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Original Article

Detection of the Timing of Human Skin Wounds by Immunohistochemical Analysis of CD14

تحديد وقت حدوث الجروح الجلدية عند الإنسان بواسطة الصبغة المناعية النسيجية الكيميائية سى دي 14

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Abstract

Determination of time of injury is one of the most important topics in forensic autopsy. Several methods have been developed to estimate wound age, but unfortunately with limited success. The aim of the present work was to evaluate the efficacy of Cluster of Differentiation 14 (CD14) as a reliable marker for estimating wound age.

The study was conducted on forty victims with different types of wound and known infliction time. Skin samples were obtained during autopsy from the center of the wound. Sections from samples were histologically examined by H & E stain. Immunohistochemical staining was done using CD14 antibody and the staining density was evaluated semi-quantitatively.

There was a statistically significant relation between wound age and percentage of CD14 expression. Expression of CD14 was 61.81±6.55 % in specimens from wounds aged less than 12 hours. It increased till reaching its maximum (96.40±3.78 %) for wounds aged between 1-3 days. Then it decreased dramatically to 14.80±3.49 % in wounds older than 3 days.

CD14 is proved to be a reliable marker for estimating wound age. It gave best results in wounds aged between 1-3 days with an overall accuracy of 100%. Accordingly, it can be used to determine wound age in medicolegal practice.

Keywords: *Forensic Science, Forensic Pathology, Wound Age, Immunohistochemistry, CD14.*

المستخلص

يعد تحديد وقت الإصابة من المهام الحيوية فى الطب الشرعي، وبالرغم من تطوير العديد من الأبحاث المتعلقة بتقدير عمر الجروح إلا إنها محدودة النجاح.

كان الهدف من هذا البحث تقييم فعالية سى دى ١٤ (CD14) كمؤشر موثوق لتقدير عمر الجرح، وأجريت الدراسة على أربعين جثه مصابة بأنواع مختلفة من الجروح ومعروف وقت الإصابة، وقد تم الحصول على عينات من الجلد من مركز الجرح خلال التشريح.

ولقد تم فحص العينات نسيجياً بطريقة صبغة الهيماتوكسيلين واليوزين، والصبغة المناعية النسيجية الكيمائية تمت باستخدام الأجسام المضادة لسى دى ١٤ . وبعد ذلك تم تقييم كثافة الصبغة بشكل نصف كمي.

وقد أظهرت النتائج أنه كانت هناك علاقة ذات دلالة إحصائية بين عمر الجرح وتعبير سى دى ١٤. فقد كان نسبته فى الإصابات التى يقل عمرها عن ١٢ ساعة ٦١,٨١ ±٦,٥٥٪ وزادت حتى وصلت إلى أقصى حد لها ٩٦,٤٠ ± ٣,٧٨٪ للجروح التي تتراوح زمن الإصابة بها ما بين ١-٣ أيام. ثم انخفضت بشكل كبير إلى ١٤,٨٠± ٣,٤٩ ٪ في الجروح التي مضى عليها أكثر من ٣ أيام.

وقد خلصت الدراسة أن سى دى ١٤ مؤشر موثوق لتحديد زمن الإصابة، فقد أعطى أفضل النتائج في الجروح التي تتراوح أعمارها بين يوم الى ثلاثة أيام (بنسبة دقة ١٠٠ ٪). ومن ثم يمكن استخدامه لتحديد عمر الجروح فى القضايا الطبية الشرعية.

الكلمات المفتاحية: علوم الأدلة الجنائية، الطب الشرعي، عمر الجروح، الصبغة المناعية النسيجية الكيمائية، سى دى ١٤.

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1. Introduction

Wound examination is amongst the most critical issues in forensic practice. It is a particularly important task for forensic pathologists to decide if an injury has vital reactions or not and to estimate time of wound infliction [1-3].

Age of wound is defined as the duration between the infliction of an injury and the time of death, or the time of examination in the case of living persons [4].

Wound age determination is frequently requested in medicolegal problems to reconstruct certain events, e.g., accidents and quarrels, and to decide whether the wounds are a result of single or split occasions [5,6].

Forensic pathologists sometimes depend on morphological appearance such as the color of contusion, or formation of scab to determine wound age; however, this method is deficient [7,8].

