HMGB1 EXPRESSION AS REGULATOR OF INITIAL INFLAMMATION COMPARED TO TNF ALPHA IN TRAUMATIC BRAIN INJURY MODEL RATS

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ABSTRACT

Introduction. Traumatic brain injury (TBI) is a serious neurological disorder, and cause of disability and death under the age of 45. One of pathological responses in TBI is neuroinflammation. Activation of neuron and glial cells is one of triggers for releasing inflammatory mediators such as HMGB1 and TNF alpha. HMGB1 is one of the nonhistone proteins attached to the nucleus. TNF alpha is a proinflammatory cytokine released at the beginning of the injury process.
Aim. This research was aimed to know expression of HMGB1 and TNF alpha in the early stages of the TBI.
Method. Using a modified Marmarau 94 method with *Rattus norvegicus* aged 4-5 months. Brain tissue was examined using RT-PCR to determine expression levels of HMGB1 and TNF alpha. Immunohistochemical staining was implemented to determine distribution of HMGB1 and TNF alpha proteins in neurons.
Results. RT-PCR of HMGB1 expression level was significantly increased since 30 minutes after TBI compared with control. TNF alpha expression was increase after 60 minutes of brain injury. Examination of HMGB1 levels with specific antibodies against HMGB1 showed similar results. This suggests that HMGBI is a proinflammatory cytokine that is first released compare to TNF alpha post brain injury.
Conclusion. HMGB1 is an early cytokine that is likely to induce further inflammatory processes.

Keywords: TBI, HMGB1, TNF alpha.

DOI: https://doi.org/10.21776/ub.jfls.2021.005.02.01

ABSTRAK

Latar belakang. Traumatic brain injury (TBI) merupakan gangguan neurologis serius yang menimbulkan kecacatan dan kematian terutama pada usia dibawah 45 tahun. Salah satu respon patologis yang terjadi pada TBI adalah munculnya proses neuroinflamasi. Aktivasi neuron, glia pada TBI merupakan salah satu pemicu pelepasan mediator inflamasi seperti HMGB1 maupun TNF alfa. HMGB1 adalah salah satu protein nonhiston yang terikat pada inti sel. TNF alfa adalah cytokine proinflamasi yang dilepaskan diawal proses injury.
Tujuan. Penelitian bertujuan untuk mengetahui ekspresi HMGB1 dan TNF alfa pada tahap awal terjadi *traumatic brain injury*
Metode. *Traumatic brain injury* (TBI) menggunakan metode Marmarau 94 yang dimodifikasi pada tikus *Rattus norvegicus* usia 4-5 bulan. Jaringan otak diperiksa menggunakan RT-PCR untuk mengetahui level ekspresi HMGB1 dan TNF alpha. Jaringan otak juga diperiksa dengan pengecatan imunohistokimia untuk mengetahui distribusi protein HMGB1 dan TNF alpha pada sel neuron.
Hasil. Ekspresi HMGB1 meningkat secara signifikan sejak 30 menit setelah TBI dibandingkan kelompok kontrol. Ekspresi TNF alfa meningkat setelah 60 menit dari TBI. Pemeriksaan kadar HMGB1 dengan antibodi spesifik terhadap HMGB1 menunujukkan peningkatan sejak 30 menit setelah TBI, sedangkan ekspresi TNF alfa terjadi peningkatan setelah 60 menit dari TBI. Hal ini menunjukkan bahwa HMGBI merupakan sitokin proinflamasi yang lebih dulu dilepas oleh jaringan otak yang mengalami injury dibandingkan TNF alfa.
Kesimpulan. HMGB1 merupakan sitokin awal yang kemungkinan dapat menginduksi proses inflamasi selanjutnya.
Kata kunci: TBI, HMGB1, TNF alfa.

INTRODUCTION

Traumatic brain injury (TBI) is a serious cause of neurological disorders and is still a leading cause of disability and death in individuals under 45 years of age (Werner and Engelhard, 2007). Every year in the United States as many as 1.7 million people experience head trauma and 1/3 of TBI cases contribute to the death of the sufferer (Algathos and Huang, 2014). In Asia and the Middle East, TBI is still a cause of high levels of cognitive, motor, behavioral and language impairments (Namas, 2008).

