Z Rechtsmed (1982) 89:81–88

Zeitschrift für
Rechtsmedizin

Histologically Demonstrable Vital Reactions to Frostbite in Guinea Pigs Dying of Hypothermia

J. Hirvonen, J. Penttinen, and P. Huttunen

Dept. of Forensic Medicine, University of Oulu, Kajaanintie 52 D, SF-90220 Oulu 22, Finland

Summary. Vital reactions in the paw skin of guinea pigs dying of hypothermia were examined using a variety of histochemical methods to find ways of distinguishing between ante-mortem and post-mortem frostbites. Some animals were treated with ethanol or diazepam.

Edema, hyperemia, and incipient accumulation of granulocytes were seen to develop within the 2–6 h for which the animals had survived in the cold (−20°C). The granulocytes were first seen in the capillaries beneath the epithelial papillae and thereafter deeper in the dermis. They mostly accumulated inside the venules in the endothelium, but there were cases in which emigration had started. The best method for demonstrating the reaction appeared to be the alkaline phosphatase reaction, which is strong in guinea pig granulocytes and highlighted the latter well against a negative background.

The conclusion is that it is possible to detect a positive vital reaction, but methodologic investigations are needed before similar changes can be visualized in cases of acute hypothermia in human beings.

Key words: Vital reactions, ante-mortem frostbites – Frostbites, vital reactions

Zusammenfassung. Vitale Reaktionen in der Pfotenhaut von an Hypothermie gestorbenen Meerschweinchen wurden unter Anwendung histochemischer Verfahren untersucht, um zu prüfen, ob es möglich ist, antemortale von postmortalen Frostschäden zu unterscheiden. Ein Teil der Tiere hatte zusätzlich Diazepam oder Äthanol erhalten.

Ödeme, Hyperämie und beginnende Ansammlung von Granulozyten waren bei Tieren zu beobachten, die 2–6 h in der Kälte (−20°C) überlebt hatten. Die Granulozyten wurden zuerst in den Kapillaren im Strat. subpapillare gesehen, später tiefer in der Dermis. Meist lagen sie gehäuft intravasal randständig am Endothel, teilweise war auch Emigration zu beobachten. Als beste Methode zur Darstellung von Granulozyten (vitale Reaktion) erwies sich die alkalische Phosphatase-Reaktion, weil sich die in den Meerschweinchen-Granulozyten stark positive Reaktion gut vom negativen Untergrund abhebt.

Offprint requests to: J. Hirvonen, MD (address see above)

0044-3433/82/0089/0081/$ 1.60

Als Schlußfolgerung ergibt sich, daß es zwar möglich ist, eine positive vitale Reaktion zu erkennen, aber weitere methodische Untersuchungen erforderlich sind, ehe solche Veränderungen in Fällen von akuter Hypothermie beim Menschen sichtbar gemacht werden können.

Schlüsselwörter: Vitale Reaktionen, antemortale Frostschäden – Erfrierungen, vitale Reaktionen – Frostschäden

Introduction

Surprisingly little is known of the vital reactions involved in frostbite or of their timing, which practically no pertinent information of these two important aspects available in the textbooks on pathology. These are, of course, typical problems of forensic pathology, even though they are of little or no interest for clinical medicine. Some experimental work on timing aspects of local frostbite has been carried out. It has been found that signs of inflammatory reactions appear in the dermis of the guinea pig within 1 h of freezing with dry ice and subsequent thawing (Laiho and Hirvonen 1971), and edema and focal hemorrhage are observed in that of the rabbit within 3.5 h of exposure to −25° C and subsequent rewarming. It has also been shown that histamine and the 5-HT content decrease in the mouse ear 10 min after freezing and thawing (Kulka 1965; Penttilä et al. 1974).

However, these results cannot be directly applied to a situation in which the animal or human being is hypothermic since the blood circulation becomes stagnated in the exposed extremities, and the vital reactions are not likely to start. Only hyperemia and edema have been found in the skin of victims of accidental hypothermia by means of routine stainings, and these are, of course, only vague signs of vital frostbite, since livores can resemble vital congestion. Differentiation between vital and postmortem freezing is important in the case of victims of hypothermia as is an estimation of the duration of exposure.

To identify some changes which might develop in exposed limbs during fatal freezing we investigated the skin on the paws of guinea pigs exposed to frost until death. The aim was to discover signs and methods which could then be applied in cases of accidental hypothermia which are often frozen postmortem and die under unknown circumstances. Since drugs are often background factors in hypothermia deaths, animals given ethanol and diazepam were included in the material. Minor signs of inflammation were demonstrable when special histochemical methods were applied.

Materials and Methods

Altogether 83 guinea pigs used in our hypothermia experiments were included in the material. Seventy-four were exposed to −20° C till death, and 17 killed without exposure, to serve as controls. Of the 74 exposed animals, 30 were acclimated to cold by keeping them at +4° C for 2 weeks or 2 months to test the cold-adaptation process. The animals were divided into the following groups.

