Research Article

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Evaluation of apoptotic caspase levels in estimation of the wound age

Yara yaşı tahmininde apoptotik kaspaz düzeylerinin değerlendirilmesi

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Abstract

Objectives: We aimed to investigate the potential use of the expression of apoptotic signaling pathway genes of rat in skin wound age estimation.
Material and methods: For this purpose, we formed cutting tool injuries using a scalpel in an experimental model. Then, we assessed Caspase 3, 8 and 9 mRNA levels by using quantitative real-time PCR and protein levels by using immunohistochemistry in rat skin wounds. In addition, we used TUNEL assay to detect apoptotic cells.
Results: We observed that Caspase 3 mRNA level significantly increased (2.1 ± 0.4 folds) on day 3 ($p < 0.05$) and Caspase 8 mRNA level significantly increased (1.8 ± 0.2 folds) on day 5 ($p < 0.05$). Caspase 9 mRNA level increased (1.9 ± 0.1 folds) on day 3 and (2.5 ± 0.4 folds) on day 5 ($p < 0.05$). The percentage values of polymorphonuclear leukocytes (PMNLs) and inflammatory mononuclear cells (IMCs) were observed after immunohistochemical staining by Caspase 3, 8, 9 antibodies. Our immunohistochemistry results were found to be consistent with the mRNA results observed. We reported a statistically significant increase in Caspase 3, 8 and 9-positive cells on days 3 and 5 after immunohistochemical staining as well.
Conclusion: Our results suggest that time-dependent features of apoptotic factors might offer a potential tool in estimating wound age.

Keywords: Forensic science; Immunohistochemistry; Real time PCR; Wound age estimation.

Özet

Amaç: Sıçan derisindeki cilt yara yaşı tahmininde, apoptotik sinyal yolağı genlerinin ifadelenmesinin potansiyel kullanımını araştırmayı amaçladık.
Gereç ve Yöntem: Bu amaçla, deneysel modelde bir neşter kullanarak kesici alet yaralanmaları oluşturduk. Daha sonra sıçan cilt yaralarında kantitatif gerçek zamanlı PCR ile Caspase 3, 8 ve 9 mRNA düzeylerini ve immünohistokimya yöntemi ile protein düzeylerini değerlendirdik. Buna ilaveten, apoptotik hücreleri saptamak için TUNEL yöntemini kullandık.
Bulgular: Kaspaz 3 mRNA düzeyinin 3. günde ($p < 0.05$) belirgin olarak arttığı (2.1 ± 0.4 kat) ve Kaspaz 8 mRNA düzeyinin 5. günde ($p < 0.05$) belirgin olarak arttığı (1.8 ± 0.2 kat) gözlendi. Kaspaz 9 mRNA düzeyi 3. günde (1.9 ± 0.1 kat) arttı ve 5. günde (2.5 ± 0.4 kat) arttı ($p < 0.05$). Polimorfonükleer lökositlerin (PMNL) ve inflamatuar mononükleer hücrelerin (IMC) yüzde değerleri Kaspaz 3, 8, 9 antikorları ile immünhistokimyasal boyama sonrasında gözlendi. İmmünohistokimya sonuçlarımızın gözlenen mRNA sonuçları ile tutarlı olduğu bulundu. İmmünohistokimyasal boyamadan sonra 3. ve 5. günlerde Kaspaz 3, 8 ve 9 pozitif hücrelerde istatistiksel olarak anlamlı bir artış olduğunu belirledik.
Sonuç: Bulgularımız, apoptotik faktörlerin zamana bağlı özelliklerinin yara yaşını tahmin etmede potansiyel bir araç olabileceğini düşündürmektedir.

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Anahtar Kelimeler: Adli tıp; Immünohistokimya; Gerçek zamanlı pcr; Yara yaşı tahmini.

Introduction

Wound examination is one of the most important areas for forensic pathologists and it requires vast amount of experience of forensic practice [1–4]. In the course of wound examination, it is essential to evaluate the causal relationship between the death and the wounds found at autopsy and to differentiate antemortem wounds from postmortem damage. Additionally, if there is an antemortem wound generated, it is also crucial to determine how long before death it was formed [5, 6]. Consequently, in order to achieve a sufficient estimation of the age of a wound in forensic practice, objective scientific evidence becomes indispensible as well as it is required.

