Cell Proliferation is Promoted by Compressive Stress During Early Stage of Chondrogenic Differentiation of Rat BMSCs

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The presence of an appropriate number of viable cells is prerequisite for successive differentiation during chondrogenesis. Chondrogenic differentiation has been reported to be influenced by mechanical stimuli. This research aimed to study the effects of cyclic compressive stress on cell viability of rat bone marrow-derived MSCs (BMSCs) during chondrogenesis as well as its underlying mechanisms. The results showed that dynamic compression increased cell quantity and viability remarkably in the early stage of chondrogenesis, during which the expression of Ihh, Cyclin D1, CDK4, and Col2α1 were enhanced significantly. Possible signal pathways implicated in the process were explored in our study. MEK/ERK and p38 MAPK were not found to function in this process while BMP signaling seemed to play an important role in the mechanotransduction during chondrogenic proliferation. In conclusion, dynamic compressive stress could enhance cell viability during chondrogenesis, which might be achieved by activating BMP signaling.


Cartilage is recalcitrant to endogenous repair and regeneration (Ryan et al., 2009). Cell-based tissue engineering is believed as a promising treatment for damaged cartilage. It’s a phased process, which begins with aggregation and condensation of mesenchymal chondroprogenitor cells, following a progression toward proliferation, prehypertrophy, and hypertrophy (Goldring et al., 2006; zuszki, et al., 2008). The presence of an appropriate number of viable cells is prerequisite of successive chondrogenesis. Richter’s study (Dexheimer et al., 2012) suggests that high cell metabolism could guarantee more cells survive the shift from 2D-proliferation to condensation and determination to the chondrogenic lineage. However, due to high cell density, low nutrient, and oxygen conditions in culture, unwanted cell loss restricts tissue engineering of cartilage in vitro. Thus prevention of early apoptosis and stimulation of cell proliferation, especially cell viability, are attractive goals to achieve better chondrogenesis.

Efforts have been made for the improvement of the chondrocyte differentiation, such as modification of culture system (Park et al., 2010; Wei et al., 2012), supplementation with chondrogenic cytokines (Opolka et al., 2012) as well as extrinsic physical stimuli (Ebisawa et al., 2004; Li et al., 2012b). Among them, mechanic loading has been widely accepted as an effective enhancement for chondrogenesis. Studies (Wu et al., 2001; Fanning et al., 2003; Haudenschild et al., 2009; Kelly and Jacobs, 2010) show that Ihh, MAPKs, and JNKs act as essential mediators in the mechano-biochemical transduction and subsequent transcriptional regulation in the process of chondrogenesis. Our previous research (Li et al., 2009) also indicates p38MAPK signaling is important in transduction of mechanical signals during chondrogenesis. Besides, by interacting with the TGF-β/Smads signaling pathway, MAPK subtypes seems to regulate chondrogenesis with a delicate balance (Li et al., 2010b).

Meanwhile, it’s reported (Ryan et al., 2009) that proliferation of chondrocyte is regulated by ERK1/2 and p38MAPK, which play opposing roles. The ERK pathway may be a negative regulator of Sox9 mRNA expression in hydrostatic pressure-induced mechanotransduction (Mio et al., 2007). Indian hedgehog (Ihh) in prehypertrophic chondrocytes has also been shown to be indispensable in regulation of chondrocyte proliferation (Long et al., 2001; Minina et al., 2002). In parallel to Ihh, BMP signaling induces chondrocyte proliferation, which is mediated by heteromeric complexes of type I (BM PR1a and

Abbreviations: BMP, bone morphogenetic protein; CDK4, cyclin-dependent kinase 4; Col2α1, collagen type II alpha 1; Cyclin D1, D-type cyclins D1; ERKs, extracellular-signal-regulated kinases; Ihh, Indian hedgehog; JNKs, c-Jun N-terminal kinases; MEK, mitogen-activated protein kinase; p38MAPK, p38 Mitogen-activated protein kinase; rBMSCs, rat bone marrow-derived mesenchymal stem cells

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BMPR I b) and type II (BMPR2) receptors. Loss of both BMPR I a and I b leads to hypoplastic cartilage anlagen, indicating BMPs as positive regulators of cell cycle progression (Wuelling and Vortkamp, 2011).