Since macroscopic and histological examination of the wound is insufficient, additional methods to determine wound age are required [8,9].

Varieties of biological substances, soluble inflammatory mediators, inflammatory cells, parenchymal cells and some extracellular matrix components are essential for wound healing. Therefore, many researchers utilized these substances as a key for wound age estimation and wound vitality [1-4,10].

Wang et al. used Vascular Endothelial Growth Factor (VEGF) for wound age estimation in mice [11].

In another study on human skin wounds, oxygen-regulated protein 150 (ORP150) was used by Ishida et al. [12].

Many immunohistochemical methods have been studied to determine skin wound age by looking at antigen-antibody reaction to verify certain proteins within the tissues [1,9].

Fronczek et al. studied 3 inflammatory cell markers, namely myeloperoxidase (MPO), Cluster of Differentiation 45 (CD45), and Cluster of Differentiation 68 (CD68) to determine wound age in living subjects [9].

Cluster of Differentiation 14 (CD14) is a protein that helps to identify bacterial lipopolysaccharide (LPS). It is present on the surface of inflammatory cells as macrophages, monocytes, and neutrophils. It is known to be a receptor for complexes of liposaccharide and its binding protein [13-16].

This protein stimulates induction of cytokines such as tumor necrosis factor alpha (TNFa), interleukin (IL)-1, interleukin (IL)-6 and interleukin (IL)-8 [15,17].

The aim of the present work was to study the immunohistochemical expression of CD14 in human skin wounds in order to evaluate its efficacy as a reliable marker for estimating the age of a wound.

2. Materials and Methods

2.1 Measurements

The study was conducted on forty autopsy cases referred to the Medicolegal Department at the Ministry of Justice, Alexandria Governorate, Egypt. The cases were victims exhibiting different types of wounds with a known post infliction time as well as known time of death. Cases with skin disease, burn, and any signs of putrefaction were excluded from the study.

After obtaining the official approval as well as ethical committee approval, the cases were chosen randomly. Consent from the relatives of decedents was acquired. Data such as age, sex, time of wound infliction and time of death were collected from the police reports. Cause of death was recorded from the autopsy report. Bodies were kept in refrigerators from the time of arrival to the morgue until samples were collected.

2.2. Skin Wound Specimens

Forty skin wound specimens were collected from the center of each wound to estimate its age. To compare between each wound, it is crucial to standardize the sampling location.

For each victim, skin specimens were collected from a noninjured area away from the traumatic lesion as control. The postmortem interval till sample collection was up to 3 days for all victims. Skin specimens were put in formalin immediately after excision.

Wound specimens were categorized into four groups according to the infliction time: group 1 (<12 hours), group 2 (12-24 hours), group 3 (1-3 days) and group 4 (> 3-10 days).

2.3. Histopathologic Examination

Four micron thick sections were cut from the formalin fixed paraffin embedded blocks. They were stained with the conventional haematoxylin and eosin (H&E) stain and examined using light microscopy for histopathological assessment.

2.4. Immunohistochemical Examination

The primary mouse monoclonal antibody CD14 Ab-2 (Ab clone 7 Lab vision Corporation Neo Marker, Fremont, USA) was applied. Immunohistochemical staining (using avidin-biotin method) was used as recommended in the manufactures protocol. The deparaffinized tissue sections were rehydrated in graded alcohols. The endogenous peroxidase was blocked using 0.3% hydrogen peroxide for 20 min. For antigen retrieval, sections were microwaved in a thermoresistant container (coplin jar) containing citrate (10mM, pH 6.0). The antibody CD14 was applied in a concentration of 1/200. The reaction product was developed using diaminobenzidine tetrahydrochloride (DAB) mixture for 10 minutes. Slides were counterstained with hematoxylin, dehydrated and mounted [7].

2.5. Semi-quantitative Assessment of CD14 Staining in Tissue Sections

Slides were screened at X100 magnification to confirm positive staining in infiltrating cells, and then numbers of positive cells were counted in four non-overlapped high power fields. The percent of staining was calculated as the mean ratio in relation to the total number of infiltrating inflammatory cells.

