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*Original Article*

# The timing of glucocorticoid-induced osteocytic cell necrosis under hypoxia

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**Abstract:** Background: Osteocytic cell necrosis is induced when dexamethasone (Dex) administration and hypoxia are superimposed. We investigated the time needed for osteocytic cell necrosis to develop in cultured osteocytic cells. Materials and Methods: Cultured murine MLO-Y4 osteocytic cells were used. After the addition of 1 μM Dex they were cultured in 1% $O_2$ (hypoxia) in an incubator for 6 h (6 h group), 9 h (9 h group), 12 h (12 h group), or 24 h (24 h group) respectively, and then cultured under normoxia (20% $O_2$) until the respective total culture time reached a total of 24 h. Viability assays were then performed using an Apoptotic/Necrotic Cells Detection Kit according to the manufacturer's instructions, and the percentages of apoptotic and necrotic cells relative to the total cell number were determined. As a Control group cultured osteocytic cells to which Dex was added and cultured for 24 h under normoxia were used. Results: The Control group, 6 h group, 9 h group, and 12 h group showed only slight increases in apoptosis, with no significant differences found between any of them. In contrast, in the 24 h group apoptosis was significantly increased, while necrosis, which did not show any increase in any of the groups up to the 12 h one, was significantly increased in the 24 h group. Discussion: In the presence of Dex and hypoxia the development of osteocytic cell necrosis appears to require a period of at least 12-24 h. This suggests that the development of osteocytic cell necrosis might be interrupted if hypoxia was suppressed or blocked within the critical time limit of 12 h.

**Keywords:** Osteonecrosis, glucocorticoid, hypoxia, necrosis, apoptosis, osteocytic cell

## Introduction

Ischemia is generally recognized to be the cause of glucocorticoid-induced osteonecrosis, but much is still unclear about its underlying pathophysiology [1, 2]. When investigating the actions of glucocorticoid administration in animal experimental models, the influence of various tissues and factors must be considered making it very complicated to elucidate in any great detail the underlying mechanisms or to focus on a single factor in isolation.

Since it is possible to observe the direct influence of glucocorticoid on the osteocytic cell in vitro, the results of such studies are also worth considering. Recently in vitro studies have also been conducted, including some experiments using cultured osteoblast cells and cultured osteocytic cells in which the combination of glucocorticoid and hypoxia was found to increase the amount of apoptosis expression [3, 4]. Scattered reports on in vitro apoptosis are seen [3-10], but almost none that mention necrosis are available. However, although recently using cultured osteocytic cells no increase in necrosis has been noted with the addition of dexamethasone (Dex) alone or hypoxia alone, the combination of Dex and hypoxia was recently reported for the first time to cause an increase in the rate of necrosis [11], suggesting that this combination is needed for the development of osteocytic cell necrosis.

On the other hand, regarding the relation between the development of osteonecrosis and duration of ischemia-hypoxia, Nishino et al. reported the development of osteonecrosis in a canine trauma model in which ischemia was induced with 9 h hip dislocation and vascular ligation [12], while no reports are available on glucocorticoid administration models. Also, Sato et al. have reported that osteonecrosis may develop after 96 h, with ischemia completely established within 1-3 days [13], while Ichiseki et al. demonstrated that oxidative

