ORIGINAL ARTICLE

Comparison of caspase-8, granzyme B and cytochrome C apoptosis biomarker levels in orthopedic trauma patients

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ABSTRACT

BACKGROUND: The primary objective of this study was to investigate whether or not apoptosis is induced following bone fracture, and if so, to investigate whether the extrinsic or intrinsic pathway of cell death is stimulated.

METHODS: A total of 30 patients who presented at our clinic and were diagnosed with bone fracture following trauma were included in the study group. A control group was formed of 37 age and gender-matched volunteers. On the day after the fracture, blood samples taken from the patients were examined for cytochrome C, granzyme B and caspase-8 with the ELISA method.

RESULTS: A total of 67 individuals were evaluated (fracture group: 30, control group: 37) in this study. Caspase-8 was found to be statistically significantly high in the patient group (0.37±0.06 ng/mL, p=0.002). No significant difference was determined between the groups in respect to cytochrome C values (p=0.173). The granzyme B values were determined to be significantly high in the patient group (52.56±8.51 pg/mL, p=0.007).

CONCLUSION: These results obtained from patients with a long bone fracture demonstrated that serum caspase-8 and granzyme B levels were higher in patients than in the control group, thereby showing activation of the extrinsic pathway. However, no significant difference was determined between the groups concerning serum cytochrome C levels. This study may guide future studies designed for better understanding of the molecular pathways that govern the events during a fracture, which will be important for the future advancement of fracture treatment.

Keywords: Apoptosis; caspase-8; cytochrome C; fracture; granzyme B.

INTRODUCTION

Fracture healing stages following trauma are separated into the phases of hematoma, inflammation, and remodelling. However, the healing process that starts in damaged tissue after trauma is more complex than this.[1] Relationships between several different molecules and cellular activities are involved in this process.[2] New cells entering the fracture site, cell proliferation, cell differentiation and cell death are a part of fracture healing.[3] Apoptosis is controlled and selective cell death for the elimination of cells in multi-cellular organisms. Apoptosis has an important role in the elimination of infected and damaged cells, which are differentiated from normal cells by vital components and have undergone mutation in development and tissue hemostasis.[4]

Caspases are the primary enzymes found in the cytoplasm that have an important role in apoptotic cell death. Caspases are members of the cysteine protease family. These proteins are inactive when synthesized and are activated with the occurrence of several cellular and morphological changes during cell death. They are activated by intrinsic or extrinsic pathways, which include events, such as endoplasmic reticulum stress, metabolic stress, excessive reactive oxygen and

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DNA damage.[5] Caspase-8 is among the activation caspases involved in signal transmission and activating killer caspases. Caspase-8 is a member of the cysteine proteases involved in apoptosis and cytokine processing. As is the case for all caspases, caspase-8 is synthesised when inactive as a zymogen procaspase single polypeptide chain and is activated with proteolytic cleavage.[4]

Granzyme B is the most important of the serine proteases stored in natural killer (NK) and cytotoxic T lymphocytes (CTL). Activated T lymphocytes express granzyme B, exposing serine proteases from granulation of cytotoxic T cells, and target cell death is induced.[6] Caspases are granzyme B substrates and their activation leads to cell death with apoptosis. In cell death induced by granzyme B, elimination of the damaged target cell occurs through a mechanism driven by NKs and CTLs.[7]

Cytochrome C is a mitochondrial protein providing vital support functions by transferring electrons to the respiratory chain to maintain ATP production. However, during the activation of apoptotic mechanisms, it is expressed from the mitochondria and activates the caspase cascade while in the cytoplasm or in the intrinsic apoptotic pathway, or provides amplification of the extrinsic apoptotic signals.[8] When cytochrome C is expressed from the mitochondria to the cytoplasm, it is an essential mediator of apoptosis. Under normal conditions, this process occurs in response to DNA damage, but because of various mechanisms, it does not function in several cancer cells.[9] While caspase-8 and granzyme B are biomarkers of the extrinsic apoptotic pathway, cytochrome C is a biomarker showing the intrinsic apoptosis pathway.