2.6 Statistical Analysis

All the data obtained from each specimen were plotted against their post infliction time. Other data, related to the victims or the wounds, were correlated with histopathological and immunohistochemical results. All statistical analysis was done using alpha error of 0.05. A *p*-value less than or equal to 0.05 was considered to be statistically significant.

The Receiver Operator Characteristic analysis (ROC curve) was employed to identify the discriminatory cut-off points for wound age. The accuracy was measured by the area under the ROC curve [18].

3. Results

3.1. Demographic Data

The majority of studied victims were males (85%). The age of the victims ranged from 16 – 68 years with a mean of 38.15 ± 16.34 years for males and 44.17 ± 17.69 years for females. No significant difference was noticed regarding age between both sexes ($p = 0.426$). About one third of the victims were in the age group of 20-30 years (Table-1).

3.2. Type and Age of Wounds

More than half of the studied injuries were stab wounds (55%) followed by lacerations (20%). Contusions were the least frequent type encountered in the present study (5%) (Table-1).

Wound age in the present work ranged from a few minutes up to 10 days. More than half of studied wounds (52.5%) were aged less than 12 hours.

Wounds aged between 12 and 24 hours were encountered in 22.5% of the victims. Group 3 (1-3 days) and group 4 (> 3 days) included 5 cases each (Table-1).

Although no significant relation was detected between the type of wounds and their ages, more than half of the stab wounds (59.1%) and the majority of abrasions (75.0%) were aged less than 12 hours.

3.3 Histopathological Examination

In the control group, the specimens showed intact epidermis with minimal lymphocytic infiltrate (Figure-1A).

Wounds aged less than 12 hours: Specimens showed early hematomas entangling inflammatory cells, predominantly neutrophils, with total loss of the epidermis in the opened wounds (Figure-1B).

The retrieved hematomas were smaller and more organized among wounds aged between 12 and 24 hours. Lymphocytes and macrophages were more prominent than acute inflammatory cells (Figure-1C).

After the first day, neutrophils markedly decreased, and

Table 1- *Demographic and injury-related data of the studied cases.*

		no.	%
Sex			
Male		34	85.0
Female		6	15.0
Age (Years)		**Male**	**Female**
Min – Max		16.0 – 68.0	22.0 – 60.0
Mean ± SD		38.15 ± 16.34	44.17 ± 17.69
p value	0.482		
Type of wound		*no.*	%
Stab wounds		22	55.0
Laceration		8	20.0
Incised wounds		4	10.0
Abrasion		4	10.0
Contusion		2	5.0
Age of wound		*no.*	%
Group 1(< 12hr)		21	52.5
Group 2 (12 - 24hr)		9	22.5
Group 3 (1- 3 day)		5	12.5
Group 4 (>3days - 10 days).		5	12.5

p; p value for Mann Whitney test, p; p value for comparing between the different categories

lymphocytes were more predominant. Later on, from the 1st to the 3rd day, macrophages were gradually increasing and becoming more prominent. The hematoma started to be absorbed (Figure-1D).

By the end of the third day the granulation tissue started to appear with newly formed capillaries and small fibroblasts (Figure-1E).

By the end of the first week the surface started to be epithelialized, and few collagen bundles appeared in the dermis with minimal inflammatory cells (Figure-1F). The inflammatory cells then gradually disappeared. Collagen was denser; it reached its maximal density by the end of the 10th day (single case).

3.4. CD14 Immunohistochemical Examination

In the control group (non-injured skin), no CD14 positive cells were detected (Figure-2A).

Positive staining was detected in inflammatory cells infiltrating the wounds with different percentages.

Wounds aged less than 12 hours: These wounds revealed hematomas entangling CD14 positive cells with a mean percentage of staining of 61.81±6.55 % (median 61.0). The stained cells were mainly polymorphs (Figure-2B).

Wounds aged between 12 - 24 hours: The CD14 immunostaining increased gradually with a mean of 83.67±3.91% (median 82). CD14 expression was observed on polymorphs and macrophages (Figure-2C).

Wounds aged between 1 - 3 days: These wounds revealed maximum CD14 immunostaining reaching up to 96.40±3.78% (median 97.0), macrophages and lymphocytes were seen diffusely infiltrating the dermis (Figure-2D).

