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HMGB1-RAGE, A USEFUL PARTNERSHIP IN VITAL RESPONSE?

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ABSTRACT

Introduction: In forensic practice, it is well known that the mechanism and dating of traumatic injuries is one of the primary responsibilities of this specialty. Currently, it is a subject still debated by researchers, and so far, an infallible marker that would objectively support their intravitam/postmortem occurrence has not yet been identified. However, studies have shown that the HMGB1-RAGE axis is rapidly activated after trauma and might be an essential element to help solve the forensic problem of wound dating.

Purpose: To compare the values of HMGB1-RAGE expression occurring in wounds produced intravitally shortly before death and in wounds produced postmortem and to quantify the differences arising between them.

Material and method: For this prospective study, skin fragments were collected from the site of wounds in autopsied cadavers at the County Clinical Service of Forensic Medicine Constanta (SCJML Constanta), wounds produced intravitally and with a maximum survival time of 60 minutes. Postmortem wounds and control fragments from volunteers undergoing surgery for skin tumours were also collected. The main conditions were: chronological documentation of the lesion and absence of neoplastic or inflammatory conditions. Ninety-six autopsy cases between 2021-2022 met the criteria for inclusion in the study. A control fragment accompanied each fragment from the wound. Routine Hematoxylin-Eosin (HE), Perls and Van Gieson Werhoeff staining, as well as immunohistochemistry with HMGB1 and RAGE markers were performed on each fragment and a score based on staining intensity was determined.

Results: Routine staining was not useful in assessing vitality in segments with survival time up to 30 min. Immunohistochemically, both markers showed increased values compared to control values ($p<0.0001$) and to lesions produced postmortem. An interesting aspect is the lack of reactivity in the lesion's margins for both markers.

Conclusions: Although further research is needed, the results of our study support the hypothesis that the HMGB1-RAGE axis is useful in assessing the vital reaction in skin wounds.

Keywords: forensic, vital reaction, immunohistochemistry, HMGB1, RAGE.

Background:

In forensic practice, it is well known that the mechanism and dating of traumatic injury is one of the primary responsibilities of this specialty. This information is essential for the authorities investigating the circumstances of a person's death, trying to reconstruct each stage/event that developed up to the occurrence of death. To do this, the judicial body is interested in knowing all the details of the injuries (bruises, lacerations, abrasions, etc.), which the forensic pathologist can provide through direct examination and laboratory analysis of the tissue fragments containing these injuries.

The skin is usually the first organ affected by a damaging agent, be it physical, chemical or biological.

In any case, as soon as an injury occurs, the skin healing process begins. This has been a

favourite target for researchers in the field of vital response, based on the premise that the healing process requires a living organism.

Skin healing consists of 3 stages:

1) inflammatory (subdivided into haemostasis and actual inflammation).

2) proliferative (re-epithelialization and angiogenesis).

3) maturation (scar remodeling) (1,2).

In each stage, numerous growth factors, cytokines and chemokines are synthesized, which will direct the whole process (3).

It goes without saying that the first stage, the inflammatory one, is the one that has aroused the interest of those who research this vital reaction phenomenon.

Therefore, many studies have focused on this stage, and an actual race has been launched in an attempt to establish a specific chronology for the release of different markers.

In an attempt to add to the vast existing literature, our study aims to address this inflammatory stage of skin healing by exploring the High mobility group box 1 (HMGB1)-Receptor for advanced glycation end-products (RAGE) relationship and its potential applications in forensic medicine.

HMGB1 is a subgroup of proteins discovered in 1973 by Goodwin et al. and is associated with DNA, performing several intranuclear roles (transcription, replication and repair)(4).

Its location is nuclear and can be identified in most tissues. (5).

HMGB1 is translocated cytoplasmically and then extracellularly following tissue injury (ischemia, trauma, infection), fulfilling the role of danger-associated molecular pattern (DAMP) or alertin. (6)

Once in the extracellular environment, HMGB1 binds to RAGE receptors or toll-like receptors (TLR2 or TLR4) and this interaction will trigger the inflammatory process by stimulating cytokine synthesis. (5)

It has been found that apoptotic cells do not release HMGB1, being retained in these cells. Necrotic cells or cells affected by some process in their vicinity will passively secrete HMGB1 (7).

It has been shown that extracellularly located HMGB1 accelerates tissue healing and regeneration by inducing the synthesis of cell growth, proliferation and differentiation factors, especially in keratinocytes and fibroblasts in injured areas, becoming an important component in the tissue healing process (8).

