The Protective Effects of Chronic Intermittent Hypobaric Hypoxia Against Osteoarthritis in Rats - Role of Nitric Oxide

Tianci Wang
Hebei Medical University Third Affiliated Hospital
Dong Ren
Hebei Medical University Third Affiliated Hospital
Yunshan Su
Hebei Medical University Third Affiliated Hospital
Jian Lu
Hebei Medical University Third Affiliated Hospital
Ming Li
Hebei Medical University Third Affiliated Hospital
Yufeng Chen
Hebei Medical University Third Affiliated Hospital
Pengcheng Wang (zhengzainingmeng@163.com)
The Third Hospital of Hebei Medical University https://orcid.org/0000-0002-4772-4538

Study protocol

Keywords: chronic intermittent hypobaric hypoxia, osteoarthritis, nitric oxide synthase, nitric oxide

Posted Date: February 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-216874/v1

Abstract

The study explored the effect of chronic intermittent hypobaric hypoxia (CIHH) on osteoarthritis (OA). CIHH conditioning was realized by exposing rats to hypobaric hypoxia environment mimicking 5,000 m high-altitude (P_B=404 mmHg, PO_2=84 mmHg) 6 h per day, once daily for 28 days. OA model was induced by surgically-induced medial meniscal tear. Male Wistar rats were randomly assigned into 5 groups: preconditioning group [CIHH + OA], postconditioning group [OA + CIHH], control group, inhibitor group [OA + inducible nitric oxide synthase (iNOS) inhibitor], blank control group. The expression iNOS, nitric oxide (NO) content levels in the joint fluid were measured at 1, 2, 3 weeks after the OA modelling. Results revealed that OA modelling induced cartilage degeneration, up-regulated iNOS expression, increased joint fluid NO content. CIHH preconditioning and postconditioning reduced cartilage degeneration, prevented the NO production. Inhibitor groups showed alleviated joint degeneration than control group, but not as effective as CIHH condition. These results suggest that both CIHH preconditioning and postconditioning plays a protective role on OA, one of the mechanism was inhibiting the overexpression of iNOS and NO production.

Introduction

Osteoarthritis [OA] is a degenerative joint disease characterized by articular cartilage destruction, synovial membrane inflammation, and subchondral bone remodeling [1]. OA is the leading cause of lower extremity disability amongst older adults the estimated lifetime risk for knee OA is approximately 40% in men and 47% in women [2]. As life expectancy increases, the prevalence of osteoarthritis is projected to increase further, resulting in a more significant healthcare burden.

The most commonly used rat OA model is the surgically-induced medial meniscal tear (MMT) model [3-5] followed by anterior cruciate ligament transection (ACLT) alone [6-8] or in combination with partial medial meniscectomy (MM) [9,10] Unilateral MMT in mature rats results in rapidly progressive cartilage degenerative changes characterized by chondrocyte and proteoglycan loss, fibrillation, osteophyte formation, and chondrocyte cloning. The MMT model offers the opportunity to evaluate chondroprotective effects of agents [4,11], cartilage repair strategies [5], and bone preserving activities since this model exhibits early subchondral resorptive and later sclerotic bone changes.

Chronic Intermittent hypobaric hypoxia (CIHH) has been proved to be beneficial to the cardiovascular system [12, 13], central nervous system [14], and the immune system [15]. It was reported that CIHH helps protect against reperfusion injuries [16]. Furthermore, CIHH has been widely used in sports training to enhance the organs and tissue's tolerance to anoxia [17]. Previous studies have shown that intermittent hypobaric hypoxia preconditioning has a protective effect against rheumatoid arthritis [18], as osteoarthritis has similar pathological changes with rheumatoid arthritis, We hypothesize that hypotension has a protective effect on osteoarthritis.

NO plays an essential role in the adaption process of CIHH. For example, CIHH preconditioning protect brain ischemia by inhibiting the overexpression of NO [19]. Exposure of rats to acute hypoxia improved papillary muscle's tolerance to hypoxia by increasing cytosolic inducible nitric oxide synthase expression and NO production in left ventricle [20]. Kanika and Geetha prove that CIHH tolerance is mediated by nitric oxide pathway [21]. NO has long been thought to be related with OA. A high NO level has been shown in patients' serum and cartilage with arthritis than in normal cases [22, 23]. NO is reported as a predominant mediator in the progression of OA and chondrocyte apoptosis [24, 25].

Taken together, the Nitrergic system plays an important role both in the pathological process of osteoarthritis and the physiological response to hypobaric hypoxia [19, 20]. This study aims to verify the protective effect of CIHH on OA and assess the role of the NO pathway in this process.

Materials And Methods

Surgical method of OA model

The surgical method of OA modelling was previously described [26, 27]. Rats were anesthetized via intraperitoneal injections of pentobarbital sodium (50 mg/kg ip) and fixed in supine position. After shaving the knee joint, the skin was disinfected with iodine. A skin incision was made over the medial aspect of the right knee, the medial collateral ligament exposed by blunt dissection, and then transected. A full-thickness cut was made through the medial meniscus to simulate a complete tear. The skin and subcutis were closed with 4-0 Vicryl suture by using a subcuticular pattern. The rats were injected with penicillin for 3 days (80000 U / 100g, once a day) after the surgery.

Induction of CIHH

Animals were placed in a hypobaric chamber. The air pressure inside was controlled using a vacuum pump and an adjustable inflow valve. The chamber was also provided with a manometer to check the experimental altitude during the process. Hypoxia was induced by down-regulating the environmental pressure to a final barometric pressure of 404 mm Hg。The temperature of the chamber was kept at 28 °C. These conditions simulate an altitude of 5000 m. Animals were placed in the chamber 5 h/day (9 AM–2 PM), 5 days/week for 5 weeks.

Animals Grouping and Management

Healthy male Sprague–Dawley rats (weighing 180–190g) were provided by Hebei Province Laboratory Animal Center (Shijiazhuang, China), and housed in a temperature and light-controlled room (24 ± 1°C, 12 h light/dark cycle), with access to food and water. All experiments were conducted accoeding to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). All animal procedure were approved by the ethics committee of The Third Hospital of Hebei Medical University (Z2018-015-1). 100 rats were randomly divided into 5 groups: preconditioning group （CIHH + OA）, postconditioning group （OA + CIHH）, control group, inhibitor group [OA + inducible nitric oxide synthase

(iNOS) inhibitor], blank control group. The preconditioning group received CIHH induction before OA surgery. The postconditioning group received OA surgery first, and then CIHH induction. The control group receive OA surgery only. The inhibitor group received OA surgery, after the surgery rats received iNOS NG-nitro-L-arginine methyl ester (MedChemExpress. America. New Jersey) by intraperitoneal injection 50 mg/kg per day for 7 days. Sham group receive the same incision as surgery modelling of OA, but with medial collateral ligament and medial meniscus intact.

Rats were sacrificed through atlantoaxial dislocation at 1, 2 and 3 weeks after OA surgery modelling. For each group, the joint fluid of rats was obtained before the execution. 10 rats of each group were killed and paraffin sections of the tibial plateau were prepared for OA evaluation by Hematoxylin and Eosin (H&E) staining and safranin O/fast green staining. The other 10 rats were killed and the tibial plateau cartilage was prepared for detecting inducible nitric oxide synthase （iNOS） expression through western blot.

Histology

After the mice were executed, the knee joint were fixed in 4% paraformaldehyde for 48 h, decalcified with 10% ethylenediaminetetraacetic acid (EDTA) solution for 15 days, and then embedded in paraffin. The samples were cut to a 5 μm thickness sagittal sections from the joint medial compartment using a microtome (Leica biosystems, USA). Afterwards, the sections were stained with safranin O/fast green or hematoxylin-eosin. The markin scoring [28] was used to evaluate the severity of the OA changes. Scoring was done by two independent investigators who are blinded to the grouping.

