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ORIGINAL ARTICLE

Immunohistochemical analysis on cyclooxygenase-2 for wound age determination

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Abstract Immunohistochemical study combined with morphometry was carried out to examine the expression of cyclooxygenase-2 (COX-2) using 60 human skin wounds of different ages: group I, 0–4 h (n=11); II, 8 h–2 days (n=21); III, 3–9 days (n=14); and IV, 12–21 days (n=14). In wound specimens aged 2 h to 2 days, anti-myeloperoxidase-positive neutrophils observed at the wound site expressed immunopositive reaction to COX-2. In wound specimens of more than 3 days, CD68-positive macrophages as well as neutrophils were positively immunostained with anti-COX-2. In group II, all 21 wound samples had COX-2-positive ratios of >40 %, and 15 out of them showed >50 %. In group III, only three wound samples with the postinfliction intervals of 3 days showed positive ratios of 40–50 % and the remaining 11 cases less than 40 %. In groups I and IV, all 25 wound specimens had COX-2-positive ratio of <40 %. With regard to the practical applicability with forensic safety, these observations suggested that a COX-2-positive ratio of >40 % indicated a wound age of 8 h to 3 days. Moreover, COX-2-positive ratios, considerably exceeding a ratio of 50 %, indicate a wound age of 8 h to 2 days. Collectively, COX-2 would be a useful marker for the determination of early wound age.

Keywords Cyclooxygenase-2 · Wound age determination · Immunohistochemistry · Forensic pathology

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Introduction

In forensic practice, wound examination is one of the most important tasks for forensic pathologists. When forensic pathologists have autopsy cases with multiple injuries such as stabbing, lacerations, and subcutaneous hemorrhage, they are always required to evaluate which wound(s) is closely related with the death [1–3]. Furthermore, it is also essential to judge how long before each wound has been sustained, so called wound age determination [1–3]. Conventionally, histological alterations in the different phases of wound healing process have been applied to wound age determination [4]. At present, immunohistochemical techniques are widely used in forensic pathological researches [5–12]. Thus, there are lots of immunohistochemical studies on wound age determination [13–21].

Wound healing is a basic biological response that involves soluble mediators, extracellular matrix components, resident cells, and infiltrating leukocytes [22–25]. The molecular mechanisms of wound repair are not fully understood but involve growth factors, cytokines, adhesion molecules, and matrix metalloproteases [26–30]. Prostaglandin E2 and prostaglandin I2 are proinflammatory mediators involved during the acute inflammatory phase [31]. These two prostaglandins are potent vasodilators that also act synergistically with other mediators to increase microvascular permeability. Cyclooxygenase (COX), the key enzyme, is

required for the conversion of arachidonic acid to prostaglandins [32]. Two COX enzymes, COX-1 and COX-2, are responsible for the production of prostaglandin H2, the first step in prostanoid biosynthesis. It appeared that COX-1 was constitutively expressed under the physiological conditions and COX-2 for the elevated production of prostanoid that occurred in sites of disease and inflammation [32]. Actually, Laulederkind and colleagues demonstrated that COX-2 was essential for skin wound healing by the use of COX-2-deficient mice [31]. In the present study, we immunohistochemically examined COX-2 expression in human skin wounds of different wound ages and discuss the practical availability of COX-2 as a marker for wound age determination.

Materials and methods

Antibodies

The following monoclonal or polyclonal antibodies (mAbs or pAbs) were used for immunohistochemical and immunofluorescence analyses in the present study: goat anti-COX-2 pAbs (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-myeloperoxidase (MPO) pAbs (Neomarkers, Fremont, CA), mouse anti-human CD 68 mAb (clone PG-M1; Dako Cytomation, Kyoto, Japan), cyanine dye 3 (cy3)-conjugated donkey anti-mouse IgG pAbs, cy3-conjugated donkey anti-rabbit IgG pAbs, and FITC-conjugated donkey anti-goat IgG pAbs (Jackson ImmunoResearch, West Grove, PA).

