

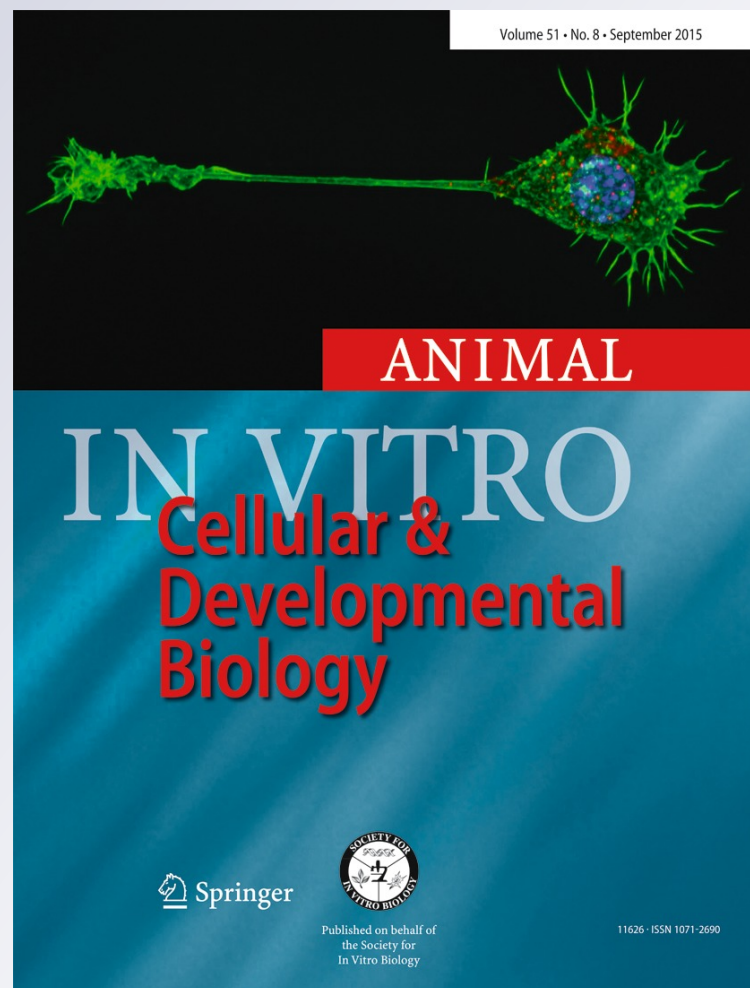
*Inflammatory response of cultured rat synoviocytes challenged with synovial fluid from osteoarthritis patients correlates with their radiographic grading: a pilot study*

**Soumya J Koppikar, Priya G Kulkarni, Dhanashri R Ingale, Dattatray Shinde, NarendraKumar Wagh, Shantanu Deshpande, Alpana S Moghe, et al.**

**In Vitro Cellular & Developmental  
Biology - Animal**

ISSN 1071-2690  
Volume 51  
Number 8

In Vitro Cell.Dev.Biol.-Animal (2015)  
51:843-850  
DOI 10.1007/s11626-015-9888-9



**Your article is protected by copyright and all rights are held exclusively by The Society for In Vitro Biology. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at [link.springer.com](http://link.springer.com)".**

# Inflammatory response of cultured rat synoviocytes challenged with synovial fluid from osteoarthritis patients correlates with their radiographic grading: a pilot study

Soumya J Koppikar<sup>1</sup> · Priya G Kulkarni<sup>1</sup> · Dhanashri R Ingale<sup>1</sup> · Dattatray Shinde<sup>2</sup> · NarendraKumar Wagh<sup>3</sup> · Shantanu Deshpande<sup>3</sup> · Alpana S Moghe<sup>4</sup> · Prabhakar K Ranjekar<sup>1</sup> · Abhay M Harsulkar<sup>1</sup>

Received: 30 October 2014 / Accepted: 5 March 2015 / Published online: 3 July 2015 / Editor: T. Okamoto  
© The Society for In Vitro Biology 2015

**Abstract** The inflammatory nature of synovial fluid (SF) of varying grade osteoarthritis (OA) patients was estimated by measuring pro-inflammatory factors and through a unique cell-challenge experiment. SF samples were collected from six OA and one non-OA patient; spanning Kellgren-

Lawrence (KL) grades were analyzed for interleukin-1-beta (IL-1 $\beta$ ), nitric oxide (NO) and its derivatives, and glycosaminoglycan (GAG). Levels of IL-1 $\beta$ , NO, and GAG in SF did not correlate with KL grades of the patients studied. In the cell-challenge experiment, cultured rat synoviocyte fibroblasts (RSFs) were challenged by the patient's SFs with and without pre-treatment of IL-1 $\beta$  and lipopolysaccharide (LPS). NO released by the cells was taken as an indicator of inflammation. SFs from KL grades 2 and 3 induced maximum inflammation in cultured RSFs (grade 2 64.61 $\pm$ 4.8 and 89.51 $\pm$ 5.6  $\mu$ M/ml after 48 and 72 h, grade 3 58.27 $\pm$ 2.7 and 64.22 $\pm$ 2.8  $\mu$ M/ml after 48 and 72 h, respectively). Similar trend was observed in RSF pretreated with either recombinant IL-1 $\beta$  or LPS suggesting that SF from patients KL grades 2 and 3 accumulates more pro-inflammatory factors. IL-1 $\beta$ -pre-treated RSFs challenged by SF for 72 h showed 234.41 $\pm$ 17.6  $\mu$ M/ml increase (patient 3, grade 3), whereas higher NO after LPS pre-treatment was recorded (118.92 $\pm$ 6.2  $\mu$ M/ml; patient 3, grade 3). Interestingly, SFs from grade 1 and non-OA patient could reduce released NO to 27.10 $\pm$ 2.2  $\mu$ M/ml showing potency to alleviate inflammation. These interesting findings, however, need to be confirmed on a wider number of patients, which may offer significant therapeutic application in treatment of OA.