Wounds aged more than three days: These wounds showed a dramatic decrease in the percentage of CD14

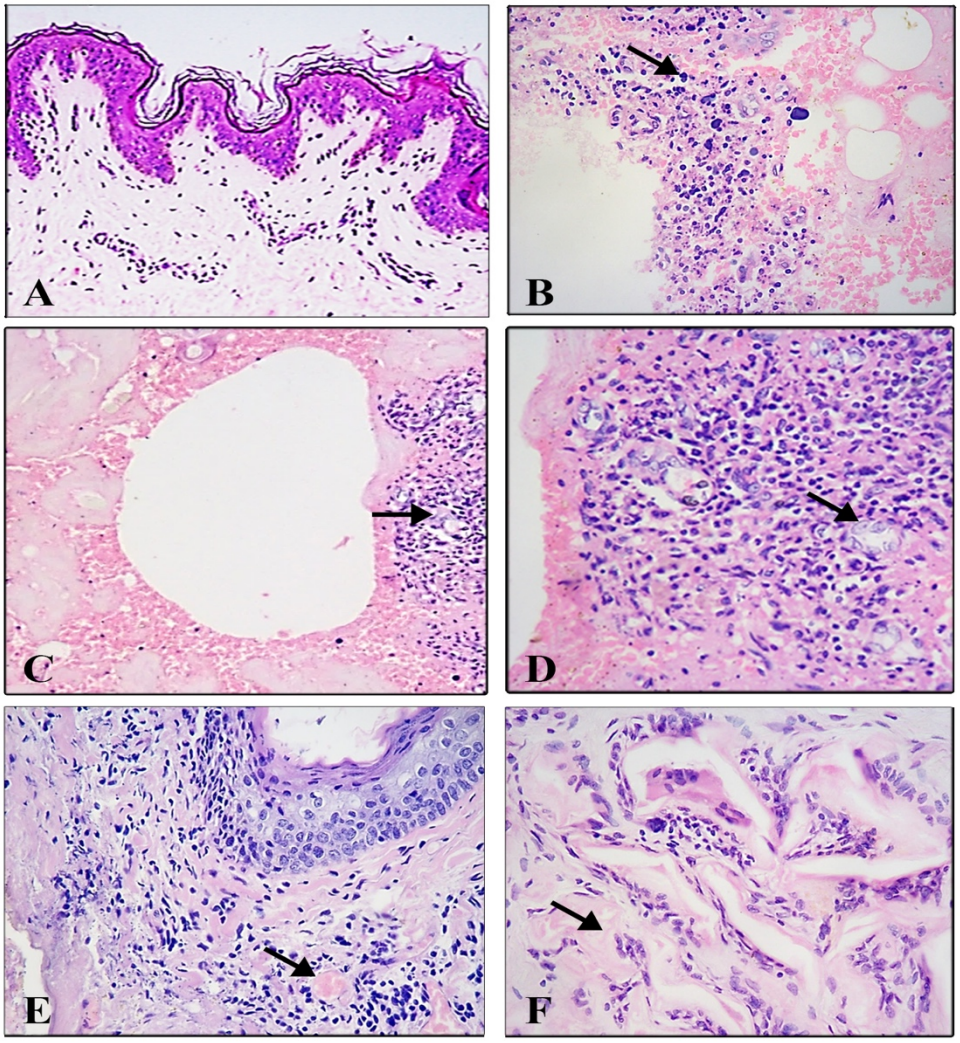

Figure 1-*Photomicrograph of skin wounds with different infliction time (HE, 100x)*

1A - *Control group, there is intact epidermis with minimal lymphocytic infiltrate.*

1B - *Laceration aged less than 12 hours: showing sloughed epidermis and a large dermal hematoma (arrow) infiltrated by inflammatory cells.*

1C - *Stab wound aged between 12 - 24 hours: showing a large organized hematoma (arrow) entangling aggregates of inflammatory cells.*

1D - *Cut wound aged between 1 - 3 days: showing granulation tissue (arrow) interspersed with inflammatory cells.*

1E - *Stab wound aged > 3 days: showing residual granulation tissue and early collagen fibers.*

1F - *Stab wound aged ≥ 1 week: the collagen fibers are denser, anastomosing and separated by fibroblasts with few lymphocytes.*

expression (Mean 14.80±3.49%, median 15), reaching its minimal density by the 10th day. Few stained inflammatory cells and fibroblasts were detected in these groups (Figure-2E, Figure- 2F).

