Soluble inflammatory mediators of synoviocytes stimulated by monosodium urate crystals induce the production of oxidative stress, pain, and inflammation mediators in chondrocytes : Secretome of synoviocytes induces chondrocyte damage

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Abstract

We hypothesized that the secretion of inflammatory mediators from synoviocytes affects the chondrocyte homeostasis of articular cartilage. This study was a preliminary attempt to elucidate the molecular mechanisms by which soluble mediators obtained from activated synoviocytes induce oxidative stress and inflammation in chondrocytes. We measured the concentrations of interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), nerve growth factor (NGF), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and nitric oxide ($NO^{\bullet}$) from articular human cells. First, we created a conditional basal medium by exposing synoviocytes (HS) to monosodium urate crystals (CBM). The chondrocytes were exposed to either CBM (CCM), urate crystals directly (CMSU), or remained untreated (CC) as a negative control. Data were analyzed by ANOVA tests; Bonferroni test was performed for multiple comparisons between groups. Interestingly, we observed that mediators of inflammation and oxidative stress were significantly higher in CCM than CMSU and CC groups ($P<0.01$). The specific concentrations were as follows: 19.85 ng/mL of IL-6, 9.79 ng/mL of IL-8, 5.17 ng/mL of NGF, and 11.91 ng/mL of MCP-1. Of note, we observed the same trend for reactive oxygen and nitrogen species ($P<0.001$). Soluble mediators secreted by synoviocytes after being activated with MSU crystals (as observed in individuals who present gout attacks) trigger chondrocyte activation intensifying the articular inflammatory, oxidative, and pain states that damage cartilage in OA; this damage is more severe even when compared to HC directly exposed to monosodium urate crystals.

Key Points

- *The molecular relation between MSU depositions and cartilage damage could be mediated by pro-inflammatory soluble mediators and oxidative molecules.*
- *The secretion of pro-inflammatory mediators by activated synoviocytes is more harmful to chondrocytes than a direct activation in the chondrocyte culture.*
- *Under this model, there is an important imbalance in the matrix homeostasis due to changes in several chemokines, cytokines, and other factors such as NGF, as well as oxidative mediators.*

Keywords

Gout. Osteoarthritis. Oxidative mediators. Pro-inflammatory Interleukins. Secretome-activated synoviocytes

Introduction

Osteoarthritis is a degenerative pathology characterized by irreversible alterations of the cartilage, synovial membrane, and subchondral bone that eventually lead to a deterioration of the joint architecture [1]. On the other hand, gout is a metabolic inflammatory condition clinically characterized by acute, self-limiting, recurrent inflammatory attacks caused by monosodium urate crystals (MSU) deposits in joints and peri-articular tissues. The chronic gouty arthritis state is characterized by tophaceous deposits and morphological-structural damage [2]. Considering that gout is characterized by a pro-inflammatory state within the joints, different studies have proposed an association between MSU deposition and osteoarthritis (OA) [3].

In addition, clinical controlled and radiographic studies have demonstrated that joints that had suffered gout attacks are more prone to show clinical evidence of cartilage damage and OA, suggesting that the localized deposition of MSU may be a predisposing factor for OA [4,5]. This evidence has been confirmed in corpses where individuals with MSU showed more readily deposits in osteoarthritic cartilage. The most commonly affected joints by both conditions, gout and OA, are the first metatarsophalangeal joint, mid-foot, knee, and the small joints of the hands [6]. Furthermore, these conditions share not only the same physical localization but also common risk factors, such as obesity and aging [7,8,9]. It is known that during a gout attack, MSU crystals activate NALRP-3 inflammasome (LRR and PYD domains containing protein) in monocytes, causing the release of interleukin-1β (IL-1β) and interleukin 8 (IL-8) [10,11]; additionally, reactive oxygen species (ROS) including the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS) such as nitric oxide ($NO^{\bullet}$) are also released. All these molecules generate a pro-oxidant, pro-inflammatory microenvironment causing injury to the joints [12]. The injury is caused not only by cytokines secreted by synovial tissue–resident cells but also by a joint-infiltrating immune cell including neutrophils and monocytes which are recruited mainly by monocyte chemoattractant protein-1 (MCP-1) in the case of monocytes [13]. These immune cells secrete pro-inflammatory molecules that generate tissue lesions, loss of function, and pain sensation characteristics of different rheumatic diseases. In this sense, it is known that the nerve growth factor (NGF) is fundamental in mediating chronic pain in individuals with OA [14]. In terms of gout, it has been observed that the production of H_2O_2 within the synovium after being exposed