NO detection

1, 2, 3 weeks after OA modelling, Normal saline (1 mL) was injected into the knee joint cavity of all the studied rats. The fluid from the joints was then collected into a test tube. The rats were killed and blood samples were collected. After centrifugation we collected the serum, the joint fluid and serum were frozen at -80°C for biochemical determination. The concentration of joint fluid and serum NO level was measured using the Griess reaction, the Griess reaction was first presented in 1879 as a colorimetric test for nitrite detection [29, 30, 31, 32]. Serum and an equal volume of Griess reagent were thoroughly mixed. The mixture was incubated for 10 min at room temperature and absorbance was recorded at 540 nm. Blank and standards were also run in parallel.

Western blot

Rats were decapitated in the indicated time as points previously mentioned and the articular cartilage of the tibial plateau was rapidly isolated, placed on ice, the tissue blocks were washed with cold phosphate buffer saline for 2-3 times to remove the blood stain, and cut into small pieces and placed in the homogenization tube. Add 2 magnetic beads, add 10 times of tissue volume lysis buffer, add protease inhibitor within minutes before use. Select the procedure of complete homogenization, 100s, and cool down intermittently. After homogenization, take out the sample tube, ice bath for 30min, shake every 5min to ensure complete tissue lysis. After centrifugation at 12000 rpm for 10 min, the supernatant was

collected, which was the total protein solution. Samples were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) loading buffer and were subsequently transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% skim milk for 1 hour at room temperature, followed by incubation with the primary antibodies and Glyceraldehyde triphosphate dehydrogenase （GAPDH） overnight at 4 ℃. Primary antibodies are produced by Hangzhou Xianzhi biology limited liability company, after washing twice with tris buffered saline tween （TBST）, the membranes were incubated with secondary antibodies (BOSTER biological technology. Wuhan China) for 1 hour at room temperature. Protein bands were detected using an electrochemiluminescence (ECL) detection kit (Thermo Scientific, 34077) and imaged.

Statistical analysis

The measurement data in accordance with normal distribution was expressed as Mean ± standard deviation. Monofactoria analysis of variance was used for comparison between groups. When there was statistical difference, Student-Newman-Keuls (SNK) method was used for pairwise comparison. The measurement data that did not conform to normal distribution was expressed as Median and quartile. Rank sum test was used for comparison between groups. Bonferroni method was used for pairwise comparison when there was statistical difference, All data were analyzed by spss25.0 statistical software. Probability values of $P < 0.05$ were considered statistically significant.

Results

The effect of CIHH on articular cartilage in rats

OA model established successfully. After 1 week of OA modeling, Mild cartilage degeneration of control group was detected characterized by superficial cellular clusters. After 2 week of OA modeling, Moderate degeneration of cartilage of control group was detected characterized by superficial cellular clusters and the less cartilage matrix staining. After 3 weeks of OA modelling, cartilage degeneration of control group extends into the deep zone and fissures into calcified cartilage layer appear. After 1（2）and 3 weeks of OA modeling, markin scores of each group were recorded (Table 1). Rats received both precondition and postcondition got better markin score than control group. Markin score of inhibitor group was lower than control group, but higher than preconditioning and postconditioning group (Fig. 1-5).

The effect of CIHH on joint lavage fluid NO

As shown in Table 2 and Fig. 6-8, at all 1, 2, and 3 week time point, joint lavage fluid NO of preconditioning group, postconditioning group and inhibitor group were lower than control group. The joint lavage fluid NO in both preconditioning group and postconditioning group rats were significantly higher than the inhibitor group.

The effect of CIHH on the expression of iNOS

In order to detect the effect of CIHH on NO, western blotting was used to analyze the expression of iNOS, as shown in Table 3 and Fig. 9-11. At each time point, there were significant differences between the five groups ($P < 0.001$). Further pairwise comparison showed CIHH downregulated the expression of iNOS in cartilage, while l-name The inhibitor had no effect on iNOS in cartilage compared with control group.

Discussion

Animal experiments show that CIHH tolerance is mediated by the heat shock response and nitric oxide pathway [21]. Many studies have shown that hypobaric hypoxia reduces NO concentration in vivo. Since oxygen is the substrate for NO production by the L-arginine pathway, the cell oxygen concentration is considered to regulate the enzymatic reaction of NO [33]. Hypoxia limits the endogenous production of no, considered an essential mechanism of hypobaric hypoxia in regulating NO concentration in vivo. However, many studies have shown that the response of NO to CIHH is different in different organs, which indicates that the effect of hypobaric hypoxia environment on NO concentration in vivo is more complicated.

CIHH reduce the concentration of NO in brain tissue by inhibiting the overexpression of Neuronal nitric oxide synthase (nNOS and iNOS) induced by cerebral ischemia in rats to improve brain tissue tolerance to ischemia [19]. CIHH increase the expression of iNOS in intestinal mucosal cells of rats with intestinal mucosal injury [34]. However, in the cardiovascular system as well as in the lung, the opposite results were obtained. CIHH increase the concentration of NO in lung tissue by increasing the expression of nNOS and iNOS in lung endothelial cells and epithelial cells [35]. CIHH induce the increase of NO expression in myocardial cells, thus playing a protective role in myocardial ischemia-reperfusion injury in rats [36]. The renovascular hypertension rat model established by the renal artery clamping method, CIHH pretreatment decreases rats' arterial blood pressure by increasing the concentration of NO in plasma [37]. Till now, no studies have shown the effect of CIHH on the concentration of no in articular cartilage.

This is the first study to investigate the protective effect of CIHH on osteoarthritis. We found that both CIHH preconditioning and postconditioning significantly reduce the pathological damage of osteoarthritis. Rats received CIHH conduction got better markin score, and CIHH conduction decrease iNOS and NO production. NO is an essential pathway for hypoxic tolerance [21], and NO has long been considered as a pathogenic factor of osteoarthritis. The present study proved that the protective effect of CIHH on OA is related to the NO pathway, and this is the first study to determine the impact of CIHH on NO of the cartilage.

CIHH alleviated the pathological damage of osteoarthritis and downregulated the NO pathway. To determine if there a causal relationship between them, we added the inhibitor group. Rats in the inhibitor group got better markin scores than the control group, but worse than the CIHH group. It proved No is involved in the protective effect of CIHH on osteoarthritis, but there must other pathways. Follow up studies are needed to determine the mechanism of CIHH on OA.

In conclusion, NO is an essential molecule in adapting to CIHH, and it also participates in the pathological process of OA. We believe that NO can protect osteoarthritis by regulating the production of no and the release of inflammatory factors in articular cartilage. This study proved that CIHH has protective effect on OA. One of the mechanisms is the NO pathway. Other mechanisms are waiting for us to explore, and it's meaningful for clinical OA treatment.

Abbreviations

OA, osteoarthritis; CIHH, chronic intermittent hypobaric hypoxia; iNOS, inducible nitric oxide synthase; NO, nitric oxide; SD, standard devariance; MMT, surgically-induced medial meniscal tear; ACLT, anterior cruciate ligament transection; pMM, partial medial meniscectomy; H&E, Hematoxylin and Eosin; EDTA, ethylenediaminetetraacetic acid; SDS PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; GAPDH, Glyceraldehyde triphosphate dehydrogenase; TBST, tris buffered saline tween; ECL, electrochemiluminescence; SNK, Student-Newman-Keuls.

Declarations

Acknowledgements Not applicable

Authors' contributions Tianci Wang and Pengcheng Wang designed the study. Tianci Wang and Dong Ren conducted the experiment. Yunshan Su, Jian Lu, and Ming Li coordinated and supervised data collection. Yufeng Chen analyzed the data. Tianci Wang drafted the manuscript. All authors (Tianci Wang, Dong Ren, Yunshan Su, Jian Lu, Ming Li, Yufeng Chen, Pengcheng Wang) contributed to interpretation of the findings. All authors participated in reviewing and editing the manuscript, and approved the final manuscript.

Funding Not applicable.

Availability of data and materials All supporting data can be provided based on request to the authors.

Ethics approval and consent to participate This retrospective study was approved by the ethics committee of The Third Hospital of Hebei Medical University (Z2018-015-1).

