Reversal of Suppressed Metabolism in Prolonged Cold Preserved Cartilage

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ABSTRACT: Chondrocytes in cold preserved cartilage are metabolically suppressed. The goal of this study was to address this metabolic suppression and seek ways to reverse it. Specifically, we examined the roles of rewarming protocols and nitric oxide (NO) in this metabolic suppression. Bovine and canine full-thickness articular cartilage explants were cultured under various temperature conditions, and NO production, proteoglycan (PG) synthesis, and cell viability were measured. Nitric oxide was shown to be negatively correlated with PG synthesis following abrupt rewarming of cold preserved osteochondral allografts. Gradual rewarming of the allograft tissue decreased NO production with higher PG synthesis. Inhibition of nitric oxide synthases (NOS) led to a decrease in NO production and a concomitant increase in PG synthesis. We were able to partially reverse metabolic suppression of cold preserved osteochondral allograft material with gradual rewarming and decrease NO production with NOS inhibition. Chondrocytes in cold preserved allograft material may be metabolically suppressed predisposing the graft to failure in vivo. Minimizing this loss of metabolic function by gradual graft rewarming and decreasing NO production by NOS inhibition at the time of graft implantation may have implications on graft survival in vivo. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 26:247–254, 2008

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INTRODUCTION

One of the most widely used options to treat symptomatic full-thickness articular cartilage defects is the use of osteochondral allografts (OCAs).[1–7] Currently, osteochondral allografts undergo a prolonged fresh cold preservation period prior to transplantation. In this method, donated tissue is stored at 4°C using standard culture medium with good cell viability for up to 14 days.[8–12] Work in our laboratory has reported maintenance of cell viability at 83% for up to 28 days.[13] Glycosaminoglycan (GAG) content is typically measured to monitor extracellular matrix (ECM) stability; with no significant decrease before 28 days.[8,9] Studies have also addressed bio-mechanical properties with results indicating no significant changes in compressive modulus.[10] Collagen network integrity was measured with indentation testing and determined that there is no significant difference in the tensile modulus.[10]

Despite good cell viability and biochemical and mechanical testing results, the main problem associated with cold preservation at 4°C is a significant decrease in metabolism of the chondrocytes. Although human cartilage can be stored in refrigeration for extended lengths of time, a gradual decline in function has been noted within 2 weeks.[14] Cold preservation of porcine cartilage is limited to 7 days.[15] Ball et al.[8] reported that after 28 days of preservation at 4°C, PG synthesis was significantly decreased from baseline. Similar results have also been reported in human,[8–12] canine,[16,17] and bovine tissue.[18] The use of cold preservation of fresh allograft material offers several distinct advantages including greater availability to surgeons; however, reduced chondrocyte

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function demonstrated with cold preservation suggests that the allograft material may be suboptimal with respect to metabolic function, and therefore predispose the graft to failure in vivo.

In the operating room setting, the donor osteochondral graft material is taken from a cold preserved state (4°C) and abruptly placed in room temperature saline or simply exposed to room temperature air (~25°C). The graft is then customized and prepared for implantation (10–20 min) and subsequently placed in the joint (~37°C). These abrupt changes in temperature are believed to affect the tissue as a whole; however, the effects of abrupt temperature change have not been studied until now. Additionally, the use of a lactated Ringer's or balanced salt solution has been used to reduce the deleterious effects of saline.[19]

Preliminary studies in our laboratory have demonstrated a concurrent suppression of PG synthesis and nitric oxide (NO) production with the abrupt rewarming of cold preserved osteochondral allograft cartilage.[20] This time line correlates with the events during surgery when the allograft is removed from the 4°C environment, abruptly warmed to room temperature (25°C) for preparation of the osteochondral plug, and then placed at body temperature (37°C). The role of NO and other reactive oxygen species (ROS) is intriguing as NO is known to suppress cell metabolism.[21–23] The abrupt rewarming following cold preservation is similar to reperfusion injury where hypoxic reoxygenated mitochondria produce excess superoxide O_2^- and release H_2O_2. Such a posthypoxic injury suggests changes in cellular injury, oxidant-generating systems, and antioxidant defense.[24,25]

In this study, we have examined the transition of cold preserved cartilage from 4 to 37°C (body temperature) with respect to NO production and its relationship to PG synthesis. Experiments were also completed to determine if the selective inhibition of NO[24] would result in improved metabolic response of the chondrocytes following cold preservation.