✉ Abhay M Harsulkar  
aharsulkar@yahoo.com

Soumya J Koppikar  
soumya.koppikar@gmail.com

Priya G Kulkarni  
pgkulkarni2610@googlemail.com

Dhanashri R Ingale  
ingaledhanashri91@gmail.com

Dattatray Shinde  
drshinde249@gmail.com

NarendraKumar Wagh  
dmarendrwagh65@gmail.com

Shantanu Deshpande  
desh75@gmail.com

Alpana S Moghe  
alpanasm@gmail.com

Prabhakar K Ranjekar  
pranjekar@gmail.com

<sup>1</sup> Department of Cell and Molecular Biology, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth University, Pune-Satara Road, Pune, Maharashtra 411 043, India

<sup>2</sup> Department of Kayachikisa, Bharati Ayurvedic Hospital, Bharati Vidyapeeth University, Pune, Maharashtra, India

<sup>3</sup> Department of Orthopedics, Bharati Medical Hospital, Bharati Vidyapeeth University, Pune, Maharashtra, India

<sup>4</sup> Department of Cell and Molecular Biology, Rajiv Gandhi institute of IT & BT, Bharati Vidyapeeth University, Pune, Maharashtra, India

**Keywords** Osteoarthritis · Synoviocytes-like fibroblast cells · Nitric oxide · Interleukin-1- $\beta$  · Glycosaminoglycan

## Introduction

Osteoarthritis (OA) is estimated as the fourth leading cause of disability (Fransen *et al.* 2000). Etiology of OA includes inflammation of synovial membrane (synovitis), subchondral bone porosis, articular cartilage degeneration, and vitiation of

synovial fluid (SF) (Sellam and Berenbaum 2010). SF mediates transport of essential nutrients, enzymes, lubricants, cytokines, and growth factors responsible for catabolic and anabolic processes. Inflection in SF, in terms of over-accumulation of pro-inflammatory cytokines, free radicals, and metalloproteinases results in cartilage degradation and disease pathology (Hassanali 2011). Synoviocytes (fibroblast-like synoviocytes and macrophages) arranged in synovial membrane in a unique cellular fashion (Smith 2011) are responsible for the synthesis and maintenance of SF (Sellam and Berenbaum 2010). These synoviocytes are sensitive towards cytokines and other molecular signals including degradation products released by cartilage and often reciprocate with disseminating inflammatory mediators, which contribute towards progressive inflammation (Wang *et al.* 2012). Studying cytokines and pro-inflammatory agents in SF and its inflammatory potential is an attractive approach towards understanding OA pathophysiology.

In the current study, we present an analysis of SF obtained from OA patients with ranging Kellgren-Lawrence (KL) grades (Table 1). Inflammatory biomarkers, such as interleukin-1-beta (IL-1 $\beta$ ), nitric oxide (NO), metabolites of NO (nitrate-nitrite), and glycosaminoglycan (GAG) were assessed. Further, in a unique cell-challenge experiment, cultured rat synoviocytes were challenged with SFs obtained from OA patients. The objective of this experiment was to study correlation in inflammation induced in synoviocytes with the disease severity.

A brief clinical background of patients is summarized as follows:

**Patient 1.** A 60-yr-old woman reported severe pain in the right knee with difficulty in walking. Her radiographs showed reduced joint space of the left knee, and no extension was possible with range of motion from 60° to 120°. Lateral patellar subluxion was further suggestive of KL grade 4 and an indication for total knee replacement (TKR).

**Patient 2.** A 63-yr-old lady approached the clinic with a complaint of difficult and painful walking of the right knee joint since the last 4 yr. The lady has undergone for TKR of the left knee in the past. Her radiographs showed reduction of joint space and lateral tibial subluxion, suggestive of tri-compartmental OA with KL grade 4. In her full leg standing X-ray, Various deformity of the right knee joint was noted and hence, TKR was performed for this patient.

**Patient 3.** A 44-yr-old man approached having complaints of right knee-joint pain, tenderness, and difficulty in walking since last the 6 mo. Antero-posterior (AP) radiograph showed reduction of joint space in the medial compartment while patella-femoral and posterior compartments were normal. A moderate osteophyte formation could be seen on the posterior side of the patella and thus diagnosed with grade 3 OA.

**Patient 4.** A 68-yr-old man with complaints of pain and swelling in the left knee joint since the last 4 yr. Patient had a long-term NSAID exposure with recurrent relapses of symptoms. Typical OA grade 2 radiograph showed tri-compartmental joint space reduction and osteophyte formation.

**Patient 5.** A 42-yr-old woman was diagnosed early OA. She complained pain in the right knee joint for the last 6 mo and an injury 3 mo back, without history of any particular disease or regular medication. Her X-rays showed joint space reduction in the medial and lateral compartment. Mild osteophyte formation was observed and thus was classified as grade 2.