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

References

1. Glyn-Jones S, Palmer AJR, Agricola R, Price AJ, Vincent TL, Weinans H, et al. Osteoarthritis. Lancet. 2015;386(9991):376-387.
2. Velasquez MT, Katz JD. Osteoarthritis: another component of metabolic syndrome? Metabolic Syndrome & Related Disorders. 2010;8(4):295-305.

3. Bendele AM. Animal models of osteoarthritis. Musculoskelet Neuronal Interact. 2001;1(4):363-376.
4. Janusz MJ, Bendele AM, Brown KK, Taiwo YO, Hsieh L, Heitmeyer SA. Induction of osteoarthritis in the rat by surgical tear of the meniscus: inhibition of joint damage by a matrix metalloproteinase inhibitor. Osteoarthritis and Cartilage. 2002;10(10):785-791.
5. Moore EE, Bendele AM, Thompson DL, Littau A, Waggie KS, Reardon B. Fibroblast growth factor-18 stimulates chondrogenesis and cartilage repair in a rat model of injury-induced osteoarthritis. Osteoarthritis and Cartilage. 2005;13(7):623-631.
6. Williams JM, Felten DL, Peterson RG, O'Connor BL. Effects of surgically induced instability on rat knee articular cartilage. Journal of Anatomy. 1982;134(1):103-9.
7. Stoop R, Buma P, Kraan PMVD, Hollander AP, Billinghurst RC, Poole AR. Differences in type ii collagen degradation between peripheral and central cartilage of rat stifle joints after cranial cruciate ligament transection. Arthritis & Rheumatism. 2000;43(9):2121–2131.
8. Karahan S, Kincaid SA, Kammermann JR, Wright JC. Evaluation of the rat stifle joint after transection of the cranial cruciate ligament and partial medial meniscectomy. Comparative Medicine. 2001;51(6):504-12
9. Hayami T, Pickarski M, Zhuo Y, Wesolowski GA, Rodan GA, Duong LT. Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. Bone. 2008;38(2):234-243.
10. Appleton CTG, Mcerlain DD, Vasek Pitelka. Forced mobilization accelerates pathogenesis: characterization of a preclinical surgical model of osteoarthritis. Arthritis Research & Therapy. 2007;9(1):2007-02-06.
11. Baragi VM, Becher G, Bendele AM, Biesinger R, Bluhm H, Boer J, et al. A new class of potent matrix metalloproteinase 13 inhibitors for potential treatment of osteoarthritis: evidence of histologic and clinical efficacy without musculoskeletal toxicity in rat models. Arthritis & Rheumatism. 2009;60(7):2008e18.
12. Zhang Y, Yang HT, Zhou ZN. The cardioprotection of intermittent hypoxic adaptation. Acta Physiologica Sinica. 2007;5:601–13.
13. Serebrovskaya TV, Manukhina EB, Smith ML, Downey HF, Mallet RT. Intermittent hypoxia: cause of or therapy for systemic hypertension? Exp Biol Med (Maywood). 2008;233(6):627–50.
14. Zhu XH, Yan HC, Zhang J, Qu HD, Qiu XS, Chen L, et al. Intermittent hypoxia promotes hippocampal neurogenesis and produces antidepressant-like effects in adult rats. J Neurosci. 2010;30(38):12653–63.
15. Jochen H, Stefanie J, Ivan I, Sylvia K, Moritz M, Fabrizio C, et al. Thirty minutes of hypobaric hypoxia provokes alterations of immune response, haemostasis, and metabolism proteins in human serum. International Journal of Molecular Sciences, 2017;18(9):1882.
16. Guo HC, Guo F, Zhang LN, Zhang R, Wang YL. Enhancement of na/k pump activity by chronic intermittent hypobaric hypoxia protected against reperfusion injury. American Journal of Physiology Heart & Circulatory Physiology, 2011;300(6):H2280.

17. Roels B, Bentley DJ, Coste O, Mercier J, Millet GP. Effects of intermittent hypoxic training on cycling performance in well-trained athletes. Eur J Appl Physiol. 2007;101(3):359–68
18. Shi M, Cui F, Liu AJ, Ma HJ, Cheng M, Song SX, et al. The protective effects of chronic intermittent hypobaric hypoxia pretreatment against collagen-induced arthritis in rats. Journal of Inflammation, 2015;12(1):23.
19. Ya-Jie H, Yu-Jia Y, Yi-Xian L, Meng-Yue Z, Jing-Ge Z, Tian-Ci, W, et al. Nitric Oxide Participates in the Brain Ischemic Tolerance Induced by Intermittent Hypobaric Hypoxia in the Hippocampal CA1 Subfield in Rats. Neurochemical Research. 2018;43:1779-1790.
20. La P, Etchegoyen M, Czerniczyniec Apiotrkowski B, Arnaiz SL, Milei J, Costa LE, et al. Cardioprotection after acute exposure to simulated high altitude in rats. role of nitric oxide. Nitric Oxide Biology & Chemistry. 2018;73:65.
21. Jain K , Suryakumar G , Ganju L , et al. Differential hypoxic tolerance is mediated by activation of heat shock response and nitric oxide pathway. Cell Stress and Chaperones, 2014;19(6):801-812.
22. Salvatierra J, Escames G, Hernandez P, et al. Cartilage and serum levels of nitric oxide in patients with hip osteoarthritis. J Rheumatol 1999;26:2015-2017.
23. Pham TN, Rahman P, Tobin YM, Khraishi MM, Hamilton SF, Alderdice C, et al. Elevated serum nitric oxide levels in patients with inflammatory arthritis associated with co-expression of inducible nitric oxide synthase and protein kinase C-eta in peripheral blood monocyte-derived macrophages. J Rheumatol. 2003;30:2529-2534.
24. Maneiro E, López-Armada MJ, Andres MC, B Caramés, Blanco FJ, et al. Effect of nitric oxide on mitochondrial respiratory activity of human articular chondrocytes. Ann Rheum Dis. 2005;64:388-395.
25. Wu GJ, Chen TG, Chang HC, et al. Nitric oxide from both exogenous and endogenous sources activates mitochondria-dependent events and induces insults to human chondrocytes. J Cell Biochem 2007;101:1520-1531.
26. Williams JM, Felten DL, Peterson RG, et al. Effects of surgically induced instability on rat knee articular cartilage. Journal of Anatomy. 1982;134(1):103-9.
27. Stoop R , Buma P , Kraan PMVD , et al. Type II collagen degradation in articular cartilage fibrillation after anterior cruciate ligament transection in rats[J]. Osteoarthritis & Cartilage, 2001, 9(4):308-315.
28. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. J Bone Joint Surg. 1971; 53A:523-537.
29. Tsikas D. Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the l-arginine/nitric oxide area of research. Journal of Chromatography B. 2007;851(1):51-70.
30. Zurcher DM, Adhia YJ, Romero JD, McNeil AJ. Modifying a known gelator scaffold for nitrite detection. Chemical Communications. 2014;50(58): 7813-7816.
31. Kunduru KR, Basu A, Tsah T, Domb AJ. Polymer with pendant diazo-coupling functionality for colorimetric detection of nitrates. Sensors and Actuators. 2017;B251:21-26.

32. Li D, Ma Y, Duan H, Deng W, Li D. Griess reaction-based paper strip for colorimetric/fluorescent/SERS triple sensing of nitrite. Biosensors and Bioelectronics.2018;99:389-398.
33. Brown DE, Beall CM, Strohl KP, Mills PS. Exhaled nitric oxide decreases upon acute exposure to high-altitude hypoxia. American Journal of Human Biology. 2010; 18(2):196-202.
34. Zhang F, Wu W, Deng Z, Zheng X, Zhang J, Deng S, et al. High altitude increases the expression of hypoxia-inducible factor 1α and inducible nitric oxide synthase with intestinal mucosal barrier failure in rats. International Journal of Clinical & Experimental Pathology. 2015;8(5): 5189.
35. Rus A, Peinado MA, Castro L, Del Moral ML. Lung enos and inos are reoxygenation time-dependent upregulated after acute hypoxia. Anatomical Record. 2010;293(6):1089-1098..
36. Xie Y, Zhu WZ, Zhu Y, Chen L, Zhou ZN, Yang HT. Intermittent high altitude hypoxia protects the heart against lethal ca^{2+} overload injury. Life Sciences. 2004;76(5): 0-572.

