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Immunophenotypical expression of adhesion molecules in vital reaction

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Abstract: The vital reaction is defined as the response of a living organism to a trauma, response that does not theoretically appear when the traumatic agent exerts its action after death. Even though in legal medicine practice hematic extravasation is considered a vital sign, in scientific literature the possibility of its appearance immediately after death has been described.

We report a case in which we attempted to explore whether immunohistochemistry can be useful in addition to usual clasic microscopy staining methods in order to distinguish between ante and postmortem injuries. We have analysed the presence and distribution of fibronectin, P–selectin and tenascin within the traumatic lesions of a newborn child found dead with limb amputations caused by dog bites.

Key Words: P-selectin; tenascin; fibronectin; vital reaction.

The vital reaction is defined as a response of a living organism to trauma. Vital reactions can be classified in: local (haemorrhage – blood infiltrate, blood clotting, tissue retraction, inflammatory reaction), general and specific [1,2]. Trauma causes an interruption of the continuity of blood vessels which leads (if the heart is stilbeating) to the appearance of bloody effusions in adjacent tissues [2].

Therefore, both in theory and in the forensic medical practice, the presence of a hematic extravasation in a tissue is highly suggestive for the lesion to be produced before death [3].

Nevertheless, experiments conducted on cadavers revealed that the appearance of bruises is also possible after death [4], in such cases the bleeding being considered a passive phenomenon [5]. Microscopically, the haemorrhage is revealed using the haematoxylin-eosin staining or the Perls reaction (Prussian blue) the latter showing blue stained red cells in the interstitium [2].

The timing of histochemical changes in skin after trauma was extensively studied by Raekallio in 1960-1970 which has analyzed, the sequence of events that occur in the healing of skin wound after injury (inflammation, proliferation, and maturation) [6]. Later on the timing began to be also studied using immunohistochemical techniques, which brought additional data allowing us to better characterize the timing of wound healing.

Case report

Scene investigation.

A newborn was found dead on the street in a disreputable area from the periphery of Bucharest. The newborn was wrapped in a plastic bag. Statements from witnesses suggested the presence of some community dogs near the body when found.

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Autopsy findings.

An autopsy was performed the next day (after 36 hours from the moment it was found) and identified a male newborn, with a weight of 3150g, apparently born at term. The external examination revealed an arm bruise, abrasions and bitemarks on the arms without macroscopically visible hematic extravasations, abrasions and bitemarks on the anterior thoraco-abdominal wall and on the head without macroscopically visible hematic extravasations, and limb amputations, also without macroscopically visible hematic extravasations, caused by dog bites (fig. 1). Internal examination revealed a liver rupture with 150 ml haemoperitoneum.