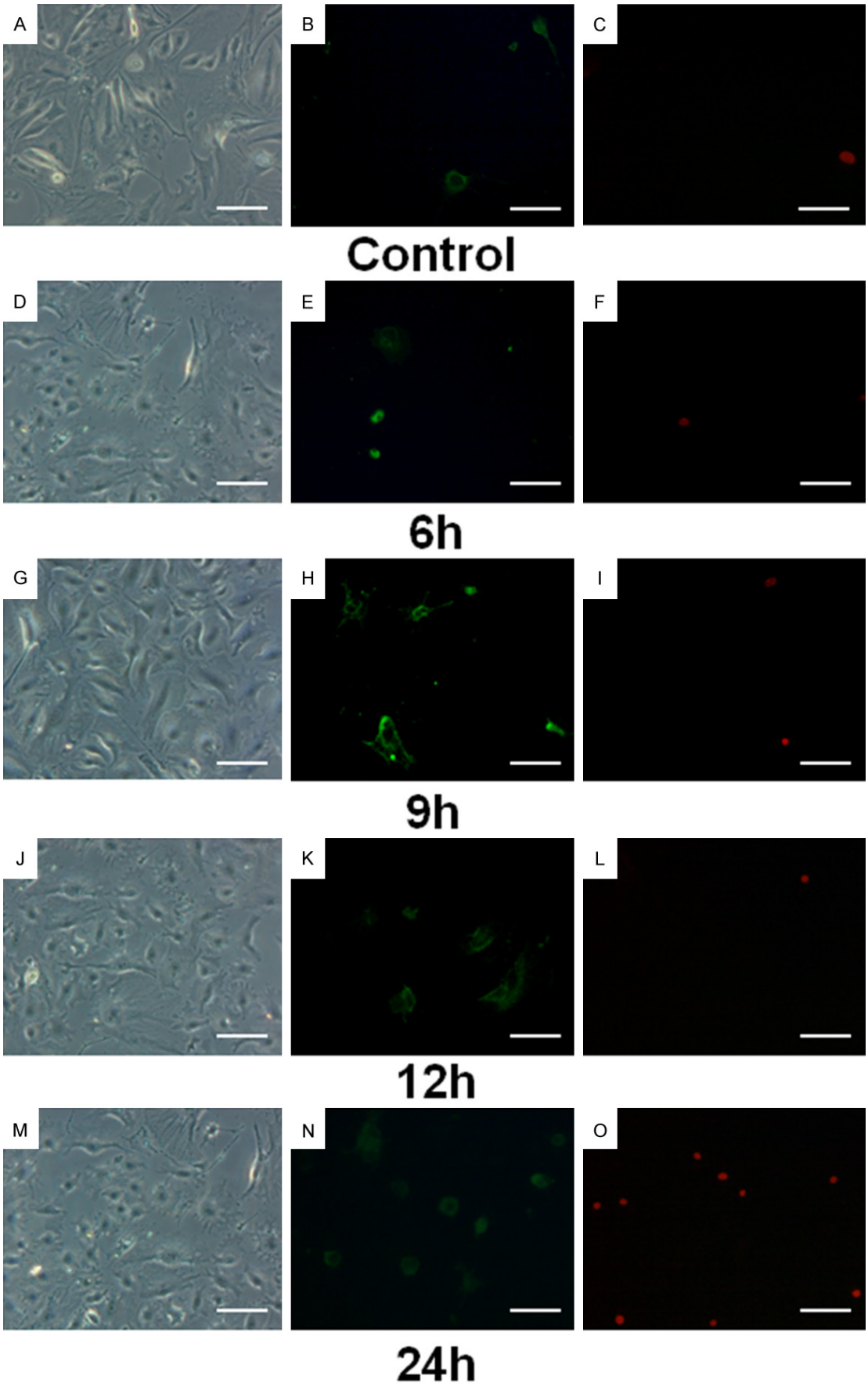
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**Figure 1.** Induction of cultured osteocytic cell apoptosis, necrosis with Dex and hypoxia. Immunostaining of apoptotic and necrotic cells using an Apoptotic/Necrotic Detection Kit as outlined in Materials and Methods. (A, D, G, J, M) Phase contrast image. (B, E, H, K, N) Detection of apoptosis (FITC-Annexin V staining). (C, F, I, L, O) Detection of necrosis (Ethidium Homodimer III staining). (A-C) Control group. (D-F) 6 h group. (G-I) 9 h group. (J-L) 12 h group. (M-O) 24 h group. Both apoptosis and necrosis were markedly increased in 24 h group (O). (Scale bar = 100 μm).

stress, a recognized cause of osteonecrosis, occurs within 12-24 h [14]. Taken together these findings emphasize that osteonecrosis develops within a relatively short time after the induction of tissue hypoxia and in vivo stress. However, in the presence of hypoxia the duration of the time span needed for injury to manifest itself at the osteocytic cell level has not yet been adequately investigated.