Wound healing process is generally comprised of inflammatory, proliferative and tissue remodeling phases [7–9]. Apoptosis is the basic cause of decreasing cellularity during the various stages of wound healing. It is a vital component in the removal of inflammatory cells and in the evolution of granulated tissue into the scar during skin wound healing [10, 11].

A special group of serin proteases, called as "caspases", are the enzymes activated at the final phase of apoptosis and play a key role in the inhibition and induction of apoptosis. Caspase activation is associated with apoptosis stimulation whereas caspase inhibition is related to apoptosis inhibition. Initiator caspases, caspases 8 and 9 convert several intra- and extra-cellular signals into proteolytic activity and thereby initiate the caspase cascade. Caspases 3, 6, and 7, which are known as effector caspases, halt specific intra-cellular polypeptide targets and maintain apoptosis mechanism. Caspase 3, at the final stage of intrinsic and extrinsic pathways of apoptosis, plays a major role in the regulation of apoptosis, such that both pathways converge at Caspase 3 [12, 13]. The extrinsic pathway, which is activated by death receptors such as Fas and tumor necrosis factor (TNF), activates Caspase 8 leading to activated effector caspases including Caspase 3, 6, and 7. The intrinsic pathway, on the other hand, is stimulated by cytochrome C released from mitochondria which activates procaspase 9 by binding caspase-activator APAF1 found in the cytosol [14]. As a result, caspase enzyme activation plays several roles in the apoptototic cell death, ranging from apoptosis induction to DNA fragmentation, plasma membrane budding, and the phospholipid asymmetry loss.

In this study, we studied the two apoptotic initiator caspases, caspase-8 and caspase-9 as well as the executioner caspase-3 in antemortem wounds in order to investigate the use of caspase levels in the determination of a wound age. mRNA and protein levels of these three caspases were comprehensively measured and analyzed in rat skin wounds formed by scalpel.

Materials and methods

Animal model of the skin wounds

Adult female albino Wistar rats (12 weeks old and 250–300 g body weight) were obtained from the Gazi University, Laboratory Animal Breeding and Experimental Research Center. All animal experimental protocols were performed in accordance with the guidelines issued by the Local Institutional Committee for the Ethical Use of Animals at Gazi University (protocol No. G.U. ET-10.064). The animals were maintained in individual polycarbonate cages at a constant temperature (21° to 24°C) and humidity of 30–49% on a 12 h light/dark cycle. Animals were fed with standard diet and water ad libitum throughout the experiment. All animals were acclimatized for a minimum period of 1 week prior to the beginning of the study.

Before the experiment, a cocktail of 50 mg/kg ketamine + 5 mg/kg xylazine was administered to the rat intramuscularly for general anesthesia. Rats' dorsal skin hair was shaved until the epidermis totally appeared and then the skin was cleared by povidone iodine. Incised wound model was formed by scalpel on the dorsal skin as described previously [15]. Thereafter, each rat was individually housed and fed with sterilized food and redistilled water. Wound, surrounding healthy tissue and control specimen were excised after the animals were sacrificed under ketamin hydrochloride and xylazine hydrochloride anesthesia at the 1st, 3rd, 5th, 7th, 17th and 21st days following the wounding. We employed 6 experimental groups and 1 control group each containing 6 study subjects, therefore a total of 42 rats.

Total RNA isolation and reverse transcriptase (RT) reaction

Total RNA was isolated from the samples using TriReagent system (peqGOLD TriFastTM, Peqlab, Erlangen, Germany) and subsequently treated with RNase-free DNase I

recombinant (DNaseI; Roche Diagnostics, Germany) to prevent genomic DNA contaminations according to the manufacturer's instructions. RNA pellets were dissolved in 10–30 μL RNase- and DNase-free water, after which they were air-dried. Isolated RNA was then stored at –80°C. Equal amounts of RNA were used for reverse transcription. First-strand complementary DNA (cDNA) was synthesized from 1 μg isolated RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol and synthesis reaction was carried out in an Eppendorf Mastercycler ep gradient S thermal cycler (Eppendorf, Hamburg, Germany). Samples were analyzed in duplicates. cDNA products were kept at –20°C.