HMGB1 also induces the synthesis of adhesion molecules, cytokines (TNF-alpha) and chemokines (IL-8) in endothelial cells (9, 10).

Plasma levels of HMGB1 have been found to be increased in stroke (11), acute myocardial infarction (12) or mechanical trauma (13).

RAGE is part of a group of transmembrane proteins and is expressed by endothelial cells, smooth muscle fibers, type II alveolar cells, monocytes, neurons and glial cells (14).

Under pathological conditions, RAGE expression is enhanced and the presence of its ligands amplifies its expression (15).

One of its most important ligands is HMGB1, but the same category also includes β-amyloid fibrils, S100, DNA and RNA (DAMPS) and PAMPs (pathogen-associated

molecular pattern molecules), such as bacterial, viral or parasitic particles (16, 17).

By binding to these molecules, RAGE can be considered as a pattern recognition receptor, such as Toll-like receptors (TLRs) and can recognize various antigens (18).

Increased expression of RAGE has been detected in different types of cancer (19), inflammatory-related diseases (20), diabetes mellitus (21) and cardiovascular diseases (22).

HMGB1-RAGE interaction will eventually lead to intracellular signaling with transcription of the pro-inflammatory factor NF-κB, with stimulation of cell proliferation and cytokine production (23).

This amplification will enhance the expression of RAGE and its ligands, in particular HMGB1, through a positive feedback mechanism (23).

This article aims to compare the HMGB1-RAGE expression values found in intravitally produced wounds shortly before death and in postmortem produced wounds, and to quantify the differences occurring between them.

Material and method

For this prospective study, skin fragments were collected from forensic cases of violent deaths autopsied at the County Service of Forensic Medicine Constanta. Survival time ranged from a period of seconds (railway accidents with decapitation) to 60 minutes. In each case 1 fragment of skin lesion (involving at least one of the margins of the wound) and a control skin fragment were collected.

Inclusion criteria were: known/documented time of injury (investigation data, video evidence, 112 calls) and the moment of autopsy within 48 hours of death.

Exclusion criteria: chemotherapy, corticosteroid or anticoagulant treatments.

History of psoriasis, lupus or other inflammatory skin disease.

96 cases autopsied between 2021-2022 met the criteria for admission to the study.

Ninety-six skin fragments from wounds and control skin fragments from incisions made during the forensic autopsy were collected. The fragments were divided into groups according to survival time(TS): Group 1: TS less than 5 min (46), Group 2: TS 5 min-30 min (24), Group 3: TS 30 min-60 min (26). In addition to the 96 skin fragments, 11 fragments from postmortem wounds (Group 4) were examined, with a time interval between 2 min and 8 hours. Seven skin fragments (Group 5) were also examined from patients who underwent skin excisions for skin tumours at the Mangalia Municipal Hospital, the fragments coming from the incisions site. Each patient filled out a consent form to be included in the study, signed by them before surgery.

Each skin fragment was placed in a container labeled with name, date and time of collection and the region from which the fragment originated, fixed in 4% buffered formaldehyde for 24-48 hours. The fragments were processed with Bioptika automated tissue processor and paraffin blocks were obtained on which routine Hematoxylin-Eosin, Pearls and Van Gieson Verhoeff staining was performed.

Immunohistochemical technique:

Anti-HMGB1 antibodies (monoclonal antibody clone GT348, dilution 1:200) from Invitrogen, ThermoFisher Scientific and anti-RAGE (clone A-9, dilution 1:150) from SantaCruz Biotechnology were used. Antigenic detection was performed by heating, boiling in citrate and for each marker the protocol recommended by the supplier was followed. 3,3'diaminobenzidine (DAB) was used as chromogen and the brown colour, considered as a positive reaction, was obtained. Mayer haematoxylin was used for counterstaining.

Interpretation of results:

For each case, slides were analysed on which standard and immunohistochemical staining was performed and a score was established taking into account the intensity of staining of epithelial cells.

HMGB1 staining quantified the staining intensity of nuclei, cytoplasm and extracellular space.	Scoring for RAGE quantified the intensity of cytoplasm and membrane staining
Nuclear intensity > cytoplasmic intensity 0	Limited to basal layer : 0
Nuclear intensity = cytoplasmic intensity 1	Weak membranous expression of spinous and granular layer 1
Moderate cytoplasmic intensity 2	Moderate membrane or cytoplasmic expression spinous and granular layer 2
High cytoplasmic intensity/ extracellular positivity 3	Strong membrane or cytoplasmic expression spinous and granular layer 3

Statistical study:

All data were entered into a Microsoft Office Excel 2007 file and statistically analyzed with Analysis Tool Pak in Microsoft Excel 2007. The means and standard deviations (SDs) were calculated for all data. Statistically significant data were evaluated with T-test with P-value < 0.05 considered significant.