Tables

Table 1 Markin scores

	1w	2w	3w
blank control group	0.00(0.00~0.00)	0.00(0.00~0.00)	0.00(0.00~0.00)
inhibitor group	2.00(1.75~2.00)	4.00(4.00~4.25)	8.00(7.75~8.00)
preconditioning group	1.00(1.00~1.25)	3.00(3.00~3.25)	7.00(7.00~7.25)
control group	2.00(2.00~2.25)	5.00(5.00~5.25)	8.00(8.00~9.00)
postconditioning group	1.00(0.75~1.00)	4.00(3.75~4.00)	7.00(6.75~7.00)
P	□0.001	□0.001	□0.001

At all 1, 2, and 3 week time point, the markin score of preconditioning group, postconditioning group and inhibitor group were lower than control group, the difference was significant (P□0.05). the markin score of preconditioning group and postconditioning group were lower than inhibitor group, the difference was significant (P□0.05).

Table 2 joint lavage fluid NO

	1w(μm)	2w(μm)	3w(μm)
blank control group	9.452±0.132	9.480±0.284	9.410±0.313
inhibitor group	8.188±0.715	14.814±0.386	20.622±0.218
preconditioning group	10.900±0.368	20.948±0.417	30.692±0.709
control group	15.968±0.290	27.600±0.549	41.334±0.971
postconditioning group	13.006±0.407	18.284±0.6098	27.988±0.148
P	□0.001	□0.001	□0.001

At all 1, 2, and 3 week time point, there were significant differences in joint lavage fluid NO among the five groups (P < 0.05), The order was as follows: inhibitor group□blank control group□preconditioning group□ postconditioning group□control group.

Table 3 iNOS of cartilage

	1w(iNOS/gapdh)	2w(iNOS/gapdh)	3w(iNOS/gapdh)
blank control group	0.177±0.015	0.227±0.018	0.241±0.025
inhibitor group	0.465±0.029	0.622±0.070	0.661±0.019*
preconditioning group	0.356±0.025	0.489±0.049	0.535±0.031
control group	0.467±0.053	0.596±0.059	0.687±0.063
postconditioning group	0.239±0.019	0.315±0.042	0.375±0.048
P	□0.001	□0.001	□0.001

At all 1, 2, and 3 week time point, iNOS of both preconditioning group and postconditioning group were lower than control group and inhibitor group, the difference was significant (P□0.05). There was no difference between inhibitor group and control group.

Figures

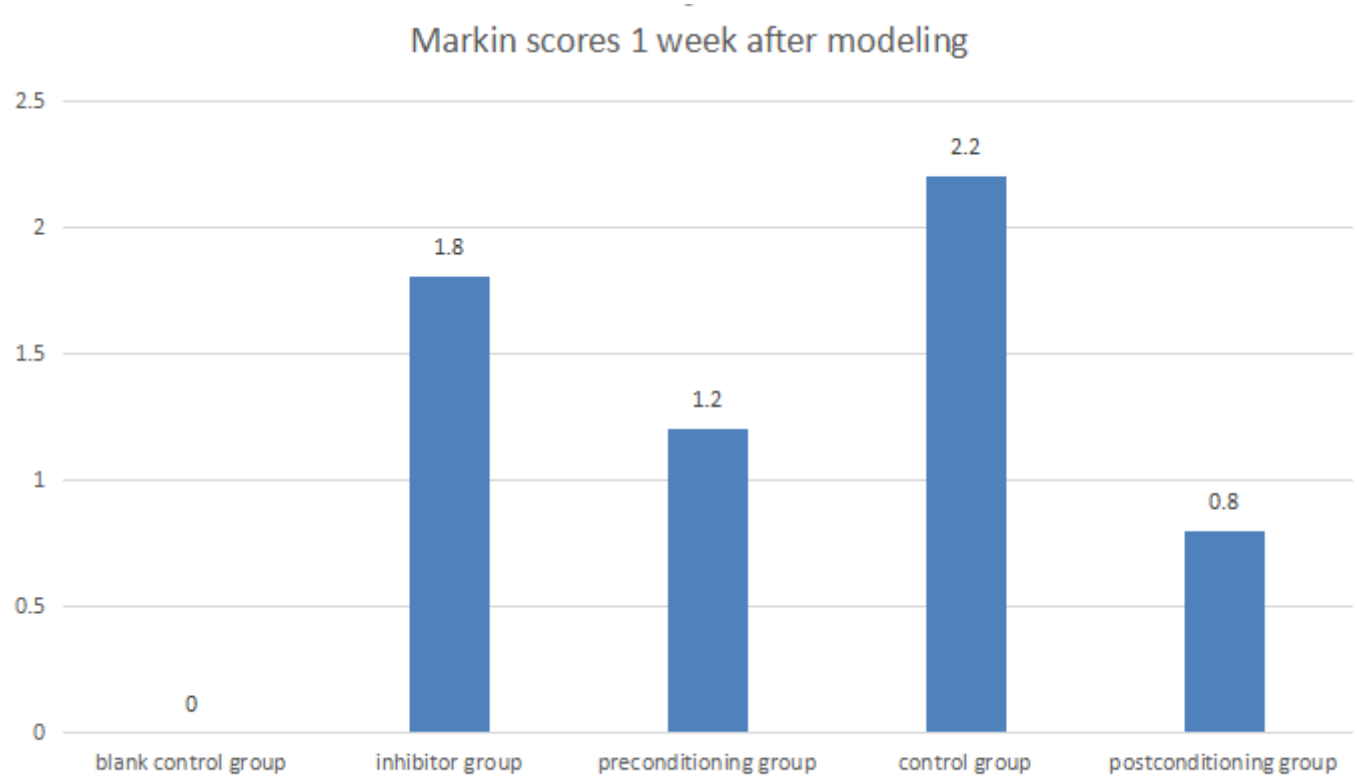

Figure 1

Markin score of inhibitor group was lower than control group, but higher than preconditioning and postconditioning group (Fig. 1-5).

