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Review

Ischemic culture of dental pulp-derived cells is a useful model in which to investigate mechanisms of post-ischemic tissue recovery

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Summary. Dental pulp is a soft tissue characterized by unique regenerative properties. It is located in the center of each tooth, and is surrounded by hard tissue (dentin). Vascular access is limited to a small foramen at the root apex. Because of this anatomical limitation, dental pulp can easily lose its blood supply, causing the tissue to become ischemic. This occurs, for example, when a tooth is dislocated by traumatic injury or is subjected to inflammation. Since ischemia is caused by a critical shortage of oxygen and nutrients, ischemic damage is usually irreversible, even when the ischemic event is transient. However, unlike ischemia-sensitive organs such as the brain and heart, dental pulp is relatively ischemia-resistant, and recovers from ischemic injury by regenerating damaged tissue. The mechanisms by which this regeneration occurs are poorly understood, but are being investigated in cell culture models that mimic *in vivo* ischemic conditions using a combination of hypoxia and nutrient deprivation. Here, we review the use of ischemic cell culture to investigate the mechanisms of post-ischemic dental pulp tissue recovery.

Key words: Ischemia, Dental pulp, Stem cells, Odontoblasts, Regeneration

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Introduction

Ischemia occurs when arterial blood flow to a tissue is restricted. Because arterial blood supplies oxygen and nutrients, ischemia is characterized by a lack of these essential elements (Shinzawa and Tsujimoto, 2003). The intensity of tissue damage depends on the length and severity of the ischemic event. Although direct comparison is difficult, tolerance to ischemia appears to differ among various tissues and organs. For example, brain tissue is highly vulnerable to ischemia, and often becomes necrotic even when only transiently exposed to ischemic conditions (Sugiyama et al., 2011). In contrast, dental pulp is relatively tolerant to ischemia and is able to survive transient ischemic events such as tooth extraction and replantation (Tsukamoto-Tanaka et al., 2006). Dental pulp recovers its functions by regenerating damaged tissue after ischemia exposure. This unique response suggests that dental pulp contains ischemia-tolerant cells that play important roles in post-ischemic tissue regeneration.

Dental pulp contains a postnatal stem cell population named dental pulp stem cells (DPSCs) (Gronthos et al., 2000). DPSCs possess multi-lineage differentiation abilities (odontogenic, osteogenic, chondrogenic, adipogenic and neurogenic lineage), and are considered potent stem cells for use in tissue engineering and regenerative medicine (Gronthos et al., 2002; Iohara et al., 2006). Although the characteristics and functions of DPSCs within the pulp remain largely unknown, these cells appear to play an important role in tissue development, homeostasis and regeneration and are a

particularly interesting target for investigations into the mechanisms of post-ischemic tissue regeneration.

Evaluating dental pulp tissue reactions during and after ischemia would ideally be undertaken *in vivo*, but this is experimentally difficult due to the anatomic location of the tissue and its complex cellular composition (Liu et al., 2006). Thus, *in vitro* ischemic culture of dental pulp-derived cells has been developed as an alternative experimental model, though studies differ in the culture conditions utilized (Agata et al., 2008; Wang et al., 2010). In this review, we discuss *in vitro* cell culture conditions that best approximate *in vivo* ischemia. Next, we evaluate the relevance of these conditions in dental pulp-derived cell culture. Finally, we examine the characteristics of dental pulp-derived cells that survive ischemic culture conditions and explore possible mechanisms of post-ischemic pulp tissue regeneration.