Results

The proposed sample for this study included skin fragments collected from injuries resulting from road traffic accidents (50%), railway accidents (14%), precipitation (12%), homicide (10%), iatrogenic (10%), other (6%).

The male/female ratio was 75/21 and the mean age was 56.45±18.33.

Routine Hematoxylin-Eosin, Perls and Van Gieson Verfoeff staining did not have a significant contribution in assessing the vital or postvital character of the lesions, except for the 30-60 minutes TS segment, where 14 cases with leukocyte margination and minimal interstitial inflammatory infiltrate were identified. All of them showed the existence of hemorrhagic wounds of different sizes, mainly located in the superficial dermis.

Perls staining revealed no haemosiderin deposits on any of the skin fragments.

Van Gieson Verhoeff showed no changes in the density of elastic fibers in the superficial or deep dermis.

Immunohistochemical staining results were summarized in Table 1.

On fragments collected from healthy volunteers, HMGB1 expression was found at the nuclear level (Figure 1) in more than 90% of keratinocytes and RAGE expression was confined mainly to the basal or focal spinous layer (Figure 2).

It was found that similar values were obtained on all control fragments, values that also corresponded to the values of group 5. Therefore, the differences between group 5 (fragments from post-mortem lesions) (Figure 6) and the control group (Figure 3) were not statistically significant.

Comparing the data from group I with the control group, highly significant statistical differences ($p<0.0001$) were identified for both HMGB1 (Figure 4) and RAGE (Figure 5).

Data from group I were also compared with data from groups IV and V and values were approximately equal to those of the control group ($p<0.0001$).

We determined the degree of correlation between groups with similar survival interval and positivity of the two markers. No statistically significant differences were found in any group, except for Group I ($p<0.001$). The highest degrees of correlation (0.45 for Group III and 0.41 for Group IV) were found for the categories with survival intervals between 30 and 60 minutes (Group III) and postmortem wounds (Group IV).