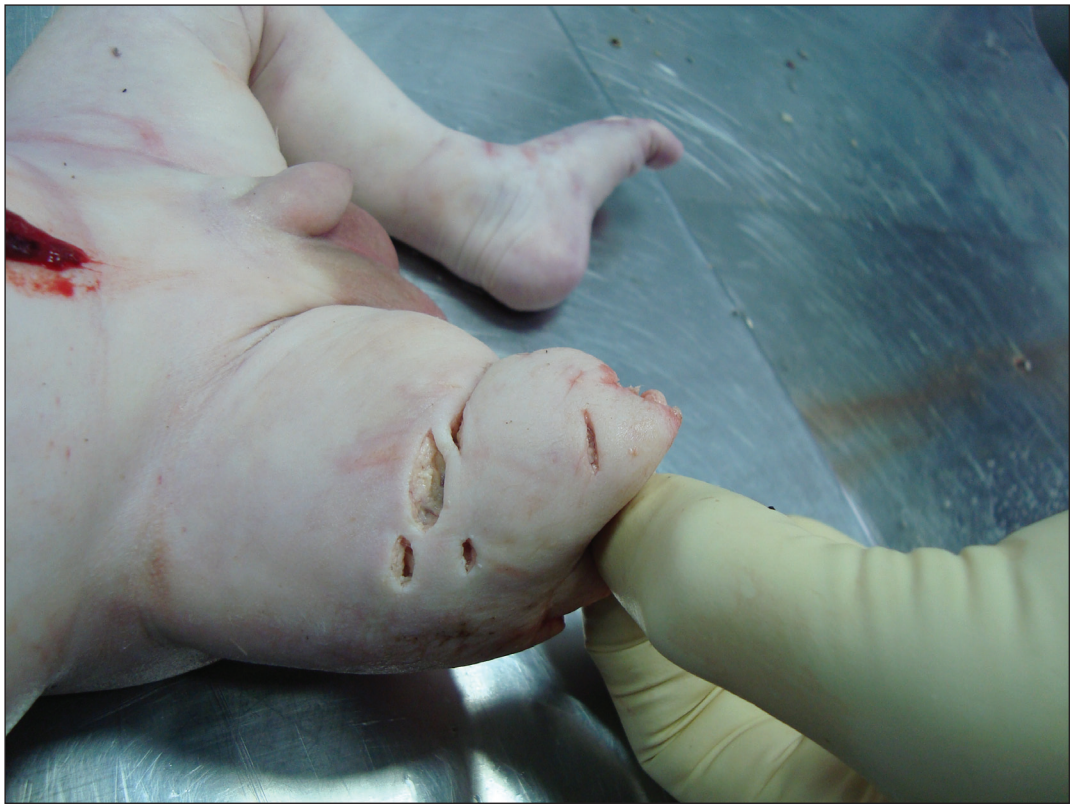

Figure 1. Lower limb amputation by dog bites.

Histopathology investigation

Material and methods

Specimens of the skin from the right thigh, right arm and occipital region were taken for histopathology investigation.

Other fragments were harvested from brain, lungs, heart, liver and kidney.

The selected tissue samples were fixed in 10% neutral buffered formalin (pH - 7) for 24–48 hours and paraffin embedded. Sections were cut at 5 μm and stained with standard HE.

In addition, special stainswere used: Elastic van Gieson and Perls.

Table 1. Antibodies used.

Antigen	Clone	Dilution	Producer	Specificity
Tenascin-X	Poly	1:50	Novocastra	Adhesion molecule
P-selectin (CD62-P)	C34	1:100	Novocastra	Activated endothelium
Fibronectin	568	1:200	Novocastra	Extracellular matrix

Immunohistochemistry

Immunohistochemical analysis (IHC) was done using sections displayed on slides treated first with poly-L-lysine. IHC was performed on 3 μm thick sections from formalin-fixed paraffin-embedded specimens, according to the indirect tristadial Avidin-Biotin-Complex method of Hsu [7], modified by Bussolati and Gugliotta [8].

Briefly, the procedure comprised: deparaffination in xylene and alcohol series, rehydration, washing in phosphate buffer saline (PBS), blocking with normal serum, for 20 min, incubation with primary antibody overnight then with standard labeled streptavidin-biotin antibody (LSAB kit, DAKO, Denmark), washing in carbonate buffer and developing in 3,3'-DAB hydrochloride/ H2O2.

The antibodies used were fibronectin, P-selectin and tenascin-X (for details, see table 1). Antigen retrieval techniques (thermal or enzymatic pretreatment) for the aforementioned antibodies were done, according to the producer's specifications. Both positive and negative controls were used.

To ensure the reliability of the experimental study, internal quality control of histopathologic and IHC techniques were performed as a part of an implemented and certified quality assurance system (ISO 9001/2008).

All slides were examined and photographed on a Zeiss AxioImager microscope (Gottingen, Germany). Digital images acquired with Zeiss Axio Vision program have been processed and analyzed with ACDSee Pro Photo Manager (Washington DC), running under Windows Vista.

Results

In all skin samples, multiple interstitial micro-hemorrhagic infiltrates were observed, predominantly perivascular and perianexial, in dermis and hypodermis (Figure 1 and 2).

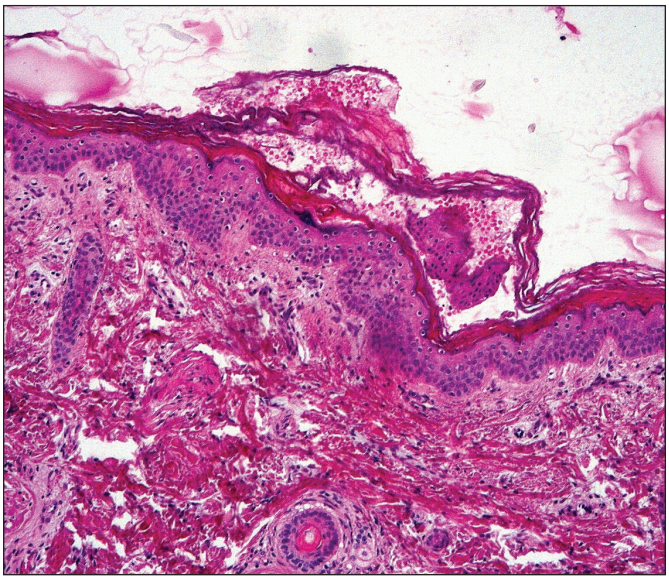

Figure 2. Skin with scattered areas of micro-hemorrhages in the superficial layers of the epidermis and in the dermis HE, 100x

