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Effect of hypoxia on generation of neurospheres from adipose tissue-derived canine mesenchymal stromal cells

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ABSTRACT

Adipose tissue-derived mesenchymal stromal cells (AT-MSCs) are good candidates for cell therapy due to the accessibility of fat tissue and the abundance of AT-MSCs therein. Neurospheres are free-floating spherical condensations of cells with neural stem/progenitor cell (NSPC) characteristics that can be derived from AT-MSCs. The aims of this study were to examine the influence of oxygen (O_2) tension on generation of neurospheres from canine AT-MSCs (AT-cMSCs) and to develop a hypoxic cell culture system to enhance the survival and therapeutic benefit of generated neurospheres.

AT-cMSCs were cultured under varying oxygen tensions (1%, 5% and 21%) in a neurosphere culture system. Neurosphere number and area were evaluated and NSPC markers were quantified using real-time quantitative PCR (qPCR). Effects of oxygen on neurosphere expression of hypoxia inducible factor 1, α subunit (*HIF1A*) and its target genes, erythropoietin receptor (*EPOR*), chemokine (C-X-C motif) receptor 4 (*CXCR4*) and vascular endothelial growth factor (*VEGF*), were quantified by qPCR. Neural differentiation potential was evaluated in 21% O_2 by cell morphology and qPCR.

Neurospheres were successfully generated from AT-cMSCs at all O_2 tensions. Expression of nestin mRNA (*NES*) was significantly increased after neurosphere culture and was significantly higher in 1% O_2 compared to 5% and 21% O_2. Neurospheres cultured in 1% O_2 had significantly increased levels of *VEGF* and *EPOR*. There was a significant increase in *CXCR4* expression in neurospheres generated at all O_2 tensions. Neurosphere culture under hypoxia had no negative effect on subsequent neural differentiation. This study suggests that generation of neurospheres under hypoxia could be beneficial when considering these cells for neurological cell therapies.

Introduction

Cellular therapy is regarded as a promising therapeutic approach in neurological disorders. Neural stem cells (NSCs) are multipotent stem cells capable of self renewal which have the potential to differentiate into three major central nervous system (CNS) cell types, namely, neurons, astrocytes and oligodendrocytes (Zavan et al., 2010). They are found in the germinal zone of the developing brain and in stem cell niches in the adult brain (Zavan et al., 2010). NSCs derived directly from CNS tissue are considered to be safe and non-tumorigenic (Schwarz and Schwarz, 2010); however, harvesting such cells directly from the CNS is an invasive procedure with ethical considerations.

Neural stem/progenitor cells (NSPCs) can be generated from adipose tissue-derived mesenchymal stromal cells (AT-MSCs) using a neurosphere culture system; generated neurospheres show characteristics of NSPCs and can subsequently differentiate into neural lineage cells (Radtke et al., 2009; Lim et al., 2010; Qian et al., 2010). AT-MSCs can be harvested and expanded from a relatively small amount of tissue and the harvesting procedure is less invasive than obtaining NSPCs directly from neural tissues (Zuk et al., 2001). Canine mesenchymal stromal cells (cMSCs) have been isolated from adipose tissues (AT) (Neupane et al., 2008; Vieira et al., 2010), successfully differentiated into neuronal lineage cells (Lim et al., 2010) and effectively transplanted into a canine spinal cord injury model that showed improved neurological function (Ryu et al., 2009).

AT-MSCs are potential cell therapy candidates for neurodegenerative disease because of the abundance and accessibility of AT. Arboleda et al. (2011) showed that neurospheres generated from AT-MSCs had enhanced expression of neural markers after neural induction and showed better survival compared to AT-MSCs when transplanted intraspinally into rats with spinal cord injury (Arboleda et al., 2011). Transplantation of either AT-MSCs or neurospheres improved hind limb motor function

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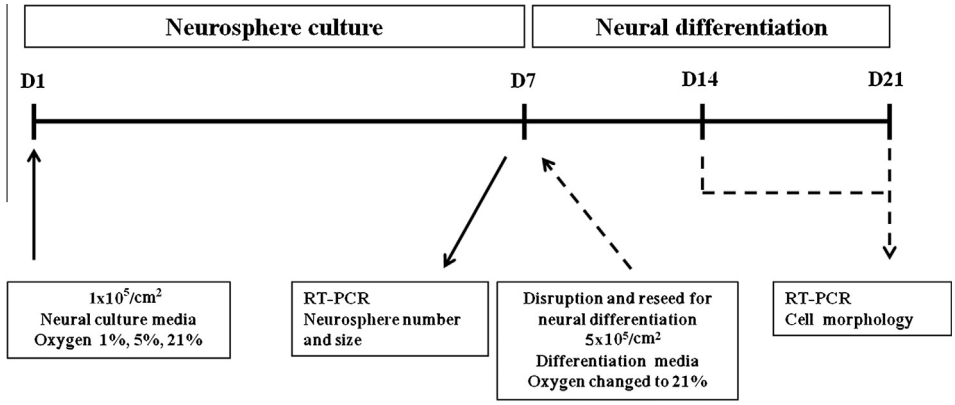

Fig. 1. Study design for neurosphere culture and neural differentiation of adipose tissue-derived canine mesenchymal stromal cells (AT-cMSCs). Neurosphere culture was conducted for 7 days and neural differentiation was initiated and observed for a further 14 days (days 7–21). Samples of total RNA for quantitative reverse transcriptase PCR (RT-PCR) were collected before induction (expanded passage 2 AT-cMSCs, not shown in the figure) and at days 7, 14 and 21. Neurospheres were counted and their sizes were measured on day 7. Differentiated neuronal cell morphology was noted on days 14 and 21.

