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Original *Article*

Does dynamic immobilization reduce chondrocyte apoptosis and disturbance to the femoral head perfusion?

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Abstract: The purpose of this study is to investigate whether the dynamic hip immobilization is more favourable for lessening ischemic injury to the immature femoral head than a static immobilization. 152 Japanese white rabbits were divided into four groups randomly, and the hips were immobilized into "human" position (group A), "frog leg" position (group B) and "dynamic frog leg" position (group C). Group D was used as control. Ten rabbits in each group were killed, and the hip specimens were harvested at 1, 2, and 3 weeks after immobilization. Bcl-2/Bax expression balance and chondrocytes apoptosis were analyzed. The remaining eight rabbits in each group were used to measure the blood supply of capital femoral epiphysis by selective vascular perfusion with Indian ink. The Bcl-2/Bax expression ratio in group C was significantly increased than that in group A and B ($p<0.001$), while that was not significantly different from control group ($p=0.0592$). At three weeks after immobilization, the average apoptotic ratio was 36.7%, 45.8%, and 26.7% in group A, B and C, respectively ($p<0.01$). There was no significant difference between group C and normal control ($p=0.0597$). The perfusion ratio was 0.03±0.03, 0.03±0.02, and 0.08±0.03 in group A, B and C respectively, and 0.12±0.04 in control group ($p<0.05$). Thus, the dynamic immobilization model exhibited a relatively less chondrocytes apoptosis and disturbance to the femoral head perfusion than other immobilizations *in vivo*, which therefore may be useful for reducing avascular necrosis following the treatment of developmental dysplasia of the hip.

Keywords: Dynamic immobilization, femoral head, blood supply, apoptosis

Introduction

The normal development of femoral head depends on the concentric relationship and dynamic interactive stimulation between femoral head and acetabulum. Stable immobilization of the hip is the most common method for maintaining the concentric reduction in the treatment of hip diseases, especially developmental dysplasia of the hip (DDH). However, avascular necrosis (AVN) of the femoral head is one of the most serious complications that can arise following the hip immobilization. Although the etiological factors of AVN have been studied in the previous reports [1-3], the molecular mechanism of AVN remains unclear. Ischemia has been investigated as one of the underling pathophysiological mechanisms of AVN following the treatment of DDH for many years [4-6]. Moreover, an increased intra-articular pressure resulted from the hip position of immobilization has also been implicated as the mechanism of AVN [7].

Based on the histopathologic studies of adult femoral head osteonecrosis, it is traditionally believed that interruption of blood supply to the femoral head induces cell death in the bone but not in the articular cartilage. In children, however, the immature or growing femoral head is comprised of articular cartilage, subarticular epiphyseal cartilage and endochondral ossification center. The findings from the studies using immature animals show that different regions of the epiphyseal cartilage are also affected by the ischemic process [8-10]. Moreover, numerous experimental studies have confirmed the cartilaginous epiphysis is vulnerable to isch-

emic injury [11-13]. One of the earliest signs of ischemic necrosis of the capital femoral epiphysis is cessation of the growth of the secondary center of ossification, and clinically, AVN following the treatment of DDH is diagnosed when the femoral head fails to ossify or to grow within 1 year after being reduced [11], suggesting the changes of chondrocytes were implicated into the early stage of AVN. In addition, the previous study has also exhibited that apoptosis of chondrocytes is the early findings in the experimental models of ischemic cartilaginous epiphysis of femoral head [14, 15]. Therefore, detection of chondrocyte apoptosis and apoptosis-related proteins, such as apoptosis-inhibiting Bcl-2 and apoptosis-inducing Bax, was helpful for investigating the ischemic status of cartilaginous epiphysis resulted from immobilization.

Although ischemia has been recognized as the substantial causes resulting in AVN, all of the previous studies are based on a static model that immobilizes the hip in a permanent position. In general, long-term static compression suppresses cellular proliferation and biosynthesis of proteoglycans and proteins in cartilage explants [16]. In contrast, dynamic compression stimulates biosynthesis of extracellular matrix components at transcriptional and translational levels in cartilage explants, suggesting that dynamic stimulation is beneficial for maintaining cartilage physiology [17]. Thus, it seems to be more reasonable if the pathology of the hip could be treated by active movement. However, the condition of dynamic immobilization of the hip has not been evaluated in the previous literatures published.

Therefore, the purpose of this present study is to investigate whether the dynamic immobilization of the hip is more useful for lessening ischemic injury to femoral head than a static immobilization. For this aim, a series of experimental models in growing rabbits with the hip various immobilizations were used to evaluate the blood supply, apoptosis and Bcl-2/Bax expression in the femoral head.

Materials and methods

Immobilizations of the hip and samples preparation

These experiments were approved by the University Committee for Animal Experimentation of China Medical University. One hundred and fifty-two Japanese white rabbits, aged 3 weeks and weighing 0.5±0.1Kg, were divided into four groups randomly, with 38 animals each group. The hips of the rabbits were immobilized by using synthetic casting tape as the following description. Group A: spica cast or "human position". The lower body, hips and the limbs of the rabbit were immobilized with the hip flexion of 90°-100°, abduction of 60°, and the knee flexion of 90° (**Figure 1A**). Group B: "frog leg" or Lorenz position, the modus was same as Group A, but placing the hip in maximal achievable abduction (80°-90°) (**Figure 1B**). Group C: "dynamic frog leg" position, the lower body and the hips were free. Only the limbs were immobilized with the hip flexion of 90°-100°, maximal achievable abduction of 80°-90°, and the knee flexion of 90°. Simultaneously, a connecting rod between the knees was used to maintain above hip position. Comparing with the "frog leg" position, the "dynamic frog leg" position immobilization allowed the hip to rotate moderately around the axis formed by the centers of bilateral acetabulum (**Figure 1C**). Group D: normal controls. No immobilization was used on the hip.