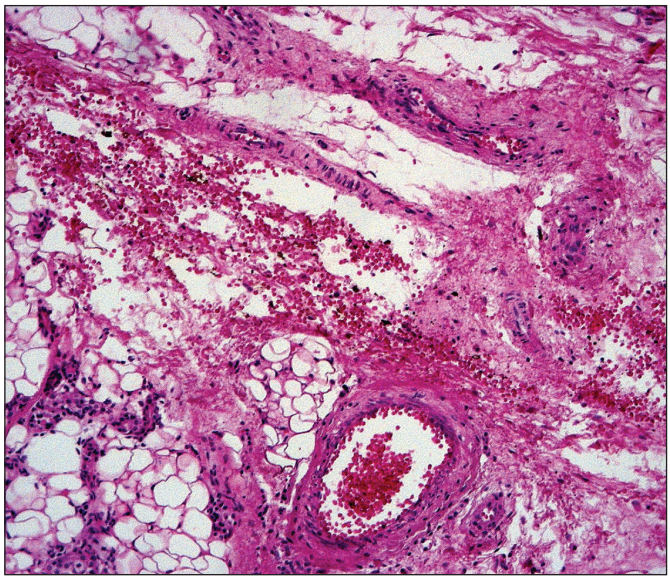

Figure 3. Interstitial and perivascular micro-hemorrhages in the connective tissue from hypodermis, HE, 100x

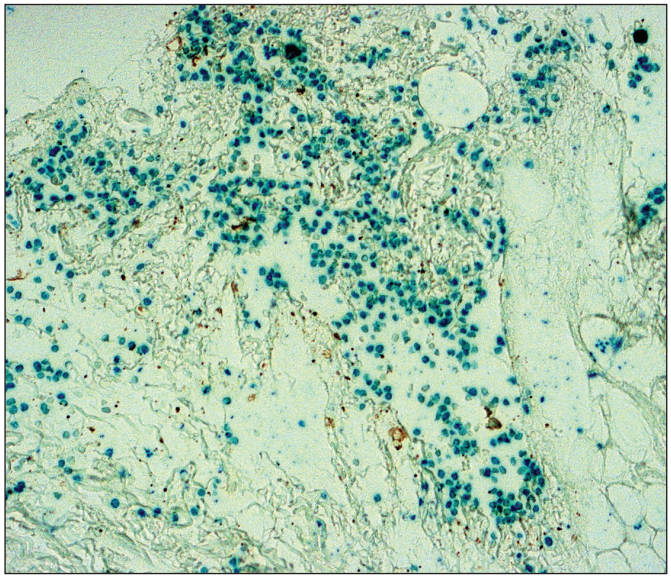

Figure 4. Positive vital reaction in a recently hemorrhagic micro-area from the connective tissue of the hypodermis, Perls stain, 200x

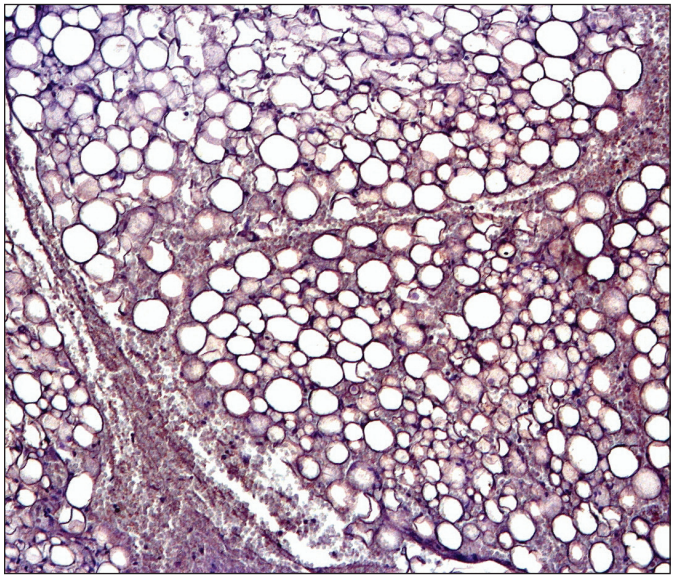

Figure 5. Hypodermis from the right arm: fibronectin positive in the extracellular matrix of the adipose tissue near micro-hemorrhagic areas, IHC, 100x