Quantitative real-time PCR (RT-PCR)

Real-time PCR experiment was performed using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. The expression levels of the following genes were investigated: Caspase 3, Caspase 8 and Caspase 9. Beta actin (ACTB) was used as a housekeeping gene in order to normalize the mRNA expression levels of the target genes. Probes and primers spanning exon–exon junction for each gene assay were designed using the Universal Probe Library (UPL) Assay Design Center (Roche Applied Science, GmbH, Mannheim, Germany). The sequences of the gene-specific primers and UPL numbers were provided in Table 1. A volume of 2 μL of cDNA product was subjected to real-time PCR in a 10 μL total reaction mixture containing 2.5 μL of LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 0.5 μL sense and antisense primers, 0.1 μL probe and template cDNA. The single step of initial denaturation at 95°C for 10 min was followed by 55 cycles of 10 s at 95°C, 20 s at 65°C, 1 s at 60°C for annealing and 30 s at 40°C for cooling. Each sample was analyzed in triplicate. The data were analyzed using LightCycler Software v 3.5 (Roche Diagnostics). Relative mRNA expressions of Caspase 3, Caspase 8 and Caspase 9 were determined using Relative Expression Software Tool 2008 (REST©) [16].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

We detected apoptotic cells in excisional biopsy materials by using the TUNEL method. TUNEL was performed using the ApopTag Peroxidase in Situ Apoptosis detection kit (S7110, Chemicon International, Inc., Temecula, CA), following the manufacturer's instructions. Peroxidase substrate 3,3′-diaminobenzidine (DAB) was used to stain apoptotic cells. Methyl green (0.5%) was used as a nuclear stain. TUNEL-positive PMNLs and IMCs were determined under a light microscope using an ocular grid measuring 0.25 mm^2 at 400× magnification (Olympus, BX51) and were grouped in the following manner. 0 = none, 1 = mild (up to 10), 2 = moderate (between 10 and 30), 3 = marked (over 30).

Immunohistochemistry

Formaldehyde-fixed and paraffin-embedded materials were sectioned at 3–4 μm thickness. After deparaffinization, they were rehydrated in distilled water. Then endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 10 min and specimens were rehydrated in distilled water. Antigen retrieval was performed in a microwave oven in 0.01 M sodium citrate buffer (pH 6.0) for 20 min. Then specimens were incubated at room temperature for 20 min. They were washed in phosphate buffer saline (PBS) (pH 7.6) for 5 min. Slides were incubated in non-immun protein blocking serum for 10 min. Immunostaining was performed using the primary antibodies of caspases 3, 8, 9 (Caspase 3 Ab-4 neomarkers RB CITRAT RTU; Caspase 9 Ab-4 neomarkers RB 1205 citrat 1:50; Caspase 8 abcam ab4052 citrat 1:50) diluted as 1:50 in TBS. The cells were incubated with these antibodies at room temperature for 1 h. The sections were rinsed in PBS

Table 1: Gene-specific primer, UPL probe numbers and sequences.

Gene	Forward primer	Reverse primer	UPL Probe No	UPL probe 5′-FAM-sequence-TAMRA-3′
ACTB	5′-CCCGCGAGTACAACCTTCT-3′	5′CGTCATCCATGGCGAACT-3′	17	CAGCTCCT
Caspase 3	5′-CCGACTTCCTGTATGCTTACTCTA-3′	5′-CATGACCCGTCCCTTGAA-3′	80	CCTGGAGA
Caspase 8	5′-AGAGCCTGAGGGAAAGATGTC-3′	5′-TCACATCATAGTTCACGCCAGT-3′	89	CAGCATCC
Caspase 9	5′-CGTGGTGGTCATCCTCTCTC-3′	5′-GAGCATCCATCTGTGCCATA-3′	10	CCACCTCC

for 5 min. They were then incubated in biotinylated secondary antibody for 20 min and rinsed in PBS for 5 min. Streptavidin conjugated with horseradish-peroxidase complex was applied to the samples and they were rinsed with PBS for 5 min. 3,3′-diaminobenzidine was used as chromogen for color development and hematoxylin was employed for counterstaining. Then the sections were dehydrated 90, 95 and 100% alcohol for 5 min and then mounted. The positive results of stained cells and their distributions were observed under a microscope of 400× magnification. The number of polymorphonuclear leukocyte (PMNL) and inflammatory mononuclear cells (IMCs) stained with caspase 3, 8, 9, antibodies in the wound area were grouped in the following manner. 0 = none, 1 = mild (up to 10), 2 = moderate (between 10 and 30), 3 = marked (over 30).