Table 1
Primers for real-time quantitative reverse transcriptase PCR.

Gene (and reference if published previously)	Direction	Sequence (5′–3′)	Size of amplicon (base pairs)	GenBank accession number
B2M (Neupane et al., 2008)	Forward	TCTACATTGGGCACTGTGTCAC	136	NM_001284479
	Reverse	TGAAGAGTTCAGGTCTGACCAG		
CXCR4	Forward	GACTCCATGAAGGAACCCTG	93	NM_001048026
	Reverse	GCCAGTCAAGAAGATGATGG		
HIF1A (Spee et al., 2005)	Forward	TTACGTTCCTTCGATCAGTTGTA	105	AY455802
	Reverse	GAGGAGGTTCTTGCATTGGAGTC		
EPOR (Zhang et al., 2007)	Forward	CTGACAGCTAGTGACTTGG	118	AY908987
	Reverse	ATCTTCTGCTTCAGAGTCC		
VEGF (Zhang et al., 2007)	Forward	TGGACATCTTCCAGGAGTACC	97	AF133248
	Reverse	GCCCTCATCATTACAGCAGC		
NES (Filioli Uranio et al., 2011)	Forward	CAGGTCCTGGAAGGTCGGCA	139	XM_547531
	Reverse	AGCCGAGAGAAGGGTTGGAACC		
CD133	Forward	TTTTGTGGCAAACCACCACC	110	XM_845738
	Reverse	GTTGATTTGCGCTGGAGTTCC		
TUBB3	Forward	ACGAGATGGAGTTCACCGAGG	112	XM_005620536
	Reverse	TCGTCGTCTTCGTACATCTCG		
VIM	Forward	CGAGGAATGGTACAAGTCC	93	XM_851385
	Reverse	CTCATTTGACTCCTGCTTCG		
GALC	Forward	GTCAATGGCTACATGACTGC	88	NM_001003238
	Reverse	GTCATCAATCCACATCGTCC		
MAP2	Forward	CTGTAGCAGTCCTGAAAGG	161	XM_856110
	Reverse	GGAGAAGGAGGCAGATTAGC		
GFAP	Forward	CTAGCTTGGATACAGAGAGG	141	XM_843285
	Reverse	CCAAGTGTATCTGGTTGCCC		

(Arboleda et al., 2011). Several studies also suggest that fully differentiated neural cells have a reduced potential to survive detachment and transplantation procedures (Hermann et al., 2004; Fu et al., 2008). Together, these studies imply that, at least for neurological implantation, AT-MSCs partially differentiated toward the neural lineage might be better therapeutic candidates in terms of differentiation potential and survival in vivo. Common markers used to confirm neuronal induction and differentiation are nestin (NES; a neural stem cell and early progenitor marker), CD133 (a marker for stem and progenitor cells, including neuronal and embryonic stem cells), tubulin, β3 class III (TUBB3; an early neuronal marker), microtubule-associated protein 2 (MAP2; a mature neuronal marker), vimentin (VIM; an early glial marker), glial fibrillary acidic protein (GFAP; a mature glial marker), and galactosylceramidase (GALC; an oligodendrocyte lineage marker) (Yu et al., 2011; Wang et al., 2013).

Another factor affecting cell survival post-implantation is tissue oxygen (O_2) tension. The normal physiological oxygen tension in the brain is low (3–5% O_2) (Panchision, 2009) compared to the O_2 tension used for standard cell culture (21% O_2). When the brain or spinal cord is injured, the O_2 tension is significantly decreased below normal tissue levels (Hukuda and Amano, 1980; Shi and Liu, 2007) due to vascular disruption and the ensuing ischemic environment (Bitar Alatorre et al., 2007). Large numbers of cells die after transplantation into hypoxic regions. Hypoxic preconditioning of embryonic stem cells, bone marrow or adipose derived MSCs prior to transplantation into regions with low tissue O_2, such as areas of cerebral ischemia and spinal cord injury, may improve survival (Theus et al., 2008; Oh et al., 2010; Wei et al., 2012). When NSPCs are isolated from brain and cultured under physiological O_2 conditions (3–5% O_2 tension) or lower, the in vitro proliferation and differentiation potential of these cells is enhanced (Horie et al., 2008). The effect of O_2 tension on the generation of neurospheres from cMSCs has not been studied.

Hypoxia-inducible factor-1, α subunit (HIF-1α) is a transcriptional activator mediating adaptive cellular responses to hypoxia (Semenza, 2012). HIF-1α is degraded under normal tissue O_2 conditions. However, under conditions of hypoxia,