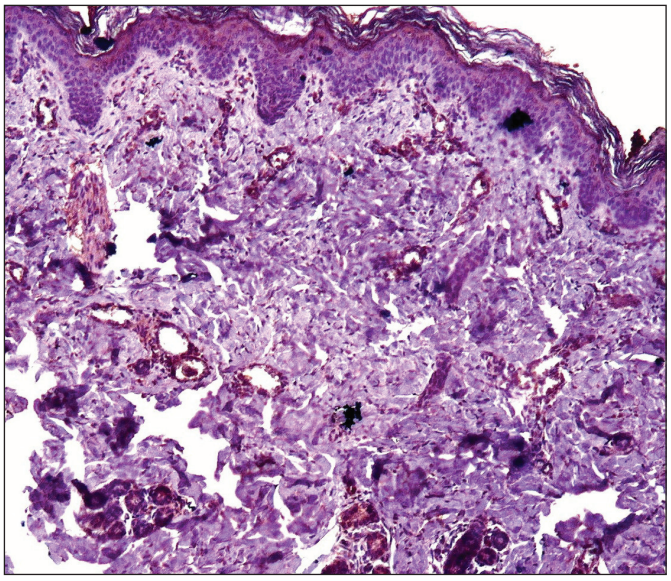

Figure 6. Skin from the right arm, P-selectin positive in frequent capillaries from dermis, IHC, 100x

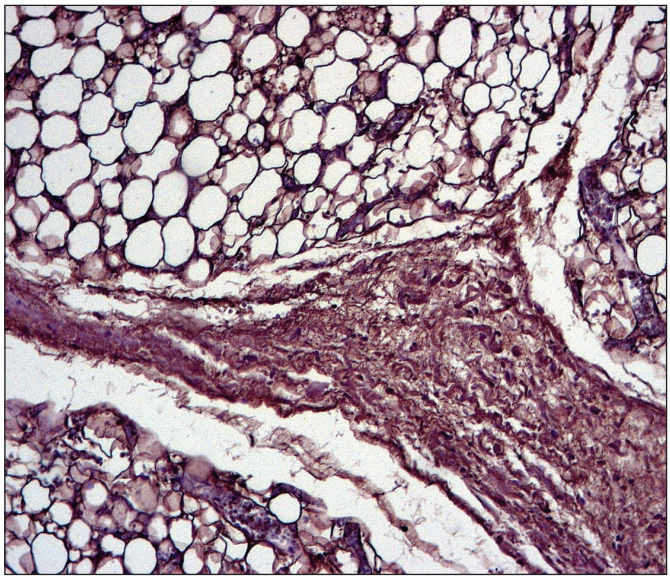

Figure 7. Hypodermis, occipital region: fibronectin positive interstitial in the connective tissue and focally on the membranes of the adipose cells, IHC, 100x

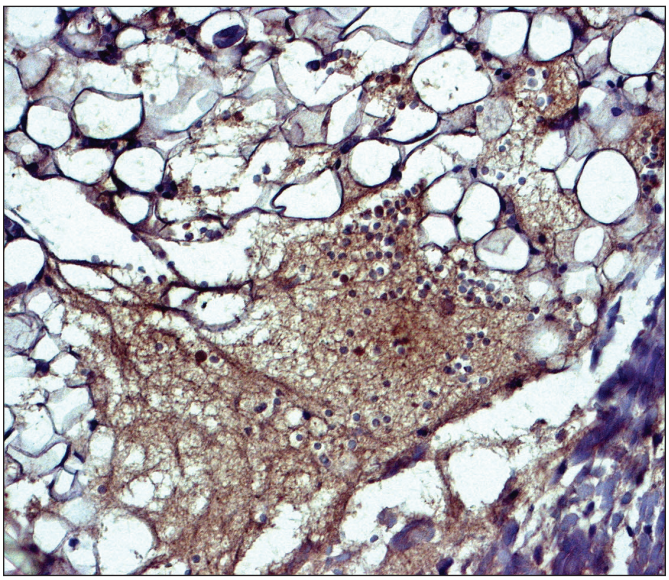

Figure 8. Hypodermis, occipital region: P-selectin positive interstitial in the connective and adipose tissue in a micro-hemorrhagic area, IHC, 200x