Ten rabbits in each experimental and control group were killed at 1, 2, and 3 weeks after immobilization. Before the animals were killed, an anteroposterior radiograph of the hip was performed to investigate whether AVN developed in the femoral head. The femoral heads with proximal femur were obtained, and were sectioned coronally into blocks of 5 mm thick. These were fixed in 10% formalin for three days, decalcified by EDTA at room temperature for eight weeks, embedded in paraffin and cut into slices of 5 μm thick. The slices through the insertion of ligamentum teres were collected for histological and apoptotic analysis.

Immunohistochemical detection of Bcl-2/Bax expression

Immunohistochemical analysis was performed using the Ultrasensitive streptavidin-peroxidase (SP) method. Dewaxed and re-hydrated sections were incubated with rat anti-rabbit Bcl-2 or Bax polyclonal antibody (Boster Biotechnology Ltd., Wuhan, China) overnight at 4°C, in accordance with the manufacturer's recommendations. All slides were counterstained with hematoxylin. Negative controls

Figure 1. Photographs of various immobilizations. A: Spica cast. B: Frog leg position. C: Dynamic frog leg immobilization. The insets show the angle of abduction (A, B) and range of immobilization (C).

were performed by omitting the primary antibodies.

The immunostained sections were examined using an Olympus microscope of ×200, connected to a computer-aided image collection system (NIS-Elements F2.30, Nikon, Japan). The captured and digitized microscopic images were quantitatively analyzed using an image analysis software of *Image-Pro® Plus 2.0* for Windows (Media Cybernetics, Maryland, USA) by measuring the optical density (OD) of positive chondrocytes. The higher the OD value was, the stronger the expression of Bcl-2 or Bax protein. Then, the ratio of Bcl-2/Bax was calculated.

TUNEL assay

For in situ visualization of apoptotic cells, we performed terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL). After a pretreatment process, the slides were stained using an *in situ* cell death detection POD kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. All slides were counterstained with hematoxylin. As a negative control, the terminal transferase was omitted. Before diaminobenzidine (DAB) coupling, a part of the slides were stained using the immunofluorescence marker included in the kit to determine whether the specimens could be stained by the TUNEL method.

Each slide was examined in five fields containing the largest number of positive chondrocytes at a magnification of ×400 for TUNEL, and each slide was read three times by a pathologist who was blinded to the animals grouping. Then, the mean number of positive chondrocytes was divided by the total number of chondrocytes to calculate the TUNEL-positive chondrocyte ratio.

Measurement of capital femoral epiphyseal blood supply

The remaining eight rabbits in each experimental and control group were used to measure the blood supply of capital femoral epiphysis by