MATERIALS AND METHODS

This study was performed in three stages. Experiment 1 was completed to establish the relationship of NO production and suppression of cartilage PG synthesis in cold preserved cartilage with an abrupt temperature change. Experiment 2 examined a more gradual rewarming of cold preserved cartilage and its relationship to NO and PG synthesis. Experiment 3 examined enzymatic inhibition of NOS and the NO production on cold preserved cartilage. Experimental designs are outlined in Table 1. All procedures involving animals were approved by the Institutional Animal Care and Use Committee.

Experiment 1—Abrupt Rewarming

Whole intact canine femoral condyles from adult male random source purpose-bred dogs (20–30 kg) were used. Animals were euthanized, after which the distal femoral condyles were exposed and removed by transecting the distal portion of the femoral shaft with a bone saw. All noncartilaginous soft tissue was dissected away from the condyles prior to placement in the storage container for cold preservation. Although the condyles were rinsed briefly with sterile saline. The femoral condyles were placed in sterile containers with 60 mL of culture media containing minimal essential medium (MEM) containing 10% fetal calf serum (FCS), glutamate, nonessential amino acids, and antimicrobial agents penicillin, streptomycin, and fungizone. Aliquots of media were removed for NO determination at each media change. The storage media was changed every 7 days for samples stored at 4°C. Media was changed every 3–4 days when cartilage was stored at 25 or 37°C. All temperatures were monitored continuously. At the end of the 21-day storage

Table 1. Experimental Designs

Samples	Temperature regimen	Total Time
Experiment 1 Design—Abrupt Rewarming		
4 condyles	21 days at 4°C	21 days
4 condyles	7 days at 4°C, then abruptly to 25°C and kept for 14 days	21 days
4 condyles	7 days at 4°C, then abruptly to 25°C for 7 days then 37°C for 7 days	21 days
4 condyles	7 days at 4°C, then abruptly to 37°C and kept for 14 days	21 days
Experiment 2 Design—Gradual Rewarming		
3 plugs	4°C	28 days
3 plugs	4°C (14 days) to 25°C (14 days)	28 days
3 plugs	4°C (7 days) to 10°C (7 days) to 25°C (7 days) to 37°C (7 days)	28 days
Experiment 3 Design—Enzymatic Inhibition of Nitric Oxide Production.		
4 plugs (+ inhibitor)	7 days at 4°C, then abruptly to 37°C and kept for 14 days	21 days
4 plugs (− inhibitor)	7 days at 4°C, then abruptly to 37°C and kept for 14 days	21 days

period full punch biopsies were obtained from the same region of the habitually loaded region of the medial femoral condyle for ^{35}S uptake. End points included PG content, PG synthesis by ^{35}S uptake, and NO production.

Experiment 2—Gradual Rewarming

We then examined the effects of a more gradual rewarming of cold preserved cartilage on NO production and PG synthesis. Using a skin biopsy punch, full thickness uniform samples (5 mm) were obtained from male bovine metacarpal phalangeal joints of animals approximately 18 months of age and randomly placed in individual sterile 12-well culture plates with 3 mL of media as described above. Media was changed every 7 days when samples were cultured at 4°C and every 3 days when cultured at 10, 25, or 37°C. Each time the media was changed an aliquot of 1 mL was saved for NO analysis. To accommodate the 10°C temperature step this experiment was carried out to 28 days. The extended periods in culture (e.g., 7 days) were to maximize results and were not meant to reflect conditions in the operating room.

Experiment 3—Enzymatic Inhibition of Nitric Oxide Production

Finally, we examined the potential of chemical inhibition of NO production in bovine cartilage that was cold preserved and then abruptly warmed. Bovine punch biopsy plugs were obtained as described in Experiment 2. NG-Monomethyl-L-Arginine (Calbiochem, LaJolla, CA), a competitive inhibitor of all three isoforms of nitric oxide synthase was added to the preservation media of fourg plugs while four control plugs were stored in media alone. All samples were stored for 7 days at 4°C and then changed to fresh media and stored at 37°C for and additional 14 days. Media was changed after the first 7 days and then every 3 days thereafter. Each time the media was changed an aliquot of 1 mL was saved for NO analysis. A concentration of 3.4×10^{-4} M NG-Monomethyl-L-Arginine was used. In addition to NO production and PG synthesis, cell viability was determined.