Human skin wound specimens

A total of 60 human skin wounds (13 stab wounds, 12 incised wounds, 20 surgical wounds, and 15 lacerations) with wound ages of a few minutes to 21 days were collected from forensic autopsies (Institute of Legal Medicine, University of Munich, Germany). The ages of the victims ranged from 7 to 83 years (mean age, 46.5 years), and the postmortem interval was less than 3 days in each case. None of the cases had suffered from severe malnutrition, malignant diseases, or metabolic disorders, and no substances such as cytostatic agents or glucocorticoids that could have influenced wound repair were administered during medical treatment. According to wound ages, the wound specimens were classified into four groups as follows: group I, 0–4 h (n=11); group II, 8 h to 2 days (n=21); group III, 3–9 days (n=14); and group IV, 12–21 days (n=14). Uninjured skin from the same individual was also taken as a control.

Immunohistochemical analysis

The wound specimens were fixed in 4 % formaldehyde solution buffered with PBS and embedded in paraffin, followed by making sections at a thickness of 4 μm. Briefly, deparaffinized sections were incubated with PBS containing 1 % normal rabbit serum and 1 % bovine serum albumin to reduce nonspecific reactions. Thereafter, the sections were further incubated with anti-COX-2 pAbs (dilution 1:100) overnight at 4°C. After the incubation of biotinylated secondary Abs, immune complexes were visualized using Catalyzed Signal Amplification System (Dako, Kyoto, Japan) according to the manufacturer's instructions. As the negative control, sections were incubated with goat normal serum instead of the primary antibody, and no positive signal could be detected, indicating the specificity of the antibody.

Double-color immunofluorescence analysis

In another series, double-color immunofluorescence staining was performed to determine the cell type expressing COX-2 [14, 17]. Briefly, deparaffinized sections were incubated with PBS containing 1 % normal donkey serum and 1 % bovine serum albumin to reduce nonspecific reactions. Thereafter, the sections were further incubated with a pair of anti-CD68 (dilution 1:100) or anti-MPO pAbs (dilution 1:100) and anti-COX-2 (dilution 1:100) overnight at 4°C. After incubation with fluorochrome-conjugated secondary Abs at room temperature for 1 h, the sections were observed under a fluorescence microscope. The immunofluorescent images were digitally merged. In order to confirm the specificity of each antibody, the sections were incubated with isotype-matched immunoglobulin instead of each primary antibody, and we could detect no positive signal, implying the specificity of each antibody.

Morphometrical analysis

According to the methods of previous studies [13–18], morphometrical analysis was performed for a semiquantitative evaluation of the immunohistochemical findings by two different investigators who had no prior knowledge of the specimens. Briefly, 10 high power microscopic fields (0.4× 0.4 mm each) were randomly selected in each section, and the number of COX-2 positive infiltrating cells and that of total infiltrating cells were counted in each microscopic field, and the ratio of COX-2-positive infiltrating cells to total infiltrating cells was calculated. The average ratio of the 10 selected microscopic fields was evaluated as COX-2 expression in each wound specimen.

Statistical analysis

In each group, the mean values of the COX2-positive ratios and standard errors (SE) were calculated. Statistical analyses were performed using one-factor analysis of variance to determine whether differences existed among the group means, followed by Scheffé's *F* test to identify significantly different means.

Results

In unwounded skin and wound of less than 30 min, leukocyte recruitment was absent, and a few resident cells (probably macrophages) in the corium rarely expressed COX-2, indicating that the evaluation of those resident cells in the corium would not be available for wound age determination likewise in previous studies [13–18]. Thus, apparent infiltrating cells such as neutrophils and macrophages were evaluated. In wound specimens aged 2 h to 2 days, neutrophils labeled with MPO were predominantly observed at the wound site, and those MPO-positive neutrophils expressed immunopositive reaction to COX-2 (Fig. 1A, B). In wound specimens of more than 3 days, CD68-positive macrophages were also recruited, and those CD68-positive macrophages were positively immunostained with anti-COX-2 pAbs (Fig. 2A, B).

Morphometrical analyses

Figure 3A demonstrates the distribution of the ratios of COX-2-positive infiltrating cells in relation to wound age. In group I, COX-2-positive ratios were very low (mean±SE, 12.2 ± 4.3 %). In wound specimens with a postinfliction interval of 8 h to 2 days (group II), the ratio of COX-2-positive cells considerably increased (mean ± SE, 58.2 ± 2.5 %). All 21 samples had the COX-2-positive ratios of >40 %, and 15 out of the 21 samples showed the positive ratios of >50 %. A 12-h-old wound in group II showed the highest value (78.3 %) among all of the 60 human skin wound specimens in the present study. Thereafter, with an increase of wound age (groups III and IV), the COX-2-positive ratio apparently decreased. Although, in group III, only three wounds samples with the postinfliction intervals of 3 days showed positive ratios of 40–50 %, the remaining 11 cases in group III and all 14 cases in group IV had COX-2 ratio of less than 40 %. Statistical analysis revealed significant differences between group II and the three other groups, between groups I and III, and between groups III and IV (Fig. 3B); however, there was no significant difference between groups I and IV.