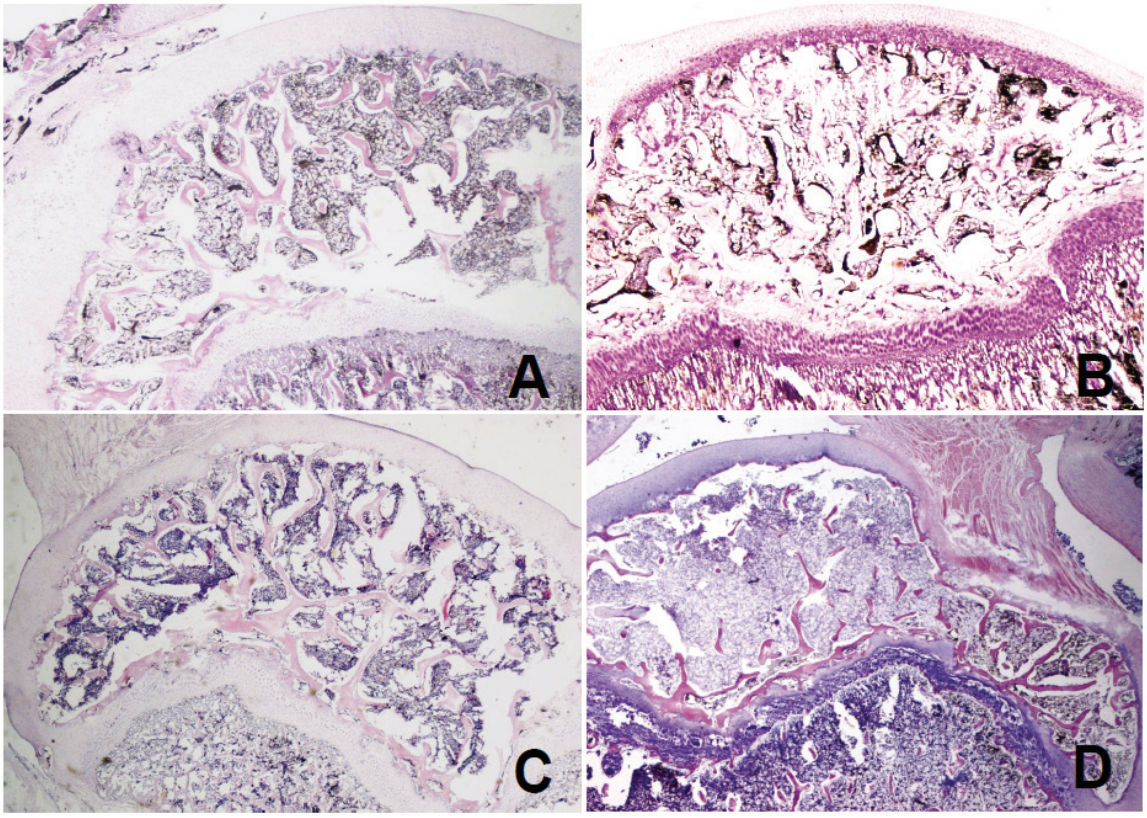

Figure 2. Photomicrographs of femoral head indicating the secondary centers of ossification perfused by Indian ink at three weeks of immobilization (HE, ×20). The area of perfusion and the area of epiphyseal nucleus can be captured by software automatically. Perfusion ratio = area of Indian ink / area of epiphyseal nucleus. A: Spica cast. B: Frog leg position. C: Dynamic frog leg immobilization. D: Normal control. 8 animals (16 hips) in each group.

selective vascular perfusion with Indian ink. The rabbit was anesthetized by intravenous injection of pentobarbital sodium. The abdominal aorta and inferior vena cava were exposed through an abdominal incision, and ligated immediately. A Y-cannula was placed in the abdominal aorta distal to the site of ligation. At the same level, a cannula was distally inserted in the vein for drainage. The abdominal aorta was irrigated with heparinized saline (50,000 units in 500ml of 0.9% sodium chloride), until the liquid flowed freely from the inferior vena cava was clear. The abdominal aorta was then perfused continuously with a solution of 10% gelatin/Indian ink (20g of gelatin in 100ml of Indian ink and 100ml of water) at pressure of 90 mmHg. When the claws of the rabbit turned black, the inferior vena cava was ligated at the distal site of the cannula. Finally, the abdominal aorta was ligated until intra-arterial pressure prevented further perfusion. Six hours after the refrigeration at -20°C, dissection was performed and the pelvis and hips were harvested. Then, the samples were fixed, decalcified, embedded and cut into slices of 10μm thick.

The sections were stained by hematoxylin and eosin, and examined using Olympus microscope of ×20, connected to a computer-aided image collection system (NIS-Elements F2.30). The ratio of perfusion was calculated with image analysis software of *Image-Pro® Plus 2.0* (Ratio of perfusion=area of Indian ink / area of epiphyseal nucleus, **Figure 2**). A higher ratio of perfusion indicated a more distribution of blood.

Statistical analysis

The data were expressed as the mean ± standard deviation, and processed with SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t*-test and analysis of variance (ANOVA) were used. A P value of <0.05 was considered statistically significant.