Cartilage PG Content and Cell Function Using $Na^{35}SO_4$ Uptake

Cartilage PG content was determined by the DMB method[26] and expressed per wet weight. Full-thickness samples of articular cartilage were placed individually in sterile 12-well plates containing 3.0 mL of culturing medium and 10 μCi/mL $Na^{35}SO_4$ for PG synthesis determination. These samples were then incubated for 24 h at 37°C under 95% air: 5% CO_2. They were then rinsed briefly in unlabeled media and weighed. Extraction of the extracellular matrix components were carried out in 2 mL of 4 M-guanidine hydrochloride/100 mM EDTA solution overnight at 4°C with continuous stirring. The extracts were dialyzed against 10 mM EDTA at 4°C to remove free nucleotides and guanidine hydrochloride. The volume of extract from each sample was determined and 200 μL was taken and subjected to scintillation counting. The counts were corrected for total volume and weight of sample and represented as counts per million (CPM) per mg of wet tissue.

Nitric Oxide Assay

Cartilage NO production was measured using the Nitrate/Nitrite Colorimetric Assay Kit (LDH-Method, Caymen Chemical Company, Ann Arbor, MI). The effects of media components on color development were accounted for by subtracting the level of nitrate/nitrite present in culture media prior to any cell growth. A standard curve was then constructed to calculate the amount of nitrate/nitrite present in the experimental samples and expressed per mg wet weight of cartilage.

Cell Viability Using the Live Cell/Dead Cell Assay[27]

Full-thickness cartilage slices (1.6 mm thick) were placed in 4 μM calcein-AM and 8 μM ethidium homodimer (in 2-mL of 0.9% saline) at room temperature with no light exposure for 30 min. Samples were then rinsed and examined on a confocal laser-scanning microscope (Nikon Eclipse TE2000) and necessary filters (fluoroscein and rhodamine). The calcein-AM and ethidium homodimer were supplied as part of a live cell/dead cell kit from Molecular Probes Inc., Invitrogen, Carlsbad, CA, which is used to identify living cells labeled with calcein-AM (green fluorescence) and dead cells labeled with ethidium homodimer (red fluorescence). Estimates of cell viability were obtained by counting the red and green cells in a full thickness area of approximately 1 mm^2 and then expressing living cells (stained green) as a percentage of the total (percent viability).

RESULTS

Experiment 1: Abrupt Rewarming of Whole Intact Canine Femoral Condyles (Fig. 1)

Cartilage PG Content

Plugs of cartilage from condyles stored continuously at 4°C had a PG content of 676 ± 51 μg PG/mg cartilage wet weight (Table 2). In contrast,

Table 2. Effects of Abrupt Rewarming of Cold Preserved Cartilage on PG Content

Cartilage PG Content (μm PG/mg Cartilage Wet Weight)		
Group	PG Content	p-Value vs. 4°C Continuous Control
4°C continuous control	676 ± 51	
Rewarming to 25°C	479 ± 79	0.0058
Rewarming to 37°C	531 ± 99	0.0048
37°C continuous	308 ± 161	0.0398

Table 3. Effects of Abrupt Rewarming of Cold Preserved Cartilage on PG Synthesis

Cartilage PG Synthesis (DPM/mg Cartilage Wet Weight)		
GROUP	PG Synthesis	p-Value vs. 4°C Continuous Control
4°C continuous control	4324 ± 476	
Rewarming to 25°C	913 ± 246	0.0010
Rewarming to 37°C	1268 ± 193	0.0100
37°C continuous	3102 ± 1316	ns

abrupt rewarming of cold preserved cartilage to 25 and 37°C reduced cartilage PG contents to 479 ± 79 ($p = 0.0058$) and 531 ± 99 ($p = 0.0048$) μm PG/mg cartilage wet weight, respectively. Similarly, cartilage from condyles kept at 37°C throughout the 21-day period had a reduced PG content (308 ± 161 μm PG/mg cartilage wet weight; $p = 0.0398$).

Cartilage PG Synthesis

Cartilage PG synthesis was 4324 ± 476 dpm/mg cartilage wet weight in condyles kept at a constant 4°C and 3102 ± 1316 dpm/mg cartilage wet weight in condyles kept at a constant 37°C (p = n.s.; Table 3). Abrupt rewarming to 25 and 37°C from 4°C reduced PG synthesis significantly to 913 ± 246 ($p < 0.001$) and 1268 ± 193 ($p < 0.01$), respectively.