Discussion

Prostaglandins are found in animals as primitive as the coelenterates and are present in a wide variety of human tissues [32]. PGs not only play a central role in inflammation but also regulate other critical physiological responses. In humans, prostaglandins are involved in diverse functions, including blood clotting, ovulation, initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, and immune responses [33].

In the synthesis of prostaglandins, two cyclooxygenase isoforms have been identified and are referred to as COX-1 and COX-2. Under many circumstances, the COX-1 enzyme is produced constitutively (i.e., gastric mucosa) whereas COX-2 is inducible (i.e., sites of inflammation) [32]. Thus, several lines of accumulating evidence demonstrated that COX-2 was closely involved in various inflammatory diseases, and eventually, novel therapeutics targeting COX-2 have been developed [33]. Actually, the inhibition of COX-2 attenuated experimental pancreatitis, indicating that COX-2 played detrimental roles in acute inflammatory disease [34, 35].

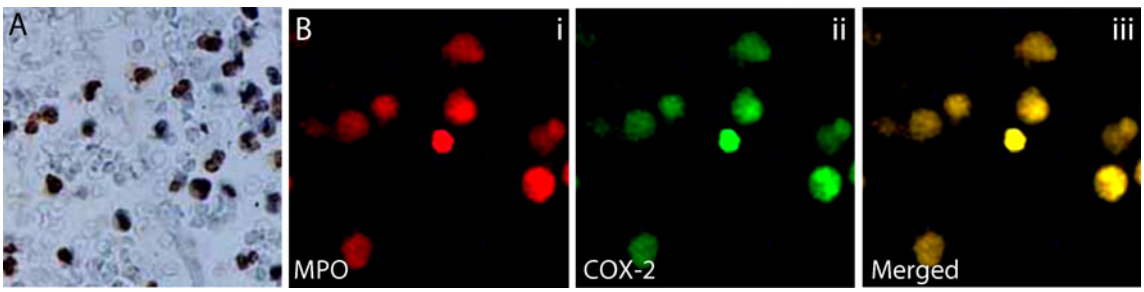

Fig. 1 *A* In a 12-h-old wound, polymorphonuclear leukocytes are immunostained with anti-COX2 pAbs (subcutaneous region at the wound sites, magnification×200). *B* Double-color immunofluorescence analysis by the use of anti-MPO and anti-COX-2. Signals in *i* and *ii* were digitally merged in panel *iii*. Representative results are shown here (subcutanenous region at the wound sites, magnification× 400)

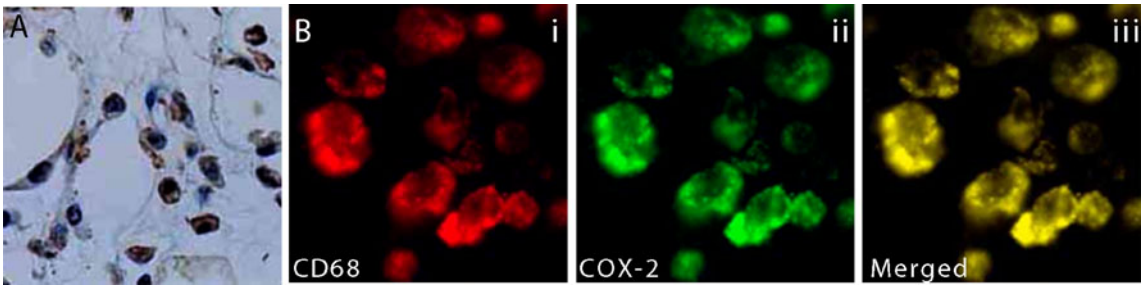

Fig. 2 *A* In a 3-day-old wound, mononuclear cells are positively immunostained with anti-COX-2 pAbs (subcutanenous region at the wound sites, magnification×200). *B* Double-color immunofluorescence analysis by the use of anti-CD68 and anti-COX-2. Signals in *i* and *ii* were digitally merged in panel *iii*. Representative results are shown here (subcutanenous region at the wound sites, magnification× 400)

Wound healing is composed of inflammatory, proliferative, and maturation phases [22, 23]. Thus, it was considered that COX-2 could be involved in inflammatory phase of skin wound healing. Our previous study indicated that COX-2 was mainly produced by neutrophils [35]. Consistent with this, MPO-positive neutrophils predominantly expressed COX-2 in human skin wound samples. Moreover, a part of CD68-positive macrophages were also immunostained with anti-COX-2. Thus, it is considered that neutrophils and macrophages should be morphometrically analyzed, in accordance with our previous studies.