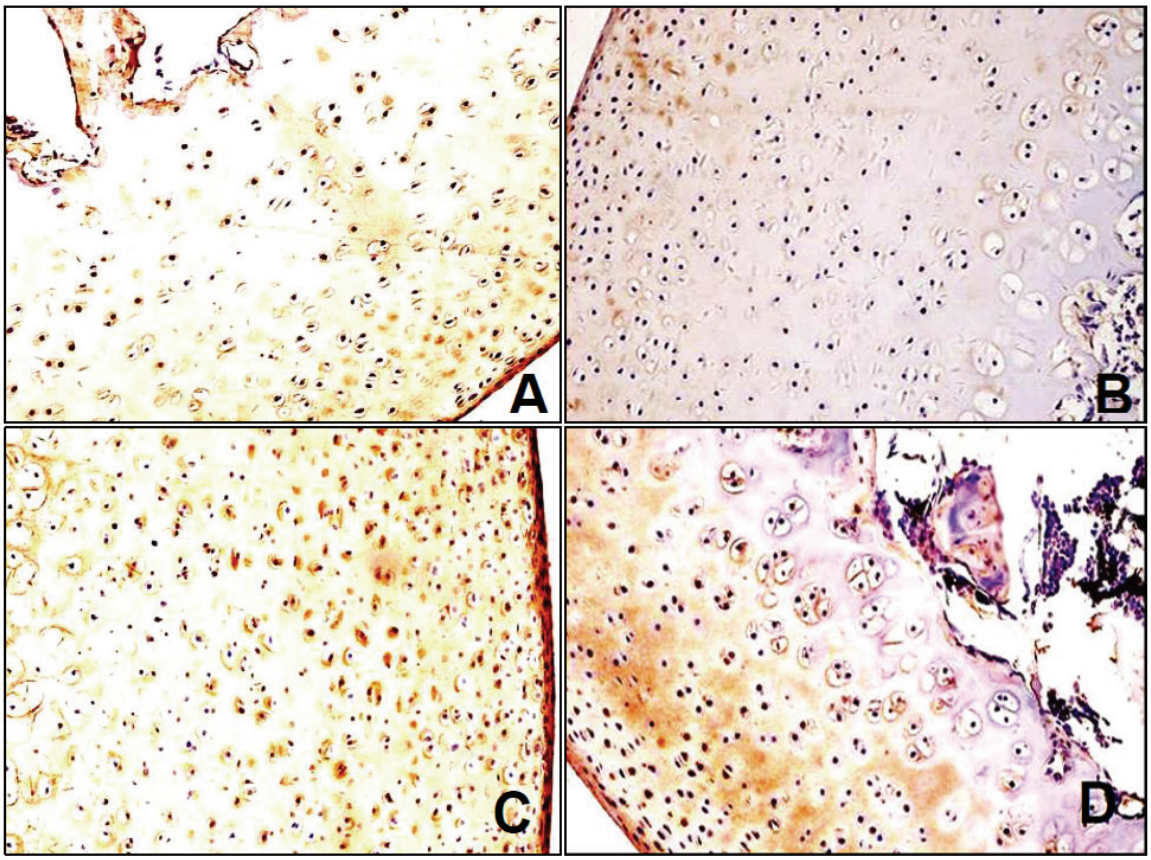

Figure 3. Immunodetection of Bcl-2 protein in the epiphyseal chondrocytes of femoral head at 3 weeks of immobilization (×200). The positive signals were detected in the cytoplasm of chondrocytes. A: Spica cast. B: Frog leg position. C: Dynamic frog leg immobilization. D: Normal control. 10 animals (20 hips) in each group.

Results

Expression of Bcl-2/Bax protein

Bcl-2 and Bax were expressed in the cytoplasm of chondrocytes from both experimental and control groups (**Figure 3** and **4**). The ratio of Bcl-2/Bax appeared not alterative with the period of immobilization in every group, except for the "frog leg" group that decreased significantly at three weeks. In group of "dynamic frog leg", the Bcl-2/Bax expression ratio trended to increase compared with that in the other experimental groups. A statistically significant difference was found between the "dynamic frog leg" and "spica cast" group, between the "dynamic frog leg" and "frog leg" group, while that was not found between the "dynamic frog leg" and control group (**Table 1**, **Figure 5**).

Apoptotic analysis

TUNEL-positive chondrocytes were observed in every group (**Figure 6**), and its percentage increased with the period of immobilization in the three experimental groups (**Table 2**, **Figure 7**). At three weeks after immobilization, the average apoptotic ratio was 36.7%, 45.8%, and 26.7% in group of "spica cast", "frog leg" and "dynamic frog leg", respectively. The "dynamic frog leg" immobilization, compared with "spica cast" and "frog leg" group, exhibited a relatively low apoptotic ratio. Although the percent of apoptotic chondrocytes in "dynamic frog leg" group was higher than normal control, no statistically significant difference was found.

Blood supply in capital femoral epiphysis

No radiographic AVN was found in all of the animals. The gross observation of the hip specimens demonstrated the acetabulums, femoral heads and soft tissue around the hip were stained black. It is interesting to note that a clear notch on the surface of femoral head resulted from the compression between femoral head and acetabulum in "spica cast" and