Approximating *in vivo* ischemic conditions in experimental cell culture systems

Under ischemic conditions, cells experience both low oxygen tension and nutrient deprivation. Hence, ischemia can be mimicked *in vitro* by exposing cells to both hypoxia and a low-glucose environment (Jones et al., 2011). These appear to be the two most influential factors for tissue survival (Acosta et al., 1978). When PC12 cells (derived from a pheochromocytoma and able to differentiate into neurons) are cultured under hypoxic conditions and in a low-glucose environment, they are severely damaged, often to the point of necrosis (Shinzawa and Tsujimoto, 2003). Unfortunately, most of the current literature on ischemic culture of dental pulp-derived cell or DPSCs is limited to investigation of the effect of low oxygen tension alone; the number of studies using both low oxygen tension and nutrient deprivation is limited (Agata et al., 2008; Wang et al., 2010) (Table 1). In fact, low nutrient supply may enhance the effect of low oxygen tension. It has been shown that caspase-independent cell death, which is commonly seen under ischemic conditions, is significantly upregulated when cells are deprived of both oxygen and glucose (Agata et al., 2008).

Another important consideration when developing *in vitro* models of ischemia is the level of hypoxia used in the experiments. Conventional cell culture experiments use approximately 20% oxygen, with a partial pressure of oxygen (pO_2) of 140 mmHg (Rodrigues et al., 2010). However, pO_2 in the arterial blood of normal human subjects ranges from 60-90 mmHg and pO_2 in bone marrow is even lower (47-49 mmHg). The discrepancy between *in vitro* and *in vivo* conditions suggests that conventional cell culture may occur in a relatively hyperoxic environment, while traditional "hypoxic" culture environments actually reproduce normal physiologic conditions. This may explain why mesechymal stem cells grow and survive better in low oxygen cell culture environments. Mesenchymal stem cells cultured under low oxygen tension (5%) have a greater number of colonies as primary isolates, proliferate more rapidly and produce more bone (Lennon et al., 2001). In fact, "hypoxic" cell culture conditions can increase proliferation rates and enhance differentiation along multiple mesenchymal lineages (Das et al., 2010), providing further evidence that the

Table 1. Effect of hypoxia on DPSC culture.

Species	Oxygen tension	culture conditions	proliferation (compared to 20% O2)	cell properties (compared to 20% O2)	References
Human	1%	Monolayer culture for 24 hours	Cell proliferaton ↑	HIF-1α↑, CXCR4↑, SDF1↓	Gong et al. 2010
Human	1%	Monolayer culture for 24 hours /Endothelial cells culture in conditioned medium from hypoxic pulp cells for 72 hours	Endothelial cell proliferation ↑	HIF-1α↑, VEGF↑, bFGF→	Aranha et al. 2010
Human	2%	Monolayer culture for 24 or 48 hours	Cell proliferation ↓	SP cells↑, ABCG2↑, Oct4↑	Wang et al. 2010
Human	2%	Monolayer culture for 24 hours after80% confluent in normoxic conditions	none	Erythropoietin↑, Erythropoinetinreceptor↑	Gong et al. 2010
Human	3%	Monolayer culture for 14 days	Cell proliferation ↑	CD133↓, STRO-1↑	Sakdee et al. 2009
Human	3%	Monolayer culture with or without osteogenic supplements for 14 days	Cell proliferation ↑	STRO-1↑, osteogenic differetiation ↓	Iida et al. 2010
Human	5%	Monolayer culture with or without osteogenic supplements for 21 days	viability ↑	OCN↑, DMP1↑, BSP↑, DSPP↑ von kossa, alizarin↑(at 21 days of culture)	Li et al. 2011
Porcine	0.1% or 5%	Monolayer culture with or without osteogenic supplements under various glucose concentrations for 24 hours /Re-oxygenation after 24 hours ano/hypoxic culture + 3 day normoxic culture	Cell proliferation; Hypoxia ↑, Anoxia ↓	Oct4 ↑, Sox2↑ (at 6 hours in non-induced cells), ALP activity↑ (at 7 days after re-oxygen)	Agata et al. 2008