MLO-Y4, which are cultured osteocytic cells, were cultured under hypoxia (1% $O_2$) after the addition of Dex. Then, the reaction was determined serially for 24 h, and their apoptosis and necrosis expression rates compared.

## Materials and methods

### *Cell culture*

MLO-Y4 murine cultured osteocytic cells, which have been used previously, were cultured [15]. The cells were plated on type I collagen-coated dishes (BD Biosciences, Bedford, USA) and cultured in α-minimal essential medium (α-MEM) supplemented with 5% (v/v) FBS, 5% (v/v) FCS, streptomycin (100 μg/ml) and penicillin (100 units/ml). Then for the hypoxia experiments, the cells were incubated for 24 h in a $CO_2$/trigas incubator (Astec, Fukuoka, Japan) set at a mixture of 5% (v/v) $CO_2$ and 1% (v/v) $O_2$ balanced with $N_2$.

### *Cell viability assay*

MLO-Y4 cells seeded in type I collagen-coated 4-chamber culture slide (BD Biosciences, Bedford, USA) were cultured overnight in an incubator under hypoxia (1% $O_2$) in the presence of 1 μM Dex (MSD, Tokyo, Japan) for 6 h (6 h group), 9 h (9 h group), 12 h (12 h group), or 24 h (24 h group) respectively. Then they were cultured under normoxia with Dex until the respective total culture time reached a total of 24 h. Viability assays were then performed using an Apoptotic/Necrotic Cells Detection Kit (PromoKine, Heidelberg, Germany) according to the manufacturer's instructions, and the percentages of apoptotic/necrotic cells relative to the total cell number were determined. In the viability assays, apoptotic cells can be detected by the staining with fluorescein-labeled annexin V (green fluorescence) and necrotic cells by the staining with Ethidiumhomodimer III, a highly positively charged nucleic acid probe, which is impermeant to live cells and early apoptotic cells, but stains necrotic cells and late apoptotic cells (entering into secondary necrosis) with red fluorescence. Fluorescence-positive cells were evaluated by phase contrast and fluorescence (470 nm and 530 nm LED modules) microscopy using Axiovert.A1 FL-LED (Carl Zeiss, Jena, Germany). In addition, as a Control group MLO-Y4 cells to which Dex was added were cultured for 24 h under normoxia (20% $O_2$).

### *Statistical analysis*

All quantified results were expressed as the mean ± SD. Statistical significance in the comparison of apoptosis or necrosis between the control and each of the experimental groups was analyzed with Dunnett's multiple comparisons test. *P* values less than 0.05 were accepted as statistically significant. The statistical analysis was performed using StatView J-5.0 software (SAS Institute, Cary, USA).

## Results

To investigate the effect of the duration of hypoxia after the addition of Dex in cultured osteocytic cells, Dex was added to murine MLO-Y4 cultured osteocytic cells, cultured under hypoxia for various durations, and then the cell viability quantified using an Apoptotic/Necrotic Detection Kit as outlined in Materials and Methods (**Figure 1**).

In the 6 h group the percentages of apoptotic cells was 8.1±2.4%, necrotic cells 1.6±0.9%; in the 9 h group 9.7±2.1% and 2.2±1.1%; in the 12 h group 7.2±2.6% and 1.8±1.7%, and in the 24 h group 17.8±2.9% and 14.6±3.0%, respectively. In the Control group apoptosis was 8.2±1.9%, and necrosis 1.8±1.2%. No significant differences were found in cell death