To the best of our knowledge, only a few studies have focused on prostaglandins and their synthases including COX in the field of forensic medicine. King and colleagues examined the levels of 19-hydroxy-prostaglandins F1 alpha/F2 alpha as a specific semen marker, in semen-contaminated vaginal swabs and semen stains [36–41]. There are only three forensic studies in wound age determination or wound vitality. The levels of leukotriene B4 but not PGFa would be useful for the differentiation between antemortem injuries and postmortem damages in autopsy samples [42, 43]. Bai and colleagues examined COX-2 mRNA expression in rabbit wound healings and showed that COX-2 gene expression would be useful for early wound age determination [44]. However, the practical usefulness of COX-2 has not been examined with human skin wounds.

From the viewpoint of forensic pathological applications, the present study shows that COX-2 is a suitable marker for wound age determination. All wound samples in groups I and IV showed the COX-2-positive ratios of <40 %. On the contrary, all 21 samples in group II had COX-2-positive ratio of >40 %. In group III, only three wounds aged 3 days showed COX-2-positive ratios of >40 %, and the remaining 11 wounds showed <40 %. Collectively, with regard to practical applicability with forensic safety, these observations suggested that a COX-2-positive ratio of >40 % strongly indicates a wound age of 8 h to 3 days. Moreover, 15 out of 21 wounds in group II samples showed >50 %, and there was no wound specimen with the positive ratios of 50 % in other three groups. Thus, COX-2-positive ratios, considerably exceeding a ratio of 50 %, indicate a wound age of 8 h to 2 days.

In line with previous studies [13–18], we examined wound samples with the postmortem intervals of less than 3 days to minimize unspecific background. Thus, there was no influence of postmortem intervals on immunostaining. Several lines of accumulating evidence implied that IL-1α would be useful for wound age determination of 4 h to 1 day

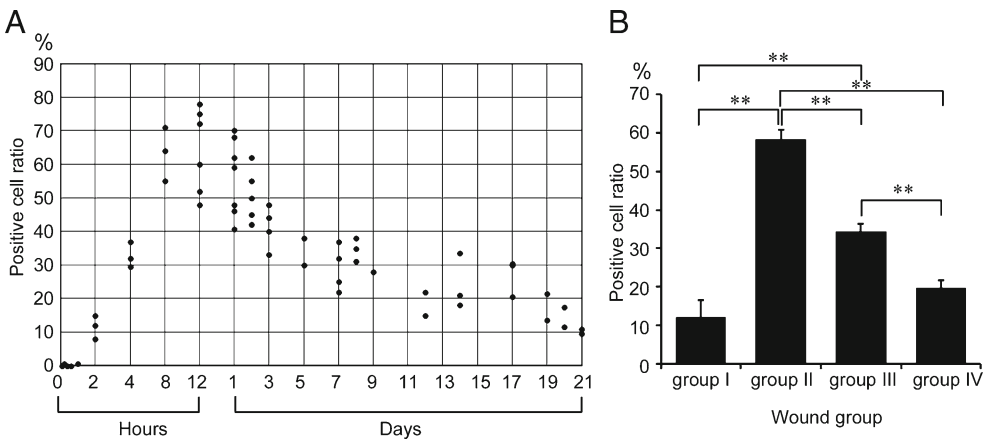

Fig. 3 **a** Ratio of COX-2-positive infiltrating cells in relation to wound age. **b** Mean value and standard error of COX-2-positive infiltrating cells in each wound group. ***p*<0.01, a significant difference was observed statistically

[16, 45]. Double-color immunofluorescence analyses of COX-2 and IL-1α or triple-color immunofluorescence analyses of COX-2 and IL-1α together with leukocyte markers (MPO or CD68) may be more useful for wound age determination at early phase after injury.