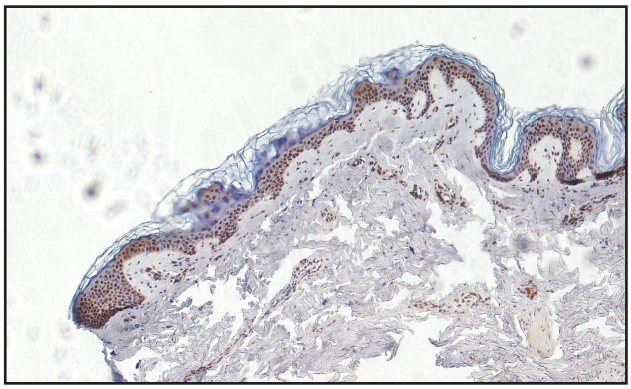

Figure 1: HMGB1 intensely positive at the nuclear level (Scor 0), Group V, 10x

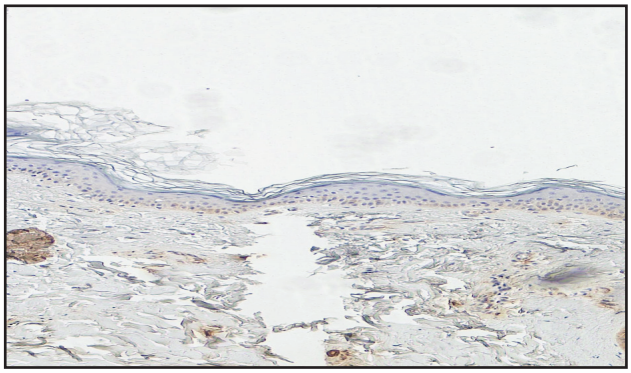

Figure 2: RAGE expression limited to the basal layer (Score 0), Group V, 10x

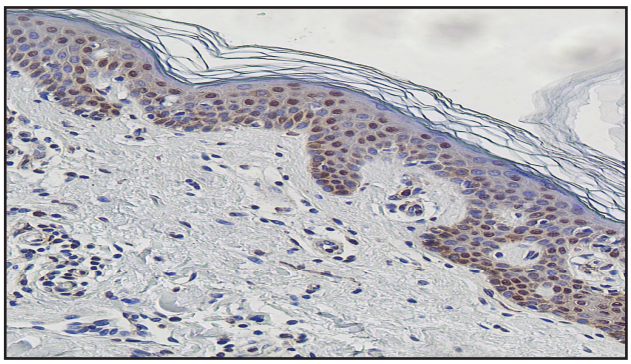

Figure 3 HMGB1 expression control group (Score 0, focal 1), 10x

Table 1 Immunohistochemical staining results

Marker	HMGB1						RAGE					
Scor	Group I	Group II	Group III	Group IV	Control	Group V	Group I	Group II	Group III	Group IV	Control	Group V
0	0	0	1	3	49	5	1	0	3	4	67	5
1	6	4	5	8	49	2	24	13	5	5	35	1
2	14	12	11	0	6	0	16	3	6	2	5	1
3	26	8	9	0	3	0	5	8	12	0	0	0

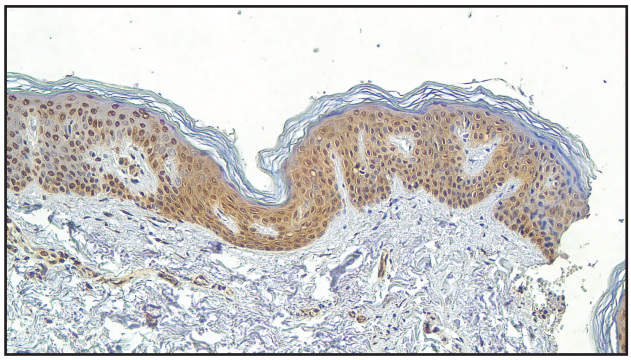

Figure 4 HMGB1 Group I expression (Score 3), score 0 on the left, 10x

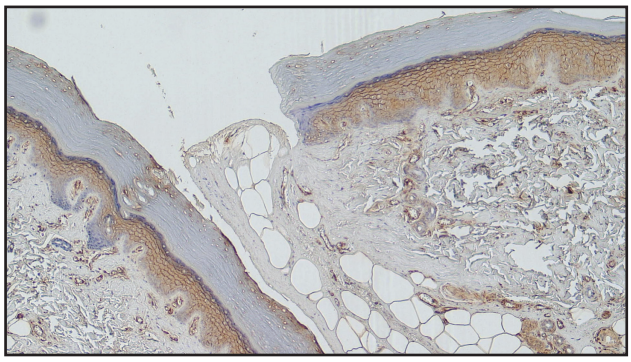

Figure 5 High RAGE expression in all epidermal layers, Group 1, Score 3, 10x

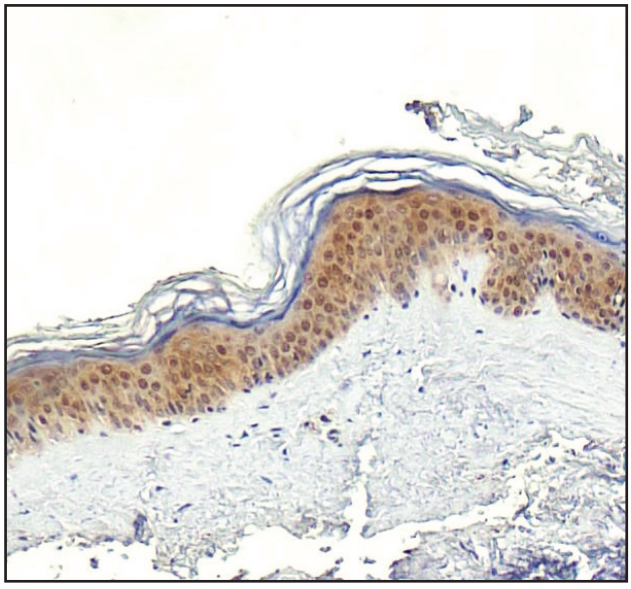

Figure 6: Postmortem lesion with nuclear expression approximately equal in intensity to cytoplasmic expression (score 1, focal 2), 10x

An interesting aspect was identified in the staining pattern of the wound's margin for HMGB1. (Figure 7) Thus, in these areas, keratinocytes do not show a reaction with the antibody (even at the nuclear level), in contrast to the surrounding regions where, in over 80% of cases, a graded reaction with a score of 2 or 3 was found. In the margins of postmortem wounds, a reaction graded 0 or 1 is found. Also, RAGE expression is much reduced in the margins of the wound. (Figure 8).