Cartilage NO Production

Cartilage NO production in condyles stored continuously at 4°C was relatively constant at 6.0 ± 3.0 μM nitrate/nitrite per mg cartilage wet weight throughout and at the end of the 21-day period the time period (Table 4). Cartilage NO production was elevated in both groups of condyles stored at 4°C and then abruptly warmed to 25°C (13.6 ± 4.1; $p = 0.0426$) or 37°C (16.8 ± 7.0; $p = 0.0223$). With respect to rewarming from 4°C cartilage NO production was significantly elevated on day 14 with abrupt rewarming to 25 and 37°C (1 week after rewarming) to 11.2 ± 1.3 ($p = 0.0085$) and 10.4 ± 0.5 ($p = 0.0024$), respectively (Fig. 1).

Table 4. Effects of Abrupt Rewarming of Cold Preserved Cartilage on NO Production

Cartilage NO Production Expressed as Nitrate/Nitrite (μM/mg Cartilage Wet Weight)		
Group	Day 21	p-Value vs. 4°C Continuous Control
4°C continuous control	6.0 ± 3.0	
Rewarming to 25°C	13.6 ± 4.1	0.0426
Rewarming to 37°C	16.8 ± 7.0	0.0223
37°C continuous	19.4 ± 6.6	0.0377

Experiment 2: Gradual versus Abrupt Rewarming of Cold Preserved Bovine Biopsy Cartilage Plugs (Fig. 2)

It was established in the previous experiment the prolonged-cold preservation method (4°C) suppressed metabolic function in chondrocytes. Data in Figure 2 shows with gradual rewarming of tissue (4–10–25–37°C), PG synthesis is significantly improved when compared to the 4°C prolonged-cold preserved samples ($p < 0.05$) and significantly suppressed when the tissue is abruptly warmed to 25°C ($p < 0.05$). Also shown in Figure 2 is data illustrating NO production of tissue kept continuously at 4°C versus cold preserved cartilage that was gradually or abruptly warmed. Abruptly rewarming the tissue significantly increased NO production ($p < 0.04$); however, gradual rewarming of the tissue resulted in significantly ($p < 0.05$) lower NO production.

Experiment 3: Chemical Inhibition during Abrupt Rewarming of Cold Preserved Cartilage (Figs. 3 and 4)

The data in Figure 3 illustrates improvement in PG synthesis in cold preserved cartilage which is abruptly warmed to 37°C when the iNOS inhibitor is present ($p = 0.04$). This is suggestive of an inverse relationship between NO and PG synthesis. Cell survival data (Fig. 4) indicated a significantly higher ($p = 0.0379$) number of living cells (76 ± 4%) when inhibitor was present when compared to samples without inhibitor added (56 ± 11%).

DISCUSSION

The results in the present series of experiments illustrate that chondrocytes in cartilage preserved for 28 days by cold preservation are metabolically suppressed. These results are consistent with other similar approaches of cartilage storage.[1,4,8–19] These results have important implications for the long-term survival of cartilage from cold preserved tissue. Currently, allografts are

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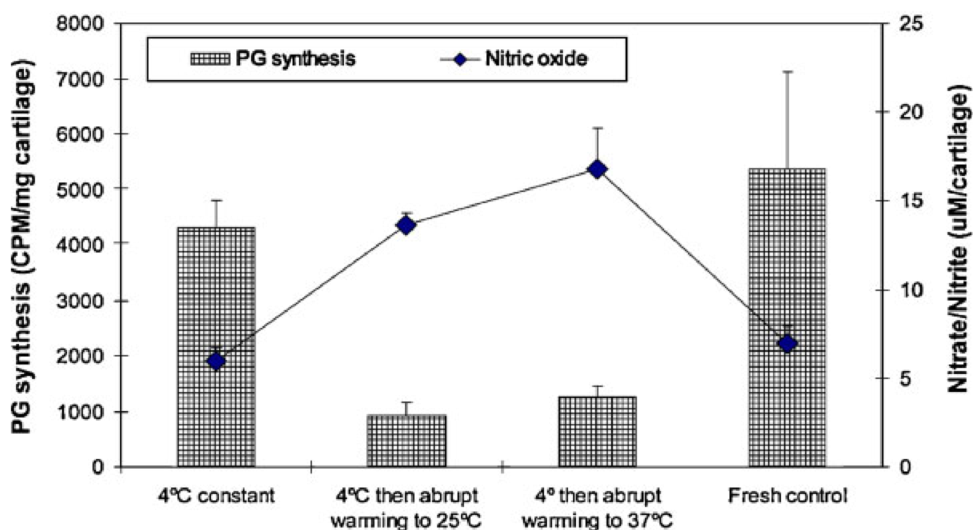