Statistical analysis

Differences between groups were interpreted using non-parametric Friedman and Kruskal-Wallis test. Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). A p value lower than 0.05 was considered significant. Statistical significance of differences in mRNA expression was analyzed by pair-wise fixed reallocation randomization test using a statistical model included in the REST© (relative expression software tool) software developed for group-wise comparison and statistical analysis of relative expression results.

Results

Caspase 3, 8, 9 mRNA levels of tissues from rat skin were determined by real-time PCR on the 1st, 3rd, 5th, 7th, 17th and 21st days after woundings. When compared with the control group, Caspase 3 mRNA level significantly increased 2.1 ± 0.4 folds ($p < 0.05$) on day 3 and Caspase 8 mRNA level significantly increased 1.8 ± 0.2 folds on day 5 ($p < 0.05$). Caspase 9 mRNA level also increased 1.9 ± 0.1 folds ($p < 0.05$) on day 3 and 2.5 ± 0.4 folds on day 5 when compared with the control group (Figure 1) ($p < 0.05$).

In the present study, we detected PMNL dominance in the wound area up to day 3. We also observed a smaller number of IMCs. Therefore, Caspase 3, 8, 9 antibodies positively stained PMNLs in this period. On days 5 and 7, PMNL and mononuclear (MNL) cells were counted equal (data not shown). Furthermore, PMNLs, macrophages and fibroblasts were positively-stained by Caspase 3, 8, 9 antibodies. On days 17 and 21, PMNL dominance decreased and IMCs became dominant (Table 2). In this period, macrophages and fibroblasts were positively stained by aforementioned antibodies. Although vascular proliferation was rarely seen until day 5, it reached a peak on days 5 and

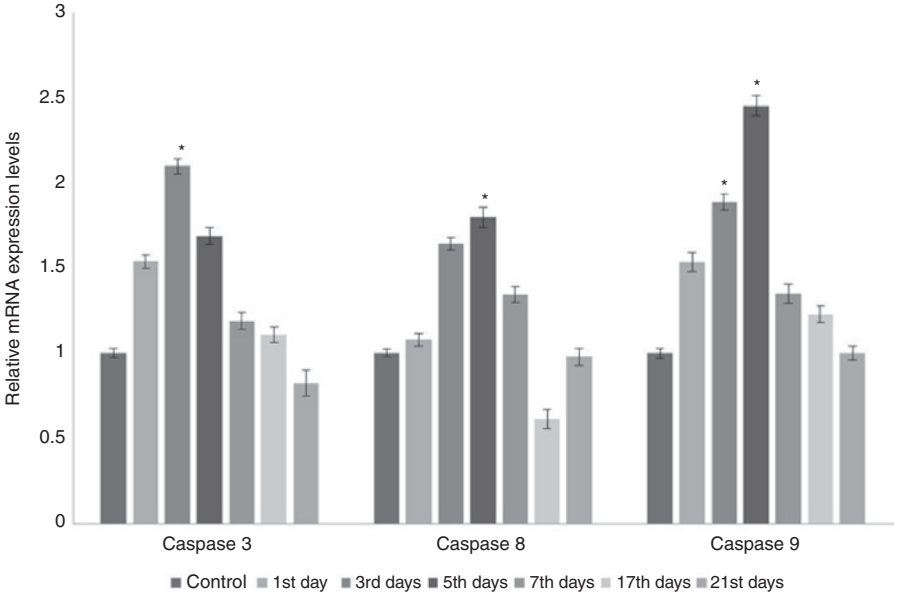

Figure 1: Differences in mRNA levels of Caspase 3, Caspase 8 and Caspase 9 in the wound formed by scalpel with respect to the control up to day 21. Non-operated tissue was used as a control. The results were normalized to ACTB – the housekeeping gene – and expressed in form of mean ± SD of at least three independent experiments. *$p < 0.05$.