HIF-1α, hypoxia-inducible factor-1α; CXCR4, CXC chemokine receptor 4; SDF1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor; bFGF, basicfibroblast growth factor; SP cells, side population cells; ABCG2, ATP-binding cassette sub-family G member 2; Oct4, octamer binding transcription factor 4; OCN,osteocalcin; DMP1, dentin matrix acidic phosphoprotein 1; BSP, bone sialoprotein; DSPP, dentin sialoprotein; Sox2, SRY-box 2; ↑, increas; ↓, decrease; →, unchanged

oxygen levels in traditional hypoxic cell culture experiments may not accurately reflect *in vitro* ischemic environments. The oxygen tension within dental pulp tissue *in vivo* is difficult to measure directly. However, it is possible that the oxygen range commonly used in "hypoxic" cell culture experiments (2-5%) may not be an accurate approximation of *in vivo* conditions, and very low oxygen tension (less than 1%) might be necessary (Agata et al., 2008).

Experimental ischemia in dental pulp-derived cell culture

Several studies have demonstrated that low oxygen tension (2-5%) promotes proliferation of dental pulp-derived cells (Amemiya et al., 2003; Sakdee et al., 2007; Iida et al., 2010; Li et al., 2011). This finding may reflect the fact that dental pulp-derived cells are exposed to relatively low oxygen tension within their normal physiologic environment. When these cells are exposed to both hypoxia (2%) and serum deprivation for 24 or 48 hours, a condition that mimics *in vivo* ischemia, proliferation rates decline (Wang et al., 2010). Cells survive in this environment even though proliferation rates decline, suggesting a complex cellular defensive response to ischemia. This response includes suppression of cell growth and induction of cellular defense systems, including upregulation of hypoxia-inducible factor 1α (HIF-1α), heat shock protein 70 (HSP 70), and AMP-activated protein kinase (AMPK) (Amemiya et al., 2003; Fukuyama et al., 2007; Agata et al., 2008; Aranha et al., 2010). These factors may even activate cell growth after the ischemic event has resolved (Ueno et al., 2006; Fukuyama et al., 2007). This complex response to hypoxia and nutrient deprivation reflects a balance between damage due to noxious stimuli and activation of cellular defense systems.

The effect of low oxygen tension on the differentiation capacity of dental pulp-derived cells is unclear. After being cultured for 14 days in 5% O_2, dental pulp-derived cells increase expression of osteonectin (ON), dentin matrix protein-1 (DMP-1),