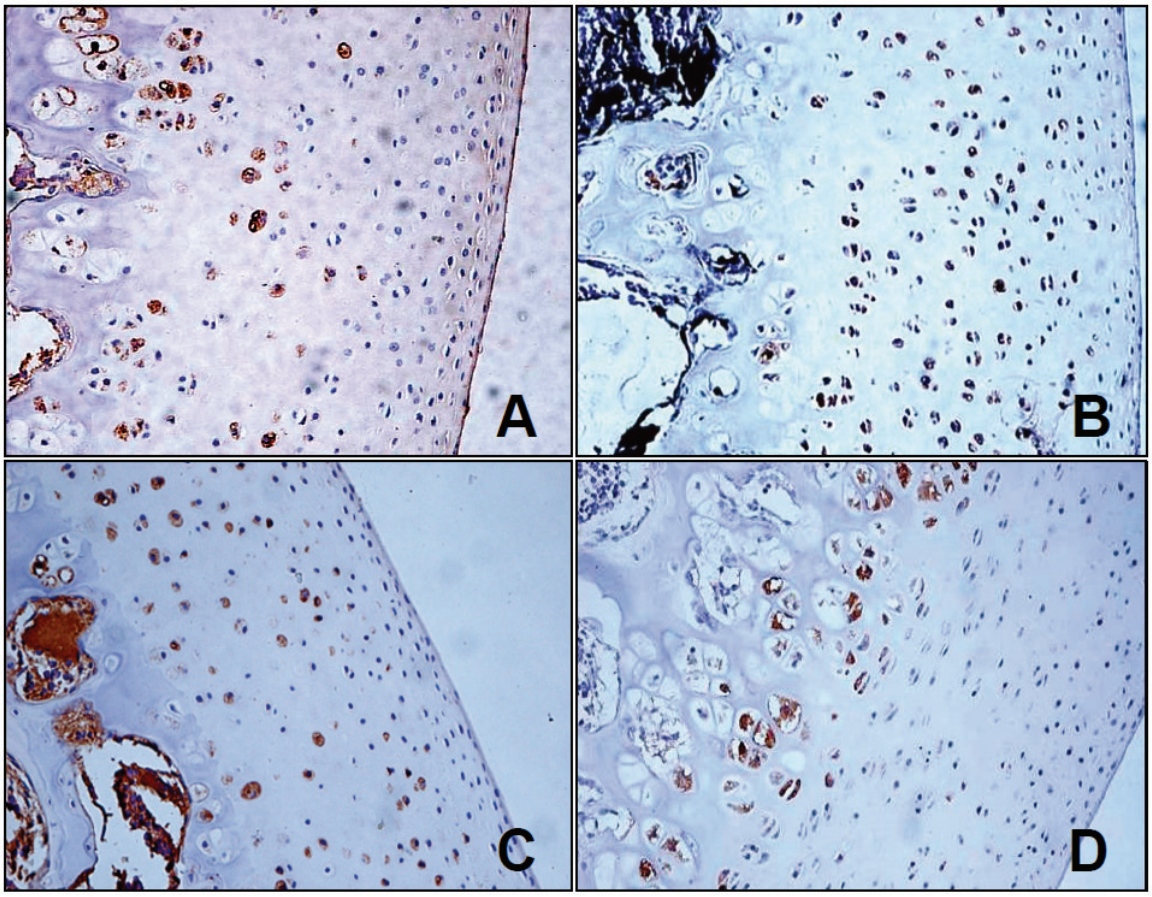

Figure 4. Immunodetection of Bax protein in the epiphyseal chondrocytes of femoral head at 3 weeks of immobilization (×200). A: Spica cast. B: Frog leg position. C: Dynamic frog leg immobilization. D: Normal control. 10 animals (20 hips) in each group.

Table 1. Ratio of Bcl-2/Bax expression in epiphyseal chondrocytes of femoral head following various immobilizations

Weeks	Spica cast		Frog leg position		Dynamic frog position		Normal control	
	Mean±SD	95%CI	Mean±SD	95%CI	Mean±SD	95%CI	Mean±SD	95%CI
1	0.70±0.43	0.40-1.01	0.80±0.31	0.54-1.06	1.33±0.66	0.72-1.94	1.67±0.89	0.85-2.49
2	0.63±0.30	0.40-0.87	0.76±0.28	0.56-0.96	1.29±0.38	0.97-1.62	1.73±0.55	1.27-2.19
3	0.75±0.30	0.53-0.96	0.40±0.13	0.31-0.48	1.38±0.47	1.08-1.68	1.70±0.54	1.36-2.04

Data was shown by mean ± standard deviation (SD) and 95% confidence interval (CI).

"frog leg" group (7/16hips and 11/16hips, respectively) (**Figure 8**). The black staining on the bisections of the hip confirmed a sufficient perfusion of Indian ink (**Figure 8**). However, the microscopic observation indicated the distribution of Indian ink was not uniform in every group. The ratio of perfusion was 0.03±0.03 in "spica cast" group, 0.03±0.02 in "frog leg" group, 0.08±0.03 in "dynamic frog leg" group ($p<0.05$, compared with other group) and 0.12±0.04 in normal control, respectively. These experimental data indicated the immobilizations decreased the blood distribution in the femoral head when the hip placed in various positions. However, the dynamic immobilization model exhibited a relatively less disturbance to the perfusion of femoral head than other immobilizations.

Discussion

In the present study, the use of the well-established experimental models immobilizing the hip in various positions provided a practical

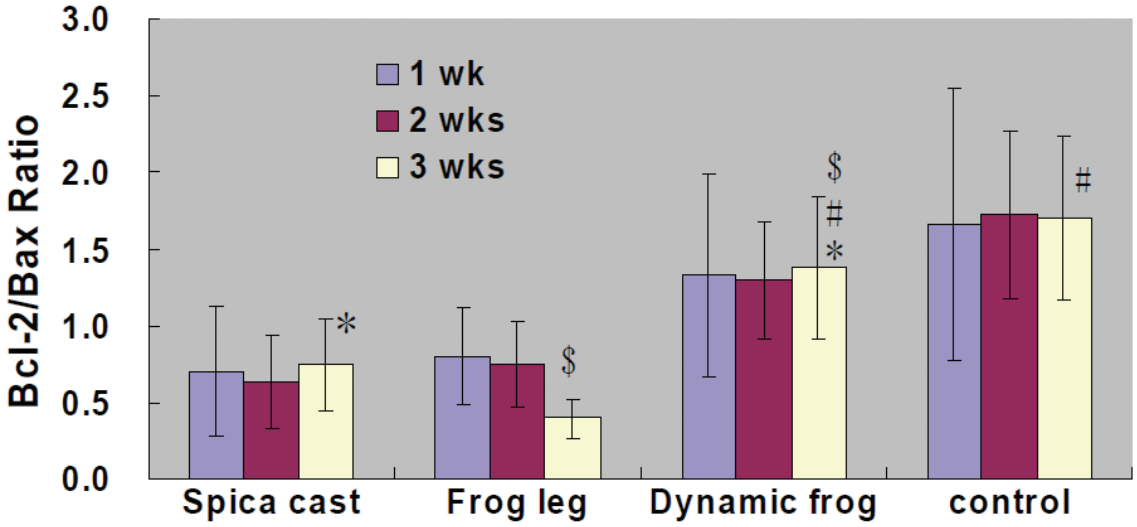

Figure 5. Ratio of Bcl-2/Bax expression in the epiphyseal chondrocytes of femoral head following various immobilizations. The result of each bar was obtained from 20 independent observations of 20 femoral heads in 10 animals. The error bars indicated standard deviation for mean. (* P<0.001, $^{\$}$ P<0.001, $^{\#}$ P=0.0592).

