RAGE Expression and Serum HMGB1 Levels in Rheumatoid Arthritis Patients and their Relations to Disease Activity

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Abstract
Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, which ultimately leads to the destruction of cartilage and bone in the affected joints. Synovial hyperplasia is a hallmark pathology of RA, and fibroblast-like synoviocytes play a critical role in RA pathogenesis by producing pro-inflammatory soluble factors or activating other immune cells. Consistent with the capacity of high-mobility group box 1 (HMGB 1) for immune activation and inducing inflammatory cytokine-like activity and the ability of single HMGB1 injection into rodent joint of inducing long-lasting destructive arthritis, this work was planned to study the serum level of HMGB 1 and the expression of its receptor RAGE in synovial tissues of patients with rheumatoid arthritis and their correlations with some clinical variables and methotrexate (MTX) therapy.

Subjects and Methods: Group IA was 15 cases of newly diagnosed RA patients without MTX therapy. Group IB was 33 cases of RA patients with MTX therapy. 28 apparently normal individuals matched for age and sex were taken as a control group (group II). In addition to routine laboratory investigations, serum HMGB 1 was measured by using commercial enzyme-linked immunosorbent assay (ELISA) kit and RAGE expression in synovial tissues was assessed using immunohistochemistry for all subjects.

Results: RAGE expression and serum HMGB1 were significantly higher in RA (group I) when compared with the control group. Also, they were significantly higher in RA patients without MTX therapy compared with those patients with MTX therapy. There were significant positive correlations between serum HMGB1 and some markers of disease activity (DAS-28, erythrocyte sedimentation rate and C-reactive protein), age of the patients and anti-CCP in group I. On the other hand, no significant correlations could be established between HMGB1 levels and duration of the disease, gender of patients and rheumatoid factor (p>0.05). However, there was a significant positive correlation between serum HMGB 1 and RAGE expression in synovial tissues in group I (p<0.01).

Conclusion: HMGB1 level and RAGE expression are increased in RA patients and may play important roles in the pathogenesis of the disease. Blockade of extracellular HMGB1 and RAGE expression may offer a novel therapeutic alternative for the treatment of RA. Further studies correlating HMGB1 and RAGE with autophagy and apoptosis are recommended.

Key Words: HMGB1 — RAGE — Rheumatoid arthritis.

Introduction
HIGH-MOBILITY group box 1 (HMGB1) is a highly conserved protein present in the nuclei of various types of cells. It has been identified as a nuclear DNA-binding protein that participates in maintaining nucleosome structure, regulating gene transcription, and modulating the activity of steroid hormone receptors [2,3]. Furthermore, HMGB1 has a role in V(D)J recombination, a mechanism of genetic recombination that is important in the generation of the immunoglobulin and T-cell receptor components of the immune system [4]. This protein is essential for survival, since HMGB1-deficient mice die due to hypoglycemia within 24h after birth [8]. On the other hand, it was clarified that HMGB1 is one of the damage-associated molecular pattern molecules and several studies have demonstrated that HMGB1 is secreted into the extracellular milieu from necrotic and inflammatory cells, but not apoptotic cells, and acts as a cytokine with multiple functions [6]. Once released, HMGB1 mediates various cellular responses including cell migration, release of pro-inflammatory cytokines, tissue repair, and angiogenesis [5,7,8]. Extracellular HMGB1 acts as a...
potent mediator of macrophage activation [5,9]. It is also secreted extracellularly by activated macrophages in response to pro-inflammatory cytokines [5,10]. Therefore, HMGB1 has a unique ability to self-amplify and prolong inflammatory response and contributes to the pathogenesis of sepsis and acute lung injury [5,9-12]. It also functions as an epigenetic modifier, mainly through regulation of NF-κB-dependent signaling pathways, to modulate the behavior of surviving cancer cells as well as the immune cells found within the tumor microenvironment [13]. HMGB1 has been reported to transduce cellular signals by interacting with at least three receptors: Receptor for advanced glycation end (RAGE) products, Toll-like receptor (TLR)-2 and TLR-4 [5]. Of receptors important for HMGB1 activity, RAGE was the first demonstrated binding partner [14]. RAGE is a transmembrane, multiligand member of the immunoglobulin superfamily. As shown in vitro, HMGB1 signaling through RAGE mediates: Chemotaxis; proliferation and differentiation of immune cells and other cells; activation of an inflammatory signaling cascade and upregulation of cell-surface receptors, including TLR4 and RAGE itself [15]. Up-regulation of RAGE is associated with sustained cellular perturbation and tissue injury and has been reported under various pathologic conditions, such as vascular injury, diabetes, neurodegenerative disorders, and inflammatory diseases [16,17].