There was a statistically significant relation between percentage of CD14 expression and age of wound (p<0.001). CD14 expression in group 3 (1-3days) was 96.40±3.78%, which was significantly higher compared to groups 1, 2 and 4. On the other hand, CD14 expression in group 4 (>3 days) (14.80±3.49 %) was significantly lower compared to the other three groups (Table-2).

On applying the same test for each type of wound separately, a significant relation was detected only in cases of stab wounds. On the other hand, the test of significance

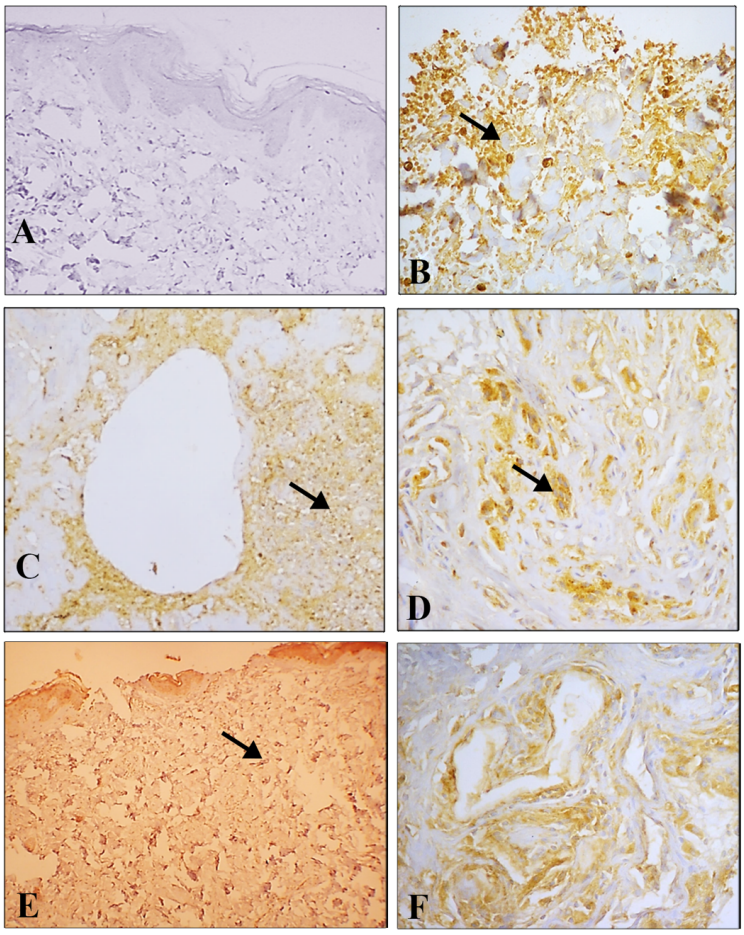

Figure 2-*Photomicrograph of skin wounds with different infliction time (immunohistochemistry) (CD14, 100x)*

2A - *Control group (uninjured skin): intact epidermis and negative for CD14 expression.*

2B - *Laceration aged less than 12 hours: CD14 expression in superficial hematoma. (arrow)*

2C - *Stab wound aged between 12 - 24 hours: moderate CD14 expression in inflammatory cellular aggregates seen within the organized hematoma (arrow).*

2D - *Cut wound aged between 1 - 3 days: dense CD14 expression seen in inflammatory aggregates disposed in-between granulation tissue (arrow).*

2E - *Stab wound aged more than 3 days: low density CD14 expression (arrow).*

2F - *Stab wound aged ≥ 1 week: few inflammatory cells expressing CD14 around newly formed capillaries.*

Table 2- *Relation between CD14 expression and age of wounds (n =40)*

	Age of wound				Kruskal-Wallis H test	*p-Value*
	<12hr (n =21)	**12 - 24hr (n =9)**	**1- 3 day (n =5)**	**>3 days (n =5)**		
CD14 expression						
Min. – Max.	50.0-70.0	80.0-90.0	92.0-100.0	10.0-19.0	32.848*	<0.001*
Mean ± SD.	61.81±6.55	83.67±3.91	96.40±3.78	14.80±3.49		
Median	61.0	82.0	97.0	15.0		

*p; p value for comparing between the different categories, *; Statistically significant at p ≤ 0.05.*

could not be applied for abrasions and contusions due to insufficient samples.