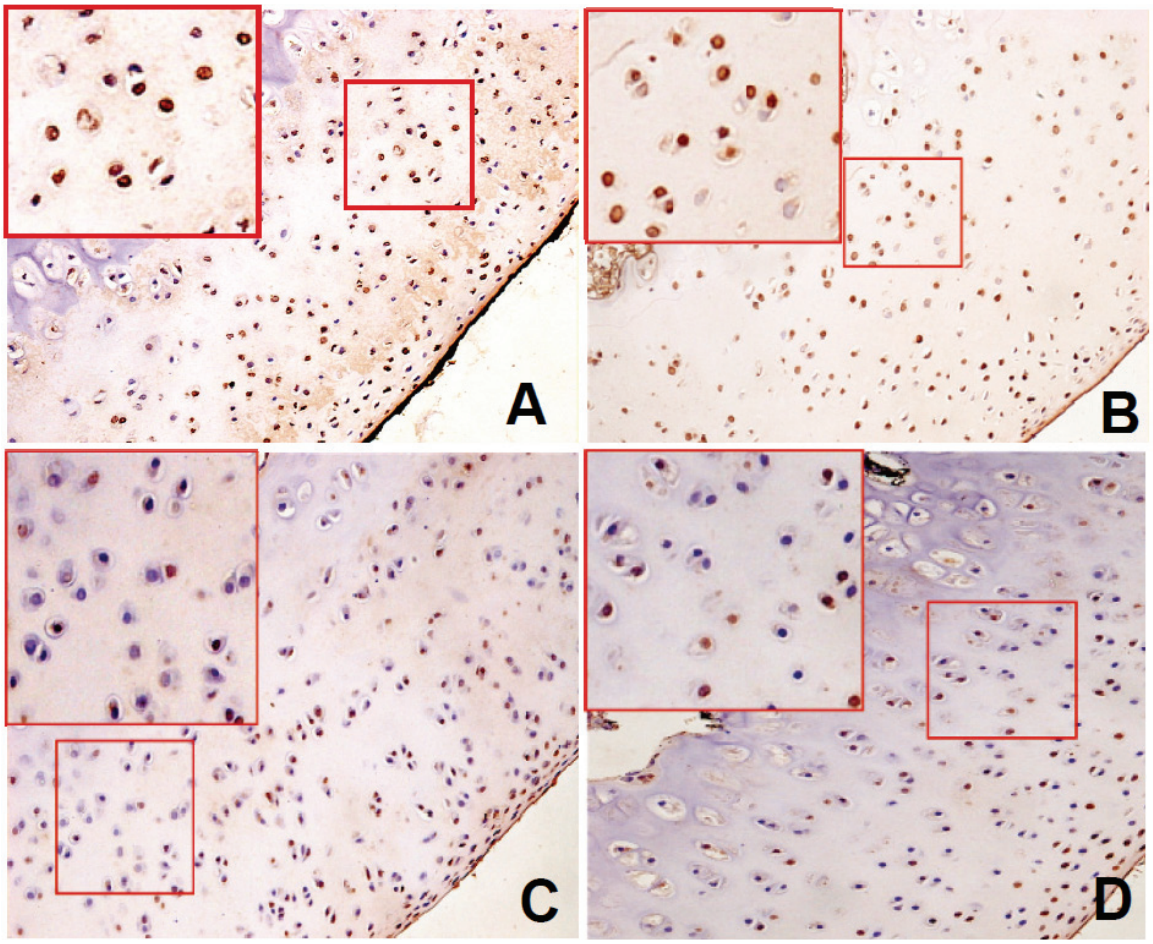

Figure 6. Photomicrographs showing the positive TUNEL reaction in the epiphyseal chondrocytes of femoral head at 3 weeks of immobilization (×200, insets: ×400). Positive chondrocytes for TUNEL reaction were detected in the cartilaginous epiphysis. A: Spica cast. B: Frog leg position. C: Dynamic frog leg immobilization. D: Normal control. 10 animals (20 hips) in each group.

Table 2. Percentage of TUNEL-positive epiphyseal chondrocytes in femoral head following various immobilizations

Weeks	Spica cast (%)		Frog leg position (%)		Dynamic frog position (%)		Normal control (%)	
	Mean±SD	95%CI	Mean±SD	95%CI	Mean±SD	95%CI	Mean±SD	95%CI
1	29.8±11.7	21.4-38.2	33.8±13.7	24.0-43.6	23.8±7.8	18.6-29.0	20.1±8.3	12.4-27.8
2	32.4±13.1	22.3-42.4	35.7±11.0	28.3-43.1	24.0±9.1	17.5-30.6	20.6±7.2	14.5-26.6
3	36.7±5.6	32.7-40.7	45.8±17.1	31.6-60.1	26.7±13.5	17.6-35.8	19.9±7.7	15.1-24.8

Data was shown by mean ± standard deviation (SD) and 95% confidence interval (CI).