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References

1. Kondo T (2007) Timing of skin wounds. Leg Med (Tokyo) 9:109–114
2. Kondo T, Ishida Y (2010) Molecular pathology of wound healing. Forensic Sci Int 203:93–98
3. Cecchi R (2010) Estimating wound age: looking into the future. Int J Legal Med 124:523–536
4. Betz P (1994) Histological and enzyme histochemical parameters for the age estimation of human skin wounds. Int J Legal Med 107:60–68
5. An JL, Ishida Y, Kimura A, Kondo T (2011) Immunohistochemical examination of intracerebral aquaporin-4 expression and its application for differential diagnosis between freshwater and saltwater drowning. Int J Legal Med 125:59–65
6. An JL, Ishida Y, Kimura A, Kondo T (2010) Forensic application of intrarenal aquaporin-2 expression for differential diagnosis between freshwater and saltwater drowning. Int J Legal Med 124:99–104
7. Nosaka M, Ishida Y, Kimura A, Kondo T (2010) Immunohistochemical detection of MMP-2 and MMP-9 in a stasis-induced deep vein thrombosis model and its application to thrombus age estimation. Int J Legal Med 124:439–444
8. Nosaka M, Ishida Y, Kimura A, Kondo T (2009) Time-dependent appearance of intrathrombus neutrophils and macrophages in a stasis-induced deep vein thrombosis model and its application to thrombus age determination. Int J Legal Med 123:235–240
9. Fracasso T, Pfeiffer H, Michaud K, Köhler H, Sauerland C, Schmeling A (2011) Immunohistochemical expression of fibronectin and C5b-9 in the myocardium in cases of carbon monoxide poisoning. Int J Legal Med 125:377–384
10. Yoshida C, Ishikawa T, Michiue T, Quan L, Maeda H (2011) Postmortem biochemistry and immunohistochemistry of chromogranin A as a stress marker with special regard to fatal hypothermia and hyperthermia. Int J Legal Med 125:11–20
11. Bohnert M, Anderson J, Rothschild MA, Böhm J (2010) Immunohistochemical expression of fibronectin in the lungs of fire victims proves intravital reaction in fatal burns. Int J Legal Med 124:583–588
12. Dressler J, Hanisch U, Kuhlisch E, Geiger KD (2007) Neuronal and glial apoptosis in human traumatic brain injury. Int J Legal Med 121:365–375
13. Kondo T, Tanaka J, Ishida Y, Mori R, Takayasu T, Ohshima T (2002) Ubiquitin expression in skin wounds and its application to forensic wound age determination. Int J Legal Med 116:267–272
14. Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2008) Expression of oxygen-regulated protein 150 (ORP150) in skin wound healing and its application for wound age determination. Int J Legal Med 122:409–414
15. Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2009) Detection of fibrocytes in human skin wounds and its application for wound age determination. Int J Legal Med 123:299–304
16. Kondo T, Ohshima T, Eisenmenger W (1999) Immunohistochemical and morphometrical study on the temporal expression of interleukin-1α (IL-1α) in human skin wounds for forensic wound age determination. Int J Legal Med 112:249–252
17. Hayashi T, Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2004) Forensic application of VEGF expression to skin wound age determination. Int J Legal Med 118:320–325
18. Kondo T, Ohshima T, Mori R, Guan DW, Ohshima K, Eisenmenger W (2002) Immunohistochemical detection of chemokines in human skin wounds and its application to wound age determination. Int J Legal Med 116:87–91
19. Ma WX, Yu TS, Fan YY, Zhang ST, Ren P, Wang SB, Zhao R, Pi JB, Guan DW (2011) Time-dependent expression and distribution of monoacylglycerol lipase during the skin-incised wound healing in mice. Int J Legal Med 125:549–558
20. Yu TS, Cheng ZH, Li LQ, Zhao R, Fan YY, Du Y, Ma WX, Guan DW (2010) The cannabinoid receptor type 2 is time-dependently expressed during skeletal muscle wound healing in rats. Int J Legal Med 124:397–404
21. Betz P (1995) Immunohistochemical parameters for the age estimation of human skin wounds. A review. Am J Forensic Med Pathol 16:203–209
22. Martin P (1997) Wound healing—aiming for perfect skin regeneration. Science 276:75–81
23. Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341:738–746
24. Gillitzer R, Goebeler M (2001) Chemokines in cutaneous wound healing. J Leukoc Biol 69:513–521
25. Martin P, Leibovich SJ (2005) Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol 15:599–607
26. Ishida Y, Gao JL, Murphy PM (2008) Chemokine receptor CX3CR1 mediates skin wound healing by promoting macrophage and fibroblast accumulation and function. J Immunol 180:569–579
27. Ishida Y, Kondo T, Kimura A, Matsushima K, Mukaida N (2006) Absence of IL-1 receptor antagonist impaired wound healing along with aberrant NF-κB activation and a reciprocal suppression of TGF-β signal pathway. J Immunol 176:5598–5606
28. Ishida Y, Kondo T, Takayasu T, Iwakura Y, Mukaida N (2004) The essential involvement of cross-talk between IFN-γ and TGF-β in the skin wound-healing process. J Immunol 172:1848-1855
29. Lin ZQ, Kondo T, Ishida Y, Takayasu T, Mukaida N (2003) Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. J Leukoc Biol 73:713–721
30. Mori R, Kondo T, Ohshima T, Ishida Y, Mukaida N (2002) Accelerated wound healing in tumor necrosis factor receptor p55-deficient mice with reduced leukocyte infiltration. FASEB J 16:963–974
31. Laulederkind SJ, Thompson-Jaeger S, Goorha S, Chen Q, Fu A, Rho JY, Ballou LR, Raghow R (2002) Both constitutive and inducible prostaglandin H synthase affect dermal wound healing in mice. Lab Invest 82:919–927
32. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE (1998) Cyclooxygenase in biology and disease. FASEB J 12:1063–1073
33. Warner TD, Mitchell JA (2004) Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. FASEB J 18:790–804
34. Foitzik T, Hotz HG, Hotz B, Wittig F, Buhr HJ (2003) Selective inhibition of cyclooxygenase-2 (COX-2) reduces prostaglandin E2 production and attenuates systemic disease sequelae in experimental pancreatitis. Hepatogastroenterology 50:1159–1162