Relevance of mechanic loading to chondrocyte proliferation might exist as the same cellular pathways and regulators are shared by cell proliferation and mechano-biochemical transduction. Indeed, a number of previous studies have demonstrated the sensitivity of chondrocyte proliferation to mechanical perturbation with divergences among them. The application of static compression inhibits the proliferation of chondrocytes cultured in vivo (Takahashi et al., 1998) and in vitro (Lee and Bader, 1997; Elder et al., 2000) whereas dynamic compression (De Witt et al., 1984; Lee and Bader, 1997) increases DNA synthesis. However, proliferation and differentiation are phase-specific and linked closely during chondrogenesis, in which proliferation would be restrained once the differentiation initiated. It’s reported by Saitoh et al. (2000) that compressive force could promote chondrogenic differentiation and hypertrophy. If the mechanic stress brought forward the differentiation, it would be still unable to achieve cartilage engineering in vitro due to unbalanced proliferation and differentiation.

Therefore, the purpose of this study is to find out that whether dynamic compression would have any effect on the cell viability in the stage of proliferation during chondrogenesis of BMSCs, as well as the underlying mechanisms.

Materials and Methods
Primary culture of rat BMSCs
Cell collection and culture were conducted according to previous study (Li et al., 2010a). Briefly, rat BMSCs were harvested from bone marrow of posterior tibias and femurs of 2-week-old SD rats. After centrifugation at 200g for 10 min, cells were resuspended in α-MEM (Gibco, Grand Island, NY) supplemented with 10% FBS (fetal bovine serum, Gibco). Non-adherent cells were removed from plates 48 h later by changing the medium. At confluence, primary cultures were detached using trypsin treatment and subcultured at 8 x 10^4 cells/cm^2. All experiments were performed according to institutionally approved guidelines for animal welfare. BMSCs were identified by detecting expression of CD34, CD44, CD73, CD90, CD105 and multipotent differentiation (data not shown).

In vitro chondrogenic induction
Chondrogenic differentiation of the BMSCs was initiated in micromass culture system (Li et al., 2010a). Briefly, passage two cells were harvested by a treatment of 0.25% trypsin and 0.02% EDTA. Suspension at a density of 2 x 10^6 cells/ml was seeded in 96-well plate with 2 ml in each well to form cell aggregates at 37 C for 2 h; then they were supplemented with α-MEM containing 10% FBS. 24 h later, culture medium was replaced with serum-free high-glucose DMEM (Gibco) supplemented with 10 ng/ml recombinant human TGF-β1 (Peprotech, Rocky Hill, NJ), 10^-7 M dexamethasone (Sigma, St.Louis, MO), 50 mg/ml ascorbic acid phosphate (Sigma), and 1% ITS-A (Gibco). Control cells were cultured in high-glucose DMEM supplemented with 1% FBS. Micromass cultures were performed for 14 days with culture media replacement of every 3 days.

Chondrocyte proliferation assays
Cell number was measured directly by cell counting. Briefly, cells were trypsinized with 0.05% trypsin and collagenase, and cell numbers were counted directly by a hemocytometer (American Optical Corp., Buffalo, NY) in triplicate. The viable cells were confirmed by trypan blue exclusion assay. Cell numbers at 1st, 3rd, 5th, 7th, 10th, 12th, and 14th day were counted and compare to those at the first day of chondrogenesis. The cell number at the first day was ascribed 100%.

Cell viability was measured in 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. At the same time on the 1st, 3rd, 5th, 7th, 10th, 12th, and 14th day, a 201 volume of MTT (Sigma) solution was added in each well at the concentration of 5 mg/ml (pH 7.4). After 4 h of incubation for MTT formazane formation, supernatant was removed and 150 l dimethyl sulfoxide (DMSO) was added with fully vibration to dissolve the formazane. Absorbance was measured at a major wavelength of 570 nm and OD value was obtained for each well. Each group included five plates and the data were repeated three times. [3H]-thymidine incorporation was also used to evaluate cell proliferation. Briefly, at the same time on the 1st, 3rd, 5th, 7th, 10th, 12th, and 14th day, 201 Ci (1 Ci = 37 GBq) [3H]-Tdr at the concentration of 50 Ci/ml (1 Ci) was added in each well in the micromass culture system. After incubation for 12 h, cells were gathered and transferred to filters, following stabilizing with 5% trichloroacetic acid, decolorizing with absolute ethanol and drying for 30 min at 80 C. Then the cells were transferred into scintillation fluid, and counted by liquid scintillation counter (counts/min, cpm). Each group included ten wells and the data were repeated three times. Cpm at the first day was ascribed 100%.