**Patient 6.** An obese woman of 45 yr had reported pain and swelling of the right knee joint for the last 6 mo. Recurrent onsets of pain and walking difficulty were noted in both knee joints. Her X-ray (lateral view) showed a smooth articular surface without osteophytes. Right knee radiograph with AP view showed good and equal tri-compartmental joint space

**Table 1** Pro-inflammatory factors in SF from knee joints of OA patient

Sr. No.	Patients	KL grading scale	IL-1 $\beta$ levels (pg/ml)	NO levels ( $\mu$ M/ml)	Nitrate/nitrite levels ( $\mu$ M)	GAG levels ( $\mu$ g/ml)
1	Patient 1	4	23.64 $\pm$ 1.8	69.11 $\pm$ 15.4	24.65 $\pm$ 0.7	437.50 $\pm$ 5.8
2	Patient 2	4	22.27 $\pm$ 7.7	62.36 $\pm$ 0.6	77.66 $\pm$ 2.6	392.50 $\pm$ 10.8
3	Patient 3	3	603.03 $\pm$ 12.7	42.35 $\pm$ 7.7	42.98 $\pm$ 1.0	253.56 $\pm$ 20.6
4	Patient 4	2	2184.85 $\pm$ 10.5	11.47 $\pm$ 1.4	66.54 $\pm$ 1.9	568.71 $\pm$ 9.7
5	Patient 5	2	642.42 $\pm$ 13.8	80.00 $\pm$ 7.6	82.68 $\pm$ 1.9	313.35 $\pm$ 9.8
6	Patient 6	1	47.27 $\pm$ 4.5	14.70 $\pm$ 0.6	23.56 $\pm$ 0.0	463.33 $\pm$ 8.3
Normal values			9.30–13.10 (Karan <i>et al.</i> 2003)	–	–	13.10 (Mattiello-Rosa <i>et al.</i> 2008)

All the values are expressed as mean $\pm$ SD ( $n=3$ )

KL Kellgren-Lawrence OA score, SF synovial fluid

with Vulgus deformity of 7–10, hence diagnosed with mild OA of KL grade 1.

## Materials and Methods

Tissue culture grade media, cytokines, enzymes, and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture plasticwares were purchased from BD Biosciences, CA, and Axygen Scientific Inc, CA.

**Selection of patients.** Patients of either sex age 30 yr or more with diagnosed knee OA on the basis of X-ray and clinical observations were selected. The study is approved by the human ethical committee of Bharati Vidyapeeth University (BVDU/MC/56). Severity of OA was determined by globally approved “Kellgren-Lawrence grading” (Kellgren and Lawrence 1957), which is based on X-ray evaluation of the patient’s knee (Table 1).

**SF collection.** Arthrocentesis was performed by an experienced orthopedic surgeon under strict aseptic precautions in a two-step process, a puncture through skin followed by puncture of synovial capsule (Mundt and Shanahan 2011). A sterile 18G needle with 10-cc syringe was used to aspirate SF. After aspiration, the fluid samples were sent to laboratory for further studies or archival.

**Isolation of rat synoviocytes fibroblast (RSF).** RSFs were isolated from synovium dissected from healthy Wistar rats according to Ding *et al.* (2003) with few modifications. Briefly, the synovium was cut into small pieces, digested in 2 mg/ml collagenase in DMEM supplemented with 1% penicillin–streptomycin in a shaking incubator at 37°C. The tissue was sheared using sterile syringe, filtered through fine sterile gauze, washed, and re-suspended in growth medium containing DMEM, 10% FBS, 2 mM L-glutamine, and 1% penicillin–streptomycin. The cells were seeded and allowed to adhere into 24-well plates with periodic replacement of growth medium. The prepared cell line was grown overnight at 37°C in 5% CO<sub>2</sub> in 96-well plates ( $1 \times 10^5$  cells/ml) and used for further studies. The protocol was approved by the animal ethics committee of Bharati Vidyapeeth University (CPCSEA/53/12).

**Validation of established RSF by studying expression of CD248.** The SuperScript® III Cells Direct cDNA Synthesis Kit (Invitrogen Co., Carlsbad, CA) was used to isolate cDNA from RSF as per manufacturer’s instructions. The quantitative real-time PCR analysis was done using an Applied Biosystems Step One Real Time PCR System (Applied Biosystems, Foster City, CA).

The TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for rats was CD248 (Cat; Rn01760170\_s1), normalized to the amount of  $\beta$ -actin (Actb; Rn00667869\_m1). Cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, 60°C for 1 min. The data was analyzed using Data Assist software ver 3.0.

**Synoviocytes challenge experiment.** The cultured rat synoviocytes were challenged with the patient’s SF that enabled assessment of cumulative action of pro-inflammatory as well as anti-inflammatory agents in SF, with progression of the disease wherein NO released by the cells was used as marker for inflammation. In another set, to mimic synovial inflammation, the cells were stimulated with human recombinant IL-1 $\beta$  (100 ng/ml) or LPS (10  $\mu$ g/ml) followed by challenging with SF (5%) for 48 and 72 h. Bell *et al.* 1995 reported treatment of cultured synovial neutrophils with increasing synovial fluid concentrations from 0, 25, 50, and 75% for 24 and 48 h and induction of apoptosis. Hence, we have chosen three concentrations (1, 5, and 10%) to standardized SF concentrations on RSF. We found that 5 and 10% were revealed best NO results as compared to 1%. The conditioned media were collected after treatment and evaluated for inflammation measured in terms of released NO.