Meanwhile, until now the discussion about the mechanisms and pathologies that occur in the brain due to TBI is still not widely known (Namas, 2008). Several processes that occur in TBI cases include primary injury or early stages that cause direct brain damage due to the trauma process itself, secondary injury which is a response to primary injury through the formation of lactate, glutamate and the presence of Ca influx. The next response is an inflammatory reaction involving toxic neurochemicals and followed by neuronal regeneration (Ray et al., 2002).

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The initial response that occurs in TBI is caused by a decrease in the amount of energy in the brain and is followed by cell death due to the excitotoxic process. This early stage usually occurs within 24 hours of the trauma that is directly related to the brain damage and physiological disturbances that occur. At this stage, neuronal damage begins with an ischemic cascade that follows TBI. This ischemic cascade will cause a decrease in the amount of energy in the brain. This reduced energy triggers a decrease in glucose use, accumulation of lactic acid and a decrease in ion pump activity, Ca influx and excitotoxicity. In the next few days (intermediate phase) a neuroinflammatory process will occur (Algathos and Huang 2014).

TBI can induce neurons to release inflammatory cytokines such as IL and TNF alpha. BBB destruction and immune cell infiltration in brain parenchyma after TBI contribute to secondary injury such as decreased CBF, cerebral edema and increased ICP (Ray et al., 2002). Neuronal cell death due to this process will occur in 2 phases, namely necrosis in the early phase and apoptosis in the next phase (Namas, 2008).

Necrosis will trigger the release of damage-associated molecular patterns (DAMPs), which will induce an inflammatory response (Maeda and Fadeel, 2014). Damage-associated molecular patterns (DAMPs) are produced by injured tissue and trigger the production of inflammatory mediators and play an important role in the inflammatory cascade. One of the molecules included in inflammatory mediators is High-mobility Group Box 1 (HMGB1) (Namas, 2008).

HMGB1 is a nonhistone protein bound in cell nuclear with a dual function. Inside the cell HMGB1 binds to DNA and plays an important role in the transcription process. HMGB1 in extra cells acts as an inflammatory cytokine (Xiangjin et al, 2014), even said HMGB1 is a key mediator in the inflammatory process. The concentration of HMGB1 was significantly increased 16 – 32 hours after exposure to lipopolysaccharide (Algathos and Huang, 2014).

A neuroinflammatory process involving a number of inflammatory mediators is known to cause secondary injury that causes aggravation of clinical symptoms in TBI cases. TNF alpha and HMGB1 are known to be early inflammatory mediators that are released when neurons are injured. So the purpose of this study was to find out the comparison of TNF alpha and HMGB 1 expression at the beginning of TBI.

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METHODS

This study uses a pure experimental design in vivo with a randomized post test only controlled group design using male rats (*Rattus norvegicus*) aged 4-5 months weighing 200-300 grams. The rats were divided into 5 groups, the control group was healthy rats without any treatment. Group 1 rats were sacrificed 30 minutes after TBI induction. Group 2 rats were sacrificed 60 minutes after TBI was induced. Group 3 rats were sacrificed 120 minutes after TBI was induced. Group 4 rats were sacrificed 240 minutes after TBI.

Rats were anesthetized with xyla (5mg/kgBW) and ketamine (50mg/kgBW) intraperitoneally. TBI induction was carried out by the Marmarou method. The hair is shaved and cleaned with 70% alcohol. A 45 gram iron cylinder (4mm diameter) is dropped from a height of 1 meter using an iron cylinder tube onto the shaved scalp. Two hours after the treatment, the mice were terminated and the brain tissue was isolated. Brain tissue was fixed using 4% paraformaldehyde for 24 hours. Brain tissue was cut to a thickness of 6 μm and stained with Hematoxylin-Eosin. RT PCR examination and Immunohistochemistry were performed on brain tissue to determine the expression of HMGB1 and TNF alpha in brain tissue. RT PCR to see the expression of HMGB1 and TNF alpha quantitatively, while TNF alpha to see the expression of HMGB1 and TNF alpha semi-quantitatively.