To our knowledge, there have been no previous studies in literature that have shown the above-mentioned apoptosis markers in vivo in patients with bone fracture following trauma. This study aims to investigate whether or not apoptosis is induced following bone fracture, and if so, to investigate whether the extrinsic or intrinsic pathway of cell death is stimulated.

MATERIALS AND METHODS

Patients in the study group were selected from the patients who presented at our clinic and were diagnosed with bone fracture caused by trauma. Exclusion criteria were defined as smoking, the use of alcohol or substances, concomitant psychiatric disease, acute or chronic disease (diabetes mellitus, hypertension, chronic renal failure etc) or the presence of a focus of infection.

Informed consent was obtained from all the study participants, and approval for this study was granted by the Local Ethics Committee.

On the basis of α=0.005, β=0.10, (1-β=0.9), it was deemed necessary to have at least 30 individuals in each group. A total of 30 adult patients were selected at random from the patients requiring surgical treatment for a diagnosis of bone fracture. For each patient in the fracture group, a record was made of the cause of trauma and information about the fracture region, in addition to other blood parameters of Wbc, Hb, Plt and blood glucose.

The control group was formed of 37 healthy volunteers, age and gender-matched to the patient group, who had no chronic disease, malignancy, autoimmune disease, or systemic infection. On the first day of hospitalisation, blood samples of approximately 5 ml were taken from the patients. The blood samples of both the patient and control groups were kept at room temperature for 5 mins, then, centrifuged at 4000 rpm for 5 mins. The serum which formed uppermost was withdrawn and portioned into at least two Eppendorf tubes, which were stored at -80°C until assay. When the number of patients required for this study was reached, all the samples were thawed then assayed in a single session in an ELISA device with human CYCS (cytochrome C), human granzyme B, and human caspase-8 tests (ELISA kits, FineTest® produced by Wuhan Fine Biological Technology, China).

Statistical Analysis

Data obtained in the study were analysed statistically using SPSS (vn 22.0 for Windows) software. For data that met parametric assumptions, the Kolmogorov Smirnov test was applied to independent groups to determine the significance between two mean values. When parametric test assumptions were not met, the Mann-Whitney U test was applied. Correlation analysis was applied and in the evaluation of the results obtained, the Chi-square test was used. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

A total of 67 individuals were evaluated, comprising 40 (59.7%) males and 27 (40.3%) females with a mean age of 62.03 years (range, 18–91 years) in the patient group and 61.62 years (range, 25–90 years) in the control group.

Fracture localisation distribution was determined as humerus in three (10%) patients, femur in 21 (70%) patients, and tibia in six (20%) patients. The mechanism of trauma was a fall in 23 (76.7%) patients, traffic accident in four (13.3%) patients, and other reasons in 3 (10%) patients. Blood parameters in the patient group were determined as Wbc: 11.26±2.98, Hb: 11.58±1.88, Plt: 246.16±81.06, and non-fasting blood glucose: 122.76±34.83 (Table 1).

No significant difference was determined between males and females. Caspase-8 was found to be significantly high in the patient group (p=0.002) (Fig. 1). No significant difference was determined between the groups in respect of cytochrome C

Table 1. Fracture localisations, mechanisms of trauma, and blood parameter results of the fracture group

	n	%	Mean	SD
Fracture localisation				
Humerus	3	10		
Femur	21	70		
Tibia	6	20		
Mechanism of trauma				
Fall	23	76.7		
Traffic accident	4	13.3		
Other	3	10		
Blood parameters*				
White blood cells			11.26	2.98
Hemoglobin			11.58	1.88
Platelet			246.16	81.06
Postprandial blood glucose			122.76	34.83

SD: Standard deviation.

values (p=0.173) (Fig. 2). The granzyme B values were determined to be significantly high in the patient group (p=0.007) (Fig. 3, Table 2).