Figure 1. Mean CPM/mg (wet weight) versus culture temperature. Assessment of cell function, as $Na^{35}SO_4$ incorporation in full-thickness whole intact canine condyles.

removed from cold storage at 4°C and then abruptly warmed to room temperature (~25°C) before being placed in the patient (~37°C). The present study has shown that cartilage stored at 4°C and then abruptly warmed to 25°C is metabolically suppressed, and that NO may play a role in further PG suppression when these allografts are abruptly rewarmed. However, in vivo studies of cartilage from improved cold preserved techniques should be pursued to evaluate fully this new method of OCA preservation.

Under conditions of abrupt rewarming, NO production peaks at 250–350% of normal, and PG synthesis is suppressed. Conversely, a more gradual rewarming regimen of 4 to 10°C to 25°C and then 37°C results in baseline NO and improved ^{35}S

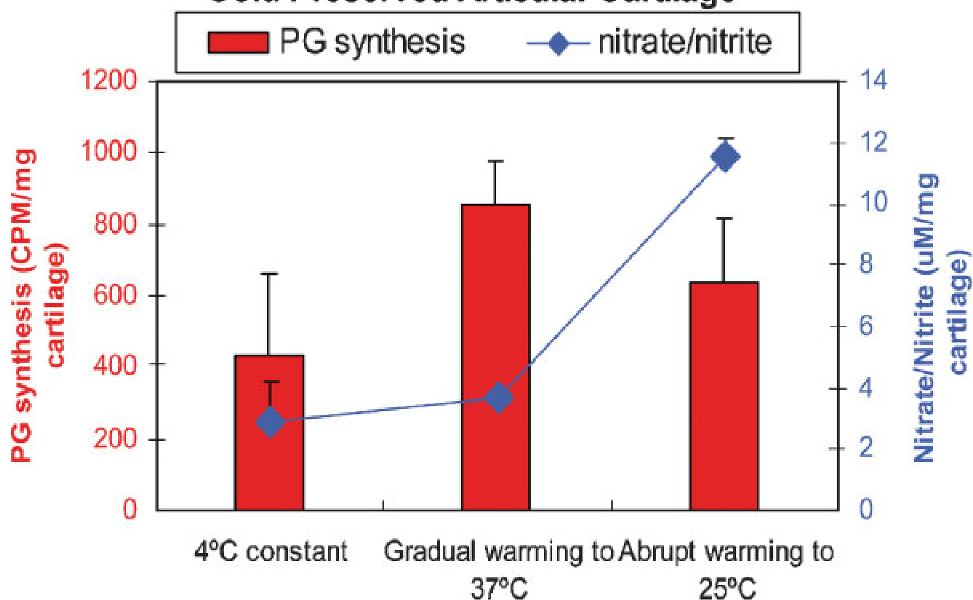

Figure 2. Gradual versus abrupt rewarming of cold preserved bovine cartilage plugs.

