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# The B-Box of HMGB1 Amplifies Nitric Oxide Production in Human Osteoarthritis-Affected Cartilage

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## Abstract

The effects of up regulated high mobility group box-1 (HMGB1) and nitric oxide (NO) contributes to pro-inflammatory activity in the synovial tissue and matrix depleting activity in human osteoarthritis (OA)-affected cartilage. The 215-amino acid HMGB1 protein has several functional domains with multiple over lapping functions. Since HMGB1 can induce NO production in cartilage, we tested the requirements of different functional areas of HMGB1 that can modulate NO in human OA-affected cartilage in *ex vivo* conditions. Recombinant HMGB1 proteins and various deletions mutants of HMGB1 were generated and purified. Human OA-affected cartilage that spontaneously released NO in *ex vivo* conditions was utilized as a model to test the effect of HMGB1 (and various HMGB1 mutants) to modulate the natural release of NO in *ex vivo* conditions. HMGB1 and deletion mutants of HMGB1 which retained the B-Box of HMGB1 had the ability to significantly augment the spontaneous release of NO in OA-affected cartilage in 24 h. Removal of the C-terminal acidic region of HMGB1 further significantly amplified NO production, whereas the A-Box of HMGB1 had no significant effect on NO-production. Furthermore, HMGB1+A-Box had no significant effect on the NO-augmenting activity of HMGB1. This paper for the first time shows that the B-Box of HMGB1 harbors the NO-augmenting activity and the C-terminal acidic tail functions as a negative regulator of HMGB1's-NO-augmenting activity in human cartilage. Therapeutic intervention of the B-Box is a promising target to deter the detrimental effects of escalated levels and effects of both HMGB1 and NO in human OA-affected joints.

**Keywords: HMGB1; B-box; Nitric oxid; Cartilage; Arthritis**

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## Introduction

The high mobility group box-1 (HMGB1) is implicated in various pathophysiological conditions [1]. These include rheumatic diseases such as osteoarthritis (OA) [2,3], rheumatoid arthritis (RA) [4], and systemic lupus erythematosus (SLE) [5]. HMGB1 is a multifunctional protein which is functional in the nucleus, cytosol and extracellular milieu [1-4]. The 30 k Da HMGB1 protein has two multipurpose DNA binding motifs: the A-Box (aa 1-79) and the B-Box (aa 89-163) [6]. The B-Box also has: (a) multiple HMGB1-receptor binding sites such as lipo polysaccharide (LPS), toll-like receptors (TLR) and receptors for advance glycosylation product (RAGE); (b) pro inflammatory activity that can induce TNFα, IL-6 and IL-8 in inflammatory cells [1,6-8]. In contrast, the A-Box may exhibit anti-inflammatory [6-8]. The N-terminal of HMGB1 has nuclear functions and anti-inflammatory activity [6-10].

We have reported that HMGB1 is upregulated in human OA-affected cartilage as compared to non-OA-normal human cartilage [2]. Immunohistochemistry of normal and OA-affected cartilage slices also showed an increased number of HMGB1-positive chondrocytes throughout the OA-affected cartilage at different stages of differentiation [2]. The HMGB1 was not only located in the nucleus and cytosol, but it was also released outside the cartilage in *ex vivo* conditions [2]. Recently,we have shown that HMGB1 could significantly induce more than 50 different transcripts (including NFκB, MMPs, cytokineschemokines, adhesion molecules, S100, HSPs and iNOS) and also inhibited transcription of over 30 essential carbohydrates and proteins, which constitute the matrix in human cartilage [10]. Nitric oxide was spontaneously released (by endogenous