to MSU crystals contributes significantly to painful and pro-inflammatory responses in a murine model [15]. Despite the well-characterized mechanisms that contribute to nociceptive pain in both conditions, the dynamics between the two different pain mediators NGF and H_2O_2 during an acute gout attack and its relationship with the development of osteoarthritis is yet poorly understood. Therefore, the aim of the present study was a preliminary approach to elucidate the molecular mechanisms that link chondrocyte activation with MSU depositions through an in vitro model.

Materials and methods

The ethics committee of the National Institute of Rehabilitation approved this study (Ref. INR-08/11). All the procedures followed the ethical standards of the Helsinki Declaration amended in 2008.

Study population

Biological samples from remnants of synovial membrane and articular cartilage were obtained from four individuals who underwent arthroscopic knee repair surgery caused by cruciate ligament damage. The participants were informed about the objective of the study; they all signed an informed consent approved by the Research Committee of the National Rehabilitation Institute (INR) (Ref. INR-08/11).

Isolation and primary cellular culture of synoviocytes and chondrocytes

Tissue samples were disaggregated by mechanical-enzymatic methods previously reported by our group [16], in order to isolate synoviocytes (HS) and chondrocytes (HC). During the fourth passage, cultures were characterized to ensure they maintained their phenotypic traits, as it has been reported [17].

Experimental model

To evaluate the effect of synoviocytes activated on the chondrocyte homeostasis, we studied the following groups:

A conditioning basal medium (CBM) was generated by exposure of HS cells to monosodium urate (MSU) crystals at a concentration of 75 µg/mL for 24 h. The MSU crystals were prepared following the same procedure as previously published by our group [16]. The concentrations of different chemokines and cytokines were measured to characterize the CBM (Table 1). The CBM medium was mixed with fresh supplemented DMEM medium in a 1:1 ration (CBM group); one human chondrocyte culture (HC) was supplemented for 24 h more (CCM group). One HC culture was exposed directly to MSU crystals in the same way as the CBM group to evaluate the direct effect of MSU on HC cultures (CMSU group). The control group was a set of HC cells cultured (CC). For both synoviocytes and chondrocytes, we started the treatments of the cell lineages at 80% confluency and kept them under their respective conditions until the end of the experiment (Fig. 1).

Pro-inflammatory protein quantification in HC culture medium

The measurement of cytokine profile from HC culture supernatant was carried out using the Milliplex Human Adipocyte Magnetic Bead Panel kit (Millipore-Merck, Darmstadt, Germany) for the soluble mediators IL-6, IL-8, IL-1β, NGF, and MCP-1. All samples, standards, and quality controls were assayed in a MAGPIX®-Luminex system (Luminex, Merck Millipore, USA) in triplicate following the manufacturer's instructions.

Intracellular measurements of reactive oxygen and nitrogen species

The ROS ($O_2^{\bullet}$, H_2O_2) and RNS ($NO^{\bullet}$) production was assessed through image cytometry (Tali, Life Technologies) following the manufacturer's instructions. The $O_2^{\bullet}$ concentration was quantified by dihydroethidium (DHE) oxidation at 530 ± 20 nm, while H_2O_2 was quantified by 5-,6-carboxy-2′,2′,7′-dichlorofluorescein diacetate (carboxy-H2DCFDA) at the same emission range. The $NO^{\bullet}$ was determined by 4 amino-5-methylamino-2,7-dichlorofluorescein (DAF-FM) at an emission of 480 ± 20 nm. Results were expressed in relative fluorescent units (RFU).