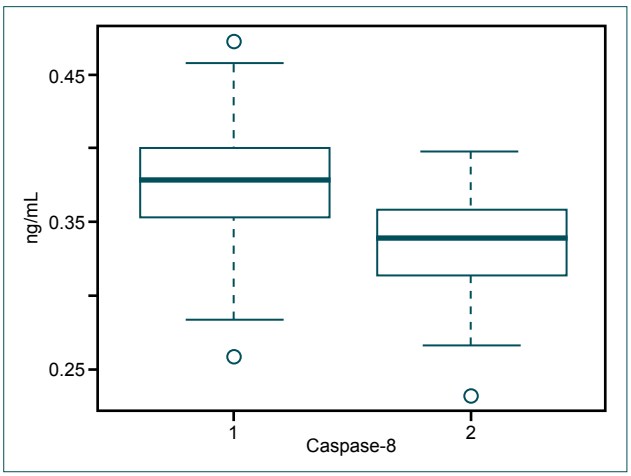

Figure 1. Box plot graph showing serum levels of caspase-8 in the fracture group (1) and the control group (2).

DISCUSSION

Following trauma, several mechanisms are triggered in the body. This study aims to investigate apoptosis in patients with a bone fracture following trauma and to investigate by which pathway apoptosis was activated. The results of this study demonstrated that both granzyme B and caspase-8 levels were higher in patients with long bone fractures than in the control group and no difference was determined be-

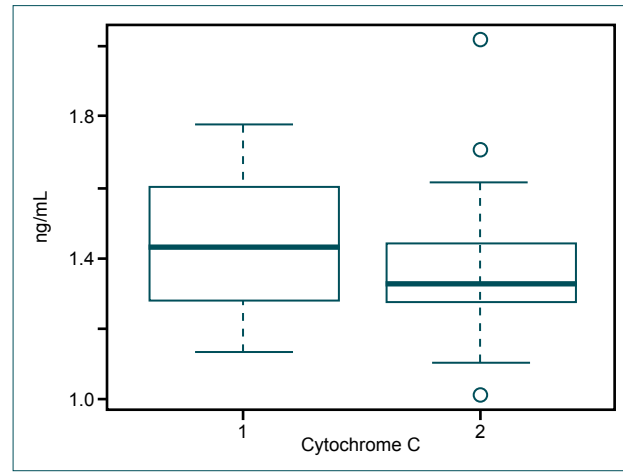

Figure 2. Box plot graph showing serum levels of cytochrome C in the fracture group (1) and the control group (2).

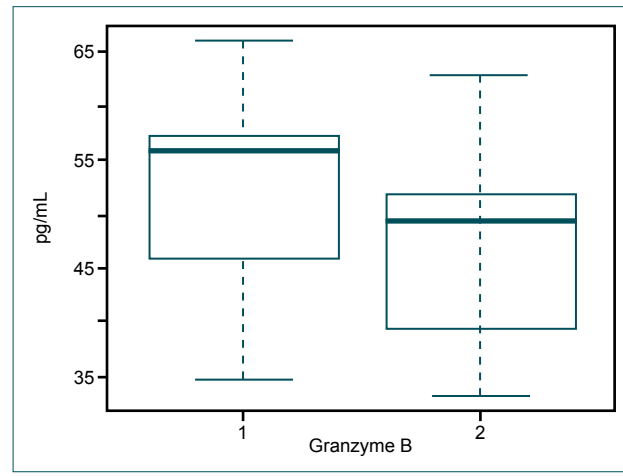

Figure 3. Box plot graph showing serum levels of Granzyme B in the fracture group (1) and the control group (2).