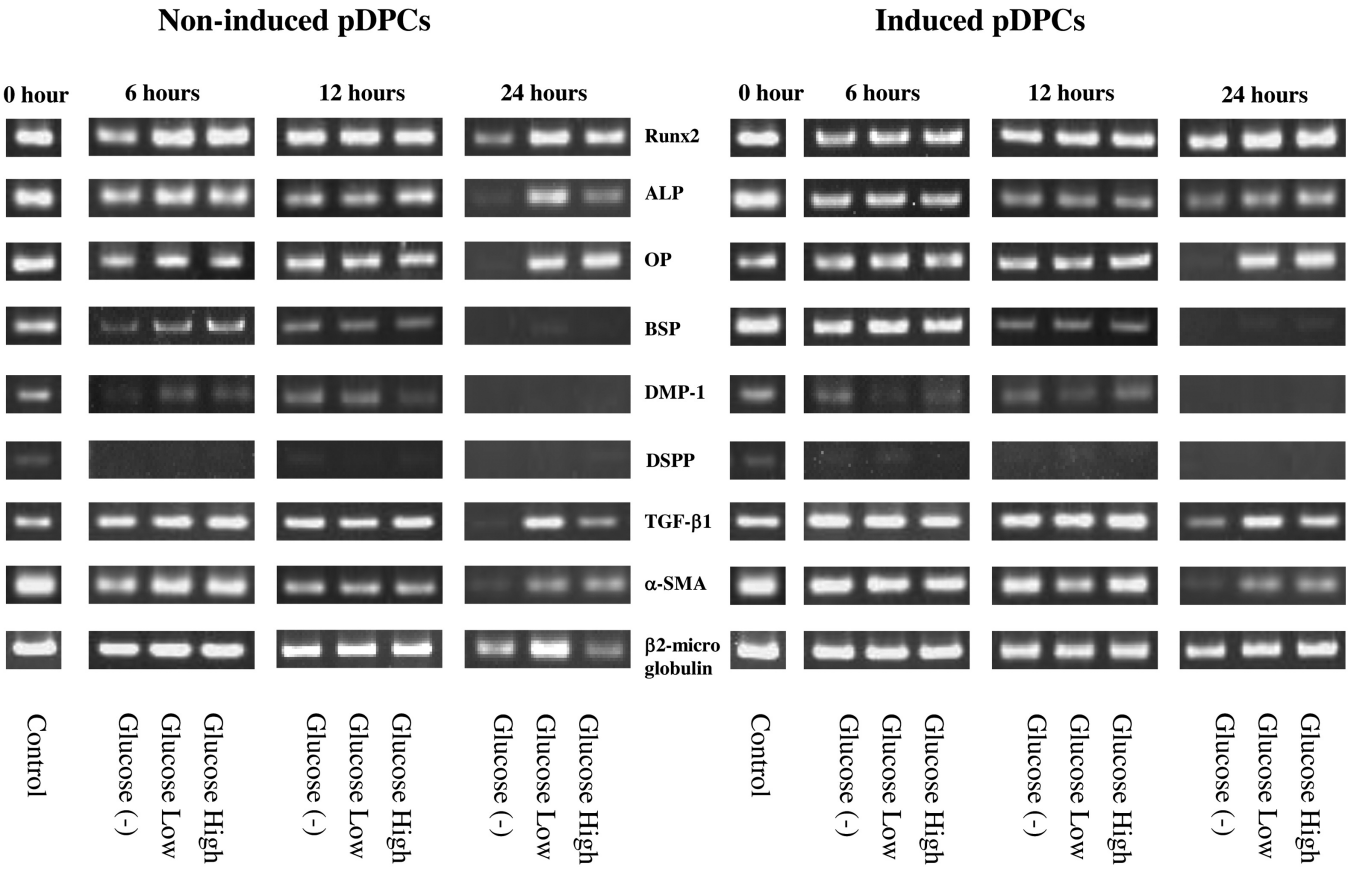

Fig. 1. Changes in the expression of odontogenic/osteogenic genes in non-induced and induced pDPCs cultured under severe-hypoxic conditions. Induced and non-induced pDPCs were cultured under severe hypoxic conditions in either low or high glucose concentration. Gene expression was analyzed by RT-PCR at 0, 6, 12 and 24 hours. Expression of Runx2, ALP, OP, TGFß1, and α-SMA was slightly inhibited in both non-induced and induced cells. The expression of BSP, DMP-1, and DSPP was strongly inhibited in both populations. Non-induced and induced pDPCs underwent de-differentiation in a time-dependent manner. (From Agata et al., 2008 with modification).

bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP), and after 21 days significantly greater calcified nodule formation is observed. Both of these factors appear to promote differentiation (Li et al., 2011). Canine dental pulp-derived cells show decreased alkaline phosphatase activity (ALP, an early marker of osteogenic (odontogenic) differentiation) after 4 days in both hypoxic (2% O_2) and normoxic culture conditions, though ALP activity in the hypoxic cells remained higher than in the normoxic cells (Amemiya et al., 2004). Other studies have reported that hypoxia has an inhibitory effect on differentiation of dental pulp-derived cells. For example, we have demonstrated that porcine dental pulp-derived cells have significantly lower expression of BSP, DMP-1, and DSPP in both differentiation-induced and non-induced cells regardless of the severity of ischemia , though expression of other marker genes is not significantly different when cells are grown in glucose-containing medium (Fig. 1). Additionally, when human dental pulp cells are cultured under 3% O_2 for 14 days, ALP activity and the expression of DMP1, DSPP and osteocalcin are suppressed (Iida et al., 2010).