Statistical analysis

Data are shown as mean ± standard deviation (SD). The levels of cytokines, NGF and MCP-1, as well as the production of ROS and RNS were analyzed among the experimental groups using one-way analysis of variance. Bonferroni test was performed for multiple comparisons. The confidence level considered was 95% (α=0.05). Statistical analyses were conducted using commercially available statistical software (Graph Pad Prism v6.01 CA, USA) and STATA v12.1 (Stata Corporation, College Station, TX, USA).

Results

Pro-inflammatory and pain mediators panel array

In the supernatant from the CMSU group, the levels of IL-6 were significantly higher (15.56 ± 0.48 ng/mL) than the CC group (6.01 ng/mL). In a similar way, the IL-8 levels observed from the CMSU group were significantly higher (7.18 ± 0.64 ng/mL) than those from the CC group (5.79 ± 0.18 ng/mL). Interestingly, when CBM was added to the chondrocyte culture for 24 h (CCM group), both IL-6 and IL-8 levels increased significantly. This increase was even higher when compared with the CBM and the CMSU groups, showing levels of 19.85 ± 2.90 ng/mL (P<0.01) for IL-6 and 9.79± 0.73 ng/mL for IL-8 (P<0.01) (Fig. 2a, b).

Likewise, there was a slight increase in the NGF levels in the CMSU group (4.1 ± 0.79 ng/mL) compared with the CC group (3.00 ± 0.08 ng/mL) without reaching statistical significance. Nevertheless, the levels of NGF in the CCM group were significantly higher (5.17± 0.47 ng/mL) than in the CMSU and CC groups (P<0.01) (Fig. 2c).

Furthermore, the levels of MCP-1 in the CMSU group were 8.43 ± 0.63 ng/mL, significantly higher than in the CC group which showed a concentration of 4.17 ± 0.31 ng/mL (P<0.01). Interestingly, the level of this chemokine in the CCM group was even higher 11.91± 0.56 ng/mL than the levels observed in the CMSU group (P<0.01) (Fig. 2d). It is important to say that IL-1β levels were below the reliable detection range of the multiplex array (data not shown).

Oxidative stress state

We also observed an increase in ROS and RNS in all the groups. In the CMSU group, the fold change of $O_2^{\bullet-}$ was 1.20, 1.28 for H_2O_2, and 1.22 for $NO^{\bullet}$; these fold changes were not significantly different when normalized and compared with the CC group ($P<0.05$). However, in the CCM group, fold changes for all these reactive oxygen and nitrogen species when normalized were significantly higher than the CC group ($P<0.001$). In detail, the fold change for $O2^{\bullet-}$ was 1.49, 1.74 for H_2O_2 and 1.44 for $NO^{\bullet}$ (Fig. 3a–c).

Discussion

It is known that in microcrystalline arthropathies such as gout, MSU crystals induce inflammation, pain, and an articular degenerative process [18]. This inflammation is mediated by cytokines and ROS/RNS that create a synergistic effect that promote a pro-inflammatory and pro-oxidant microenvironment in the joints [19]. Furthermore, it has been observed that a dysregulation of hypoxia and reoxygenation levels in the joint induces a secretory phenotype of the pro-inflammatory molecules senescence-associated [20]. In the clinical practice, there is an increase in the number of individuals with gout and coexistent OA, suggesting an association between the two diseases [3,4,5]. Although clinical evidence linking gout with OA has increased, the specific molecular pathway that explains this phenomenon remains unknown.

A potential pathway is through the secretome, where molecules secreted by a specific tissue can affect adjacent tissues or even have a systemic effect [21]. Under this premise, we evaluated and compared the molecular array differences of chondrocytes exposed to molecules secreted by MSU-stimulated synoviocytes.

We found that the secretion of pro-inflammatory mediators by synovial cells participates in the chondrocytes activation process by increasing the production of key cytokines and chemokines such as IL-6, IL-8, and MCP-1, as well as free radicals and the nociceptive pain modulator NGF.