**Determination of NO.** NO levels were measured using Griess reaction (Sumantran *et al.* 2011). The conditioned media and SFs were appropriately diluted, with addition of 1% sulphanilic acid and 0.1% N, N-naphthylethylenediaminedihydrochloride (NEDD) prepared in 5% phosphoric acid in 1:1 ratio. After incubation for 10 min at room temperature, absorbance was measured at 540 nm in a plate reader (Biorad, Hercules, CA). Further calculations were done from a standard curve made with linear concentrations of sodium nitrate.

**Determination of nitrate-nitrite (NN).** The nitrate-nitrite activities were determined by using kit (Cayman, Ann Arbor, MI) according to the manufacturer’s instructions. In brief, for measurement of nitrate, 200  $\mu$ l assay buffer was added to the blank wells without adding any reagents. Add 80  $\mu$ l of sample or sample dilutions (1:5 in the current case). Add 10  $\mu$ l of the enzyme cofactor to each wells followed by addition of 10  $\mu$ l of nitrate reductase mixture. Incubate at room temperature for 1 h. After the required incubation time, add 50  $\mu$ l of Greiss Reagent R1. Immediately add 50  $\mu$ l of Greiss Reagent R2 and allow color to develop for 10 min at room temperature. Read the absorbance at 540 nm. For measurement of nitrite, add 200  $\mu$ l of assay buffer to the blank wells. Add 100  $\mu$ l of sample in 1:5 dilutions in present case. Add 50  $\mu$ l of Greiss Reagent R1 followed by of Greiss Reagent R2 and allow the

color to develop for 10 min; measure the absorbance at 540 nm.

Nitrate-nitrite was calculated using following formula:

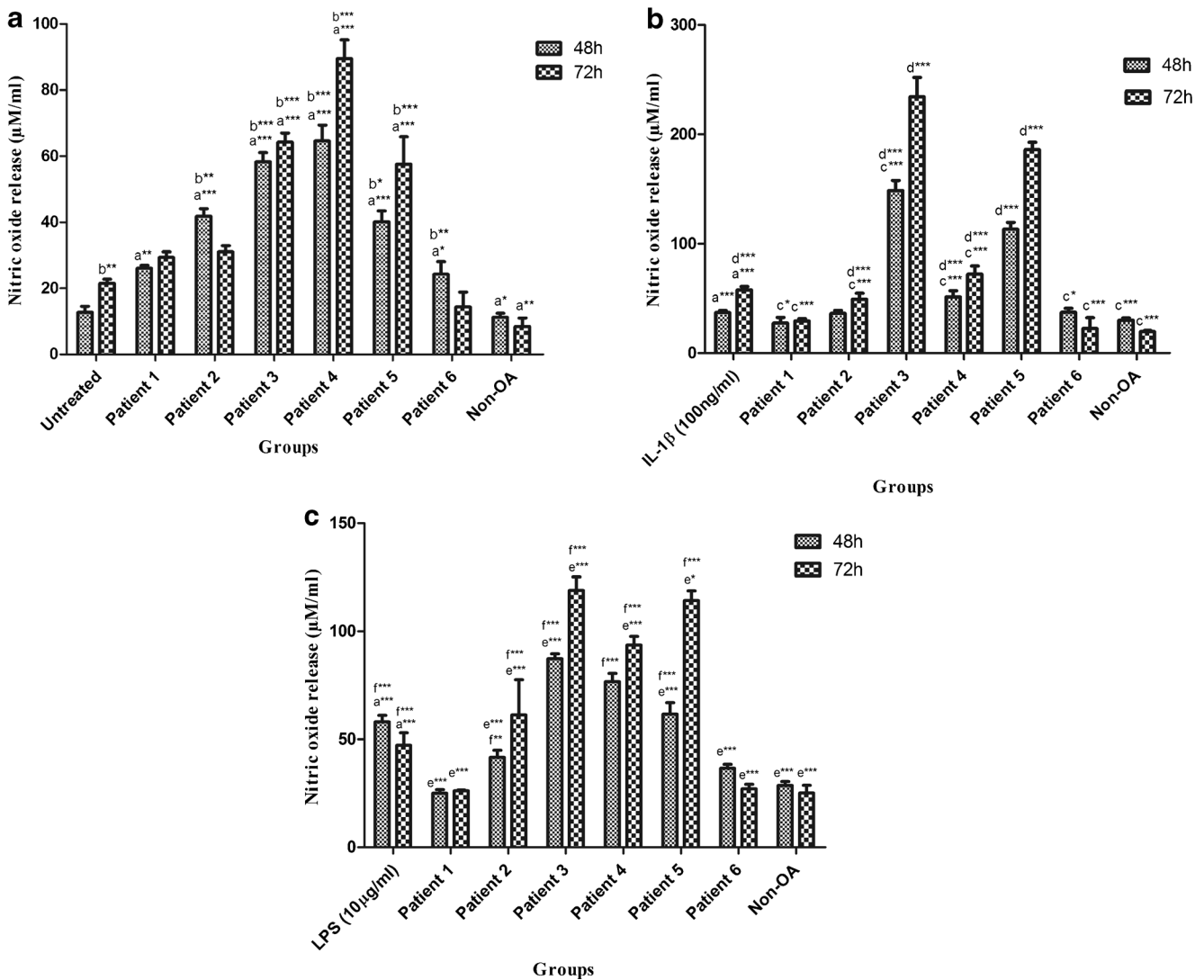
$$[\text{Nitrate} + \text{Nitrite}] (\mu\text{M}) = (A_{540} - y\text{-intercept} / \text{slope}) \times (200 \mu\text{l} / \text{volume of sample used } (\mu\text{l})) \times \text{dilution}$$