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, which ultimately leads to the destruction of cartilage and bone in the affected joints. Synovial hyperplasia is a hallmark pathology of RA, and fibroblast-like synoviocytes (FLS) play a critical role in RA pathogenesis by producing pro-inflammatory soluble factors or activating other immune cells. Evidence is accumulating that, once initiated, the inflammatory process in the synovial tissue is dominated by activated monocytes/macrophages and fibroblasts. Cytokines derived from these cell types are abundantly expressed, and it is commonly accepted that tumor necrosis factor (TNF) and interleukin-1 (IL-1) are pivotal mediators in the pathogenesis of RA, providing validated targets for successful therapy [18,19]. Disease-modifying antirheumatic drugs (DMARD) are the primary treatment for RA. They have been found to improve symptoms, decrease joint damage, and improve overall functional abilities [20]. They should be started very early in the disease as when they result in disease remission in approximately half of people and improved outcomes overall [21]. Methotrexate (MTX) is considered to be the most important and useful DMARD and is usually the initial line of treatment [22]. The precise way in which MTX operates in terms of anti-inflammatory activity is not fully understood. Consistent with the capacity of HMGB1 for immune activation and inducing inflammatory cytokine-like activity and the ability of single HMGB1 injection into rodent joint of inducing long-lasting, destructive arthritis [23], this work was planned to study the serum level of HMGB1 and the expression of its receptor RAGE in synovial tissues of patients with rheumatoid arthritis and their correlations with some clinical variables and MTX therapy.

**Subjects and Methods**

This study included 76 subjects categorized into two groups:

- Group I: 48 patients with RA diagnosed according to the diagnostic criteria of the American college of Rheumatology for classification of RA [24]. They were classified into:
  - Group IA: 15 newly diagnosed RA without starting Methotrexate therapy. They were 6 males and 9 females. Their ages range from 28-48 years. The duration of their disease ranges from 5-30 months.
  - Group IB: 33 patients with RA received MTX therapy. They were 14 males and 19 females. Their ages range from 28-60 years. The duration of their disease ranges from 2-19 years.

- Group II: 28 apparently normal individuals, with matched age and sex, served as a control group. They were 12 males and 16 females. Their ages range from 27-59 years.

All individuals were subjected to the following:

1. Full history taking and complete clinical examination.

2. Routine laboratory investigations including:
   - Complete blood count using Sysmex S.F 3000 (Kobe, Japan), with examination of Leishman stained films.
   - Liver and Kidney function tests using Dimension autoanalyzer (Dade Behring, Illinois, USA).
   - Erythrocyte sedimentation rate (ESR) using Westergren method.
   - Rheumatoid factor (RF) using latex agglutination (Spinreact, Ctra. Santa Coloma, Spain). RF was considered to be positive when the serum titer was 10IU/mL.
- Anti-cyclic citrullinated peptide antibodies (anti-CCP) using ELISA kit (Immunoscan RA Anti-CCP test kit; Euro Diagnostica, Sweden). The sample is considered positive when its level lies above the cut-off level of the kit (25U/mL).
- C-reactive protein using latex agglutination (Spinreact, Ctra.Santa Coloma, Spain).
- Plain X-ray for hands.

3- Disease activity measurement using DAS-28:

Disease Activity Score-28 (DAS28) includes a 28 tender joint count, a 28 swollen joint count, erythrocyte sedimentation rate, and a general health assessment. The level of RA disease activity can be interpreted as low (DAS28 = 3.2), moderate (DAS28 = >3.2 but 5.1), or as high disease activity (DAS28 = >5.1). A DAS28 of 2.6 corresponds with being in remission.