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RESULTS

1. Real Time-PCR HMGB1 and TNF-alpha

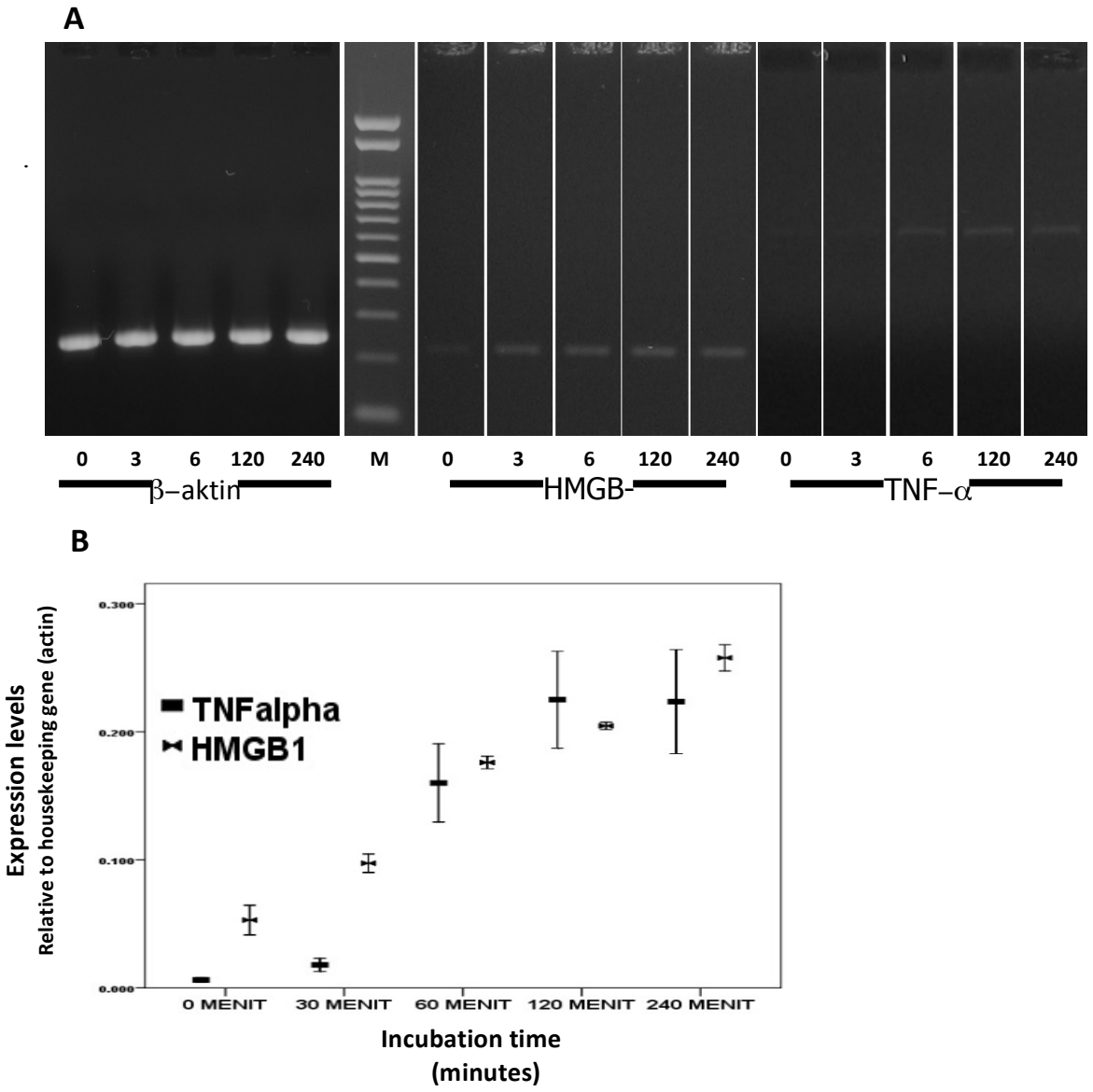

Figure 1. Reverse Transcriptase (RT)-PCR semi-quantitative analysis. (A) Representation of agarose gel electrophoresis results RT-PCR product of HMGB1 and TNF-alpha. (B) Graph of agarose gel electrophoretic densitometry analysis for HMGB1 and TNF-alpha using NIH Image-J. The X axis shows the incubation time group after TBI. And Y shows the relative expression of HMGB1 and TNF-alpha, which is the average of 3 times the PCR of the same cDNA template. The relative expression of HMGB1 and TNF-alpha is the ratio of each gene band to housekeeping gene (actin) during the incubation period and the same dose group.