No statistical significant correlation was found between percentage of CD14 expression in all groups and age of victims (p = 0.354) (Table-3).

Receiver Operator Characteristic (ROC) Analysis of CD14 Expression:

ROC curve analysis was used to assess the discriminant ability of the tested variable. Accuracy is measured by calculating area under the ROC curve.

A cut-off value of 90% was set to differentiate between wounds aged less than 24 hours as well as more than 3 days and those aged between 24 hours and 3 days.

At this cut-off value, the sensitivity of CD14 as a discriminating test of wound age was 100% and the specificity was 100%. The overall accuracy was 100% (Figure-3, Table-4).

On the other hand, when a cut-off value of 70% was set to differentiate between wounds aged less 24 hours and those aged between 24-hours up to 10 days (Figure-4, Table-5), the result was insignificant and the overall accuracy was 65.0%.

4. Discussion

Table 3- *Relation between CD14 expression and age of wounds (n =40).*

	n	Percentage of CD14 expression			Test of sig.	*p-Value*
		Min - Max	Mean. ± SD	Median		
Age of victims (Years)						
<20	4	10.0 – 80.0	55.0 ± 31.09	65.0		
20 ≤ 30	12	10.0 – 100.0	61.67 ± 34.33	65.0		
30 ≤ 40	3	70.0 – 80.0	73.33 ± 5.77	70.0	χ^2 = 5.538	0.354
40 ≤ 50	8	60.0 – 100.0	72.50 ± 15.81	65.0		
50 < 60	6	10.0 – 70.0	55.0 ± 22.58	60.0		
60+	7	60.0 – 100.0	80.0 ± 15.28	80.0		

χ^2; Chi square test, p; p value for comparing between the two groups

Table 4- *Agreement (sensitivity, specificity) for CD14 to predict time of wound infliction (groups 1,2,4 versus group 3).*

	AUC	*P*	95% C.I	Cut off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
CD14	1.000	<0.001*	1.0-1.0	>90	100	100	100	100	100

*AUC; Area under the curve, p value; probability value, CI; Confidence Interval, *; Statistically significant at p ≤ 0.05, PPV; Positive Predictive value, NPV; Negative Predictive value.*

Table 5- *Agreement (sensitivity, specificity) for CD14 to predict time of wound infliction (groups 1, 2 versus groups 3, 4).*

	AUC	*P*	95% C.I	Cut off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
CD14	0.500	1.000	0.190-0.810	70	50.0	70.0	35.7	80.8	65.0

*AUC; Area under the curve, p value; probability value, CI; Confidence Interval, *; Statistically significant at p ≤ 0.05, PPV; Positive Predictive value, NPV; Negative Predictive value.*