Dynamic compressive loading
Dynamic compressive stimulation was delivered to cells via a custom-made, computer-operated pressure system, in which compressive pressure was implemented by increasing gaseous tension above the supernatant media in a sealed chamber. The details of the device were described in our previous reports (Li et al., 2009, 2012a). Cells under chondrogenic induction were exposed to sinusoidal dynamic force at 10–40 kPa. A daily regimen was delivered at 1 h per day for the loading group, and the loading experiments were conducted for 1, 3, 5, 7, 10, 12, and 14 consecutive days. Cultures without mechanic loading were served as controls.

Before the mechanical loading experiment, a preliminary test of frequency gradient was performed to choose optimal frequency for compressive loading: 0.125, 0.25, 0.5, and 1 Hz were implemented, respectively. All experiments were repeated three times for reliable data.

Pharmacological inhibition test
The participations of MEK/ERK, p38MAPK, and BMP in cell proliferation were explored in this study. The pharmacological inhibitors of MEK/ERK (PD98059, Sigma), p38 MAPK (SB203580, Sigma) and BMP (recombinant human noggin, Prospec) were applied. Cells after chondrogenic induction were divided into four groups: (a) no-load without inhibitor; (b) no-load with inhibitor; (c) loaded without inhibitor; and (d) loaded with inhibitor. The inhibitors were added 2 h before mechanic loading. The final concentration of PD98059 was 10 mM; SB203580, 2 mM; and noggin, 1 g/ml. Each groups contained five samples. Tests were conducted at 1st, 3rd, 5th, and 7th day respectively and repeated for three times.

Real-time quantitative PCR (RT-PCR) for Col2a1 and Ihh
Expression of Col2a1 and Ihh were determined by RT-PCR as described previously (Li et al., 2009). Briefly, cells were harvested at indicated time points by trypsin and dissolved into Trizol Reagent (Invitrogen, Carlsbad, CA). Total mRNA was extracted and reverse transcribed into cDNA by MmLV reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Each real-
time PCR was conducted using the SYBR Prime Script™ RT-PCR kit (Takara, Dalian, China) in an ABI PRISM 7300 Real-Time PCR System. The primers and reaction system were the same with our previous study (Li et al., 2010a). The sterilized ddH$_2$O was used as negative control. The housekeeping gene GAPDH was amplified in each sample and used as a control for RNA loading. The cDNA of control cells at the first detected time point was ascribed a fold induction of one.

**Western blot assay**

Expression of cyclin D1 and CDK4 were determined by western blotting. Briefly, cells were harvested at indicated time points, and then dissolved into a lysis buffer supplemented with inhibitors of proteases and phosphatases (CST, Boston). Protein concentration was determined by a BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA). After transfer, membranes were blocked and then incubated overnight at 4°C with appropriate antibodies Endogenous levels of cyclin D1 and CDK4 were determined by anti-cyclin D1 (ab1224, Abcam, HK), Anti-CDK4 antibody (ab7955, Abcam). All primary antibodies were applied at a 1:1,000 dilution. Blots were further incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell signaling) at 37°C for 1 h. The immune complexes were detected using a chemiluminescence kit (Millipore) and visualized via the ChemiDoc XRS Gel documentation system (Bio-rad, CA). The gray intensity was measured by the software of Quantity One (Bio-Rad).

**Statistical analysis**

Data of evaluation parameters were expressed as mean ± SE, and analyzed by factorial analysis of variance (ANOVA) followed by Newman–Keuls test. $P < 0.05$ was considered statistically significant.