Table 2. Comparison of serum caspase-8, cytochrome C and granzyme B levels between groups

	Group	Minimum	Maximum	Mean	Standard deviation	p
Caspase-8 (ng/mL)	Fracture	0.26	0.47	0.37	0.06	0.002
	Control	0.23	0.40	0.33	0.04	
Cytocrome C (ng/mL)	Fracture	1.13	1.78	1.44	0.18	0.173
	Control	1.01	2.02	1.37	0.19	
Granzyme B (pg/mL)	Fracture	34.76	66.09	52.56	8.51	0.007
	Control	33.19	62.84	46.67	8.80	

tween the groups in respect of cytochrome C. In a previous study that examined cell proliferation and apoptosis in a mouse femoral fracture model, it was seen that despite being opposing processes, these two events were coupled in fracture healing.[10] It has been reported that inhibition of apoptosis signal-regulating kinase 1 (ASK1) could be a potential therapeutic option for fracture healing and the prevention of osteoarthritis progression.[11] The data of those studies partly support the findings of the current study. Both previous research and the current study show that apoptosis is a part of the healing processes following trauma or fracture.

Although there have been many previous studies on apoptosis during fracture healing, those reports have been in vitro or experimental animal models. The current study was especially designed to compare orthopaedic trauma patients with a control group. Apoptosis is a fundamental biochemical process for the selective and controlled elimination of cells within multicellular organisms.[4] Previous studies have demonstrated that apoptosis plays a critical role during embryonic limb development, skeletal maturation, and adult bone turnover through modelling and remodelling processes, and during fracture healing and bone regeneration. In humans, increased osteocyte apoptosis has been correlated with sites of rapid bone turnover, and osteoblast apoptosis plays an important role in bone development and maintenance.[12] It is estimated that 60–80% of the osteoblasts that originally assemble at the resorption pit die by apoptosis.[13] Moreover, chondrocyte apoptosis is an important event in the transition from cartilage to bone during fracture healing and the growth of long bones.[14] The two major pathways of apoptosis are the extrinsic and intrinsic pathways.[15] The results of the blood samples taken on the first day from the patients with long bone fracture were important in respect of showing that the extrinsic pathway of apoptosis was active.

Kim et al.[16] observed high levels of chondrocyte apoptosis following intra-articular fracture, and they suggested that apoptosis inhibition may be effective in the setting of acute trauma to prevent chondrocyte loss. In a study by Li et al.,[10] the relationship between cell proliferation and apoptosis during fracture healing was demonstrated in a mouse femoral fracture model. Cell proliferation and apoptosis were stated to be indispensable components of fracture repair. These findings are consistent with the results obtained in this study. The apoptosis mechanism following musculoskeletal system trauma has not been fully explained.[17] However, apoptosis is known to be a critical part of the pathological and physiological processes in bone, just as it is for all tissues. The apoptotic process is triggered by two pathways; the intrinsic pathway and the extrinsic pathway.[18]

Once granzyme B is delivered into the cytosol, it can proteolytically attack different protein substrates and initiate programmed cell death.[6] When granzyme B has entered the cell, it activates apoptotic procaspases, including caspase-8, which is an important regulator of cell viability.[19] Several diseases are associated with aberrant apoptosis. Caspases undertake a key role in osteoblast apoptosis.[18] Cytochrome C is a well-known mitochondrial protein with significant roles within the mitochondria, including in the electron transport chain, antioxidant defenses, and cell death.[20] The function of cytochrome C changes radically when apoptosis starts.[8] By stimulating different pathways, apoptosis affects bone diseases, such as osteoporosis, Paget's disease, osteoarthrosis, hyperparathyroidism and malignant osteolysis.[18]

There has been shown to be an increase in apoptosis following intra-articular fractures or in chondrocytes exposed to high-energy trauma.[21] A good understanding of apoptosis is important to resolve the problems brought by these diseases. The present study is important in respect of showing which pathway was active on the first day after trauma. A better understanding of the mechanism of bone apoptosis is important for bone health and to reduce the effects of aging.[13] The use of anti-apoptotic after muscle injury has been shown to have a positive effect on healing.[22] It is possible that in the future, the intra-operative use of apoptosis inhibitor agents could be a treatment option,[21] such as biphosphonates as anti-apoptotic drugs in the treatment of bone loss.[18] Prevention of osteoblast apoptosis is a crucial mechanism for the anabolic effects of PTH on the bone.[23]

In the light of all this information, the results of the blood samples taken from the patients with long bone fractures on the day after the trauma showed that the apoptosis extrinsic pathway was active.