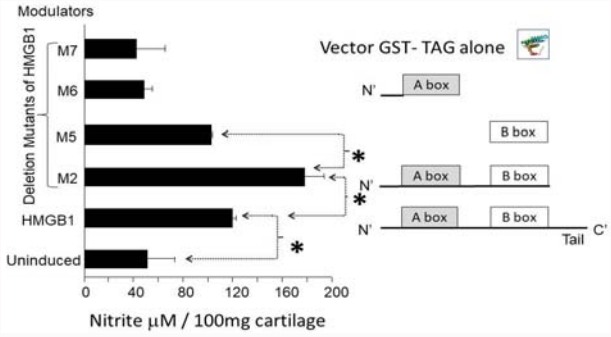


**Figure 1:** Effect of HMGB1 and its mutants on NO production in human OA-affected cartilage.

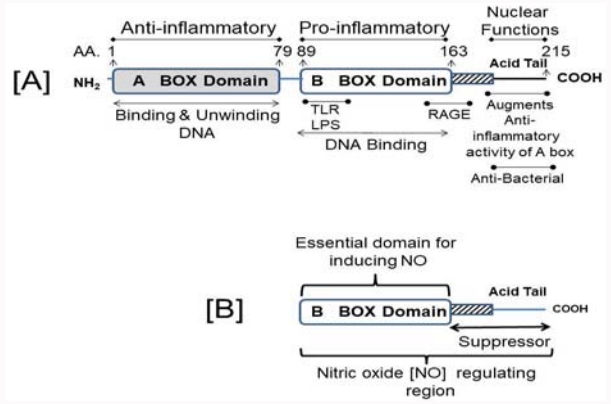


**Figure 2:** Schematic diagrams of functional domains of HMGB1.

stimulation) from human OA-affected cartilage in *ex vivo* conditions [11,12]. The excessive amounts of nitric oxide can exert detrimental effects on chondrocyte functions, during arthritis by the generation of peroxynitrite, inflammation (e.g., increased levels of MMPs and prostaglandins), inhibiting collagen and proteoglycan synthesis and enhancing chondrocyte apoptosis, leading to impaired cartilage homeostasis [12]. Together over expression of both HMGB1 and NO has adverse effects on human OA-affected cartilage on inflammation, cartilage degradation and repair [2,3,10]. Injection of HMGB1 into joints of mice induces arthritis, which could be blocked by neutralizing antibodies to HMGB1 [6-9] and iNOS$^{-/-}$ mice are immune to laboratory-induced arthritis [12].

Although recombinant HMGB1 is shown to induce mRNA of iNOS and nitric oxide in human OA-affected cartilage and chondrocytes [2,10], the specific region(s) responsible for regulation of NO by HMGB1 remains elusive in human OA-affected cartilage. In the current study, we further mapped the functionally active regions of HMGB1 that modulates the spontaneous release of NO in human OA-affected cartilage in *ex vivo* conditions.

## Materials and Methods

### Reagents

All endotoxin-free cell culture media and FCS were obtained from Gibco BRL.

### Generation and purification of native HMGB1 and mutant HMGB1

The purified native recombinant HMGB1 and mutants were provided by Dr. Kevin Tracy's Laboratory (The Feinstein Institute for Medical Research-LIJ School of Medicine, NY). Briefly, the endotoxin-free native recombinant HMGB1 and their mutants were cloned into the M7-GST vector. The recombinant HMGB1 was purified as previously reported [2,13,14] and tested for endotoxin contamination [2,13-15]. The proteins were also run on an SDS-PAGE gel to ascertain their purity.

### Human OA-affected cartilage and demographics

We only utilized cartilage from human OA-affected individuals based on the American College Rheumatologists (ACR) symptomatic criteria. These criteria included but not limited to radiographic findings, pain, morning stiffness, bony tenderness, and enlargement, ESR, RF, and finally the histology of the cartilage by the pathologist. Potential donors were diagnosed with OA by the Rheumatologists. This cartilage was obtained from donors who were undergoing knee replacement surgery for OA. All the tissues were made available after permission from the Institution Review Board for the use of human tissues in clinical research from New York University's Hospital for Joint Diseases. The OA-affected individual donors were about 51% Hispanics, 21% Caucasians, 17% African Americans, and 11% Asians with an average donor age of 60-85. All the clinical samples were administered via the Pathology Laboratory at HJD. OA-affected cartilage samples with a range of an OARSI score of 3-5 were used in the study [2,16]. We utilized about 20 samples of OA-affected cartilage in these studies. The OA-affected cartilage samples were not sufficient for a single experiment, and hence the cartilage sample were pooled and prepared for assays as described below. The classification of cartilage was based on OARSI scores [2,16].

### Human OA-cartilage explant assay for nitric oxide production

Human OA-affected cartilage from several donors was isolated, minced, mixed and distributed equally in 24 well plates containing Ham's F-12 medium (2 mL). The Ham's F12 media also contained 1% endotoxin-free human albumin (which was devoid of any oxidizing or reducing agent) in the presence and absence of 5 µg/ml of different samples of HMGB1 and/or its mutants [2,16]. Nitric oxide was estimated after 24 h for their stable end product, nitrite, from the cartilage supernatant. The medium or supernatant from each well was removed and evaluated for the levels of NO using a Griess assay [2,16].

### Data presentation and statistical analysis

The Student's t-test was utilized for statistical calculation [10,11]. the *p-value ≤ 0.05 was considered significant. The data are represented as µM/100 mg cartilage ± standard deviation (SD) where n was a minimum of 3 [10,11].