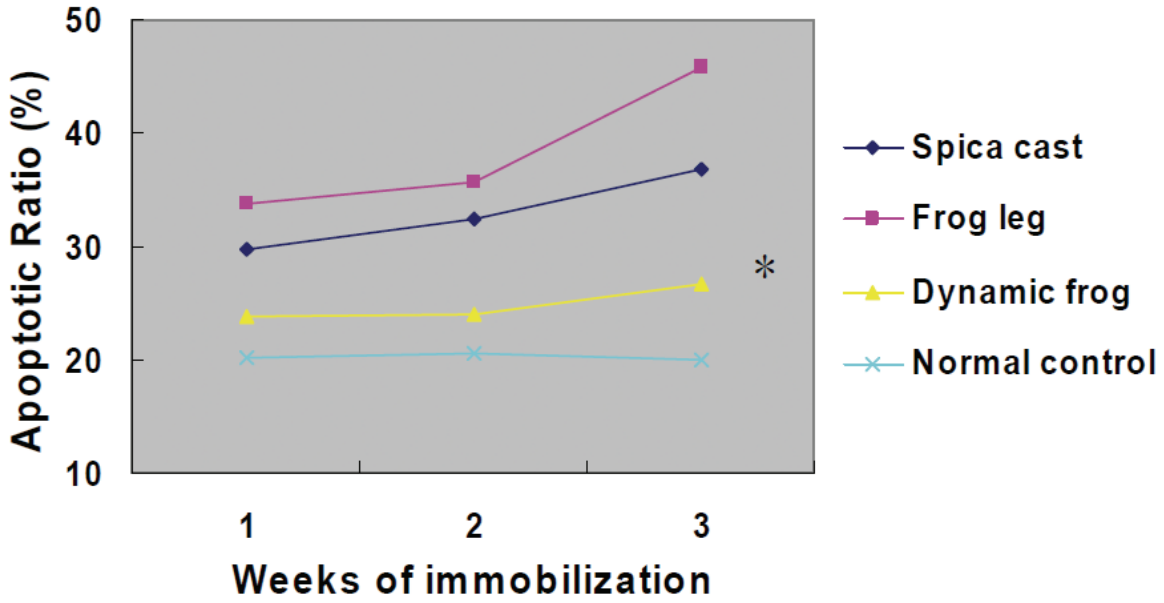

Figure 7. Percentage of apoptotic chondrocytes in the cartilaginous epiphysis of femoral head following various immobilizations. The result of each time-point for immobilization was obtained from 20 independent observations of 20 femoral heads in 10 animals. *There are significantly different from the other experimental groups at 3 weeks of immobilization (p<0.01), but no significantly different from the normal control group (p=0.0597).

method of determining the development of the immobilized femoral head *in vivo*. The methods using vascular perfusion combined with microscopic observation to evaluate the blood supply of capital femoral epiphysis is well accepted in other study [18-20]. Under the aid of computed software, the investigators can measure the blood distribution in the epiphysis quantitatively. In the current study, the authors used this method to quantitate the ratio of perfusion and to define the effect of various hip immobilizations on capital femoral epiphyseal blood supply in the immature femoral head of rabbits.

A significant decrease in blood supply in the femoral head was noted in the extreme abduction position in the experimental hips ("frog leg" group) when compared with the normal hips. This is in coincidence with the previous reports by Schoenecker et al. [21] utilizing the hydrogen-washout technique and by Jaramillo et al. [22] using gadolinium-enhanced MR imaging. It has been considered that some aspects of hip immobilization must be associated with compromise of blood flow resulting in ischemia. The vascular anatomy of this epiphysis and the vascular contribution to the femoral head has been described by several authors [18, 23-25]. An extreme position of immobilization can potentially block the extracapsular vascular perfusion, and the vessels obstruction in that position has been confirmed by angiographic study on autopsy [6, 21, 22, 26]. In addition, Salter et al. [11] suggested that compression of the soft pre-osseous cartilage of femoral head against the acetabulum can cause vascular obstruction. Naito et al. [27] reported the blood flow rate of the femoral head was significantly decreased with compression applied to the hip by experimental study in puppies. A direct pres-