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2. Immunohistochemistry Expression of HMGB1

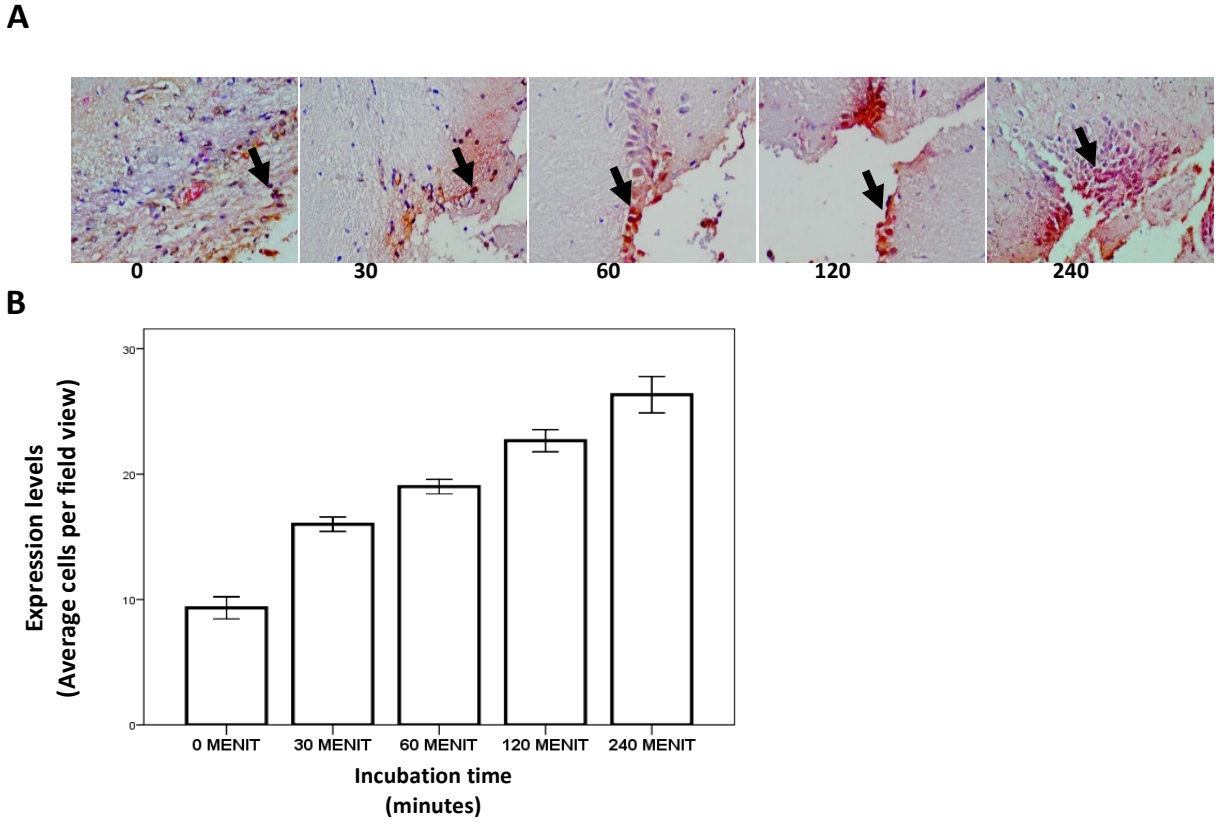

Figure 2. Results of HMGB1 immunohistochemistry staining, using specific antibodies against HMGB1 using the immunoperoxidase technique, visualized with AEC substrate (red color). A. Photomicrograph of HMGB1 smeared brain tissue in the cortex (TBI drop area). HMGB1 was observed in the cortical part of the brain neuron cells (arrows) in the cytoplasm and the cell nucleus. Photographed using a Panasonic DMC-G3 on an Olympus Microscope at 400x magnification. B. Histogram graph representing the calculated HMGB1 expression calculated according to Soini et al (1998) and Pizem and Cor (2003), shows an increase in HMGB1 expression at 30 minutes after exposure to TBI.