Investigations into the effect of low oxygen tension on the ability of dental pulp-derived cells to differentiate differ in terms of the species from which the cells were isolated and the conditions under which the cells were cultured (i.e. level and duration of hypoxia, and the status of cell differentiation), making direct comparisons difficult. However, accumulating evidence indicates that ischemic conditions do affect the differentiation of dental pulp-derived cells, and the type and magnitude of the effect may correlate with the severity of ischemia. Detailed cellular analyses are required to understand the mechanisms underlying this phenomenon.

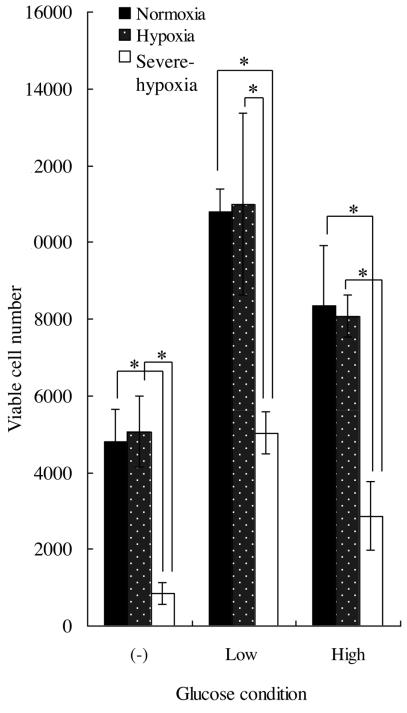

Fig. 2. Viability of porcine dental pulp-derived cells (pDPCs) in cell culture depends on ischemic conditions. To investigate the effect of differential ischemic conditions, pDPCs were cultured under a range of O_2 concentrations (normoxia, hypoxia, or severe-hypoxia) and glucose concentrations (high glucose, low glucose, or glucose (-)) in serum-free media. Viable cell number was calculated as the percentage of surviving cells. Significantly more cells survived under normoxic and hypoxic conditions than under severe-hypoxic conditions. No significant difference was observed between normoxic and hypoxic conditions. pDPCs cultured in low glucose media were significantly more viable than cells cultured in high glucose or glucose (-) media. Error bars represent the mean ± standard deviation for six separate experiments. Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test (From Agata et al., 2008 with modification).

Characteristics of dental pulp-derived cells that survive under ischemic cell culture conditions.

Dental pulp tissue often recovers its function after an ischemic event, and ischemia-surviving cells likely contribute to tissue recovery. As previously discussed, traditional low oxygen culture conditions may not accurately reflect hypoxic environments *in vivo*. Accordingly, very low oxygen tension (<0.1% O_2, or even anoxia) is required for culture conditions to truly approximate physiologic environments (Fig. 2) (Agata et al., 2008). Furthermore, these cultures may require "ischemic conditions", which involves not only very low oxygen tension but also nutrient deprivation (Agata et al., 2008; Wang et al., 2010).

Investigations into the mechanisms by which dental pulp-derived cells recover from ischemia have focused on determining whether cellular differentiation affects survival. However, the number of experiments performed with very low oxygen tension (hypoxia) or ischemic conditions (hypoxia plus nutrient deprivation) is limited. We have shown that differentiation-induced and non-induced cells (possibly stem/progenitor cells) survive at equivalent rates under a range of ischemic conditions. Hence ischemia-tolerance is comparable between differentiated and non-differentiated cells (Agata et al., 2008). Despite these similarities, differences between the two cell populations do exist. Upregulation of the pluripotent stem cell markers octamer-binding transcription factor 4 (Oct4) and Sox2 is observed only in non-induced cells under ischemic conditions (Fig. 3). This finding suggests that ischemia-surviving non-odontogenic cells (undifferentiated cells) may be able to de-differentiate, acquiring greater growth and differentiation potential for post-ischemia tissue regeneration. In contrast, de-differentiation of ischemia-surviving odontogenic (differentiated) cells may allow them to re-acquire mitotic potential (pulp-resident odontoblasts are post-mitotic cells that are not able to divide or repair damaged dentin) (Liu et al., 2006).