4- Special investigations:

Synovial tissue biopsies were done for all patients. For controls, synovial tissue samples were obtained during joint replacement surgery of the hip or arthroscopy of the knee joint, samples showing inflammatory synovitis on histopathological examination were excluded from the study. The specimens were immediately fixed by immersion in 10% buffered formalin solution for 4-8 hours at room temperature, dehydrated with 70%, 90%, and 100% alcohol respectively. The tissues were immersed in xylene, embedded in paraffin at 58°C, and sectioned into 7-gm-thick slices. Gelatin-coated slides were prepared, dried overnight at room temperature. Slides with paraffin-embedded sections were stored at room temperature.

Immunohistochemistry:

Anti-RAGE polyclonal antibody was purchased from R & D Systems, (R & D Systems, Inc., MN, USA). Immunohistochemical analysis for RAGE was carried out by biotin-streptavidin method. Briefly, Tissues were rehydrated before commencing staining protocol by immersing the slides in xylene and 100%, 95%, 70%, and 100% alcohol respectively. The tissues were immersed in xylene, embedded in paraffin at 58°C, and sectioned into 7-gm-thick slices. Gelatin-coated slides were prepared, dried overnight at room temperature. Slides with paraffin-embedded sections were stored at room temperature.

Detection of serum levels of HMGB1 by ELISA (IBL International GMBH-Hamburg, Germany):

HMGB 1 ELISA is a Sandwich-enzyme immunoassay for the quantitative determination of HMGB 1 in serum and plasma. The wells of the microtiter strips are coated with purified anti-HMGB 1 antibody. HMGB 1 in the sample binds specifically to the immobilized antibody and is recognized by a second enzyme marked antibody. After substrate reaction the HMGB 1 concentration is determined by the colour intensity. The intensity of the blue colour was proportionate to the amount of HMGB1 bound in the initial step. The colour development was stopped, and the intensity of the colour was measured and compared with a standard curve. Reading was done at 450nm wavelength.

Statistical analysis:

Results were expressed as Mean±Standard deviation (SD) and were analyzed statistically by using analysis of variance (ANOVA) and t-tests. Least significant difference (LSD) was done to test the difference between the different studied groups. The Chi Square statistic was used to compare the number RAGE expression between groups. Correlation analysis was performed with Pearson correlation test. p-values below 0.05 were considered to be significant. Data were tabulated statistically and analyzed using SPSS version 20.0 for windows (SPSS Inc., Chicago, Illinois, USA).
Results

All studied patients received an ultimate diagnosis based upon clinical and laboratory data.

Some data of the RA group (group I) and the control group (group II) were illustrated in Table (1). In group IA, DAS-28 of 3 patients were (20%), 9 patients were 3.2-5.1 (60%), and 3 patients were >5.1 (20%). In group IB, DAS-28 of 19 patients were (57.6%), 8 patients were 3.2-5.1 (24.2%), and 6 patients were >5.1 (18.2%).

By RAGE immunohistochemistry, 10 specimens of group IA (66.7%) showed 2+, 3 specimens (20%) showed 1+, and 2 specimens (13.3%) showed ± staining. 17 specimens of Group IB (51.5%) showed 2+, 9 specimens (27.3%) showed 1+, and 7 specimens (21.2%) showed ± staining. Twenty five specimens (89.3%) of the control group showed negative staining while 3 specimens (10.7%) showed ± staining. (Table 2 & Figs. 1,2). The immunopositivity did not correlate with age of patients, duration of the disease, gender of patients, ESR, CRP, WBCs count, rheumatoid factor, DAS-28 and anti-CCP in all groups. In group I, the immunopositivity had significant positive correlation with serum HMGB1 level (p<0.01). (Table 3) The mean values of serum HMBG1 were 7.1±3.7ng/ml in RA patients without MTX therapy (group IA), 4.6±2.49ng/ml in RA patients with MTX therapy (group IB), and 0.725±0.356ng/ml in the control group (group II). There were highly significant differences among the studied groups (p<0.001). (Table 4 & Fig. 3). Table (5) and Figs. (4-9) show that there were significant positive correlations between serum HMGB1 and ESR, CRP, anti CCP, age and DAS-28 in group 1. There were no significant correlations between HMGB 1 levels and duration of the disease, gender of patients and rheumatoid factor (p>0.05).