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3.Immunohistochemistry expression of TNF alpha

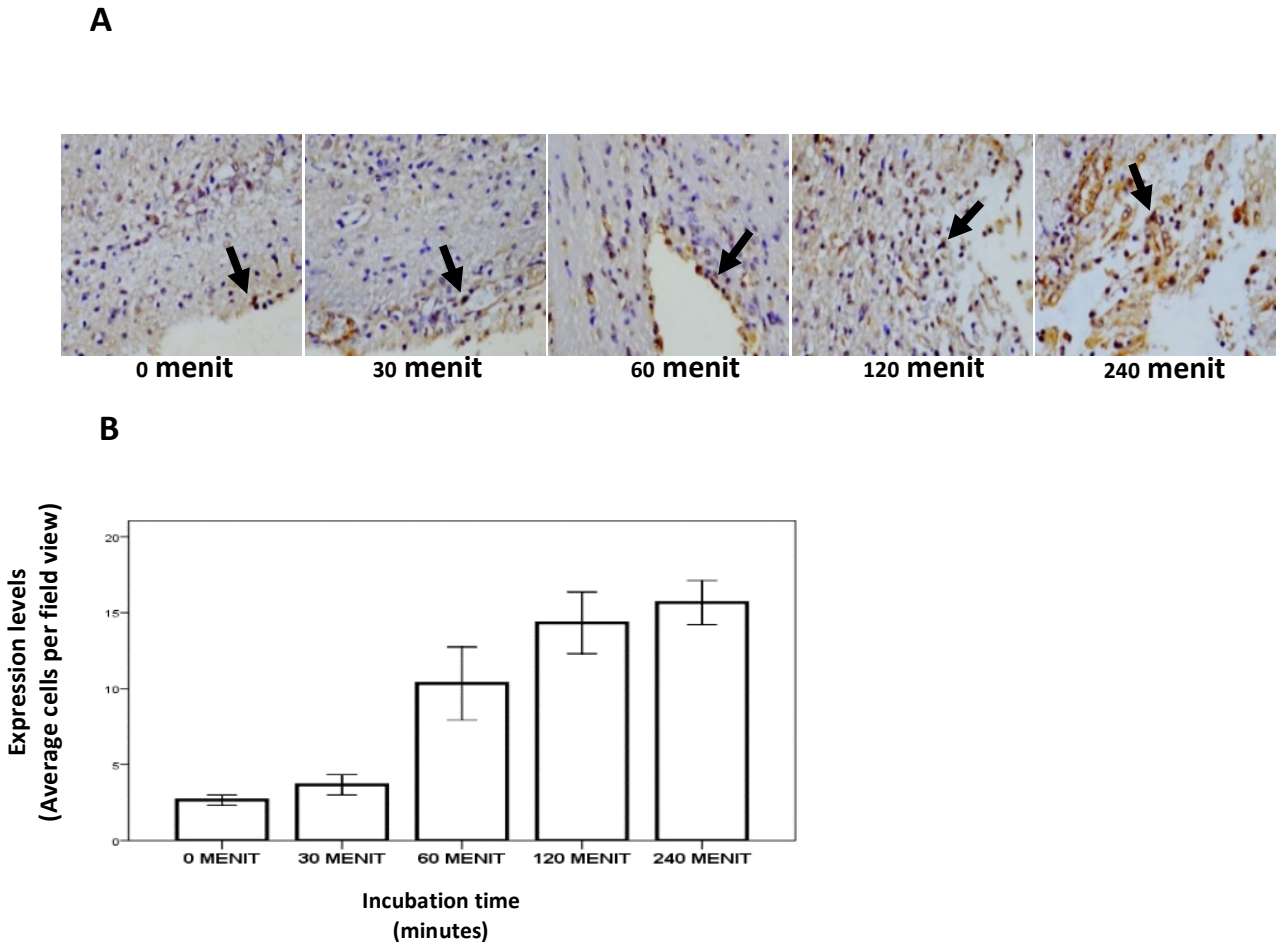

Figure 3. Immunohistochemical staining results of TNF-a, using specific antibodies against TNF-a using the immunoperoxidase technique, visualized with DAB substrate (brown color). A. Photomicrograph of TNF-alpha smeared brain tissue in the cortex (TBI drop area). TNF-alpha was observed in the cortical part of the brain neuron cells (arrows) in the cell cytoplasm. Photographed using a Panasonic DMC-G3 on an Olympus Microscope at 400x magnification. B. Histogram graph representing TNF-alpha expression calculated according to Soini et al (1998) and Pizem and Cor (2003), shows an increase in TNF-alpha expression at 60 minutes after exposure to TBI.

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Based on RT-PCR results on the expression level of HMGB1, there appears to be a significant increase since 30 minutes after exposure to TBI compared to the control group. This increase will continue after 240 minutes of exposure to TBI. The expression level of TNF-a, showed an increase after 60 minutes of exposure to TBI. At 30 minutes, there was an increase but did not show significant significance, compared to the control group. An increase in TNF-a expression level occurred between 60-120 minutes after exposure to TBI.

Immunohistochemical staining results visualized using AEC (Amino ethyl Carbazole), in post-TBI brain tissue, it appears that HMGB1 expression is shown in red (arrow). HMGB1 expression showed a significant increase at 30 min after exposure to TBI compared to the control group (figure 2B). And it was shown that an increase in HMGB1 expression appeared to occur up to 240 minutes after exposure to TBI.

DISCUSSION

High mobility group box 1 (HMGB1) is a protein in the cell nucleus that can be released by activated monocytes, macrophages, neutrophils, platelets. The release of HMGB1 is thought to be relatively slower than other cytokines such as TNF alpha and IL 1b. HMGB1 is also said to be a late mediator during the inflammatory process (Luan et al., 2010). However, in this study it was found that HMGB1 levels began to appear 30 minutes from the injury process in the brain, while TNF alpha began to be expressed after 60 minutes from the injury process. This suggests that HMGB1 may be released earlier by injured cells than TNF alpha.