Hence cells that survive ischemic insult may de-differentiate under ischemic conditions and obtain more stem cell-like characteristics, thereby contributing to the regeneration of damaged tissue.

Insights into the mechanisms of dental pulp tissue recovery after ischemic damage

Cellular damage caused by ischemia is generally irreversible even when the ischemic insult is transient. Dental pulp tissue can recover its functions after transient ischemia, suggesting that this tissue may undergo cellular regeneration rather than cellular recovery. The physiologic function of dental pulp (protection, nutrition, and tooth sensation) are supported by multiple cell types, hence post-ischemic pulp recovery has previously been attributed to multiple cell populations (Sloan and Smith, 2007). However, with the discovery of dental pulp stem cells (DPSCs) which are able to differentiate into multiple cell lineages (angiogenic, vasculogenic, neurogenic, chondrogenic, and osteo/odontogenic) a new paradigm has emerged in which a single stem cell population may be all that is required for pulp tissue recovery. Thus, it is of interest to investigate whether the post-ischemic recovery of pulp function is mediated solely by DPSCs.

One of the most important functions of dental pulp is to protect the tooth from noxious stimuli through dentin formation. Reparative dentin formation is frequently observed in teeth that survive ischemic insults (Spahr et al., 2002). The cell types responsible for this restorative process are unknown. Dental pulp contains terminally differentiated odontoblasts, but these cells are considered post-mitotic and thus not able to divide and form dentin (Liu et al., 2006). Additionally, odontoblasts may become necrotic under ischemic conditions. This has led to the assumption that reparative dentin formation following an ischemic event might be solely mediated by DPSCs (though other functions appear to be recovered by multiple cell populations) (About and Mitsiadis, 2001; Liu et al., 2006). However, as stated above, results from ischemic culture of induced dental pulp cells