Fig. (1-A): Low power X 10 (group Ia).
Fig. (1-B): High power X 20 (group Ia).
Fig. (1-C): High power X 40 (group Ib).
Fig. (1): RAGE expression in group 1.
Fig. (2): RAGE expression in group 11.
Fig. (3): Serum HMGB 1 levels in the studied groups.

Fig. (4): A significant positive correlation found between the S. HMGB1 & anti-CCP in group Ia (r=0.55; p<0.01).

Fig. (5): A significant positive correlation found between the S. HMGB1 and ESR in group lb (r=0.51; p<0.05).

Fig. (6): A significant positive correlation found between the S. HMGB1 and CRP in group lb (r=0.53; p<0.05).

Fig. (7): A significant positive correlation found between the S. HMGB1 and age in group lb (r=0.50; p<0.05).

Fig. (8): A significant positive correlation found between the S. HMGB1 and DAS-28 in group lb (r=0.56; p<0.01).
Fig. (9): A significant positive correlation found between the S. HMGB1 and DAS-28 in group Ia (r=0.53; p<0.01).

Table (1): Some findings of the studied groups.

<table>
<thead>
<tr>
<th></th>
<th>Group Ia (N=15)</th>
<th>Group Ib (N=33)</th>
<th>Group II (N=28)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y.)</td>
<td>39.4±6.5</td>
<td>44.1±10.3</td>
<td>41.1±8.9</td>
<td>F=1.6</td>
</tr>
<tr>
<td>ESR (mm)</td>
<td>64.5±32.6ab</td>
<td>42.3±21.5b</td>
<td>7.7±3.5</td>
<td>F=43.3</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>61.6±30.5</td>
<td>35.27±29.73</td>
<td>1=2.8</td>
<td>0.007 HS</td>
</tr>
<tr>
<td>WBC count (X1000/u1)</td>
<td>9.3±4.5</td>
<td>8.9±3.6</td>
<td>7.3±3</td>
<td>F=2.1</td>
</tr>
<tr>
<td>Rheumatoid factor (U/1)</td>
<td>266.7±212.6</td>
<td>196.2±24.5</td>
<td>1=1.9</td>
<td>0.06 NS</td>
</tr>
<tr>
<td>Anti-CCP (U/ml)</td>
<td>130.5±60.1ab</td>
<td>81.9±51.4b</td>
<td>14.8±5.5</td>
<td>F=38.7</td>
</tr>
<tr>
<td>DAS-28</td>
<td>4.3±0.85</td>
<td>3.63±1.09</td>
<td>1=2.1</td>
<td>0.04 S</td>
</tr>
</tbody>
</table>

NS: Not Significant.  
S: Significant.  
ESR: Erythrocyte sedimentation rate.  
CRP: C-reactive protein.  
ab: p<0.01 when compared with group Ib.  
b: p<0.0001 when compared with group II.

Table (2): Number and Percentage of RAGE expression in the studied groups.

<table>
<thead>
<tr>
<th></th>
<th>Group Ia (N=15)</th>
<th>Group Ib (N=33)</th>
<th>Group II (N=28)</th>
<th>X²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>2+</td>
<td>10 (66.7%)</td>
<td>17 (51.5%)</td>
<td>0</td>
<td>25.5</td>
<td>&lt;0.0001 HS</td>
</tr>
<tr>
<td>1+</td>
<td>3 (20%)</td>
<td>9 (27.3%)</td>
<td>0</td>
<td>8.7</td>
<td>0.013 S</td>
</tr>
<tr>
<td>+</td>
<td>2 (13.3%)</td>
<td>7 (21.2%)</td>
<td>3 (10.7%)</td>
<td>1.34</td>
<td>0.512 NS</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>25 (89.3%)</td>
<td>63.9</td>
<td>&lt;0.0001 HS</td>
</tr>
</tbody>
</table>

NS: Not Significant.  
S: Significant.  
HS: Highly Significant.