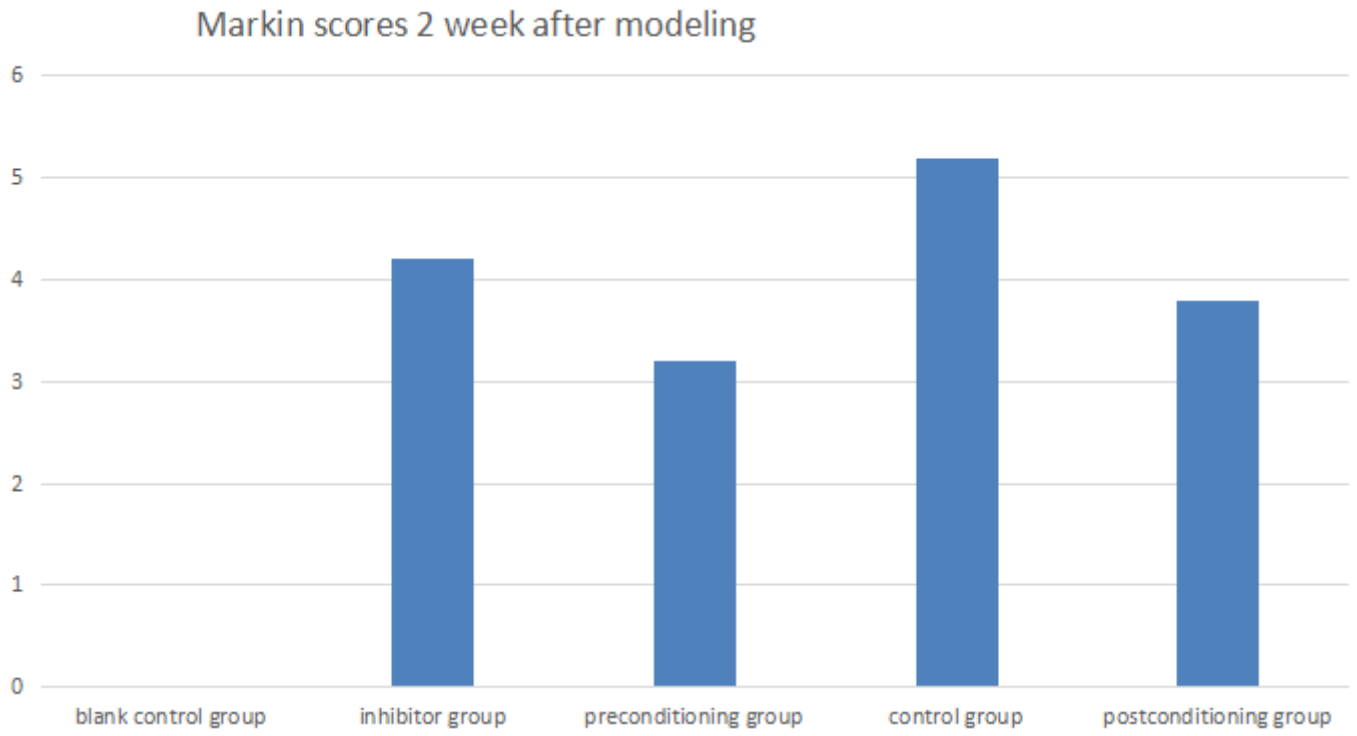

Figure 2

Markin score of inhibitor group was lower than control group, but higher than preconditioning and postconditioning group (Fig. 1-5).

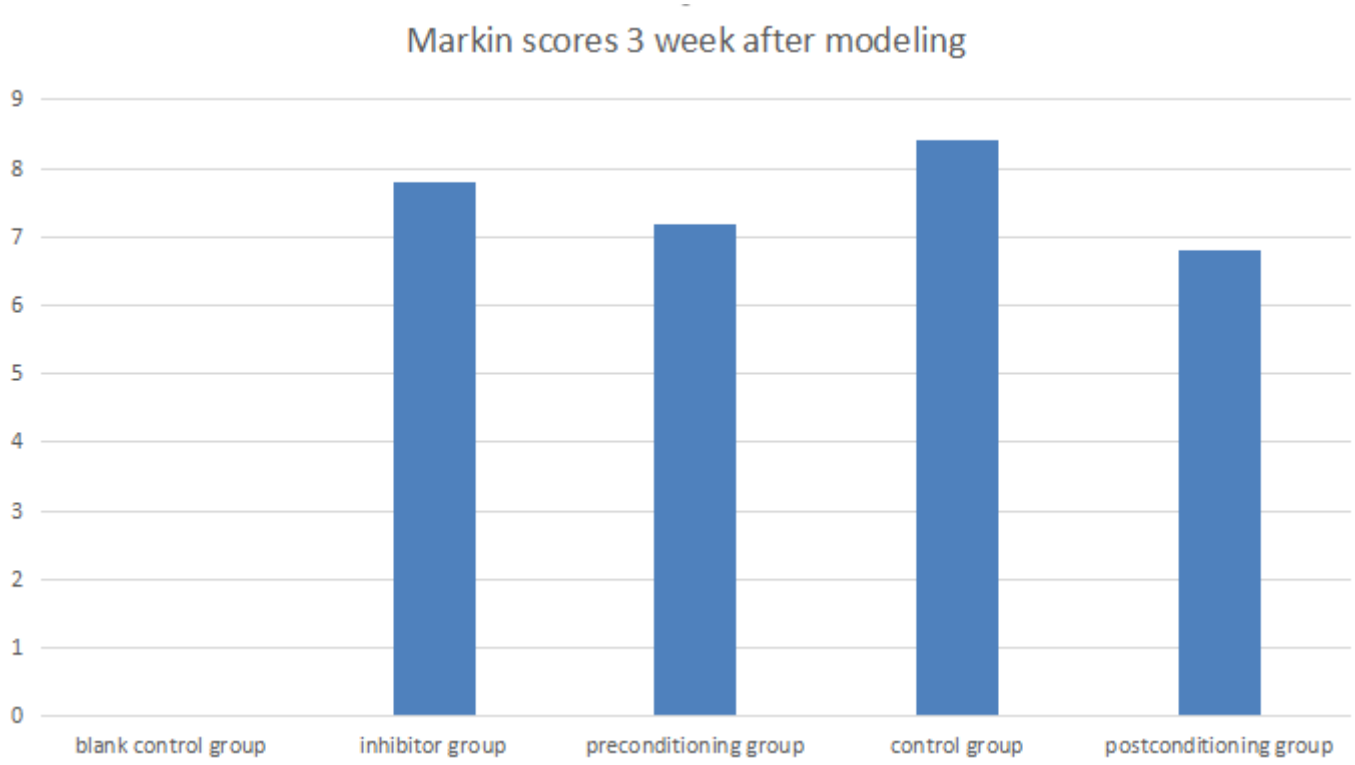

Figure 3

Markin score of inhibitor group was lower than control group, but higher than preconditioning and postconditioning group (Fig. 1-5).

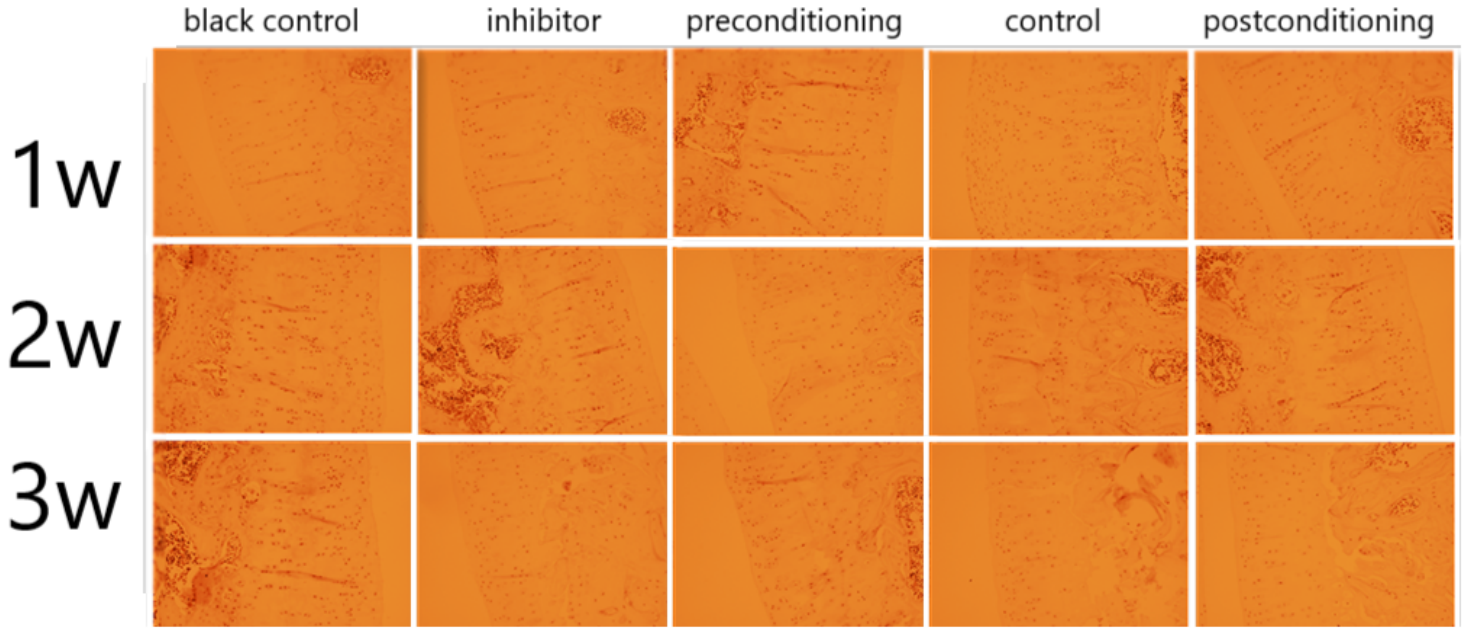

Figure 4

Markin score of inhibitor group was lower than control group, but higher than preconditioning and postconditioning group (Fig. 1-5).