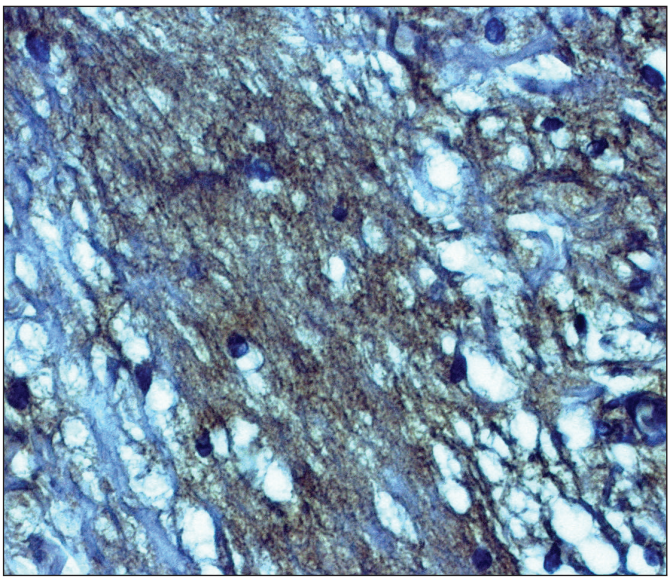

Figure 9. Fibronectin positive interstitial, with a reticular pattern, in the peri-hepatic connective tissue, IHC, 400x

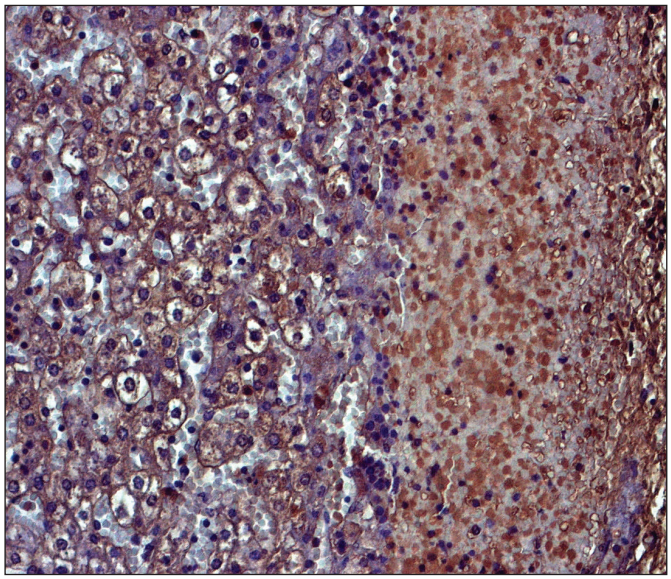

Figure 10. Subcapsular hepatic hemorrhage, fibronectin positive on the membranes of the adjacent hepatocytes, IHC, 100x

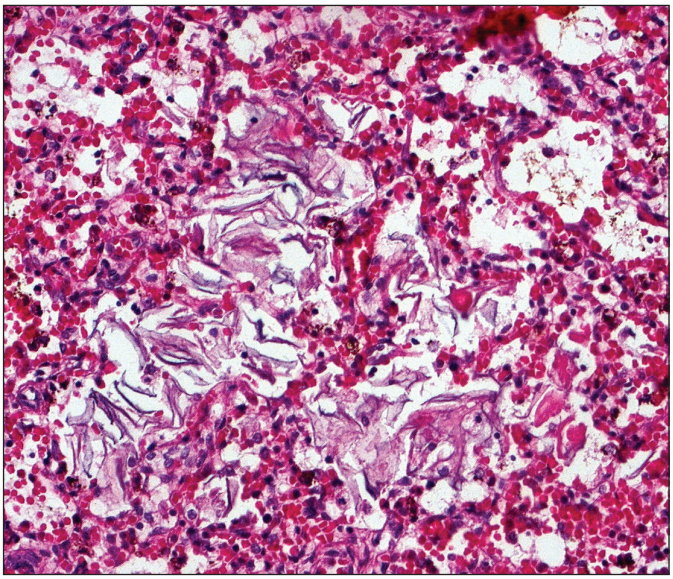

Figure 11. Amniotic fluid aspiration with keratin lamellae, septal congestion and intra-alveolar micro-hemorrhages, HE, 200x

These findings were supported by Perls stain, which has revealed a positive reaction in the hemorrhagic infiltrates from the connective tissue of hypodermis (Figure 3).