Table (3): Correlations between RAGE expression and some findings in the studied groups.

<table>
<thead>
<tr>
<th></th>
<th>Group Ia (N=15)</th>
<th>Group Ib (N=33)</th>
<th>Group II (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y.)</td>
<td>0.31</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>Duration of disease (y.)</td>
<td>0.22</td>
<td>0.20</td>
<td>0.03</td>
</tr>
<tr>
<td>Gender</td>
<td>0.19</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>ESR (mm)</td>
<td>0.33</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.30</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>WBC count (X1000/u1)</td>
<td>0.22</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>Rheumatoid factor (U/1)</td>
<td>0.31</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>Anti-CCP (U/ml)</td>
<td>0.33</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>DAS-28</td>
<td>0.28</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>S. HMGB1 (ng/ml)</td>
<td>0.59**</td>
<td>0.55**</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**Highly Significant=p<0.01.
Inflammation is the common driving force leading to cartilage, bone, and soft tissue destruction in rheumatoid arthritis. Many factors involved in the regulation of normal tissues, in particular cartilage and bone, are deregulated in arthritic diseases [25]. Also, synovial angiogenesis is thought to be a critical component in RA pathogenesis, contributing to pannus proliferation, infiltration of inflammatory leukocytes, as well as osteophyte formation [26]. Because HMGB1 initiates endothelial growth as well as endothelial cell migration and sprouting, it has also been identified as an angiogenetic switch molecule [27]. It has a unique ability to self-amplify and prolong the inflammatory response [5]. Rheumatoid arthritis FLS resists apoptosis following stress by autophagy formation, which may contribute to resistance against rheumatoid arthritis treatments [28]. Tang, Kang and their coworkers have shown that HMGB1 is a novel Beclin 1-binding protein important in sustaining autophagy [29,30]. Also, reducible HMGB1 binds to RAGE, induces Beclin 1-dependent autophagy. Moreover, RAGE sustains autophagy and limits apoptosis [31,32]. In this context, this work was planned to study the level of serum HMGB1 and the expression of its receptor, RAGE, in synovial tissues in patients with rheumatoid arthritis. In our study, we found increased level of serum HMGB1 in patients with rheumatoid arthritis compared to normal individuals. This result is in agreement with Pullerits, Goldstein, Taniguchi, and their coworkers who found an increase in extra cellular HMGB1 expression in blood, synovial tissue and synovial fluid in RA [33-35]. Similarly, studies of RA synovial biopsies from patients with RA indicate aberrant HMGB1 expression in the pannus tissue at the cartilage-bone interface as well as in areas with tissue hypoxia [36]. Palmblad et al., demonstrated that cytoplasmic and extracellular HMGB1 appears early in the development of arthritis. They speculate that HMGB1 might be a major contributor to pannus formation in chronic arthritis. In addition, at the time point of arthritis onset, a substantial number of the infiltrating inflammatory cells expressed HMGB1 in their cytoplasm, apparently more than cells expressing TNF or IL-10. Their data indicate that activated synovial macrophages, fibroblasts and vascular endothelial cells both respond to and secrete HMGB1 in synovitis, suggesting diverse sources of HMGB1 production and target activity [231. However, the HMGB1 dependence of the recruitment of inflammatory cells into damaged tissues was already shown in several animal models, most notably in heart ischemia/reperfusion [37], in peritonitis [38], and in hepatitis [39,40]. In our study, the levels of HMGB1 in patients with RA without MTX therapy were significantly higher than that of patients with MTX therapy. Kuroiwa et al., demonstrated that HMGB1 is a direct binding protein of MTX and binding of MTX to RAGE-binding region in HMGB1 inhibited the HMGB1/ RAGE interaction at the molecular and cellular levels. These data might explain the molecular basis underlying the mechanism of action for the anti-inflammatory effect of MTX [41].