**Results**

**Cell proliferation during chondrogenesis**

To define the proliferative stage of chondrocytes in vitro, cell number was counted from 1st to 14th day after seeding. In control group, the cell number increased immediately after seeding. After a sharp increase in the first 5 days, the growth slowed down and remained in a high level, about two times of the initial level. Cells under chondrogenic induction remained in a remarkably lower level than the control group throughout the 14 days of culture. After a mild increase during the first 3 days, a sharp increase followed until reaching the peak on the 7th day, increasing by 70%. Then a gradual decrease followed. The result revealed that the quick increasing period of cell number distributed from the 1st to 7th day regardless of chondrogenic induction (Fig. 1A).

The result of [3H]-thymidine incorporation indicated that there was a gradual decrease of DNA replication activity since chondrogenic induction initiated, compared to cells in control group. However, it remained constant after the 7th day, at the level of 70% of the first day (Fig. 1B).

We also tested the cell viability by MTT assay. For cells in control group, cell viability fluctuated to reach to the peak on the 7th day, after that, it decreased gradually until the 14th day, at almost the same level of 1st day. In the meantime, viability of chondrogenic cells remained in a low level throughout the period. After reaching a peak on the 3rd day, cell viability fell back to the 1st day’s level on the 7th day. Slight increase was observed at the 9th day, though, the cell number decreased gradually after that (Fig. 1C).

![Fig. 1](image-url)
Cell proliferation upon mechanical regulation

A preliminary frequency-gradient test was conducted. Different frequencies of dynamic compression as well as static compression were applied to the chondrocytes in medium respectively to choose the optimal frequency of dynamic loading. Culture without loading was served as control group. MTT assay demonstrated that under static compression (0 Hz) cell viability decreased slightly compared to the control group, whereas dynamic compression enhanced cell viability to various degrees relative to different frequencies. Among them, cells viability was the highest under dynamic compression of 0.5 Hz. Thus 0.5 Hz was chosen as the optimal frequency of stress in the following experiment (Fig. 2A).

After application of cyclic stress of 0.5 Hz, cells under chondrogenic induction showed increased cell viability than those in control group (Fig. 2B). Both groups showed similar tendency with peak on the 3rd day, then a decline followed throughout the period, except a sudden rise on the 10th day in control group. [3H]-thymidine incorporation showed DNA replication was more active in loaded group than those without stress (Fig. 2C). These results indicated that dynamic compression could enhance the proliferation of cells under chondrogenic induction significantly.

Col2α1 expression increased under dynamic stress

The immunohistochemical staining in chondrocyte showed the expression of Col2α1 increased significantly under dynamic compression. As the culture time prolonged, cell morphology turned round, and increased cells overlapped in micromass with deepening staining color (Fig. 3A).

Gene expression of Col2α1 in both groups increased gradually from the 1st day to the 14th day (Fig. 3B). Compared to control samples, dynamic compression enhanced Col2α1 expression significantly (P < 0.05), especially on the 10th and 14th day (P < 0.01).

Cell cycle regulators increased under stress

Western blotting showed that in control group without stress, protein expression of Cyclin D1 as well as Cyclin-dependent kinase 4 (CDK4) increased in early time, after achieving to a peak in the 5th day, it declined gradually. Dynamic compression increased their expression significantly with similar trends (Fig. 4A).

In control group without stress, expression of Ihh increased quickly when chondrogenic induction initiated. After reaching the peak on the 5th day, it falls gradually to the 14th day. Dynamic stress increased Ihh expression significantly with similar tendency (P < 0.05), especially on the 1st and 5th day (P < 0.01). After the 7th day, Ihh expression under dynamic compression fell to the same level as the control group (Fig. 4B).

MEK/ERK and p38 MAPK were not involved in cell proliferation

MEK/ERK and p38 MAPK were accepted as important regulators for chondrogenic differentiation, so we tested whether cell proliferation was regulated by the two key MAPK pathways in stress-induced chondrogenic process. Dynamic compression increased cell viability significantly compared to the non-load groups (P < 0.01), and this increase was not influenced by the supplement of PD98059 (P > 0.05). Besides, this inhibitor did not bring any significant difference between groups under the same loading conditions. The results indicate MEK/ERK may not participate in the chondrocyte proliferation (Fig. 5A).
immunohistochemical staining of Col2α1 expression in chondrogenic induction at 3rd, 7th, and 14th day under dynamic compression. The expression of Col2α1 is up-regulated by dynamic compression. Subpart (A) shows immunohistochemical staining of Col2α1 and (B) demonstrates gene expression of Col2α1 in the process of chondrogenic induction. * Indicates significant difference from corresponding control group, $P < 0.05$; ** Indicates $P < 0.01$.