I. Ethanol Groups

1. Thirteen guinea pigs were acclimated at +4°C for 2 weeks and divided into two subgroups:
   1.1. Seven animals were given a single dose of ethanol (2 g/kg b.wt.) i.p. 30 min before exposure to −20°C.
   1.2. Six animals were given 0.9% NaCl i.p. instead of ethanol before exposure to −20°C.

2. Twenty-nine guinea pigs were kept at +20°C for 2 weeks and divided into two subgroups:
   2.1. Sixteen animals received 2 g ethanol/kg b.wt. i.p. 30 min before exposure to −20°C.
   2.2. Fourteen animals received 0.9% NaCl i.p. before exposure to −20°C.

II. Diazepam Groups

1. Fifteen guinea pigs were kept at +4°C for 2 months to achieve a proper state of acclimation and were then divided into two subgroups:
   1.1. Nine animals were given 15 mg diazepam/kg b.wt. i.m. 30 min before exposure to −20°C.
   1.2. Six animals were given 0.9% NaCl i.m. before exposure to −20°C.

2. Fifteen guinea pigs were kept at room temperature for 2 months and divided into two subgroups:
   2.1. Ten animals were given 15 mg diazepam/kg b.wt. i.m. 30 min before exposure to −20°C.
   2.2. Five animals were given 0.9% i.m. before exposure to −20°C.

III. Control Groups

Seventeen control guinea pigs were neither acclimated nor given any drug, nor exposed to cold, but killed directly by a blow on the neck.

Parameters Studied

Rectal temperature was measured every hour during exposure and upon death from hypothermia, i.e. when the heart stopped beating.

Two samples of the skin from the front paw were excised for histological study. One piece was fixed in neutral 4% formalin and processed in the usual way to obtain paraffin blocks, sections from which were then stained with Movat's pentachrome (AFIP manual 1968) and HE. The other piece was fresh-frozen in liquid nitrogen and sectioned at 10 µm in a cryostat to test its reactions with alkaline phosphatase, naphthylamidase, non-specific esterase, and lactate dehydrogenase (Barka and Anderson 1965).

Results

The survival times were longer in the groups which had been cold-acclimated at +4°C, and it was only in the ethanol-treated guinea pigs that the difference between the acclimated and non-acclimated animals failed to be significant. Both ethanol and diazepam shortened the survival time, diazepam to a greater extent. Real comparison is impossible, however, without dose-dependent tests (Table 1).

Microscopy of the Paw Skin

The epidermis appeared intact, and not even any loss of enzyme reaction was visible. There were areas in the dermis which were faintly stained and appeared loose, i.e., sites of apparent edema. Mild hyperemia was present in some sections.

Table 1. Survival time and presence of granulocytes in the paw skin of guinea pigs with or without acclimation to cold at +4°C and with or without administration of ethanol or diazepam before exposure to frost (−20°C)

	N	Survival, min			HE		AlPase	
		Mean ± SD			+	−	+	−
I. *Ethanol* (2 g/kg b.wt.) groups								
1. Acclimated at +4°C for 2 weeks								
1.1. Ethanol + exposure	7	405 ± 248			6	1	6	1
1.2. NaCl + exposure	6	672 ± 129*		NS	4	2	5	1
2. Kept at +20°C for 2 weeks			***					
2.1. Ethanol + exposure	16	275 ± 74			13	3	14	2
2.2. NaCl + exposure	14	351 ± 54**			11	3	11	3
II. *Diazepam* (15 mg/kg b.wt.) groups								
1. Acclimated at +4°C for 2 months								
1.1. Diazepam + exposure	9	202 ± 74			7	2	8	1
1.2. NaCl + exposure	6	610 ± 295**	**	*	5	1	4	2
2. Kept at +20°C for 2 months								
2.1. Diazepam + exposure	10	140 ± 42			9	1	9	1
2.2. NaCl + exposure	5	269 ± 98**			4	1	4	1
III. No drug, no exposure	17	—			2	15	3	14

HE = Hematoxylin and Eosin, AlPase = Alkaline phosphate, NS = not significant

Student's *t*-test

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

There was no change of colour in the collagen fibres with the Movat's pentachrome staining.

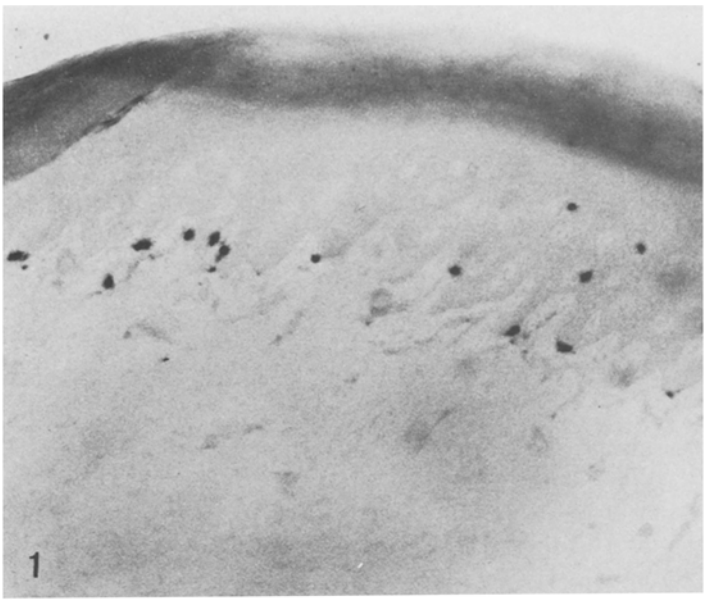
Incipient inflammatory reaction was visible in the most exposed animals, amounting to 81% of the whole material with HE staining and 84% when assessed from the reaction with alkaline phosphatase (AlPase). Three control animals of 17 had a few granulocytes visible below the base of the papillae (Table 1).