## Results and Discussion

HMGB1 and other DAMPS not only bind to a variety of primary receptors (such as TLR, LPS, RAGE) but also co-binds to several components of ECM such as heparin, proteoglycans, syndecan-1, sulfoglycolipids and phospholipids during signal transduction [1-3,10]. Furthermore, the redox conditions surrounding the HMGB1 modulate the effects of HMGB1 [1,3,8,10]. The avascular cartilage exhibits reduced oxygen tension. The gradient of $O_2$ in the cartilage can vary from 6% $O_2$ in the superficial zone to 1% $O_2$ in the deep zone of cartilage [12,17]. The generation of NO is more pronounced at 1% to 5% oxygen tension as compared to 20% oxygen tension within the cartilage [12,17]. Therefore, we utilized NO-producing organ cultures of human OA-affected cartilage (with the essential matrix

**Table 1:** Effect of A-Box (M6) on HMGB1's-NO augmenting activity in human OA-affected cartilage in ex-vivo conditions..

| Experimental Conditions | Nitrite µM/100 mg cartilage ± SD | P value / Cont* | P value / Expt$^{\#\theta}$ |
|---|---|---|---|
| M7-GST vector protein (5µg/ml)-Control (Cont) | 19 ± 6* | - | - |
| HMGB1 (5µg/ml) | 92 ± 9*$^{\theta}$ | ≤0.003 | ≤0.3$^{\theta}$ |
| HMGB1 (5µg/ml) + M7-GST vector protein (5µg/ml) | 113 ± 28*$^{\theta}$ | ≤0.004 | ≤0.3$^{\theta\#}$ |
| HMGB1 (5µg/ml) + A-Box-M6 (5µg/ml) | 136 ± 16*$^{\#}$ | ≤0.003 | - |

components and 3-D environment conditions) where HMGB1 may interact with chondrocytes and the critical matrix components to modulate NO production at a reduced oxygen tension [3,10,17].

The human OA-affected cartilage (uninduced) exhibits the spontaneous release of NO in *ex vivo* conditions as shown in Figure 1. There was no significant difference in the accumulation of NO in the presence of the GST-Vector alone (M7) as compared to the "uninduced" OA-affected cartilage. As expected, 5 µg/ml of HMGB1 (but not GST-vector tag) could significantly augment NO accumulation above "uninduced"-basal levels in 24 h (Figure 1). The recombinant B-Box (M5) alone, but not A-Box (M6) showed a significant increase in the NO levels as compared to "uninduced"-Basal levels of NO. Human OA-affected cartilage was incubated in a 24 well plate in triplicates in the presence and absence of HMGB1, various deletion mutants of HMGB1 (M2, M4, M5, M6) and vector GST-TAG alone (M7) and assayed for NO after 24 h. Data were expressed as µM/100 mg cartilage ± SD, where n=3 for each experiment. The P values were compared to between two conditions as shown as dotted arrows: (···>) where (*) designates significance of ≤ 0.05 (Figure 1). These observations suggested that the B-Box was essential for augmenting the levels of NO in human OA-affected cartilage. The effects of the B-Box on the induction of NO in cartilage are analogous to induction of TNFα and IL-6 by HMGB1 or B-Box in macrophages [6-8]. HMGB1 may also co-function with or via NO. For example, HMGB1 or B-box caused an alteration in gut barrier function in the presence of NO and 0N00$^{-}$ [18]. The functional role of B-Box *in vivo* (in inflammation) was further validated when injection of neutralizing antibodies raised against the B-Box significantly inhibited the lethal endotoxemia (or sepsis in mice) that was induced by cecal perforation [19]. These experiments suggest that the B-Box could recapitulate the lethality (of sepsis) similar to the full-length HMGB1 in Balb/C mice. These observations infer that the B-Box of HMGB1, which shares the highly conserved DNA-binding region and cytokine inducing activity [19] of HMGB1 and also harbors NO-augmenting activity as shown throughout this study.

Previous studies have demonstrated the inhibition of HMGB1-induced IL-6, TNFα, and efferocytosis by the C-terminal acidic tail of HMGB1 [9,20]. The C-terminal acidic region of HMGB1 also exhibits antibacterial and DNA-bending activity [21,22]. The HMGB1 constructs M2: (with the N' terminal+A-Box+B-Box), but devoid of the C-terminal tail showed a significant increase in NO as compared to HMGB1 treated cartilage (Figure 1). Our observations indicate that the C-terminal acidic region of HMGB1 may function as a suppressor for induction and/or augmentation of NO within HMGB1. These observations highlight the broad range of functions of the C-terminal acidic region of HMGB1 [9,20-22] and NO-suppressive activity as shown in this study.

The common functional denominator in HMGB1, and (M2 and M5 mutants) was the B-Box, which could augment NO production in human OA-affected cartilage. There was no significant effect of A-Box (M6) alone (Figure 1) or HMGB1 + M6 on (Table 1) or when human OA-affected cartilage was incubated with A-Box (M6) or vector GST-TAG (M7) for 24 h in triplicates in the presence and absence of HMGB1 [10] and assayed for NO. Data in Table 1 were expressed as µM/100 mg cartilage ± SD, where n=3 for each experiment. Student's t-test was used at the significant level of $p \leq 0.05$. p values show the comparison between control vector and other experimental condition. The p values between HMGB1+M7-GST vector and HMGB1$^{\theta}$ or HMGB1+M6$^{\#}$ was not significant ($p \leq 0.3$).