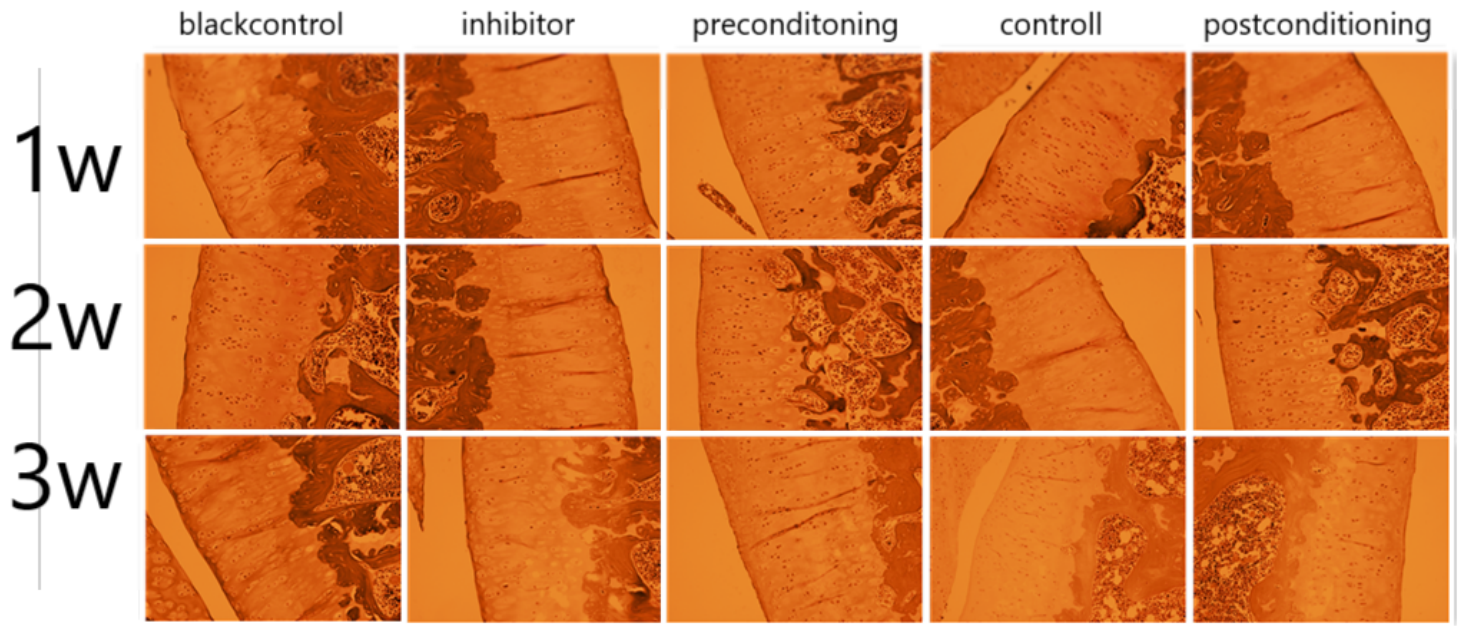

Figure 5

Markin score of inhibitor group was lower than control group, but higher than preconditioning and postconditioning group (Fig. 1-5).

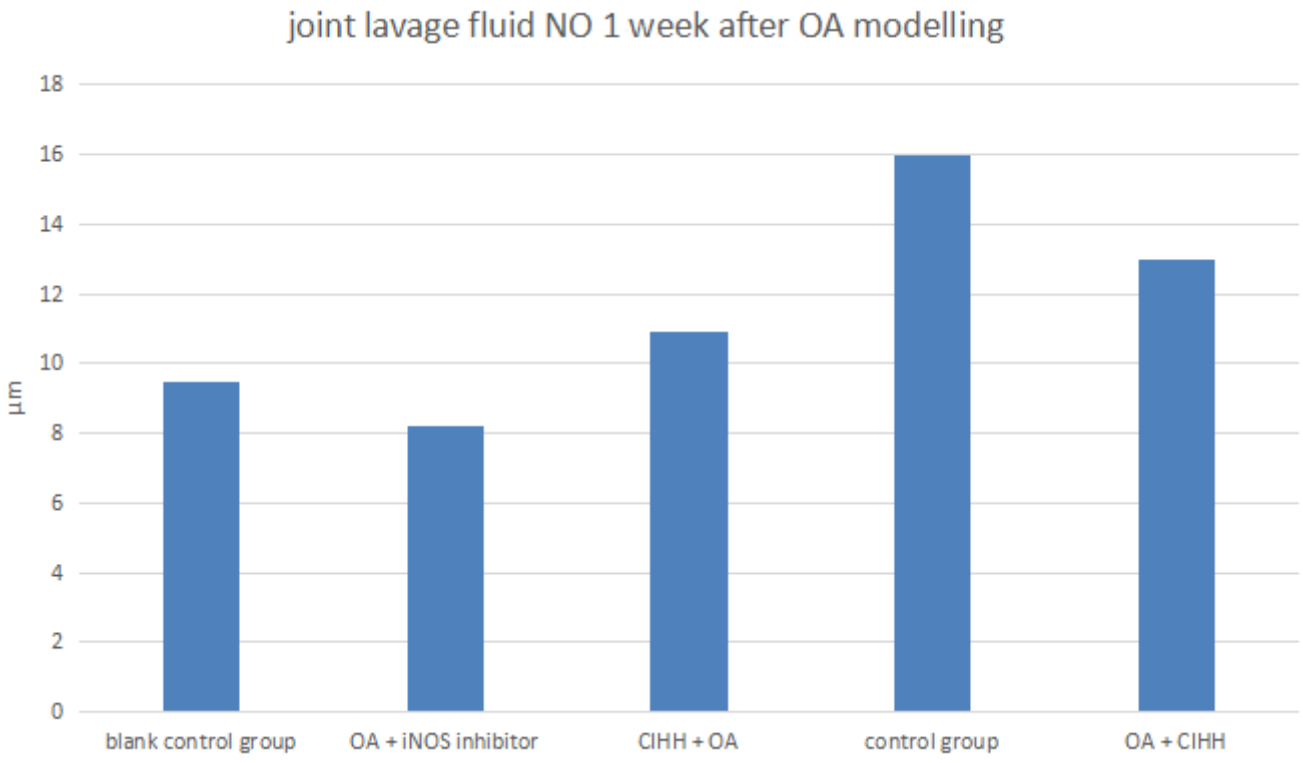

Figure 6

at all 1, 2, and 3 week time point, joint lavage fluid NO of preconditioning group, postconditioning group and inhibitor group were lower than control group. The joint lavage fluid NO in both preconditioning group and postconditioning group rats were significantly higher than the inhibitor group.