In particular, we observed a significant increase in IL-6 cytokine and IL-8 chemokine production in chondrocytes that were treated with CBM. In the case of IL-6, its action is relevant in the inflammatory process as well as in the activation of the hypoxia 2-α inducible factor associated with articular cartilage degeneration [22]. The increase of IL-

8 has also been associated with hyperalgesia states and macrophage recruitment that intensify the inflammatory process, resulting in both degeneration and loss of the articular cartilage, as it is observed in individuals with OA [23].
In terms of the recruitment of inflammatory cells, a state commonly reported in individuals with gout and OA, we found higher levels of MCP-1 in chondrocytes treated with CBM. These results agree with previous studies that have demonstrated that chondrocytes activated with either IL-1, TNF-α, or LPS have the ability to secrete MCP-1 [24]. Interestingly, higher levels of MCP-1 have been associated with worse degrees of RA, confirming the key role of this chemokine in other rheumatic inflammatory diseases [24].
The higher level of cytokines observed in chondrocytes stimulated with CBM was paired with an intracellular increase of ROS and RNS, which jointly could trigger severe, irreversible cartilage damage [25]. The inflammation and oxidative stress states that we observed, agree with a previous study where major disturbances in the extracellular matrix such as an oxidation profile of type II collagen-like proteins, inhibition of proteoglycan synthesis, as well as major damage to lipids and DNA were observed [12].
Our results suggest that the pro-inflammatory mediators stemming from activated synoviocytes may have a larger impact on articular degeneration mediated no only by chemokines and cytokines but also by free radicals, when compared to the effect of MSU crystals alone. In this sense, it has been widely reported that ROS ($O_2^{\bullet-}$, H_2O_2) plays an essential role in cartilage deterioration through chondroptosis [25,26]. Likewise, an increase in $NO^{\bullet}$ as observed in chondrocytes exposed to the CBM might be a powerful cartilage damage pathway, as it inhibits the synthesis of the proteoglycans required to maintain homeostasis in the ECM [27]. Furthermore, $NO^{\bullet}$ expression has been reported to be key in signaling pathways for macrophages and other inflammatory cells that intensify the inflammatory and degenerative articular state [28].
Besides the pro-inflammatory and pro-oxidant response, our results show preliminary in vitro evidence, of the very intense pain suffered during a gout attack that, to our knowledge, is yet poorly understood. In our experiments, we observed that in chondrocytes exposed to the CBM, the protein responsible for triggering nociceptive pain NGF, increased significantly, in contrast to the chondrocytes directly exposed to MSU, where this did not happen. We hypothesized that the NGF production in chondrocytes

might regulate pain signaling by overexpressing neural pro-inflammatory molecules, namely, substance P, the calcitonin-gene-related peptide (CGRP), and serotonin, among many others [14]. Additionally, Walsh et al. [29] and Bonnet et al. [30] proved that NGF favors the angiogenesis process in articular cartilage bolstering the inflammatory process [29]. It is important to note that the angiogenesis process derived from NGF overexpression could mark the beginning of a cartilage degenerative process due to the oxidant system imbalance in the hypoxic cells of the joints, as they are sensitive to changes in oxygen tension [30].

In addition to the NGF overexpression, the H_2O_2 production also increased in chondrocytes exposed to CBM. This free radical regulates the production of CGRP, a neurotransmitter that takes part in the nociceptive pain conduction process [31].

To our understanding, the relation between the initiation of OA and MSU deposits is still obscure. In conclusion, our preliminary findings suggest that upon activation by MSU crystals, synoviocytes secrete inflammatory, and pro-oxidant molecules that activate cells in adjacent tissues (like those in the articular cartilage), thus initiating the articular degenerative process and OA development. Additionally, this locally inflammatory microenvironment could be boosted by the recruitment of pro-inflammatory leukocytes by MCP-1. Finally, we stress the fact that synovial secretome fosters the release of NGF and H_2O_2 in chondrocytes, cartilage cells that may be responsible for the local nociceptive pain caused during an acute gout attack.