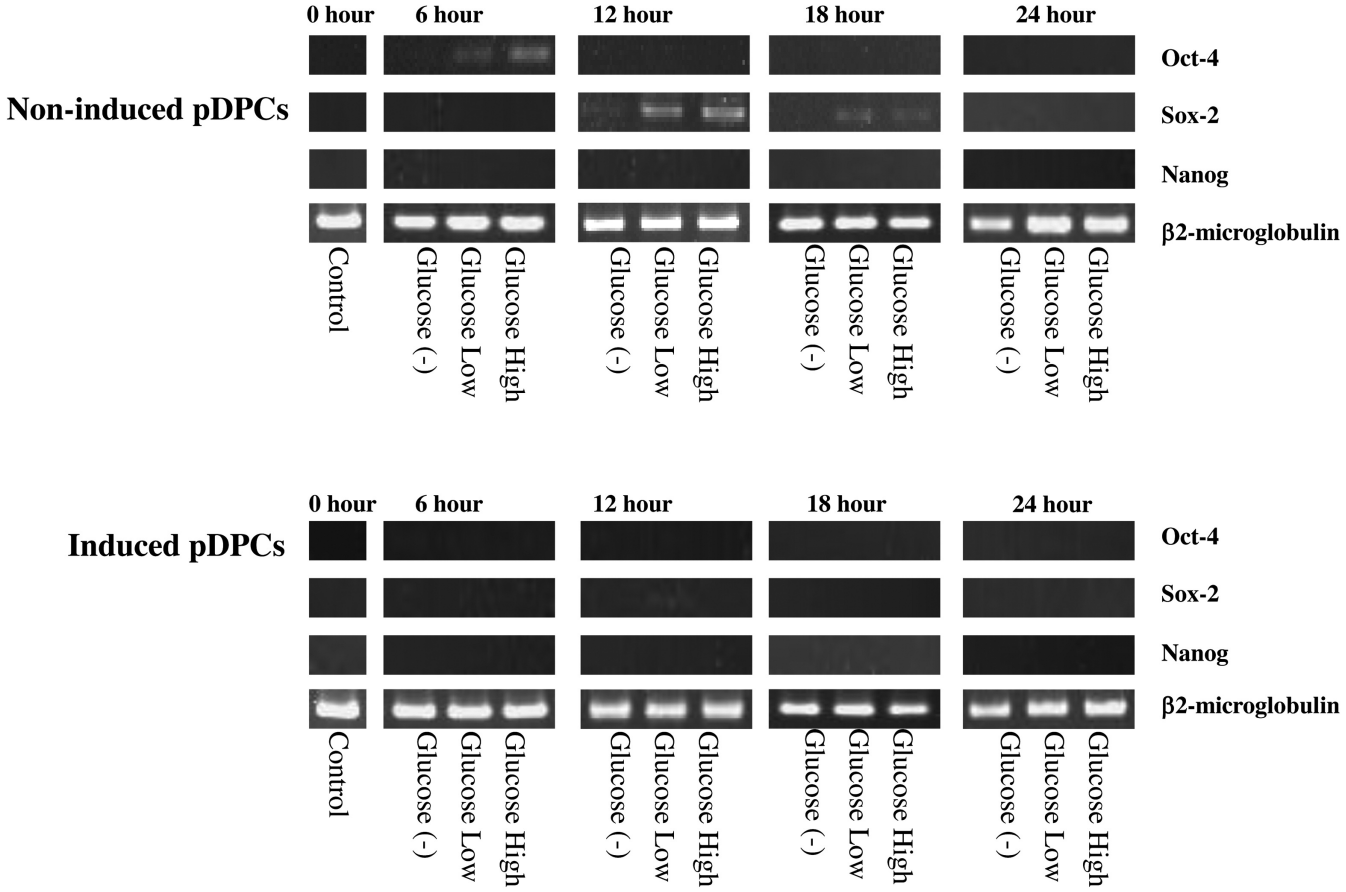

Fig. 3. Expression of pluripotent stem cell markers in induced and non-induced pDPCs cultured under severe-hypoxic conditions. RNA from induced and non-induced pDPCs cultured under severe-hypoxic conditions was extracted at 0, 6, 12, 18, 24 hours. Upregulation of the pluripotent stem cell markers Oct-4 and Sox-2 was observed only in non-induced pDPCs. Oct-4 was transiently activated at 6 hours, followed by the activation of Sox-2 from 12 to 18 hours. Nanog expression was not observed under any conditions. (From Agata et al., 2008 with modification).

(odontoblast-like cells) indicate that resident committed odontoblasts/precursors may de-differentiate and re-acquire mitotic potential during ischemia (Fig. 1). Therefore, post-ischemic reparative dentin formation may in fact be mediated by both DPSCs and de-differentiated odontoblasts, though further investigation is required to confirm this. If pulp-resident odontoblasts do contribute to post-ischemic reparative dentin formation, their ability to form dentin may be lower than that of DPSCs, because ALP activity of de-differentiated cells is lower in induced cells than in non-induced cells (which possibly contain DPSCs) (Fig. 4A,B).

Investigations into the post-ischemic recovery of other physiologic functions of dental pulp tissue are rare (e.g. nutrition and tooth sensation), but studies in other tissues demonstrate reparative roles for multiple cell types, including endothelial and neural cells (Sheridan and Bonventre, 2000; Bernert et al., 2003). Thus a more complete understanding of the mechanism of post-ischemic pulp tissue recovery should include analysis of endothelial and neural cell populations residing in dental pulp tissue. Finally, angiogenic factors, such as vascular endothelial growth factor (VEGF), are induced under hypoxic conditions, which may support the durability of dental pulp during hypoxia *in vivo* (Amemiya et al., 2003; Aranha et al., 2010).

Conclusion

It has long been known that dental pulp recovers its function after transient ischemia, but the mechanisms underlying this phenomenon have not been fully investigated, in part because *in vivo* monitoring of this tissue is anatomically difficult. Cell culture is emerging as a useful model for examination of cellular responses to ischemia and may become a valuable approach for investigation into the mechanisms of post-ischemic pulp tissue recovery.