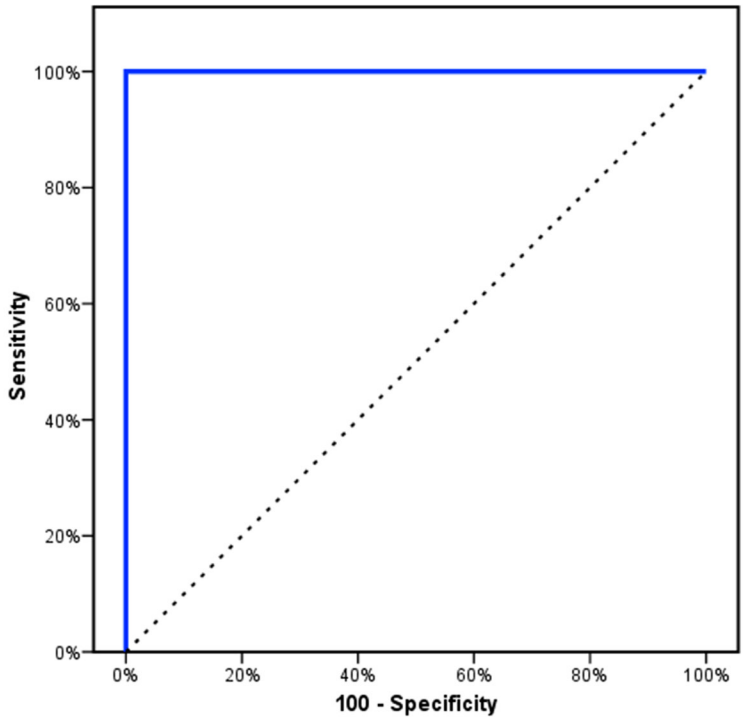

Figure 3-*ROC curve assessment of sensitivity and specificity of CD14 expression groups 1,2,4 versus group 3)*

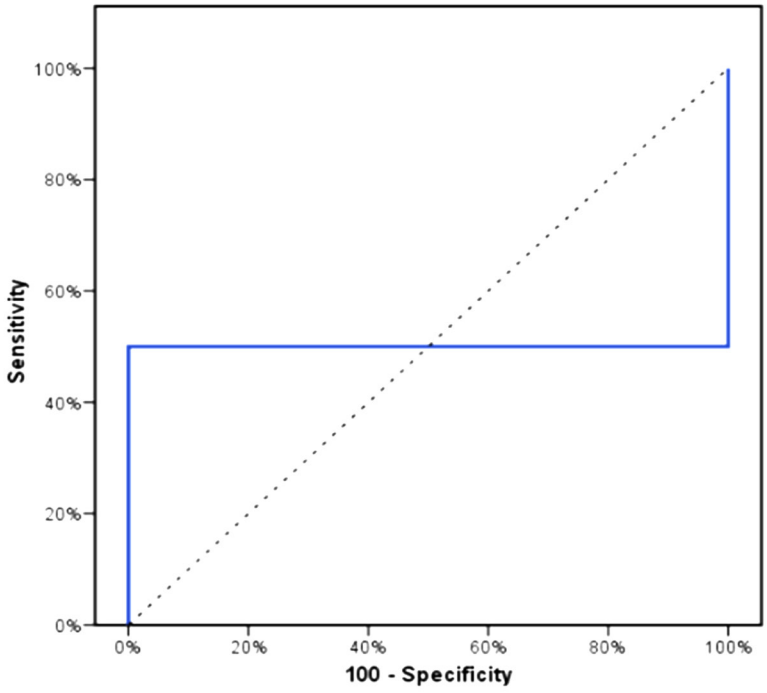

Figure 4-*ROC curve assessment of sensitivity and specificity of CD14 expression (Groups 1,2 versus group 3,4).*

Estimation of wound age is amongst the most critical issues and indispensable areas in forensic pathology [1-3,19]. Wound age is especially important to verify if a certain wound is related to the cause of death or not and to reconstruct the sequence of certain events [1-3,5,6].

CD14 is a membrane bound protein present on the surface of inflammatory cells such as macrophages, monocytes and neutrophils. It is a clue in the immune response, as it is a ligand to LPS [13-16,19]. It may be present in two forms, either membrane bound receptor (ligand to LPS) or secreted form (soluble CD14 SC14) (transporter to LPs) [20,21]. The binding of LPS to CD14 initiates production and release of interleukin (IL)-1, interleukin (IL)-6, interleukin (IL)-8, interleukin (IL)-18 and tumor necrosis factor-α [15 ,17,19,22,23].

Some studies elucidated that CD14 is expressed in patients with immune diseases and serum of patients with severe burns [19,24-26]. However, there have been few reports on the expression of CD14 in human skin wounds from the viewpoint of wound age estimation.

Males outnumbered females in the studied victims with sex ratio of 5.7:1. About one third of them were in the age group 20-30 years. Sharma et al. recorded similar results in their study on dead victims [27]. Males in this age group are more liable to injuries due to road traffic accidents, falls, labor work accidents as well as assaults. They are also more exposed to violence and struggle [28].

Stab wounds were encountered in 55% of the studied victims. Only 5% of them had contusions. In the study of Fronczek et al., which was conducted on living subjects, contusions were the predominant wounds while stab wounds were the least encountered ones [9].