Although these results are promising, this study was limited as we were not able to measure metalloproteinases or other key molecules related to chondrocyte hypertrophy such as IL-1β that could corroborate the degenerative state of the chondrocytes in vitro. The relevance of IL-1β in models of inflammation triggered by the secretion of other neighboring cells have been studied by Bao and colleagues, where they found that they could suppress the inflammatory state that characterizes OA development by the supplementation of synoviocytes cultures with oleanolic acid, via de NF-kB axis [32]. Additionally, it is necessary to corroborate these findings with deep in vitro studies such as co-cultures in order to elucidate the mechanism of damage in the cartilage and relate these data to the phenomena observed in the clinic where the majority of individuals with gout show symptoms of local OA along with severe pain.

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Author notes

Alberto López-Reyes and Daniel Medina-Luna contributed equally to this work.

Informed consent

A written informed consent was obtained from all participants prior to the collection of biological samples.

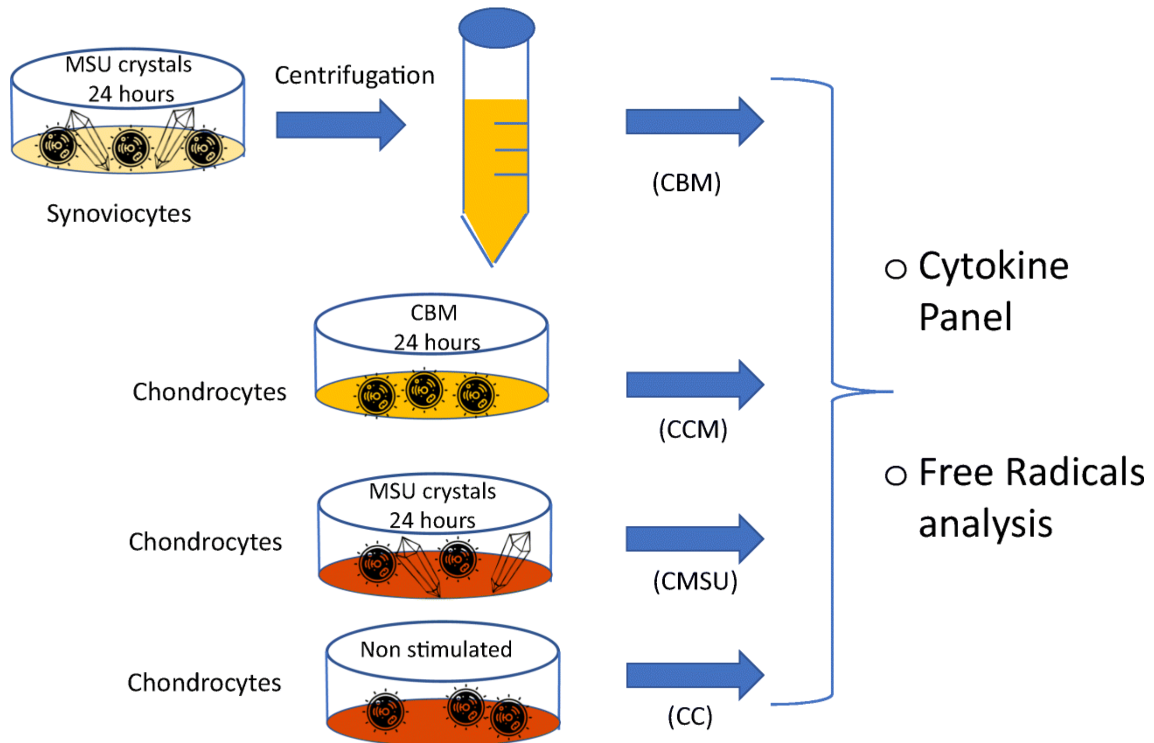
Disclosures

None.