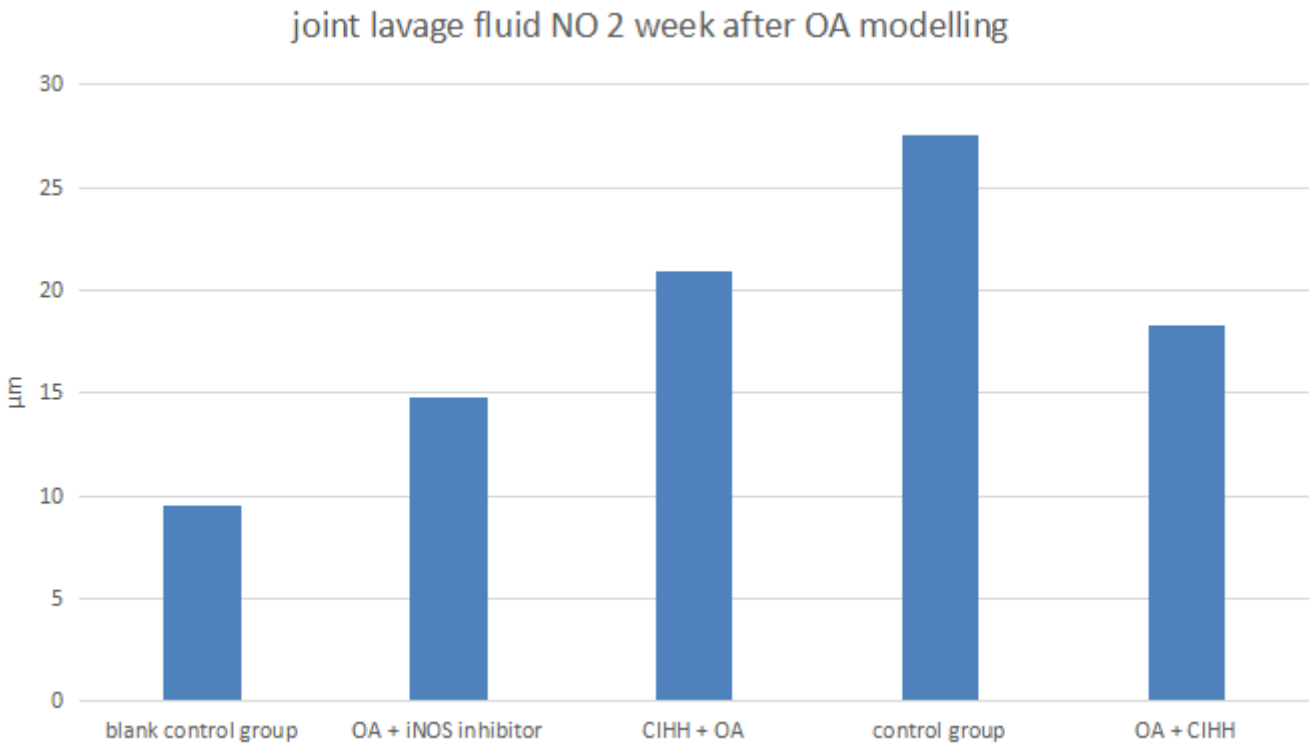

Figure 7

at all 1, 2, and 3 week time point, joint lavage fluid NO of preconditioning group, postconditioning group and inhibitor group were lower than control group. The joint lavage fluid NO in both preconditioning group and postconditioning group rats were significantly higher than the inhibitor group.

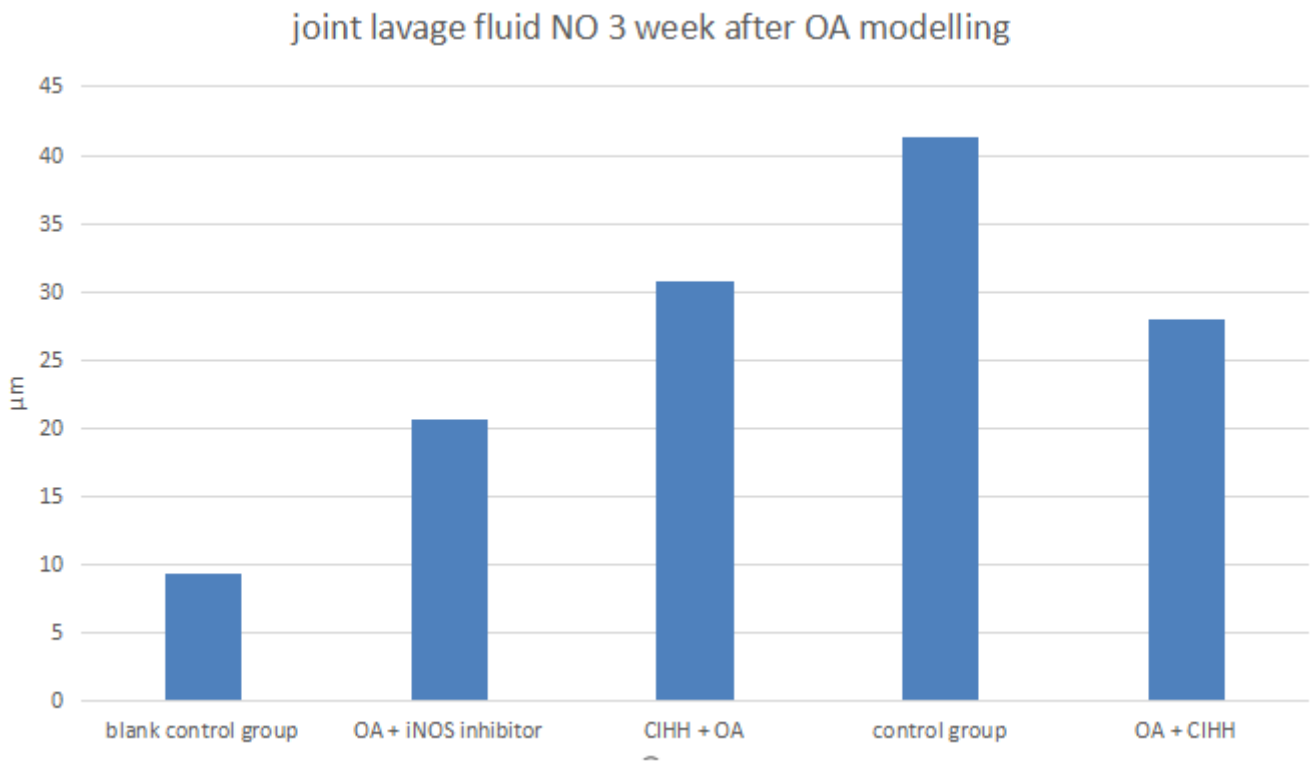

Figure 8

at all 1, 2, and 3 week time point, joint lavage fluid NO of preconditioning group, postconditioning group and inhibitor group were lower than control group. The joint lavage fluid NO in both preconditioning group and postconditioning group rats were significantly higher than the inhibitor group.