In our study, there were significant positive correlations between serum HMGB1 levels and ESR, CRP, DAS-28 and anti-CCP. Thus, HMGB1 might be used as an additional marker for the assessment of disease activity. Abdulahad, Hayashi, Li and their coworkers demonstrated that HMGB1 contributes to the pathogenesis of inflammatory and autoimmune diseases, especially SLE [42-44]. Outside the cell, HMGB1 plays a critical role as a pro-inflammatory cytokine that induces the release of TNF and IL-1 from macrophages and dendritic cells [9,10,45,46]. HMGB1 can form complexes with IL-1α and IL-1β to enhance immune responses [47-60]. These complexes might be particularly relevant for joint inflammation and disease activity, where all three factors are present. The importance of this pathway is supported by obser-
vations that single intra-articular HMGB1 injections in mice deficient for the receptor of IL-1 fail to generate synovitis [49]. As HMGB1 can bind other partner molecules, such as bacterial DNA, viral RNA, or endotoxin, the resulting complexes might also induce proinflammatory cytokine production and increase the disease activity. As a signal of tissue damage, HMGB1 mediates tissue regeneration by inducing mesoangioblast migration and proliferation [51]. Synovial tissue has a strong capacity to regenerate, and not surprisingly mesenchymal stem cells have been isolated from synovium [52]. Thus, we conclude that HMGB1 is a comprehensive cytokine that is able to orchestrate the regulation of both inflammation and tissue regeneration to promote wound healing. Also, in our study, we found a significant positive correlation between HMGB1 and age. As previously mentioned, the major characteristics of aging process are the increased expression of genes associated with inflammation in tissues [53,54] and augmented levels of cytokines, for example, IL-6 and TNF-a, in serum [55]. Interestingly, aging is also linked to a clear decline in autophagy [56-58] as well as apoptosis [59,60]. In our study, we observed that RAGE expression was far stronger in RA synovium than in synovial tissues of the control group. We found a significant positive correlation between RAGE expression and serum HMGB1 level in the RA group. This finding agrees Heo et al., who found that RAGE was overexpressed in RA synovial tissues [61]. The up-regulation of RAGE in RA synovium may be related to the abundance of inflammatory cytokines in RA synovial tissue. Heo and his colleagues observed that IL-1b and IL-17 have stimulatory effects on RAGE expression and production in RA-FLS [61]. Drinda et al., also detected RAGE expression in the synovial lining, sublining, and stroma in RA. They found that many T cells and some macrophages showed positive immunostaining for RAGE, whereas B cells were mostly negative [62]. Sunahori and his colleagues reported that RAGE mRNA expression is augmented by various cytokines, most potently by IL-1b [63]. We conclude that extracellular HMGB1 is one of the important factors causing increase in RAGE expression in synovial tissues of RA patients and both are important factors in the pathogenesis of the disease. However, we found increase in RAGE expression in patients without MTX therapy than that of patients with MTX therapy and this may be attributed to the binding of MTX to RAGE-binding region in HMGB1 which inhibits the HMGB1/RAGE interaction at the molecular and cellular levels [41]. As previously mentioned, HMGB1 signal-ing through RAGE mediates upregulation of cell-surface RAGE itself [15]. HMGB1-1 also stimulates the invasiveness of RA-FLS by activation of RAGE [64]. As such, RAGE may be an interesting target for therapy directed at the inhibition of synoviocyte activation. Further evidence of the role of HMGB1 and RAGE in joint inflammation comes from studies indicating disease attenuation by agents (including Polyclonal and monoclonal anti-HMGB1 anti bodies, recombinant A box domain of HMGB1 and soluble RAGE) which antagonize the activity of HMGB1 [65-72]. In conclusion, HMGB1 level and RAGE expression are increased in RA patients, especially in patients without MTX therapy, and may play important roles in the pathogenesis of the disease. Blockade of extracellular HMGB1 and RAGE expression may offer a novel therapeutic alternative for the treatment of RA. Further studies correlating HMGB1 and RAGE with autophagy and apoptosis are recommended.

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