Table 1 Basal levels of the cytokines in the conditioning basal medium

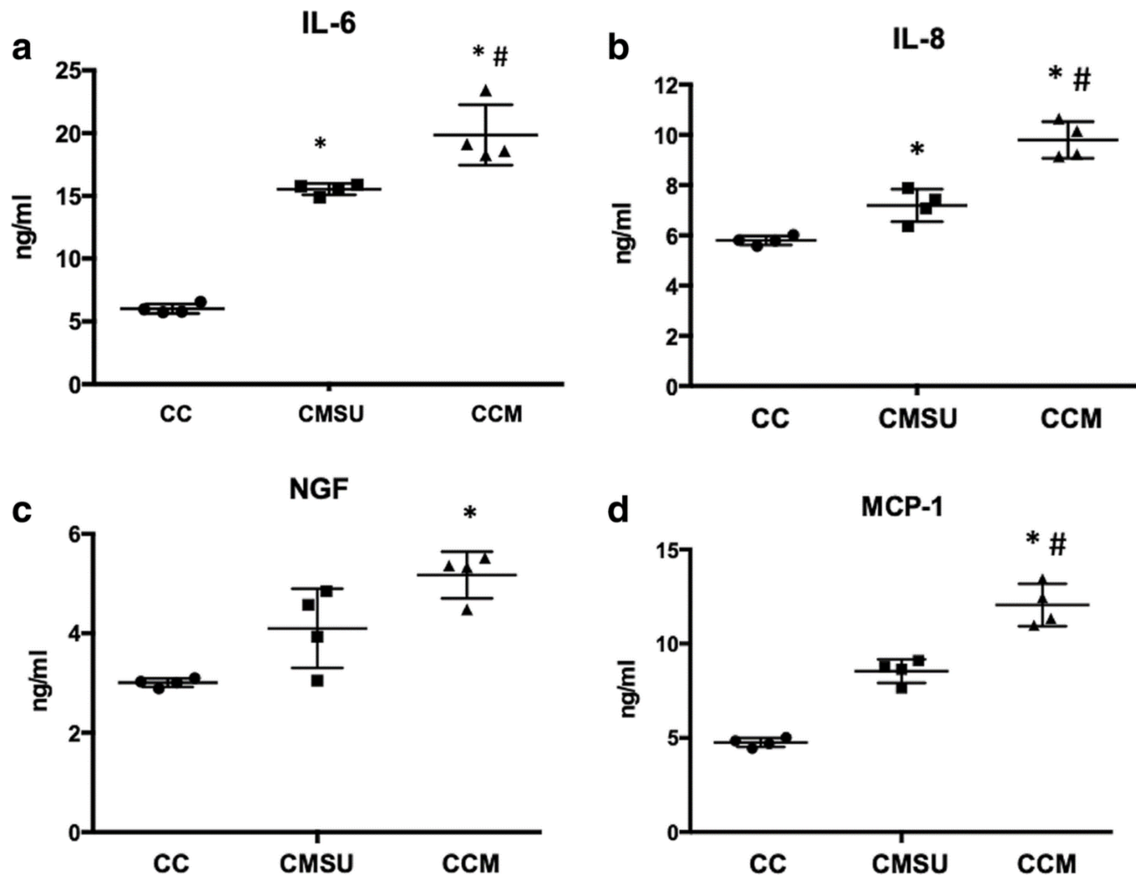
	IL-1 (ng/mL)	IL-8 (ng/mL)	MCP-1 (ng/mL)	NGF (ng/mL)
CBM	16.98 ± 0.03	8.4598 ± 0.08	7.67± 0.01	3.92± 0.19