Tenascin-X was negative and P-selectin was irrelevant.

At the right arm, fibronectin was positive interstitial, in the connective tissue from the dermis – hypodermis junction. Also, it was positive focally in hypodermis, near micro-hemorrhagic areas (Figure 4). P-selectin was positive in frequent capillaries from dermis and isolated in the interstitium of hypodermis (Figure 5). Tenascin-X was negative.

At the occipital region, fibronectin was positive focally in the extracellular matrix (Figure 6). P-selectin was positive isolated, in scattered capillaries from subcutaneous tissue, and sometime interstitial, in the hypodermic connective tissue, near micro-hemorrhagic areas (Figure 7). Tenascin-X was negative.

In the liver, hepatic laceration with subcapsular hemorrhage was noticed, associated with interstitial peri-hepatic hemorrhagic infiltrates in the connective tissue.

Fibronectin was positive focally with a reticular pattern, in peri-hepatic connective tissue (Figure 8) and on the membranes of hepatocytes (Figure 9), near hemorrhagic areas. P-selectin and Tenascin-X were negative.

In lungs, amniotic fluid aspiration with keratin lamellae were noticed in frequently distended alveoli, on a background of congestion (Figure 10).

The other organs showed congestion, edema and incipient autolytic lesions.

Discussions

The main problem of this case was to identify whether the dog bites were produced intravitam or

after death. The presence of a partially ventilated lung containing amniotic aspirate asociated with a liver rupture and secondary haemopaeritoneum suggested the possibility for the liver rupture and arm bruise to be caused by trauma associated to brutal manoeuvers during an unassisted delivery, with a short survival of the newborn which led to the hypothesis that the newborn was already dead when bitten by stray dogs.

The pathological examinations (HE and Pearls stains) revealed microscopic hematic extravasations at the level of the limb amputations therefore raising doubts about this initial hypothesis, and suggesting the possibility that the newborn was either alive or in an agonic state when attacked by animals. As immunohistochemistry was proven to be more sensitive in differentiating between ante and postmortem lesions we have also studied the presence in fibronectin, P-selectin and tenascin in the traumatic lesions

Fibronectin is multifunctional cell adhesion protein found in blood and some tissues. It displays a tendency to form networksin tissues at the level ofvessel lesions with subsequent hematic extravasations. It is involved in the adhesion and migration of the fibroblasts, keratinocytes and endothelial cells during the healing process of a lesion [9]. P selectin is a cell adhesion molecule, present on the activated surfaces of the vascular endothelia in the initial phases of inflammation [10]. Tenascins are glycoproteins from the extracellular matrix with roles in the healing process (cell adhesion, migration, growth) [11].

Research conducted by Betz et al. 1992 revealed a positive reaction for fibronectin in human skin wounds longer than 30 minutes after the production of a lesion and a negative reaction in postmortem induced lesions [12]. Other authors [13] report positive reactions for fibronectin in 20 minutes-old wounds, they should however be analyzed with cautions as other studies warn about false-positive reactions due to autolysis [14] .

Experiments on mice incised 5, 10 and 15 minutes, respectively before being sacrificed, described fibronectin and tenascin nets within the incisions margins [15] while other studies only reveal tenascin two days after a trauma [16].

It has to be mentioned that another study on animals in which lesions were induced 0-5 minutes after heart activity ceased noticed the appearance of the fibronectin net in some cases [17] raising some doubts about the value of fibronectin as a marker of vitality for a lesion. Studies analyzing the detection of selectines in skin lesions revealed positive reactions for P selectin even afterthree minutes after the induction of the lesion [18, 19] but Ortiz-Rey at al.

Also identified their presence postmortem lesions [20]. In our case all skin samples were negative for tenascin. In the skin fragment sampled from the bruise at the arm level we identified P-selectin in the vascular endothelium which could be regarded as an argument for a vital lesion compared with the other skin fragments where P-selectin was only focally positive in the interstitium i.e the hypodermic adypose tissue. A positive fibronectin reaction was identified in both the bruise in the arm and within the bitten areas.

A positive fibronectin staining in our case, which is mainly focal in the bitten areas may have been caused by the presence of an incipent autolysis. This, together with the presence of only small foci of positive P-selectin in the bitten areas and a negative tenascin reaction suggests the possibility of a positive postmortem reaction.

Conclusion

Immunohistochemical vital reactions must be regarded with caution when interpreting borderline cases. Its usefulness has not yet been fully established and before being used in forensic practice more, larger scale studies are needed.

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