Table 2: The percentages of total polymorphonuclear leukocytes (PMNLs) and inflammatory mononuclear cells (IMCs) immunohistochemically stained with Caspase 3, 8 and 9 antibodies in the wound area.

Time after injury	Caspase 3	Caspase 8	Caspase 9
24 h	Up to 10%	None	Up to 10%
3 days	10–30%	Up to 10%	10–30%
5 days	Up to 10%	10–30%	30–50%
7 days	None	None	None
17 days	Up to 10%	None	None
21 days	None	Up to 10%	Up to 10%

7 followed by a gradual decrease on days 17 and 21. The number of PMNLs and IMCs were observed after immunohistochemical staining by Caspase 3, 8, 9 antibodies are shown in Table 2.

In the first day of wound, only a few cells was immunoreactive with caspase 3 and 9 and there was no caspase 8 expression. Moderate caspase 3 and caspase 9 immunoreactivity and few cells was positive with caspase 8 was seen on the third day of wound. On the fifth day, there was a peak expression in Caspase 9, moderate and few cells were positive with caspase 3. Interestingly, on day seventh day, caspase 3, 8 and 9 immunreactivity was not seen. In the seventeenth day of wound, there was no immunoreactivity for caspase 8 and 9 and few cells were positive with caspase 3. On the twenty first day of wound, caspase 8 and caspase 9 were positive in few cells and caspase 3 was negative (Figures 2–4).

On the third, fifth and seventh days of wound, the number of apoptotic PMNL and IMC cells stained with the TUNEL technique was found to be high when compared to control group. Besides, apoptotic cells was not seen on days 1, 17 and 21 when compared to control group (Figure 5).

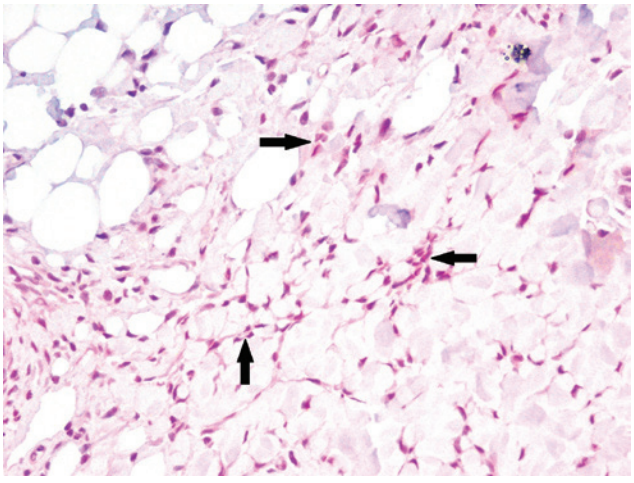

Figure 2: Caspase 9 positivity in 30–50% of PMNLs and IMCs on the 5th day (×400).

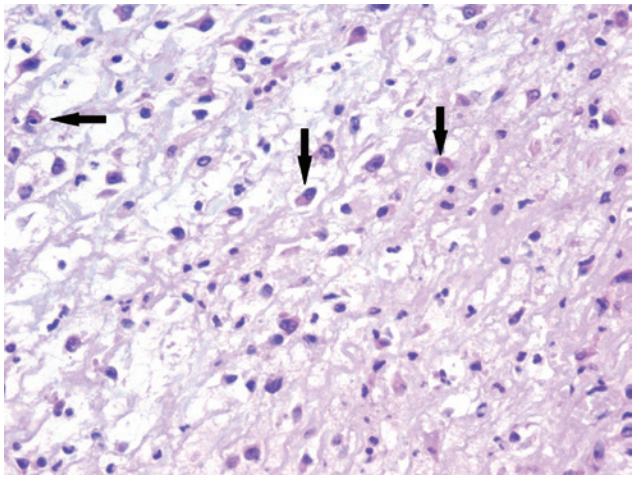

Figure 3: Caspase 8 positivity in 10–30% of PMNL and IMCs on the 5 th day (×400).