Fig. 1



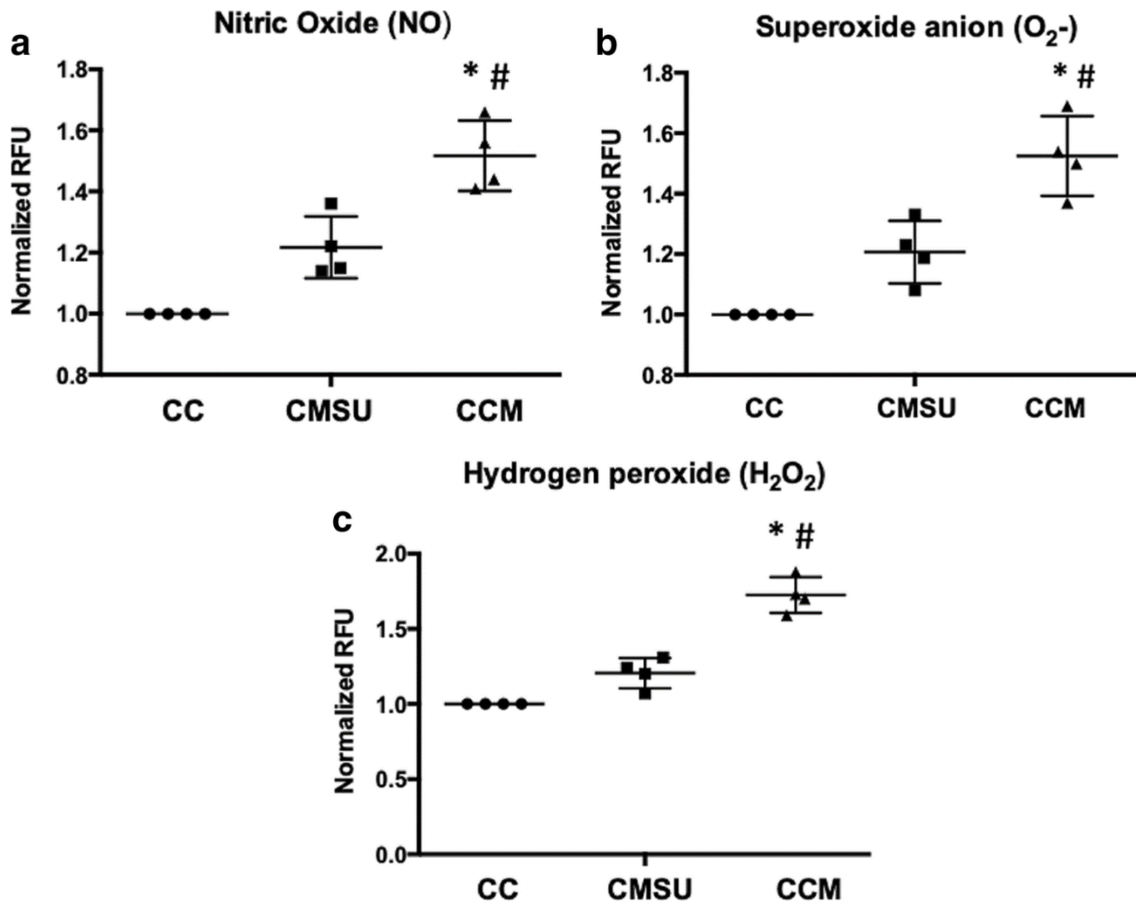
Conditioning medium transfer system diagram. A synoviocyte culture was incubated for 24 h with MSU crystals. The supernatant was transferred to a clean tube and centrifuged at 1000×*g* for 15 min, then it was used as a conditioning basal medium (CBM). Human chondrocytes were incubated with 50% CBM and 50% fresh culture medium for 24 h, the media from the human chondrocytes cultured with CBM was then collected and analyzed (CCM). As positive control, a separate culture of human chondrocytes was exposed for 24 h to MSU crystals and the supernatant was harvested (CMSU). As negative control, we culture some human chondrocytes and left them without any exposure (CC)

Fig. 2



IL-6 (**a**), IL-8 (**b**), NGF (**c**), and MCP-1 (**d**) concentrations in the supernatant. CC, non-stimulated chondrocytes; CCM, chondrocytes + conditioning basal medium; CMSU, chondrocytes + MSU crystals. *P<0.05, data compared to CC; # P<0.05, data compared to CMSU

Fig. 3



Intracellular production of ROS, superoxide anion (**a**), hydrogen peroxide (**b**), nitric oxide (**c**). CC, non-stimulated chondrocytes (normalized to 1); CCM, chondrocytes + conditioning basal medium; CMSU, chondrocytes + MSU crystals. Results are expressed as fold change. *$P<0.05$, data compared to CC; # $P<0.05$, data compared to CMSU