Fig. 3. Morphology changes of cells in micromass (200 ×) and Col2α1 expression in chondrogenic induction at 3rd, 7th, and 14th day under dynamic compression. The expression of Col2α1 is up-regulated by dynamic compression. Subpart (A) shows immunohistochemical staining of Col2α1 and (B) demonstrates gene expression of Col2α1 in the process of chondrogenic induction. * Indicates significant difference from corresponding control group, $P < 0.05$; ** Indicates $P < 0.01$.

Similar situation was also found in groups adding SB203580, the inhibitor of p38 MAPK. Mechanic loading seemed to be the factor making the cell viability different regardless of the presence of SB203580 (Fig. 5B). This inhibitor could decrease cell viability in groups under the same loading condition; however, these differences were insignificant ($P > 0.05$).

**BMP inhibitor partially inhibited proliferation of cells**

Noggin decreased cell viability significantly in all groups (Fig. 6A). The inhibitory effect of Noggin was observed since the first day and it maintained throughout the period. Dynamic stress boosted cell viability remarkably, but this increase was offset by the supplement of Noggin, which decreased the raised cell viability to almost the same level as control group. However, cell viability in group-(d) was still higher than that in group-(b).

Gene expression of Ihh increased steadily to the peak on the 5th day and then declined on the 7th day, and it was not influenced by Noggin in non-load groups (Fig. 6B). Dynamic compression raised Ihh expression level significantly ($P < 0.01$), though; this increase was offset by Noggin. Noggin significantly reduced Ihh expression in loading group to almost at the same level as in control group ($P < 0.01$). However, in presence of Noggin, Ihh expression stimulated by stress was still higher than that in no-load groups.

**Discussion**

Our study shows that appropriately applied dynamic compressive stress could increase cell viability by increasing the expression of Col2α1, Ihh, Cyclin D1, and CDK4 in early stage of chondrogenic induction, in which BMP signaling might play a role.

The chondrogenic differentiation is a phased process in which proliferation is in early stage. We found that when chondrogenic induction initiated, cell proliferation was slightly increased, however, it is lower than cells without chondrogenic induction. The presence of appropriate number of viable cells is prerequisite of successful tissue engineering, thus promotion of cell viability is important while maintaining its capacity of chondrogenesis. Previous studies have demonstrated the sensitivity of chondrocyte to mechanical perturbation. Wang and Mao (2002) study shows that application of cyclic mechanical stimuli is sufficient to up-regulate chondrocyte proliferation in vivo. Dynamic compression and cyclic stretch at physiological frequencies induces DNA synthesis of articular chondrocytes (Lee and Bader, 1997). The results of our study demonstrate that dynamic compression increases cell number and viability, as well as the expression of Ihh, Cyclin D1 and CDK4. As one of the key regulators of endochondral bone formation, Ihh is expressed in prehypertrophic chondrocytes, which directly activates proliferation. Increased Ihh expression resulted in an upregulation of Cyclin D1, a positive regulator of cell cycle (Minina et al., 2002). Cyclin D1 belongs to the D-type cyclins (D1, D2, and D3), which controls progression through the G1 phase of the cell cycle in complexes with CDKs 4 and 6 (Beier et al., 2001). The increased expression of Ihh, Cyclin D1, and CDK4 indicates that cell proliferation is active during the early stage of chondrogenesis, and it is promoted by dynamic compression. The gene of collagen type II (Col2α1) is expressed primarily in chondrocytes, which is accepted as important markers for phenotypic chondrocyte in numerous previous studies (Krebsbach et al., 1996; Lu et al., 2008; Seki et al., 2003; Szabova et al., 2009). It is suggested that chondrogenesis involves a transient proliferation phase appearing simultaneously with start of collagen type II deposition (Dexheimer et al., 2012). Previous studies suggest that the cyclic compression (Park et al., 2006) as well as cyclic tension (Wong et al., 2003) could up-regulate the expression of type II collagen. In our study, the expression of Col2α1 has increased gradually since the chondrogenic induction initiated, which implies these cells are in determination to chondrogenic lineage, and dynamic stress could promote its expression in the early stage. Furthermore, it is found in our study that dynamic stress did not shift the peaks of cell number and viability forward or backward, as well as gene expression between the groups, implying that the dynamic stress does not break the balance between proliferation and differentiation during chondrogenesis. This is coincident with Wu and Chen (2000) study that mechanical signals stimulate the differentiation extent by increasing the peak of marker synthesis instead of altering the speed of differentiation.