Wounds in the present work were classified into 4 groups according to their ages. More than half of wounds were in group 1 (less than 12 hours). The least number of cases were in group 3 and 4 (1-10 days).

The histological examination of the studied wounds by H&E revealed that hematomas evident before 12 hours contained acute inflammatory cells (mainly neutrophils). Sharma et al. observed hemorrhage as early as 10 minutes after infliction which remained till 48 hours in specimens collected from deceased persons [27]. Between 12 - 24 hours, lymphocyte and macrophage became more prominent within more organized hematomas.

After the first day, the acute inflammatory cells started to be replaced partially by macrophages as well as lymphocytes within the dermis. Later on, from the 1st to the 3rd day the number of macrophages reached its maximum value.

In 2004, a study was conducted by Oehmichen on human skin wounds. He observed macrophages from 3-7 hours outnumbering neutrophils from 20 hours. They reached a peak around 1-2 days [29].

The presence of a predominant number of neutrophils in the current study indicated that the wound age is most probably less than 12 hours. On the other hand, the outnumbering of macrophages over the acute inflammatory cells means that the wound age is between approximately 1-3 days.

Unmesh and Rema conducted a study on specimens collected from a deceased person. They observed maximum concentration of lymphocytes after 48 hours, which remained as such for days and then gradually declined. Macrophages were noted in increased numbers from the fourth day onwards [30].

In the current study, granulation tissue, small fibroblasts and newly formed capillaries started to appear by the end of the 3rd day. Few collagen bundles appeared by the end of the 1st week and increased markedly by the end of the 10th day. In contrast to our study, a previous study on dead victims reported that fibroblastic proliferation occurred after 19 hours and collagen formation was observed by the third day [27].

The present study revealed a statistically significant relation between percentage of CD14 expression and age of wound. The mean percentage of CD14 positive cells increased gradually to reach its maximum by the end of the 3rd day (96.40±3.78%) then decreased dramatically to 14.80±3.49 % by the end of the 10th day.

Yagi et al. [19] studied expression of CD14 in human skin wounds taken during autopsy. They reported that the positive ratio of CD14 in wounds aged between 1 and 5

days significantly exceeded those aged less than one day and those more than 7 days with specificity (87.2%) and sensitivity (100%) [19].

Kagawa et al. studied expression of the CD14 gene in mice after excisional wounds. They proved that the CD14 gene peaked from 12 to 24 hours. This is in contrast to the results of the present study, which may be explained by the earlier expression of mRNA relative to its protein that requires further time. The high stability of protein against degradation after death clarifies why protein expression persists longer than mRNA [31].

In an earlier study, serum CD14 (sCD14) level was measured in polytrauma patients. Its level decreased after trauma then increased again within 6 days. The study also reported that sCD14 sustained elevated levels until 14 days of trauma [19]. This did not coincide with the result of the present study due to different kinetic behaviour of membrane and soluble CD14.

By using ROC curve analysis, the study proved an overall accuracy of 100% for CD14 as a discriminating test for wound age when setting a cut-off value of 90%.

The mean percentage of expression of CD14 of more than 90% correctly diagnosed that the age of the wound is between one to three days in 100% of the cases.

5. Conclusion

The histopathological examination of the studied samples helped to a certain extent in dating the age of the skin wounds.

On the other hand, immunohistochemical examination of CD14 proved to be a reliable marker for determination of wound age. It gave best results in wounds aged between 1-3 days with overall accuracy of 100%.

Limitations & Recommendations

- Although only cases with known infliction time were included in the study, the exact time may not be recorded accurately in police reports.
- The number of cases in the study was relatively small due to many obstacles such as the availability of human samples with known wound infliction time and ethical issues regarding their use in research.
- To yield more reliable results that could be replicated in forensic practices:
  - Further studies with larger samples size under different environmental conditions using samples with different range of shorter PMI to elucidate the effect of PMI on timing of wounds are recommended.
  - In addition, the same study for each type of wound should be conducted separately.
- Combined use of histopathology and immunohistochemistry is recommended for dating of the wounds.

Conflict of Interest

None

Source of Funding

None

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