Several studies have revealed that apoptosis is an essential process of the human body. However, in this highly sophisticated series of physiological events, as occurs in patients with long bone fractures, the complicated activation processes have not yet been fully understood. Furthermore, this study also highlights the difference in the extrinsic and intrinsic pathways.

There were some limitations to this study primarily that this study was conducted in a single centre and small groups were compared. There is a need for further studies of larger patient populations. More importantly, only a single measurement was taken following the trauma. With repeated measurements, changes in the serum levels could be observed, but the surgical treatment and drugs administered could also affect the serum levels of apoptosis markers. Therefore, it was decided to evaluate serum apoptosis marker levels on the first admission and to compare these with a matched control group to determine the serum apoptosis marker levels. The main strength of this study was that, to our knowledge, this is the first in vivo study in the literature to determine serum

apoptosis marker levels in trauma patients in comparison to healthy individuals. Further studies of large cohorts are needed to reach a better level of evidence.

Conclusion

The results of this study demonstrated that serum caspase and granzyme levels were higher in patients with long bone fracture, thereby showing the activation of the apoptosis extrinsic pathway. However, no significant difference was determined between the groups concerning serum cytochrome C levels. This study may guide future studies designed to better understand the molecular pathways that govern the events during fracture, which will be important for the future advancement of fracture treatment. Elucidation of these mechanisms and the development of new therapeutic agents to control apoptosis may play an important role in the future of fracture management.

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Conflict of Interest: None declared.

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ORİJİNAL ÇALIŞMA - *ÖZET*

Ortopedik travma hastalarında kaspas-8, granzim B ve sitokrom C apoptoz biyobelirteçlerin düzeylerinin karşılaştırılması

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AMAÇ: Bu çalışmanın amacı kırık sonrası apoptozisin indüklenip indüklenmediğinin belirlenmesi ve eğer öyleyse ekstrensek ya da intrensek hangi apoptotik yolağın aktif olduğunun gösterilmesidir.
GEREÇ VE YÖNTEM: Kliniğimize başvuran ve travma nedeniyle kırık tanısı konulmuş olan 30 kişi, hasta grubu ve hasta grubu ile benzerlik gösteren gönüllülerden oluşan sağlıklı 37 bireyden kontrol grubu oluşturuldu. Kırık sonrası birinci gün hastalardan alınan kanlardan ELISA yöntemi ile sitokrom C, granzim B ve kaspas-8 testleri çalışıldı.
BULGULAR: Çalışmaya toplam 67 kişi dahil edildi (kırık grubunda 30, kontrol grubunda 37 kişi). Hasta grubunda ortalalama yaş 62.03 (min: 18, maks: 91), kontrol grubu ortalama yaşı 61.62 (min 25, maks: 90). Kaspas-8 hasta grubunda anlamlı yüksek bulundu 0.37 ng/mL (Std: 0.06), (p=0.002); gruplar arasına sitokrom C ölçüm değerleri arasında fark izlenmedi (p=0.173). Granzim B hasta grubunda anlamlı olarak daha yüksek ölçüldü 52.56 pg/mL (Std: 8.51), (p=0.007).
TARTIŞMA: Uzun kemik kırıkları olan hastalarda ekstrensek apoptotik yolak aktivasyonunu gösteren serum kaspas-8 ve granzim B düzeyleri yüksektir. Ancak, serum sitokrom C düzeyleri açısından gruplar arasında anlamlı bir fark bulunamadı. Bu çalışma, kırık sonrası olayları yöneten moleküler yolakların daha iyi anlaşılması için tasarlanacak gelecek çalışmalara rehber olabilir ve kırık tedavisinin gelecekteki ilerlemesi için önemlidir.
Anahtar sözcükler: Apoptoz; granzim B; kaspas-8; kırık; sitokrom C.

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