *CASP9*), sobrevivência celular (*DKC1, HDAC1* e *DNMT1*), imunomodulação (*HGF, IDO, PGE2* e *IL-6*), proliferação e diferenciação (*FGF2*), angiogênese (*MMP2* e *VEGFA*), reguladores do ciclo celular (*Cyclin-D1* e *TP53*) e regulador celular de resposta adaptativa a hipóxia (*HIF1-*α). As cAT-MSCs mostraram durante e ao final do cultivo morfologia fibroblastóide e aderência ao plástico. No entanto, os grupos tratados apresentaram maior distanciamento entre as células aderidas. Após 144 horas de cultivo, todos os grupos tiveram comportamento similar quanto a curva de proliferação celular, com diminuição da concentração nas primeiras 48 horas, seguido por uma fase de manutenção e finalizando com um ligeiro crescimento. A expressão gênica revelou que as cAT-MSCs tratadas com IFN-γ apresentaram

um incremento na expressão do gene pró-apoptótico *Casp9* quando comparado com o grupo IFN-γ/DFO; o gene *FGF2* importante para os processos de proliferação e diferenciação celular foi aumentado no grupo IFN-γ quando comparado aos outros grupos tratados; os genes *DKC1* e *TP53* envolvidos com a sobrevida celular e supressão tumoral respectivamente, tiveram um incremento no grupo IFN-γ quando comparado com o grupo DFO; a expressão do gene relacionado com os processos de angiogênese, o *VEGFA*, foi maior nos grupos onde o DFO foi usado. Concluímos que condicionar as cAT-MSCs com a citocina pro-inflamatória IFN-γ estimulou um aumento na sobrevivência, proliferação celular e angiogênese mediada por *MMP2*. Já a mimetização de hipóxia, levou a uma diminuição da expressão de genes relacionados a estas propriedades associadas a um estímulo para a angiogênese dependente de *VEGF*. Apesar disso, os genes relacionados a imunomodulação não foram influenciados pelas condições de cultivo. Os resultados obtidos indicam que as propriedades de indução da regeneração tecidual foram mais afetadas pelas condições de cultivo, em comparação às propriedades imunomodulados das cAT-MSC.

Palavras-chave: imunomodulação, inflamação, expressão genica.

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SUMMARY

Mesenchymal stem cells (MSC) have been highlighted as candidates for use in regenerative therapies. However, it has been shown that not all clinical conditions can be favored by the presence of MSCs, as their properties are influenced by the conditions of the microenvironment where it will be applied, conditions that are usually accompanied by the presence of inflammatory and ischemic factors. So, this study aimed to evaluate the *in vitro* effects of the addition of DFO and/or IFN-γ in the culture medium of mesenchymal stem cells (MSCs Mesenchymal Stem Cells) from canine adipose tissue (cAT-MSCs). MSCs from 6 bitches and belonging to a cell bank in the third passage were used. Samples were divided in 4 groups: Control (MSCs under standard culture conditions in maintenance medium); DFO group (50 μM of DFO in maintenance medium), IFN-γ Group (50 ng/mL of IFN-γ in maintenance medium) and IFN-γ/DFO group (50 μM of DFO and 50 ng/mL IFN-γ in maintenance medium). Proliferation test was performed for 144 hours of culture and viability, apoptosis and gene expression were evaluated after 48 hours of culture. In gene expression, transcripts related to apoptosis (*BCL2, CASP8* and *CASP9*), cell survival (*DKC1, HDAC1* and *DNMT1*), immunomodulation (*HGF, IDO, PGE2* and *IL-6*), proliferation and differentiation (*FGF2*), angiogenesis (*MMP2* and *VEGFA*), cell cycle regulators (Cyclin-D1 and *TP53*) and cellular regulator of adaptive response to hypoxia (HIF1-γ) were analyzed. The cAT-MSCs showed fibroblastoid morphology and plastic adherence during and at the end of the culture. However, the treated groups showed greater distance between adhered cells. After 144 hours of culture, all groups had a similar behavior regarding the cell proliferation curve, with a decrease in concentration in the first 48 hours, followed by a maintenance phase and ending with a slight growth. Gene expression revealed that cAT-MSCs treated with IFN-γ showed an increase in the expression of the pro-apoptotic *Casp9* gene when compared to the IFN-γ/DFO group; the *FGF2* gene important for cell proliferation and differentiation processes was increased in the IFN-γ

group when compared to the other treated groups; the *DKC1* and *TP53* genes involved with cell survival and tumor suppression respectively, had an increase in the IFN-γ group when compared to the DFO group; the expression of the gene related to the angiogenesis processes, *VEGFA*, was higher in the groups where DFO was used. We conclude that conditioning cAT-MSCs with the pro-inflammatory cytokine IFN-γ stimulated an increase in survival, cell proliferation and *MMP2*-mediated angiogenesis. The mimicry of hypoxia, on the other hand, led to a decrease in the expression of genes related to these properties associated with a stimulus for *VEGF*-dependent angiogenesis. Despite this, genes related to immunomodulation were not influenced by culture conditions. The results obtained indicate that tissue regeneration induction properties were more affected by culture conditions, compared to the immunomodulated properties of cAT-MSC.

Key words: immunomodulation, inflammation, gene expression.

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