The NO production in the cartilage explant model was used for this study. The A-Box has been reported to function as an HMGB1 antagonist in experimental autoimmune encephalomyelitis, sepsis and macrophages [1,4-10,23]. In summary, our studies indicate that the A-Box (M6) alone or (A-Box+HMGB1) or (A-Box+B-Box) did not exhibit HMGB1-antagonistic activity on the spontaneous release of NO in OA-affected cartilage. These experiments suggest that the A-Box does not harbor the anti-NO-activity nor NO-augmenting activity in human OA-affected cartilage. Fu et al. [24] has shown HMGB1-A-Box inhibits IL-1β induced MMPs, NO and $PGE_2$ in human chondrocytes. This observation by Fu et al. [24] may make some sense because we have demonstrated that HMGB1 can induce mRNA for IL-1β in cartilage and chondrocytes [10]. However, the spectrum of gene expression arrays (pro and anti-inflammatory mediators) in human chondrocytes incubated with HMGB1 or IL-1β under the same conditions is distinct. Figure 2A show the various structural and functional domains of HMGB1 in macrophages as described [5-9]. The A-Box exhibits the anti-inflammatory and DNA-interacting region (Figure 2). The B-Box represents the pro-inflammatory area of B-Box, DNA binding regions and the sites of various receptors (TLR, LPS, and RAGE) binding regions. The acidic tail region which has other nuclear functions can also collaborate with the A-Box to unveil its anti-inflammatory activity. The acidic tail region also has anti-microbial activity [5-9]. Figure 2B summarizes the essential domain (B-Box) of human HMGB1 that is a prerequisite for augmenting the spontaneous release of NO in human OA-affected cartilage. The tail region seems to act as a suppressor for the regulation of NO by HMGB1.

Furthermore, B-Box induced the same common inflammtory mediator (such as MMPs, NO, and $PGE_2$) but via separate signaling pathways and several receptors. The lack of an effect on NO by HMGB1-A-Box in our system may be because of some distinct differences in the experimental approach: (a) use of monolayers of chondrocytes against whole cartilage samples, which involve matrix components (and other DAMPS) [1-3]. (b) Moreover, the difference in inducers, IL-1β *vs.* unknown endogenous inducer(s) of NO in OA-affected cartilage which facilitates the spontaneous release of NO. Indeed, A-Box+C-terminal tail-fusion proteins seem to augment inhibition of LPS induced cytokines (as compared to A-Box alone) in THP-1 cells [9]. Wang et al. [25] showed that multifunctional miR-142-3p inhibited HMGB1-mediated inflammation (IL-6, IL-1β, and TNFα) in chondrocyte via the NF-κB signaling pathway. Furthermore, the over expression of miR-142-3p inhibited the

progression of OA progression in mice *in vivo* [25]. It is entirely possible that miR-142-3p may also inhibit HMGB1-mediated NO production in human OA-affected chondrocytes. Since HMGB1 is differentially expressed in (a) normal and OA-affected cartilage, (b) different zones of cartilage and sub-cellular distribution of chondrocytes in various zones of the cartilage [2]. It would be more interesting to learn the differential expression of miR-142-3p in different types of Chondrocytes in normal and human OA-affected cartilage. It will give a better understanding of the role of miR-142-3p and the action of HMGB1 in cartilage.

In summary, we propose that the B-Box of HMGB1 is essential, and the C-terminal acidic tail may function as a repressor for optimization of the NO-inducing activity of HMGB1 in human OA-affected cartilage. The A-Box's anti-inflammatory activity (as observed in other cells) does not include NO modulating activity in human OA-affected cartilage. Given the pro-inflammatory, chemokine signaling, cartilage homeostasis, and matrix-depleting activity of HMGB1 in cartilage by chondrocytes in different stages of development [10], the multifunctional role of HMGB1 in human cartilage is complexand requires further studies. HMGB1, like NO, is a double edge sword with productive and destructive activity depending upon their location, the timing of development and concentration of HMGB1 and/or NO in the tissue milieu and the surrounding cells [1,11,12].

## Contribution

The project was performed in ARA's laboratory who also takes responsibility for the integrity of this research. All the authors were involved in the interpretation of the data and preparation of the manuscript.

## Competing Interests

ARA's salary was partially supported by a Translational Research and Target Discovery Contract in collaboration with Yamanuchi (Astellas) Pharmaceuticals.

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