$$[\text{Nitrite}] (\mu\text{M}) = (A_{540} - y\text{-intercept} / \text{slope}) \times (200 \mu\text{l} / \text{volume of sample used } (\mu\text{l})) \times \text{dilution}$$

$$[\text{Nitrate}] (\mu\text{M}) = (\text{Nitrate} + \text{Nitrite}) - (\text{Nitrite})$$

**GAG assay.** GAG was measured by a spectrophotometric dye binding assay using 1,2-dimethylmethylene blue (DMMB) dye

using chondroitin sulfate as a standard (Sumantran *et al.* 2011). The levels of GAG were expressed as microgram equivalents



**Figure 1** (a) Nitric oxide released from RSF after challenging with SF from OA patients at 48 and 72 h. All the values are expressed as mean ± SD (n=3). *a* denotes significance when compared with untreated cells control. *b* denotes significance when compared with cells treated with SF from non-OA patient. \**p*<0.05; \*\* *p*<0.01; \*\*\* *p*<0.001. (b) NO released from RSF pre-treated with IL-1β and challenged by SFs from OA patients at 48 and 72 h. All the values are expressed as mean ± SD (n=3). *a* denotes significance when compared with untreated cells control. *c* denotes significance when compared with cells pre-treated with IL-1β

whereas *d* denotes significance when compared with cells pre-treated IL-1β and challenged with SF from non-OA patient. \**p*<0.05; \*\* *p*<0.01; \*\*\* *p*<0.001. (c) NO released from RSF pre-treated with LPS and challenged by SFs from OA patients at 48 and 72 h. All the values are expressed as mean ± SD (n=3). *a* denotes significance when compared with untreated cells control. *e* denotes significance when compared with cells pre-treated with LPS whereas *f* denotes significance when compared with cells pre-treated by LPS and challenged with SF from non-OA patient. \**p*<0.05; \*\* *p*<0.01; \*\*\* *p*<0.001.

of chondroitin sulfate per milliliter of SF sample.

**IL-1 $\beta$  assay.** IL-1 $\beta$  levels were quantified for using human enzyme-linked immunosorbent assay (ELISA) kit (Abnova, Walnut, CA) according to the manufacturer's instructions. The optical density was estimated at 450 nm using ELISA-microplate reader (Biorad, Hercules, CA).

**Statistical analysis.** All cell experiments were performed in triplicates and data was presented as mean $\pm$ SD. Two-way ANOVA was applied followed by Bonferroni post-tests to determine significance of differences using GraphPad Prism 5.0 (San Diego, CA).

## Results

Isolated RSF cells were validated by studying expression of CD248 gene marker using real-time (RT)-PCR. CD248 is fairly selective biomarker for synoviocytes cell line (Hardy *et al.* 2013). Our RT-PCR results demonstrated a CT value of 25 for CD248, whereas reference gene ( $\beta$ -actin) showed CT value of 32 at passage 7. At passage 4, the CT value for CD248 was 26 (CT for  $\beta$ -actin was 17).

As summarized in Table 1, IL-1 $\beta$  levels were higher in all SF samples compared to normal values obtained from published literature (Karan *et al.* 2003). KL grade 2 (patient 4 2184.85 $\pm$ 10.5 pg/ml and patient 5 642.42 $\pm$ 13.8 pg/ml) showed higher IL-1 $\beta$  than KL grade 3 (patient 3 603.03 $\pm$ 12.7 pg/ml). KL grade 1 matched with the lowest IL-1 $\beta$  (patient 6 47.27 $\pm$ 4.5 pg/ml). No correlation was observed with NO levels and its derivatives in SF with radiological KL grade of OA patients. The highest value of NO and NN were calculated in KL grade 2 (patient 5 80.00 $\pm$ 7.6  $\mu$ M/ml; 82.68 $\pm$ 1.9  $\mu$ M, respectively), which corresponds with the recent history of knee injury reported by this patient. The lowest values

was seen in KL grade 1 (patient 6 14.7 $\pm$ 0.6  $\mu$ M/ml; 23.56 $\pm$ 0.0  $\mu$ M), which corroborated with lower IL-1 $\beta$ . Interestingly, despite of highest IL-1 $\beta$ , patient 4 revealed the lowest NO level. However, it should be noted that the same sample revealed comparatively higher NO derivatives, a more stable product of NO (Feelisch 2008).