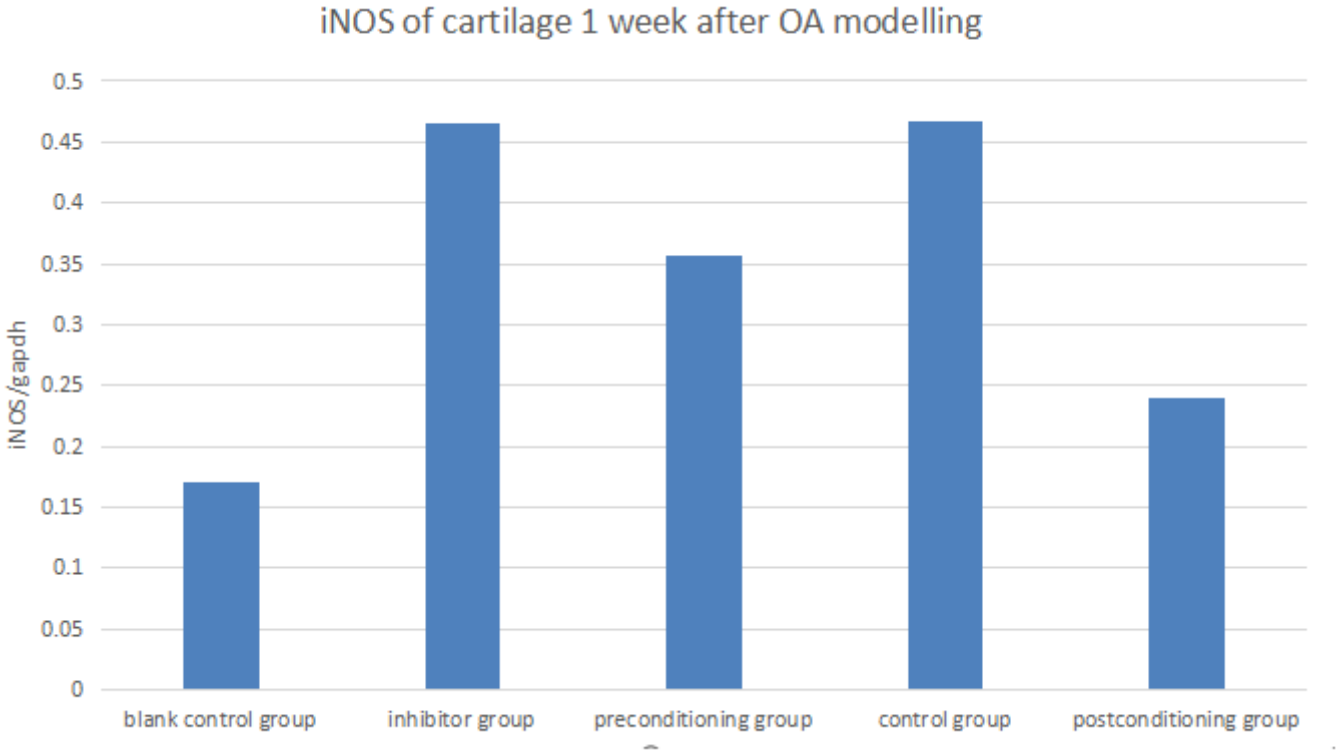

Figure 9

In order to detect the effect of CIHH on NO, western blotting was used to analyze the expression of iNOS, as shown in Table 3 and Fig. 9-11.

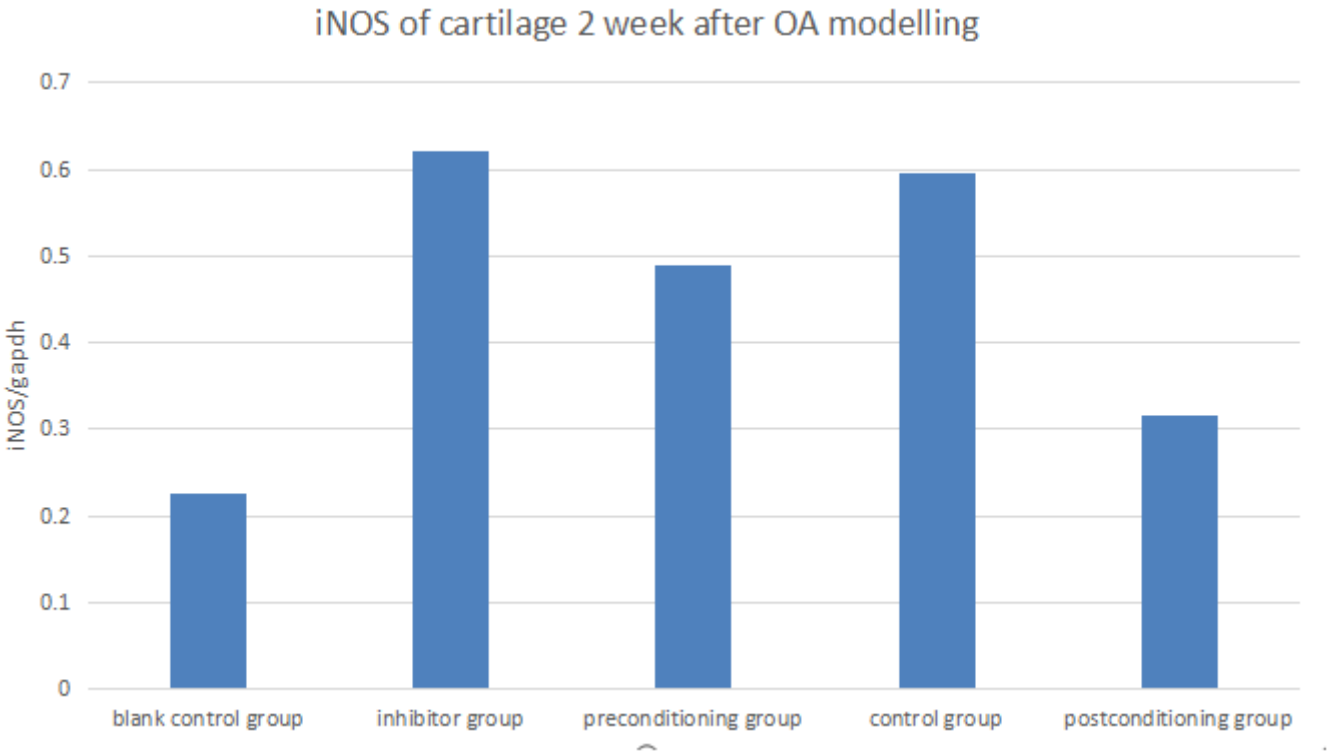

Figure 10

In order to detect the effect of CIHH on NO, western blotting was used to analyze the expression of iNOS, as shown in Table 3 and Fig. 9-11.

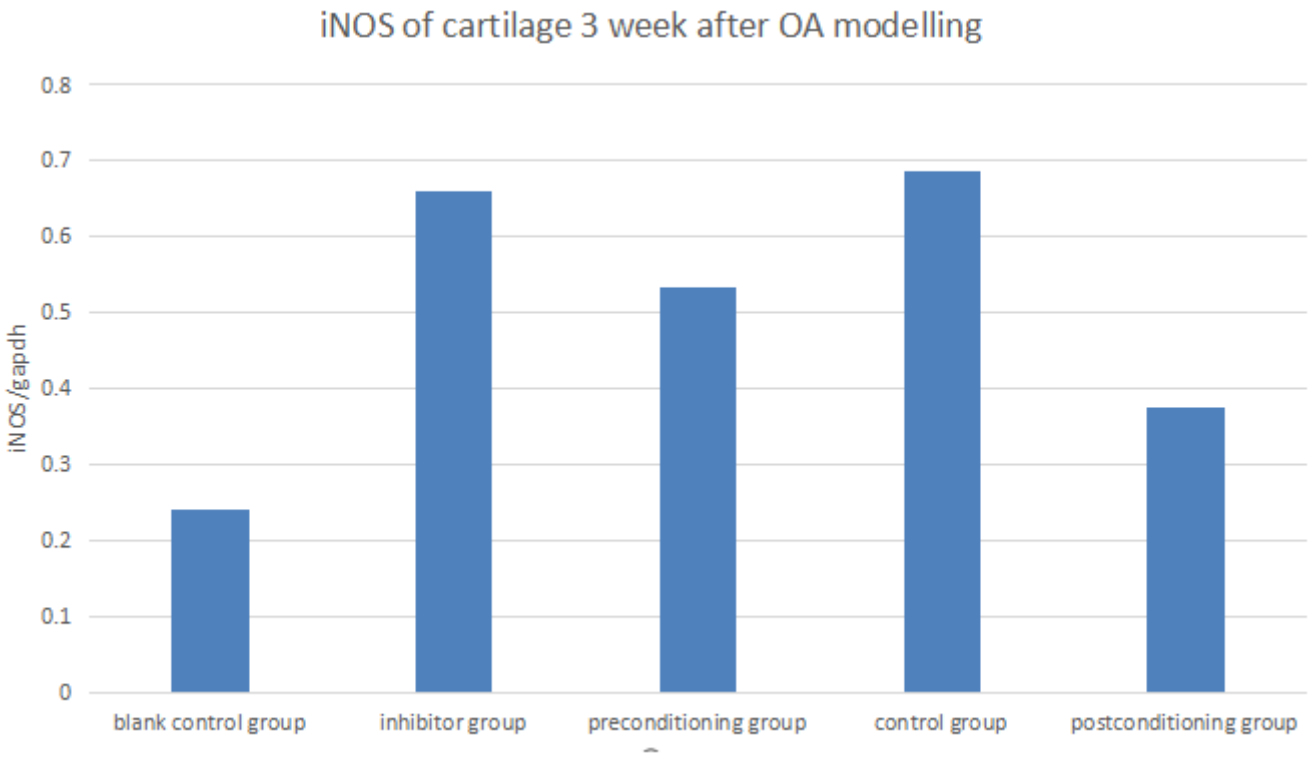

Figure 11

In order to detect the effect of CIHH on NO, western blotting was used to analyze the expression of iNOS, as shown in Table 3 and Fig. 9-11.