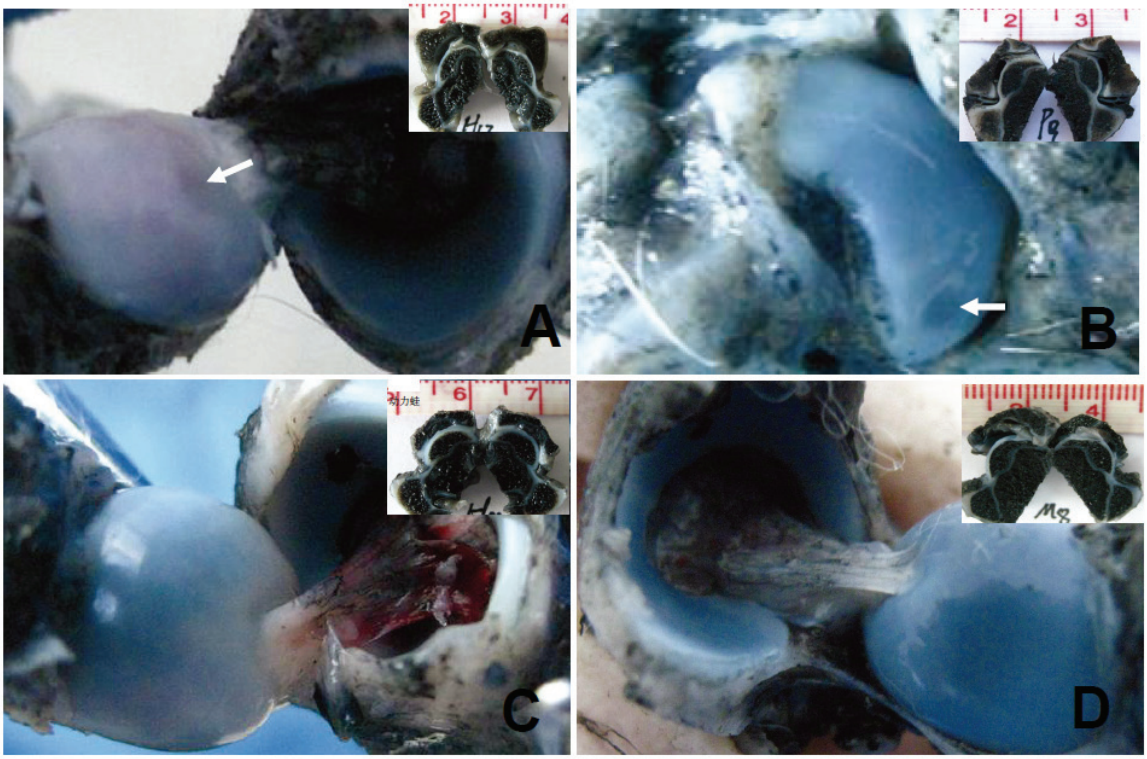

Figure 8. Gross observation of the hip specimens. The photographs demonstrated the acetabulums, femoral heads and soft tissue around the hip were stained black. A clear notch (white arrows) on the surface of femoral head is noted in "spica cast" (A) and "frog leg" group (B), but not in "dynamic frog leg" (C) and control group (D). The inserted photographs of the bisections of the hip confirm the perfusion of Indian ink is sufficient.

sure of the femoral head against the acetabulum resulted from a persistent immobilization may compress the vascular canals and cause ischemia by blocking the vessels within the epiphysis.

Unexpectedly, the ratio of perfusion of femoral head in "dynamic frog leg" group was considerably increased than that in "spica cast" or "frog leg" group, although which was decreased than normal control. The dynamic immobilization used in this study seemed to play an active role in diminishing ischemia of capital femoral epiphysis. To our knowledge, this is the first time to apply a dynamic immobilization model to investigate the blood distribution in a growing femoral head. Being different from the stable static immobilization previously, the dynamic frog leg immobilization provides the hip a chance for more spontaneous flexion after an unforced flexion of 90°-100°, and allows the hip to rotate moderately around the axis formed by the centers of bilateral acetabulum. We presume that at least three advantages of the dynamic immobilization contribute to the prevention of severe decrease of blood perfusion to the epiphysis. First, the limited motion of the hip within the cast may relief the obstruction of extracapsular vessels, and the blood supply to the femoral head may be recovered with the hip motion intermittently. Second, due to the active contraction of pericapsular muscles, the effect of muscular pump may be helpful to enhance the perfusion. Finally, unlike the stable static immobilization, the dynamic immobilization keeps the compressed position of femoral head against the acetabulum altering with the allowed motion of the hip, which makes the vascular canals within the cartilaginous epiphysis open but not occluded permanently. The most direct evidence of compression on femoral head resulted from the static immobilization is that the characteristic notch was seen in the "spica cast" and "frog leg" group, while that was not found in the "dynamic frog leg" group. Overall, as far as the dynamic immobilization, even though the maximal achievable abduction of the hip, the dynamic stimulation is relatively

beneficial for remolding and blood perfusion to the capital femoral epiphysis.

Apoptosis, as a subsequent result of ischemic injury to the femoral head has been recognized for many years. The majority of previous studies have shown that the expression of Bcl-2 plays an apoptosis-inhibiting role, while the expression of Bax plays a contrary role [28, 29]. The expression balance of Bcl-2/Bax determines death or survival of the cells. In this study, the highest incidence of apoptotic chondrocytes was coincident with the decreased ratio of Bcl-2/Bax expression, and that was seen in "frog leg" group and also in "spica cast" group. These data confirms the correlation between apoptosis and ischemia again, since the lowest ratio of blood perfusion was detected in those two groups. Interestingly, the "dynamic frog leg" immobilization demonstrated a relatively low percentage of apoptosis. Although the reasons for this result were unclear and need to further investigate, we believe it is considerable that the distinctive characteristic of dynamic immobilization in lessening disturbance to the femoral head perfusion in the largest extent. Meanwhile, the no-permanent compression may also contribute to the decrease of apoptosis, because *in vitro* study demonstrated the chondrocyte apoptosis can be induced by injurious mechanical compression [7, 30]. In addition, the limited motion of the hip joint within the cast provides the interactive dynamic stimulation between acetabulum and femoral head, which is also beneficial for development of chondrocytes [31-33].

In conclusion, taking together, the dynamic immobilization showed a notable effect in lessening disturbance to the femoral head perfusion and decreasing chondrocyte apoptosis *in vivo* of rabbits. Therefore, it may be effective for reducing AVN following the hip immobilization. Clinically, the stable static immobilization, such as spica cast, has been used for many years in the treatment of DDH. However, AVN of the femoral head is still inevitable. The hip joint is an organ of movement and its pathology must be treated by active movement [34]. Nevertheless, the dynamic immobilization has never been used in the current treatment of DDH. We believe the philosophy of dynamic immobilization the first used in this study should be useful for providing a new understanding and leading to a new strategy in the treatment of DDH.

Acknowledgments

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