Relatively, higher GAG was noted in KL grade 2 (patient 4 568.71 $\pm$ 9.7  $\mu$ g/ml and patient 5 313.35 $\pm$ 9.8  $\mu$ g/ml), which correlated with elevated IL-1 $\beta$  calculated in the same samples. Patient 1 and patient 2 showed high GAG levels (437.50 $\pm$ 5.8  $\mu$ g/ml, 392.50 $\pm$ 10.8  $\mu$ g/ml, respectively) with lower IL-1 $\beta$  values. Patient 3 and patient 6 did not show any correspondence between KL grade and GAG levels (253.56 $\pm$ 20.67  $\mu$ g/ml, 463.33 $\pm$ 8.37  $\mu$ g/ml, respectively). The lowest GAG was measured in patient 3 despite of its higher KL grade, while higher GAG value was estimated in patient 6, a case of early OA.

**RSF challenged with SF.** Interestingly, inflammatory response in terms of NO release was substantially increased when RSF were challenged with SF from OA patients (Fig. 1a), with a peculiar trend. SF from patients with KL grades 2 and 3 could induce maximum inflammatory response [patient 3 KL grade 3; 48 h ( $p$ <0.001), 72 h ( $p$ <0.001); patient 5 KL grade 2, 48 h ( $p$ <0.05), 72 h ( $p$ <0.001)] as summarized in Table 2. Patient 4 was noted with the highest NO release for 48 and 72 h, respectively ( $p$ <0.001,  $p$ <0.001). Interestingly, SF from KL grade 1 could not induce hyper inflammatory response [patient 6 KL grade 1, 48 h ( $p$ <0.05)]. This result was supported by the SF from non-OA control [72 h ( $p$ <0.001)]. Surprisingly, KL grade 4 (patient 1 and patient 2) [48 h ( $p$ <0.01)] showed lower NO release.

**RSF pre-treated with IL-1 $\beta$  and challenged with SF.** The NO release from RSF pre-treated with IL-1 $\beta$  (100 ng/ml,

**Table 2** Fold increase in NO released from RSF challenged with SF

Sr. No.	Patients	NO release after 48 h ( $\mu$ M/ml), mean $\pm$ SD	NO fold increase after 48 h		NO release after 72 h ( $\mu$ M/ml), mean $\pm$ SD	NO fold increase after 72 h	
			Untreated control	Non-OA patient		Untreated control	Non-OA patient
1	Untreated control	12.75 $\pm$ 1.8	1.0	1.1	21.53 $\pm$ 1.2	1.0	2.5
2	Patient 1	26.08 $\pm$ 0.9	2.0	2.3	29.14 $\pm$ 1.6	1.4	3.4
3	Patient 2	41.76 $\pm$ 2.4	3.3	3.7	30.98 $\pm$ 1.9	1.4	3.7
4	Patient 3	58.28 $\pm$ 2.7	4.6	5.2	64.22 $\pm$ 2.8	3.0	7.6
5	Patient 4	64.61 $\pm$ 4.8	5.1	5.7	89.15 $\pm$ 5.6	4.1	10.6
6	Patient 5	40.10 $\pm$ 3.3	3.1	3.6	57.55 $\pm$ 8.3	2.7	6.8
7	Patient 6	24.31 $\pm$ 3.8	1.9	2.2	14.35 $\pm$ 4.5	0.7	1.7
8	Non-OA patient	11.24 $\pm$ 1.3	1.1	1.0	8.47 $\pm$ 2.5	0.4	1.0

All the values are expressed as mean $\pm$ SD ( $n$ =3)

**Table 3** Fold increase in NO released from RSF pre-treated with IL-1 $\beta$  and challenged by SF

Sr. No.	Patients	NO release after 48 h ( $\mu$ M/ml), mean $\pm$ SD	NO fold increase after 48 h			NO release after 72 h ( $\mu$ M/ml), mean $\pm$ SD	NO fold increase after 72 h		
			Untreated control	IL-1 $\beta$ control	Non-OA patient		Untreated control	IL-1 $\beta$ control	Non-OA patient
1	IL-1 $\beta$ control	37.16 $\pm$ 2.0	2.9	1.0	1.2	58.04 $\pm$ 3.0	2.5	1.0	2.9
2	Patient 1	27.45 $\pm$ 5.3	2.1	0.7	0.9	29.61 $\pm$ 1.9	1.3	0.5	1.5
3	Patient 2	36.47 $\pm$ 2.7	2.8	1.0	1.2	49.41 $\pm$ 5.6	2.1	0.9	2.5
4	Patient 3	148.53 $\pm$ 9.3	11.4	4.0	4.9	234.41 $\pm$ 17.6	10.1	4.0	11.8
5	Patient 4	51.63 $\pm$ 5.4	4.0	1.4	1.7	72.25 $\pm$ 7.5	3.1	1.2	3.6
6	Patient 5	113.24 $\pm$ 6.2	8.7	3.0	3.8	186.18 $\pm$ 6.7	8.0	3.2	9.4
7	Patient 6	37.45 $\pm$ 3.5	2.9	1.0	1.2	22.55 $\pm$ 10.1	1.0	0.4	1.1
8	Non-OA patient	30.16 $\pm$ 1.6	2.3	0.8	1.0	19.85 $\pm$ 0.9	0.9	0.3	1.0

All the values are expressed as mean $\pm$ SD ( $n=3$ )

24 h) and further challenged with SF is summarized in Fig. 1b. SF from patient-3 (KL grade 3) showed 4.9-fold and 11.4-fold increase in NO after 48 and 72 h, respectively, when compared to SF from non-OA control [48 h ( $p<0.001$ ), 72 h ( $p<0.001$ )]. These results are summarized in Table 3. In comparison with non-OA control, patient 5 revealed 3.8-fold ( $p<0.001$ ) and 9.4-fold ( $p<0.001$ ) NO increase after 48 and 72 h, respectively. This NO increase can be correlated with recent traumatic history reported by this patient. Surprisingly, SF from early OA (patient 6) and non-OA control SF, found to reduce inflammation induced by IL-1 $\beta$ . On the other hand, KL grade 4 patient's SF generated lesser amount of NO, compared to non-OA patient.