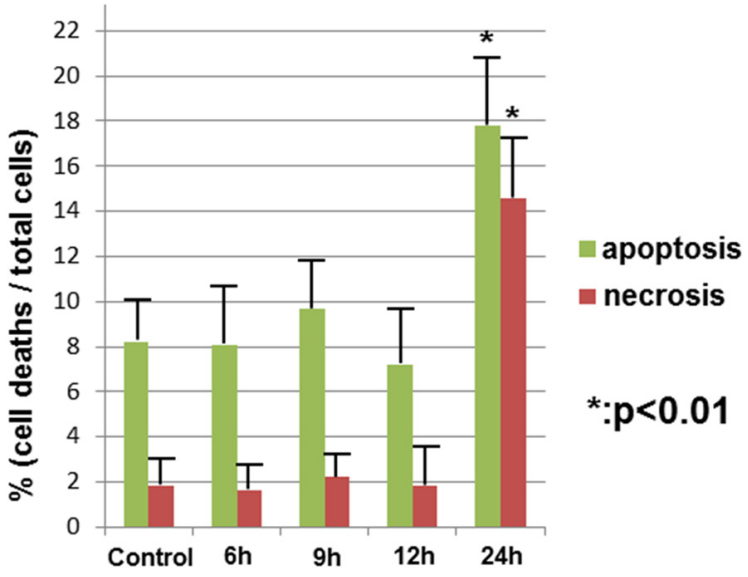


**Figure 2.** Percentage of cultured osteocytic cell apoptosis, necrosis with Dex and hypoxia. The Graph indicates the percentages of apoptotic and necrotic cells exposed to hypoxia for the respectively indicated durations. The numbers of apoptotic cells and necrotic cells relative to the total cell count were determined. Columns and bars indicate means and S.D. respectively (n = 5). (*: P<0.01 vs. Control, 6 h, 9 h and 12 h group). Apoptotic and necrotic cells were significantly increased in the 24 h group.

between any of the 6 h, 9 h, or 12 h groups, whereas in the 24 h group cell death was significantly increased as compared to the Control, 6 h, 9 h, and 12 h groups (P<0.01) (**Figure 2**).

## Discussion

Not only in osteocytic cells as used in the present study, but also in various other cells and tissues reported elsewhere the administration of Dex has been shown to increase apoptosis. Zhu et al. reported that in osteoblasts the administration of Dex alone or hypoxia alone induced a further increase in apoptosis as compared with Dex and Hypoxia [4]. Furthermore, like in osteocytic cells, it has been reported that necrosis first becomes significantly increased only after culture in the presence of Dex and hypoxia for 24 h.

In this experiment, apoptosis was only slightly increased at 12 h, with no increase in osteocytic cell necrosis found. However, consistent with previous reports after culture for 24 h there was a significant increase in osteocytic cell necrosis [11]. Moreover, although in the osteocytic cell hypoxic stress is considered to induce mitochondrial swelling and dysfunction leading to apoptosis [16, 17], its ultimate fate as apoptotic or necrotic is determined by multiple factors such as the degree and duration of hypoxic stress and type of cell [3]. Namely, the present results demonstrated that culture with Dex and hypoxia for 12-24 h increased not only osteocytic cell apoptosis but also necrosis, and that for the osteocytic cell to become necrotic at least 12-24 h of Dex and hypoxia is required. And since these findings are consistent with those of a traumatic osteonecrosis study using dogs that recommended intervention within 12 h [18-20], we also conclude that treatment must be initiated within a short time. Accordingly, from the aspect of prevention, the hypoxic state must be improved within at most 12 h.

Hitherto, considering that injury is induced by oxidative stress and hypoxia within a short time osteonecrosis has been reported to be preventable with antioxidants and antivasospastic agents [21-24], or that because of the involvement of a lipid disorder by the timely administration of lipid-lowering agents such as pitavastatin [25-29]. In addition to these, if hypoxia could similarly be reversed by the administration of blood flow improving drugs or other interventions we consider it important that the intraosseous environment be normalized within at most 12 h.

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## Disclosure of conflict of interest

None.

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