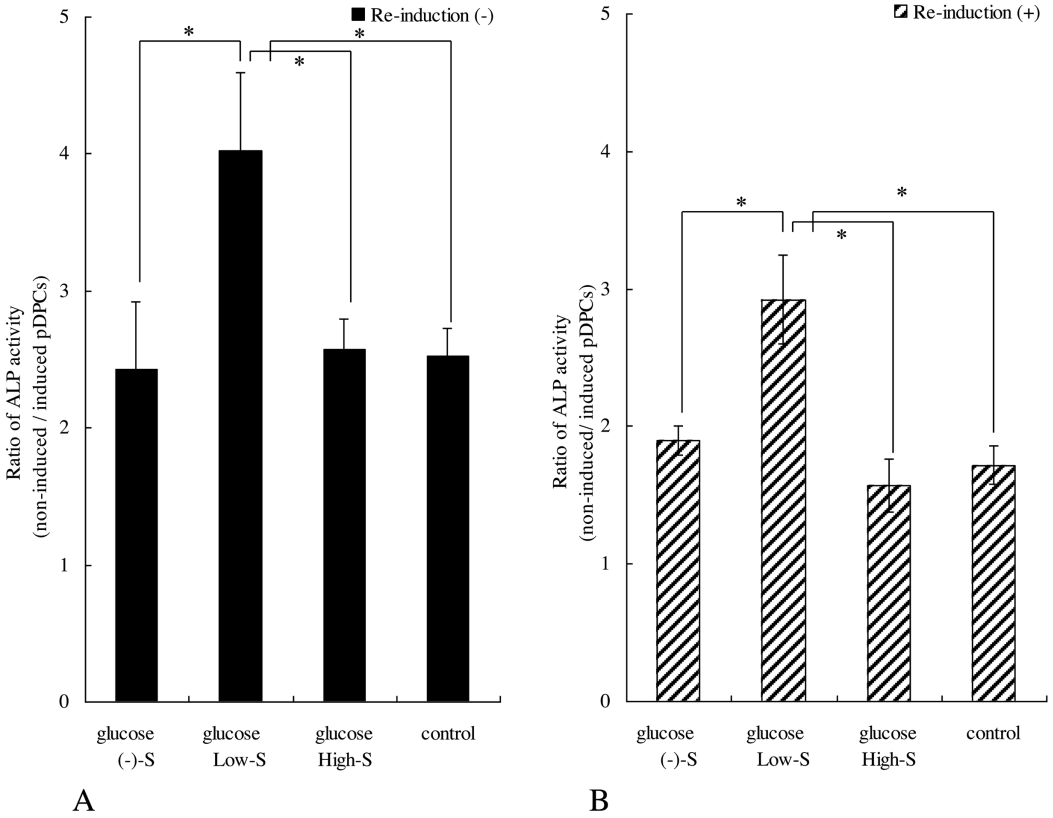

Fig. 4. Differentiation potential of odontogenic cells after ischemic de-differentiation. Induced and non-induced pDPCs were cultured in serum-free D-MEM at various glucose concentrations and exposed to severe-hypoxia for 24 hours. Surviving cells were then "re"-differentiated. Controls consisted of induced and non-induced pDPCs, respectively, cultured in serum containing D-MEM with high glucose under normoxia. ALP activity after "re"-differentiation was measured and compared with that before "re"-differentiation in both non-induced pDPCs groups (re-induction (-), **A**) and induced pDPCs groups (re-induction (+), **B**). Although ALP activity was increased after "re"-differentiation in both groups, the differentiation plasticity was observed to be higher among non-induced pDPCs groups (re-induction (-), **A**) than that of induced pDPCs groups (re-induction (+), **B**). S: Severe-hypoxia. Error bars represent the mean ± standard deviation for three separate experiments. Statistical analysis was performed using one-way ANOVA and Tukey-Kramer multiple comparison test (From Agata et al., 2008 with modification).

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