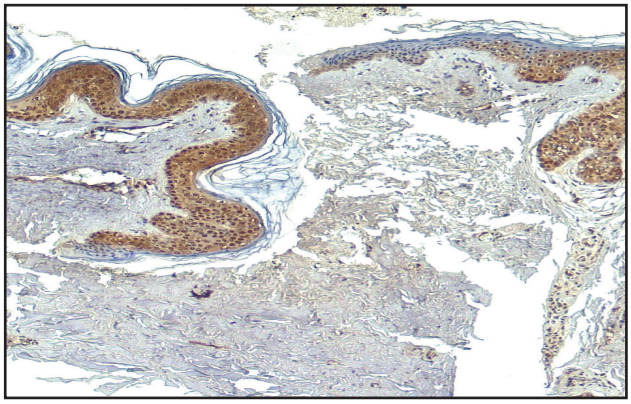

Figure 7: High HMGB1 expression (score 3), group 1, with loss of reactivity in keratinocytes in the margins of the wound, 10x

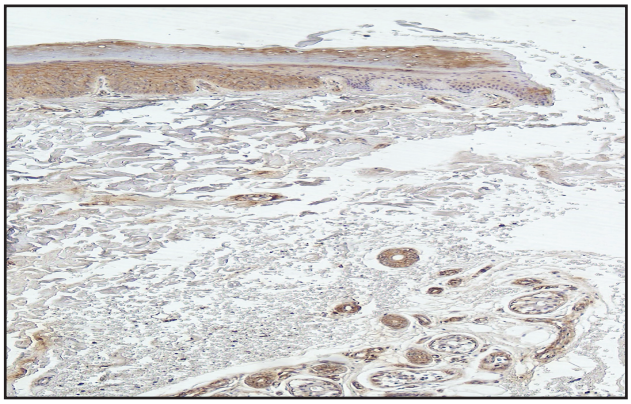

Figure 8: Increased RAGE expression in the vicinity of the lesion (Score 3), with reduced reactivity at the wound's margin (Score 0), Group 2, 10x

Discussion:

Our hypothesis for this study was that, following damage due to mechanical forces, HMGB1 will be released from keratinocyte nuclei and will function as a DAMP. Being released into the extracellular environment, HMGB1 will bind to various receptors, including RAGE, enhancing its expression.

As expected, traumatic injury produced translocation of HMGB1 from the nucleus into the cytoplasm and then into the extracellular environment.

Kiyoshi et al. used serum levels of HMGB1 in a batch of laboratory animals to estimate the

time interval after termination and concluded that HMGB1 might be helpful in forensic practice (24).

In a study led by Eman Ahmed Alaa El-Din et al., they determined both serum levels and immunohistochemical expression of HMGB1 in the skin of laboratory animals and human cadavers. They found increased expression of HMGB1 directly proportional to the time elapsed after death. Determination of HMGB1 expression was performed on the uninjured skin. (25)

In another study by Tie-Lei Gao et al., it was found that in the first 30 minutes after brain injury, HMGB1 is released from the nuclei of neurons, with an absence of expression in the center of the lesion (the area that absorbs the greatest impact force). In our study we also found an absence of expression in keratinocytes in the margins of the wound, with preservation of expression in postmortem wounds. RAGE expression was also shown to be enhanced in the first 6 hours after trauma. (26)

A study that monitored plasma levels of HMGB1 in patients with major trauma demonstrated a 30-fold increase in protein levels immediately after trauma ($P < 0.003$ vs. healthy volunteers). Also, plasma levels of HMBGB1 in those who did not survive after trauma were higher than those of survivors (13).

In cell culture, it has been shown that RAGE expression is induced by amphoterin (HMGB1) (27).

Our study evaluated, for the first time, the expression of HMGB1 in skin wounds and revealed that translocation of the protein intracytoplasmically or even extracellularly occurs from the first minutes of lesion occurrence.

Also, RAGE expression became more pronounced in group III, being directly correlated with HMGB1 expression.

A clear difference in the expression of the two markers was revealed by comparing postmortem wounds with survival time wounds.

A shortcoming of the study was the relatively small number of samples collected from postmortem lesions.

Conclusions: Following immunohistochemical evaluation of HMGB1 in skin wounds, it was found that it may be useful in differentiating postmortem from intravitally produced lesions. RAGE expression, associated with HMGB1 expression, can be useful in assessing the chronology of wound occurrence, provided that these markers are also determined in control fragments, which are essential for a reliable analysis of vital reactions.

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