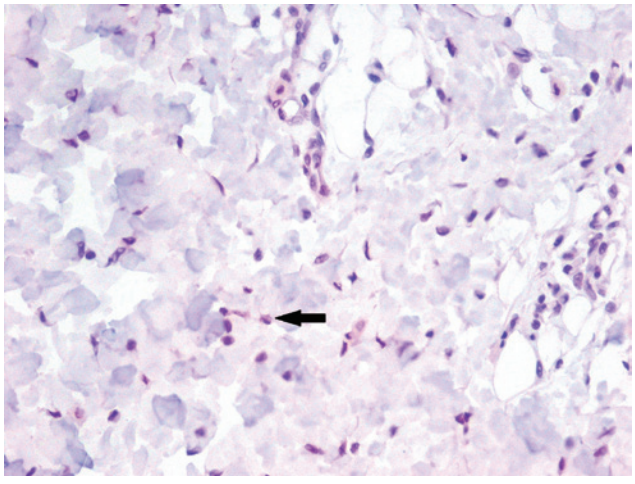

Figure 4: Caspase 3 positivity in up to 10% of PMNLs and IMCs on the 5th day (×400).

Discussion

Wound age estimation has been one of the most popular themes in forensic research. In forensic medicine practice, physicians usually encounter cutting tool injuries. For this purpose, we formed cutting tool injuries using scalpel in an experimental model and we aimed to investigate the apoptotic effects of the skin wound.

Excessive scar is formed as a result of aberrations in physiologic wound healing and may arise following

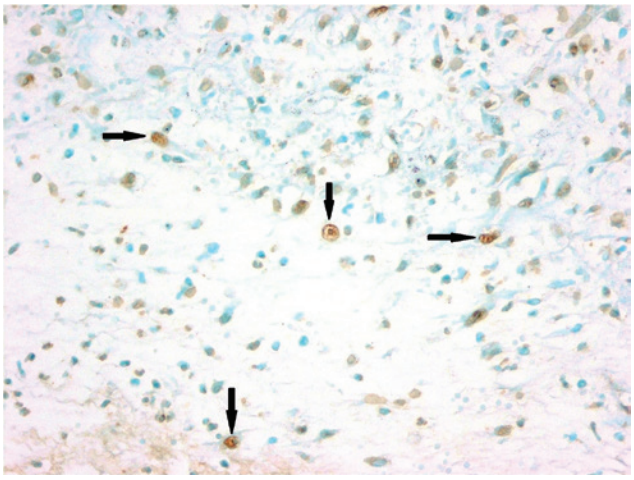

Figure 5: Apoptotic cells in the scalpel wound area in the 3rd day (TUNEL ×400).

a damage to the deep dermis. The physiologic response to wounding in adult tissue is the formation of a scar, a process that can be temporally grouped into three distinct phases; inflammation, proliferation and remodeling [17]. During the wound healing process, scar tissue forms due to decreased cellularity of the hypercellular granulation tissue. In wound healing, cell proliferation and apoptosis are well harmonized. Reduction of cellularity due to apoptosis plays a major role in the transformation of the granulation tissue into scar tissue. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar [18].

Some recent studies also suggest that Caspase 3 mRNA levels significantly increased after formation of burn margin skin [19–21].

When our results were compared to those of others which showed the effects of apoptotic process on wound healing, not only were they found to be consistent with aforementioned studies but they were also providing some new evidence for the increase in the expression levels of the apoptosis-inducer caspases. In our study, Caspase 3, Caspase 8 and Caspase 9 mRNA levels markedly increased on day 3 and day 5 in the scalpel injury groups when compared with uninjured control group. In compatible with the mRNA levels, Caspase 3 protein levels were significantly upregulated on day 3 when compared with the control group.