**RSF pre-treated with LPS and challenged with SF.** Figure 1c shows NO release pattern when LPS pre-treated (10  $\mu$ g/ml, 24 h), RSFs were challenged with SF. Patient 3 showed 3-fold ( $p<0.001$ ) NO rise after 48 h and 4.7-fold ( $p<0.001$ ) NO increase at 72 h compared to non-OA patient (Table 4). Patient 4 and patient 5 (KL-2) doubled NO release at 48 h

and 4-fold NO increase after 72 h ( $p<0.001$  and;  $p<0.001$ , respectively). SF from patient-4 (KL grade 2) and non-OA SF reduced LPS-induced NO release. Similarly, patient 1 (KL grade 4) showed no response after 48 and 72 h whereas patient 2 estimated 1.5-fold and 2.4-fold elevated NO levels.

## Discussion

SF obtained from OA patients revealed enhanced levels of pro-inflammatory agents like IL-1 $\beta$ , NO, and GAG, which play a key role in initiation and progression of OA pathology (Goldring 2000; Taylor and Gallo 2006). SF repositors plethora of biochemicals in the form of signals, transmitted between chondrocytes and synoviocytes. Proteomic analysis of SF from OA patients revealed abundance of pro-inflammatory as well as anti-inflammatory factors (Goldring and Otero 2011; Sohn *et al.* 2012). A delicate balance between these factors constitutes homeostasis in healthy knee joint and also thought to be critical to maintain cartilage integrity and

**Table 4** Fold increase in NO released from RSF pre-treated with LPS and challenged by SF

Sr. No.	Patients	NO release after 48 h ( $\mu$ M/ml), mean $\pm$ SD	NO fold increase after 48 h			NO release after 72 h ( $\mu$ M/ml), mean $\pm$ SD	NO fold increase after 72 h		
			Untreated control	LPS control	Non-OA patient		Untreated control	LPS control	Non-OA patient
1	LPS control	58.04 $\pm$ 3.0	4.5	1.0	2.0	47.25 $\pm$ 5.7	2.0	1.0	1.8
2	Patient 1	25.10 $\pm$ 1.7	1.9	0.4	0.9	26.27 $\pm$ 0.3	1.1	0.6	1.0
3	Patient 2	41.77 $\pm$ 3.3	3.2	0.7	1.5	61.17 $\pm$ 16.3	2.6	1.3	2.4
4	Patient 3	87.35 $\pm$ 2.2	6.7	1.5	3.0	118.92 $\pm$ 6.2	5.1	2.5	4.7
5	Patient 4	76.65 $\pm$ 3.8	5.9	1.3	2.7	93.63 $\pm$ 4.0	4.0	2.0	3.7
6	Patient 5	61.67 $\pm$ 5.3	4.8	1.1	2.2	114.26 $\pm$ 4.5	4.9	2.4	4.5
7	Patient 6	36.66 $\pm$ 1.9	2.8	0.6	1.3	27.10 $\pm$ 2.2	1.2	0.6	1.1
8	Non-OA patient	28.65 $\pm$ 1.8	2.2	0.5	1.0	25.19 $\pm$ 3.5	1.1	0.5	1.0

All the values are expressed as mean $\pm$ SD ( $n=3$ )



healthy turnover. It could be hypothesized that advancement of OA progressively vitiates SF with accumulation of pro-inflammatory factors as evident from Table 1, leading to inflammation and progressive cartilage degeneration.

To test the above mentioned hypothesis, a cell-challenge experiment was designed with cultured primary RSFs, which were challenged by SF obtained from OA patients. This experiment allowed us to evaluate cumulative action of inflammatory and anti-inflammatory factors present together in SF. Induced NO released from these challenged RSF was measured as indicator of inflammation. A marked increase in inflammation upon SF treatment was seen over the base level of untreated cells, endorsing our hypothesis. Interestingly, a clear trend of increasing inflammation in RSF matched with the patient's KL score, which reiterates increasing accumulation of inflammatory factors with progression of OA. In other words, SF tends to become increasingly inflammatory to synoviocytes with severity of the disease. The present experiment revealed interesting facts; as seen in Table 1, NO levels in SF did not correlate with patient's radiological grade. However, inflammatory response in RSF induced by the same SFs was found in coherence with the patient's KL grade. This suggests plurality in inflammatory factors present in SF.

Though accumulation of pro-inflammatory markers in SF is evident from the earlier studies; SF from KL grade 4 failed to trigger the inflammation in RSF. This could be explained considering that the cartilage is completely degenerated at this stage and the residual chondrocytes are too less to release enough pro-inflammatory factors.