Another study conducted by Luan Z.G et al., which examined HMGB1 as a stimulator of TNF alpha production, showed that 4 hours after incubation of human umbilical vein endothelial cells (HUVEC) with HMGB1 showed a significant increase in TNF alpha levels compared to the control group and The peak increase in TNF alpha levels occurs within 24 hours after incubation. In this study, the induction effect of HMGB1- on nuclear factor kappa B (NF-kB) in HUVECs was also carried out. The results of this study found that NF-kB appeared to increase after 30 minutes of HMGB1 stimulation. Based on these results, it is suspected that HMGB1 is a key inflammatory mediator that can induce an inflammatory response during the injury process (Luan et al., 2010). In this study, it was found that HMGB1 was released first after the cell injury process compared to TNF alpha. However, in this study it cannot be concluded that HMGB1 induces the release of TNF alpha.

Based on research by Luan Z.G that HMGB1 can induce translocation of NF-KB which is a transcription factor for inflammatory proteins such as TNF alpha, interleukin. So it is possible that HMGB1 can induce the release of TNF alpha through NFKB activation. The mechanism of HMGB 1 in inducing NFKB is thought to be through the HMGB1 receptor, namely RAGE which has a binding site for NFKB (Luan et al., 2010).

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CONCLUSSIONS

The TBI process will induce an inflammatory response that can contribute to the worsening of clinical symptoms in TBI cases. The neuroinflammation that occurs is triggered by the release of inflammatory mediators such as TNF alpha, IL, or HMGB1. In this study, it was found that HMBG 1 is an inflammatory mediator that is released first after the injury process compared to TNF alpha.

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