Moreover, Jadhav et al. [22] showed that increased Caspase 3 mRNA and protein levels were observed in the combined radiation and burn injury group in comparison to thermal injury alone. Johnson et al. [23] demonstrated significant differences in the gene expression levels of key mediators of both the intrinsic and extrinsic apoptosis pathways in oral wound healing in comparison to skin wound healing. They found that Caspase 3, Caspase 8 and Caspase 9 expressions were significantly higher in uninjured skin, and that as opposed to uninjured tissues, they displayed significant increases at 24 h postinjury during both skin and tongue wound healing, with skin showing greater expression levels than tongue [23]. In addition, Chodon et al. [24] analyzed normal and keloidal fibroblasts and demonstrated that Caspase 3 was activated in only normal fibroblasts following the induction of apoptosis by a pharmacological agent.

In our study, we analyzed the apoptotic PMNL and IMC counts using the TUNEL method in serial sections obtained from the injury tissues. Our analysis results show that following scalpel-induced injury, there were no significant differences in the TUNEL-positive cell counts of ten injury sites randomly selected at 24 h, on days 17 and 21. However, on day 3, 5 and 7, TUNEL-positive cell counts as the number of apoptotic cells stained with TUNEL was found to be significantly higher when compared to other groups and the control group. Our results pointed to a high level of apoptosis due to the high number of apoptotic cells. Chen et al. [25] showed no statistically significant increases in the TUNEL-positive cell count up to 1 h following reperfusion. However, the authors reported a peak at 12 h marked with a 3.3-fold increase in the apoptosis rate in the intestinal mucosa following reperfusion [25]. Another study showed an increase in the TUNEL-positive cell count in the inflamed colon epithelium of patients with ulcerative colitis. The authors showed that Caspase 8 and 9 protein levels increased on day 3 and day 5 in the scalpel injury groups when compared with the control group [26].

The initiator (Caspase 8 and 9) and effector (Caspase 3, 6 and 7) caspases are activated during apoptosis. Real-time PCR analysis and immunohistochemical staining confirmed that Caspase 3, 8 and 9 were activated and their expressions were enhanced at some posttraumatic intervals, which provides evidence to some extent that extrinsic and intrinsic pathways both play pivotal roles in monocytes and fibroblasts apoptosis because both pathways are linked and the molecules in one pathway can influence the other [27]. Through the experiments performed in our study, it was observed that caspase 3, 8, and 9 were all positive in all injured samples, suggesting that may be contributing to PMNs apoptosis. Zhao et al. [8] showed that in injured sections, IMCs accounted for the Caspase 8-positive cells while Caspase 6, 7 and 9 were detected in PMNs and IMCs near the wound site. The authors found that Caspase 8-positive cells on days 1 and 5 and Caspase 9-positive cells on day 3 were at their

highest levels after immunohistochemical staining [8]. Another study revealed that the changes in Caspase 3 activity were assessed by imunohistochemistry, western blot and Caspase 3 colorimetric assay. It showed that expression level of Caspase 3 in the peripheral area of contused rat skeletal muscle increased gradually and peaked on the fifth day after injury, and then decreased gradually [7]. Additionally, Du et al. [28] looked into the expression of Caspase 3 during skin wound healing and explored the applicability of Caspase 3 to determination of wound age. The ratio of the Caspase 3-positive cells was low in the wound specimens aged between 0–3 h, and peaked in the wound specimens aged 3 days. Thereafter, the ratio decreased and reached a minimum in the specimens aged 14 days [28]. In accord with the findings of the aforementioned study, we reported a statistically significant increase in the Caspase 3, 8 and 9-positive cells on days 3 and 5 after immunohistochemical staining in the scalpel-induced injury groups. Although changes in gene expression levels do not necessarily translate into protein expression or function, our findings show that Caspase 3, Caspase 8 and Caspase 9 protein levels follow similar trends to that of gene expression.

In conclusion, in context of data obtained, forensic pathologists, in tackling chalenging cases characterized with sharp injuries, would be able to distinguish antemortem and postmortem injuries, and perform age estimation of the injury based on the expression levels of the related genes involved in the apoptotic pathway. From the viewpoint of forensic pathology, the time-dependent characteristics of these factors provide a potential for wound age estimation. Moreover, our findings may help physicians make correct decisions in differentiation of pre and postmortem traumatic changes. However, further investigations using human wounded skin with known postinfliction intervals are necessary to better comprehend the role of mRNAs in wound examination.

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Conflict of interest: The authors declare that they have no conflict of interest.

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