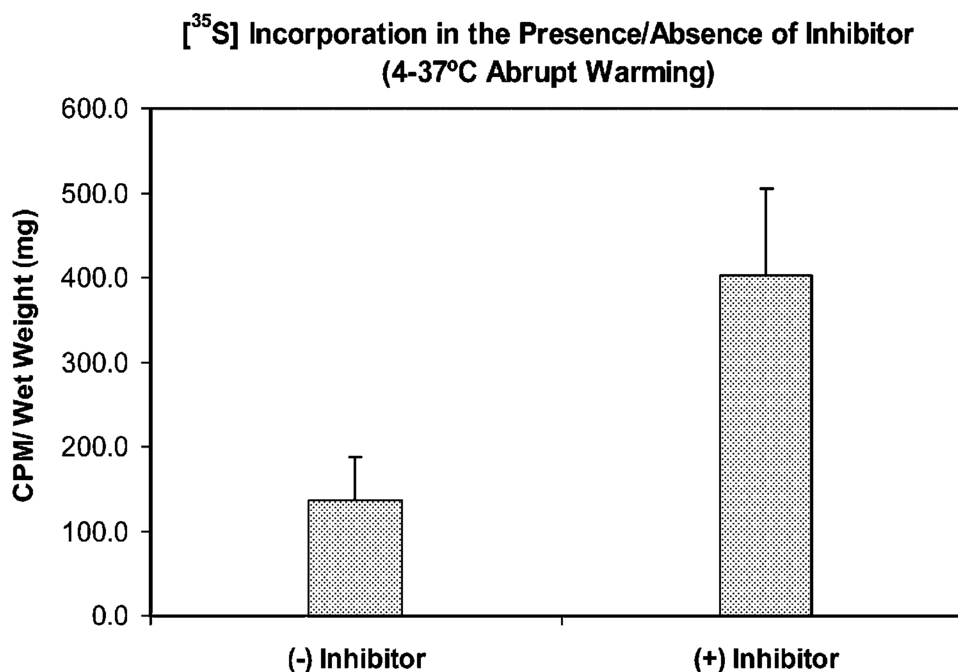

Figure 3. Mean CPM/mg (cartilage wet weight). Assessment of cell function, as $Na^{35}SO_4$ incorporation in full-thickness bovine biopsy punches, with and without inhibitor.

uptake, suggesting improved PG synthesis and subsequent reversal of chondrocyte metabolic suppression. This is important because decreased chondrocyte metabolism suggests that the cell maybe unable to effectively maintain the extracellular matrix, including catabolism and anabolism of PGs, noncollagenous proteins, and collagen. Reversing the metabolic suppression ensures that the chondrocytes are able to maintain the cartilage matrix and the transplanted tissue may be less prone to failure following implantation.

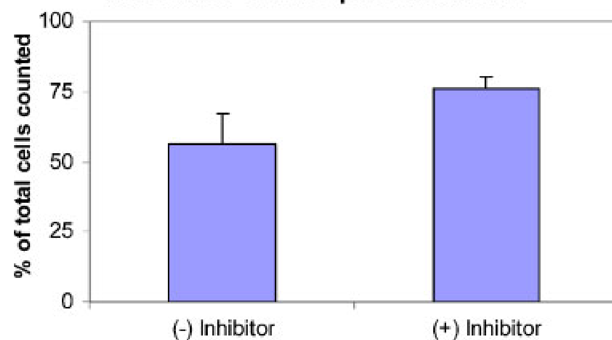

Figure 4. Percentage of cell survival in bovine cartilage slices.

The mechanism of metabolic suppression of chondrocytes that have been cold stored is not well known. One possibility is simply that tissue stored at a cold state of 4°C shifts to a minimal metabolic state. Results from this study support that notion. Additionally, the results in the present study suggest a role for NO production during abrupt rewarming that could reduce chondrocyte metabolism, which is consistent with other studies demonstrating that NO does inhibit cartilage PG synthsesis.[28–32] The exact mechanism of NO inhibition of cartilage PG synthesis is not completely understood, and it cannot be assumed that it is the only factor. Other ROS species, besides NO, may be generated in the tissue due to an increase in metabolic activity during the abrupt rewarming protocols. The increase in temperature could induce a state of oxidative stress and thus producing ROS such as OH· (superoxide anion). Superoxide in the presence of NO forms peroxynitirate, which is a known inducer chondrocyte apoptosis.[32,33] In addition, studies have also indicated that NO plays a key role in progression of osteoarthritis and inflammatory arthritis.[33–35]

The results of this study demonstrate a significant increase in NO production with concomitant significant decrease in PG synthesis and PG content when the samples were abruptly rewarmed compared to those that were gradually warmed. The warming regimen used in this study was

chosen to illustrate the cause/effect relationship and not to mimic operating room times. Suggesting changing operating room protocols to include lengthy rewarming protocols would be vastly impractical. With this in mind, we examined the NO inhibition effect on PG synthesis of abruptly rewarmed cartilage we examined the chemical inhibition of a key enzyme pathway for NO production using the NO inhibitor N_G-Monomethyl-L-Arginine. The increase in PG synthesis in samples exposed to the inhibitor suggests that NO does play a key role in PG synthesis suppression when the tissue is abruptly rewarmed and improves cell survival. Such results warrant further study of this phenomenon of cartilage metabolism and NO production as a more practical way to ensure optimal metabolic activity of allograft cartilage prepared from cold stored osteochondral tissue.

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