In another set of experiments, RSF pre-treated with IL-1 $\beta$  and LPS for 24 h were further challenged with the patient's SF for 48 and 72 h. Pre-treatment of IL-1 $\beta$  and LPS simulated inflammation in synoviocytes, similar to arthritic knee, injury, and infection. SFs from patients with severe OA (grade 3) further enhanced inflammatory response in RSF (Fig. 1b, c). More surprisingly, SF from patient 6 (KL grade 1) reduced the inflammation induced by IL-1 $\beta$  and LPS both. This trend was confirmed as well by using SF obtained from a non-OA patient (Tables 3 and 4). These results suggested that SF from non-OA and early OA patients can potentially shield synovial inflammation. Different types of proteins like anti-TNF (Mcneaney *et al.* 2004) and IL-1Ra, anti-proteinase, anti-trypsin agents, etc. were detected in SF collected from OA as well as RA patients (Sohn *et al.* 2012), which perhaps was responsible for buffering the inflammatory effects of cytokines as observed here. With progression of OA, levels of these anti-inflammatory agents may deplete initially giving way to low-grade inflammation that progress further with OA severity. These interesting findings, however, need to be confirmed on a wider number of patients, which may offer significant therapeutic application in treatment of osteoarthritis.

**Acknowledgments** We are thankful to Pallavi Nirmal and Shama Aphale for preparation of rat synoviocytes in-house. This work was supported by funding from the Bharati Vidyapeeth University, Pune, India.

## References

- Bell AL, Magill MK, McKane R, Irvine AE (1995) Human blood and synovial fluid neutrophils cultured in vitro undergo programmed cell death which is promoted by the addition of synovial fluid. *Ann Rheum Dis* 54:910–915
- Ding CH, Li Q, Xiong ZY, Zhou AW, Jones G, Xu SY (2003) Oral administration of type II collagen suppresses pro-inflammatory mediator production by synoviocytes in rats with adjuvant arthritis. *Clin Exp Immunol* 132:416–423
- Feelisch M (2008) The chemical biology of nitric oxide—an outsider's reflections about its role in osteoarthritis. *Osteoarthritis Cartilage* 16: S3–S13
- Fransen M, Bridgett L, March L, Hoy D, Penserga E, Brooks P (2000) The epidemiology of osteoarthritis in Asia. *Int J Rheum Dis* 14:113–121
- Goldring MB (2000) Osteoarthritis and cartilage: the role of cytokines. *Curr Rheumatol Rep* 2:459–465
- Goldring MB, Otero M (2011) Inflammation in osteoarthritis. *Curr Opin Rheumatol* 23:471–478
- Hardy RS, Hülso C, Liu Y, Gasparini SJ, Fong-Yee C, Tu J, Stoner S, Stewart PM, Raza K, Cooper MS, Seibel MJ, Zhou H (2013) Characterisation of fibroblast-like synoviocytes from a murine model of joint inflammation. *Arthritis Res Ther* 15:R24
- Hassanali SH (2011) Osteoarthritis: a look at pathophysiology and approach to new treatments: a review. *East Afr Orthop J* 5: 51–57
- Karan A, Karan MA, Vura P, Erten N, Tascioglu C, Aksoy C, Canbaz M, Oncel A (2003) Synovial fluid nitric oxide levels in patients with knee osteoarthritis. *Clin Rheumatol* 22:397–399
- Kellgren JH, Lawrence JS (1957) Radiological assessment of osteoarthritis. *Ann Rheum Dis* 16:494–502
- Mattielo-Rosa SMG, Cintra Neto PFA, Lima GEG, Pinto KNZ, Cohen M, Pimental ER (2008) Glycosaminoglycan loss from cartilage after anterior cruciate ligament rupture: influence of time since rupture and chondral injury. *Rev Bras Fisioter* 12:64–69
- Mcneaney T, Baethge BA, Cao S, Alam R, Lisse JR, Westlund KN (2004) Excitatory amino acids, TNF $\alpha$ , and chemokine levels in synovial fluids of patients with active arthropathies. *Clin Exp Immunol* 137:621–627
- Mundt LA, Shanahan K (2011) Synovial fluid: Graff's textbook of routine urinalysis and body fluids, 2nd edn. Lippincott Williams & Wilkins, a Walter Kluwer business, Walnut Street, pp 253–263
- Sellam J, Berenbaum F (2010) The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis. *Nat Rev Rheumatol* 6:625–635
- Smith MD (2011) The normal synovium. *Open Rheumatol J* 5:100–106
- Sohn DH, Sokolove J, Sharpe O, Erhart JC, Chandra PC, Lahey JL, Lindstorm TM, Hwang I, Boyer KA, Andriacchi TP, Robinson WH (2012) Plasma proteins present in osteoarthritic synovial fluid can stimulate cytokine production via Toll like receptor 4. *Arthritis Res Ther* 14:R7
- Sumantran VN, Joshi AK, Boddul S, Koppikar SJ, Warude D, Patwardhan B, Chopra A, Chandwaskar R, Wagh UV (2011) Antiarthritic activity of a standardized, multiherbal, ayurvedic formulation containing boswellia serrata: in vitro

- studies on knee cartilage from osteoarthritis patients. *Phytother Res* 25:1375–1380
- Taylor KR, Gallo RL (2006) Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. *FASEB J* 20:165–169
- Wang J-H, Shih K-S, Liou J-P, Wu Y-W, Chang AS-Y, Wang K-L, Tsai C-L, Yang C-R (2012) Anti-arthritic effects of magnolol in human interleukin 1 $\beta$ -stimulated fibroblast-like synoviocytes and in a rat arthritis model. *PLoS One* 7:e31368