35. Hayashi T, Ishida Y, Kimura A, Iwakura Y, Mukaida N, Kondo T (2007) IFN-γ protects cerulein-induced acute pancreatitis by repressing NF-κB activation. J Immunol 178:7385–7394
36. King SJ, Werrett DJ, Harrison DT (1989) The evaluation of an ELISA for semen-specific 19-OH prostaglandin F1 alpha/F2 alpha using ex-casework swabs and stains. Forensic Sci Int 42:103–123
37. King SJ, Kelly RW, Sutton JG (1989) The development of an enzyme-linked immunosorbent assay for 19-OH PG F1 alpha/F2 alpha. Forensic Sci Int 40:211–216
38. King SJ, Sutton JG, Trewsdale LA (1989) Radioimmunoassay detection limits for 19-OH F1 alpha/F2 alpha prostaglandin in normal, infertile and vasectomized semen stains. Analysis of saliva, sweat and urine for possible non-specific or matrix effects. Forensic Sci Int 40:221–229
39. King SJ, Sutton JG (1989) A survey of the concentration of 19-OH F1 alpha/F2 alpha prostaglandins in the semen of fertile, infertile and vasectomized men and their stability in both liquid semen and semen stains. Forensic Sci Int 40:217–220
40. Sutton JG, King SJ, Taylor M (1987) The application of a simple RIA technique for the detection of 19-OH F1 alpha/F2 alpha prostaglandin, a specific semen marker, in semen contaminated vaginal swabs: time since intercourse studies. Forensic Sci Int 34:143–153
41. Sutton JG, Kelly RW, Morris BA (1987) Evaluation of the 190H analogues of prostaglandins E1, E2, F1 alpha and F2 alpha as specific markers for the identification of human semen in body fluid mixtures. Forensic Sci Int 33:103–116
42. He L, Zhu J (1996) Distinguishing antemortem from postmortem injuries by LTB4 quantification. Forensic Sci Int 31:11–16
43. Hernández-Cueto C, Vieira DN, Girela E, Marques E, Calvo MD, Villalobos M, Oliveira de Sà F, Villanueva E (1994) Prostaglandin F2a (PGF2a): an inadequate marker of the vitality of wounds? Int J Legal Med 106:312–314
44. Bai R, Wan L, Shi M (2008) The time-dependent expressions of IL-1beta, COX-2, MCP-1 mRNA in skin wounds of rabbits. Forensic Sci Int 175:193–197
45. Kondo T, Ohshima T (1996) The dynamics of inflammatory cytokines in the healing process of mouse skin wound: a preliminary study for possible wound age determination. Int J Legal Med 108:231–236