It is observed in our study that inhibition of both MEK/ERK and p38 MAPK do not bring significant differences to cell viability in non-loaded condition, as well as to the boosted cell viability brought by dynamic stress. It is suggested by Lemonnier et al. (2000) that ERK and p38 MAPK mediates proliferation and differentiation of chondrocytes respectively, indicating this two pathways function in a time-specific way. It is
also argued by Ryan et al. (2009) that ERK1/2 inhibitor could block the increased chondrocyte proliferation induced by acute static mechanical compression. However, our result indicates that cellular pathways of MEK/ERK and p38 MAPK may not function during early proliferation of chondrogenesis, and the enhanced cell proliferation induced by dynamic compression may not take effect via the two pathways. Different frequencies and magnitudes of stress as well as cell sources may be responsible for the divergences between our results and previous studies.

Our study found that inhibitor of BMP signaling could remarkably decrease cell viability, and dynamic stress could make up for this reduction to a certain extent. It’s suggested by Wuelling and Vortkamp (2011) that maintaining a normal proliferation rate requires BMP and Ihh acting in parallel, and the role of BMP signaling is independent of the Ihh/PTHrP pathway (Minina et al., 2002). This is evidenced by our study that Ihh expression is not influenced by noggin under conditions without stress; however, the increased Ihh expression induced by stress is partially offset by BMP antagonist. It is speculated that BMP signaling not only participate in chondrogenic proliferation, but also may intervene in the expression of Ihh signaling under compression. It is suggested by Wu et al. (2001) that Ihh mediates the
mechanotransduction process in BMP-dependent and parathyroid hormone-related peptide-independent manner. BMP antagonist might inhibit mechanical stimulation of chondrocyte proliferation through the induction of Ihh. Furthermore, the inhibition of Noggin could not completely neutralize the increased Ihh expression induced by stress, which implies that other cellular pathway might implicate in transduction of mechanic signaling to Ihh expression. The mechanical stimulus transmitted from extracellular to intracellular depends on various pathways (Wu and Chen, 2000). Studies show that mechanical signals are transduced from extracellular matrix to inside of the cell via cation channels (Duncan and Turner, 1995).

In summary, the present study is focused on rapid proliferative stage in chondrogenic differentiation, which spans from 1st to 7th day. During this period, dynamic stress of 0.5 Hz could remarkably promote cell proliferation, evidenced by increased cell viability, expression of chondrogenic-specific markers and cell cycle regulators. MEK/ERK and p38 MAPK may not participated in early stage of chondrogenesis while BMP signaling plays an important role in stress-induced cell proliferation, which might be achieved by regulating the expression of Ihh.

**Fig. 5.** Influence of inhibitors of MEK/ERK and p38MAPK on cell viability under compressive conditions. The medium is added with 10 mM PD98059 and 2 mM SB203580, respectively before compressive loading. The MTT assay shows viability of cells under chondrogenic induction is not influenced by PD98059 (A) nor SB203580 (B). * Indicates significant difference from corresponding control group, \(P < 0.05\); ** Indicates \(P < 0.01\).

**Fig. 6.** Effects of BMP antagonist on cell viability and gene expression of Ihh under compressive stimulation. Noggin at concentration of 1 c\(^{-9}\)g/ml is added into cell culture 2 h before mechanic loading for 1 and 7 days. The MTT assay shows that cell viability is affected by noggin in different degrees under compressive conditions (A). RT-PCR analysis shows Ihh expression was not influence by noggin in non-loaded condition whereas it is suppressed under compression (B). * Indicates significant difference from corresponding control group, \(P < 0.05\). ** Indicates \(P < 0.01\).

**Literature Cited**


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