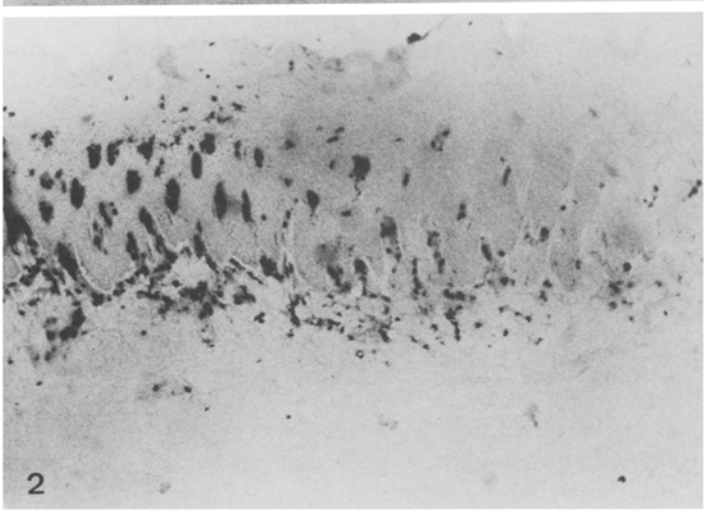
In the first phase such granulocytes seemed to gather in the capillaries beneath the epidermis, while at a more advanced phase they were also found in and around the deeper dermal capillaries and in the subcutis. In many samples the granulocytes were located at the vessel endothelium (margination phenomenon; Figs. 1–3).

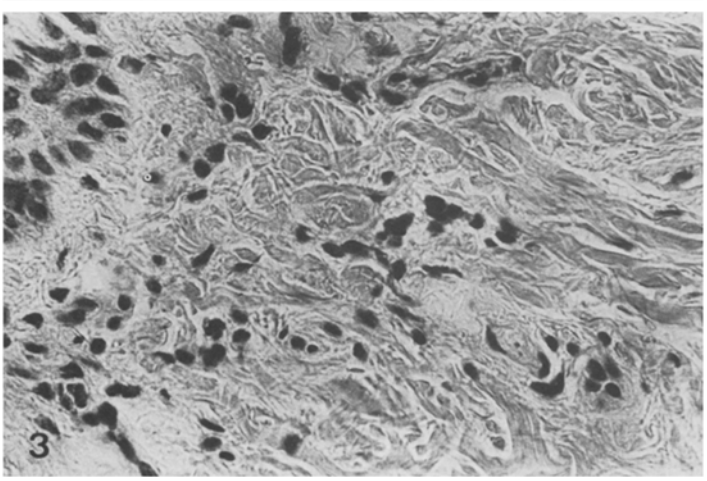
Fig. 1. Paw skin from a control guinea pig. There are a few highly positive granulocytes at the junction of the epidermis. Alkaline phosphatase reaction, × 53

Fig. 2. Paw skin from a guinea-pig which died in the cold. There are a large number of granulocytes at the epidermal junction and also in the dermal extensions. Alkaline phosphatase reaction, × 45

Fig. 3. Paw skin from another guinea pig which died in the cold. Incipient emigration of granulocytes into the dermis is visible. Focal mild edema is seen in the periphery. HE, × 264


1


2


3

The granulocytes were best visualized with the AlPase reaction since the cells have a strong reaction with this enzyme, and the epidermal and dermal cells are negative. The other enzyme reactions did not give such a clear differentiation, as the epidermal and dermal cells reacted as strongly as the granulocytes with non-specific esterase, naphthylamidase and lactic dehydrogenase.

There was no correlation between the mean survival time and the presence of inflammation within the groups, but a survival time of 2–3 h seemed to be enough for the early inflammatory reaction to become visible. Ethanol and diazepam did not seem to modify the inflammation. No thrombosis of the venules or capillaries was observable.

Discussion

The present experiments demonstrated one vital reaction which may be helpful in the investigation of hypothermia deaths, in which these signs are scarce, i.e., the mild inflammatory reaction which appeared in the paw skin during the average of 5–6 h for which the guinea pigs survived at −20° C. The reaction consisted of edema and granulocytes which gathered at the endothelium of the venules or emigrated beyond the vessels in a more advanced state. Edema is difficult to evaluate in single cases and none of the stainings used—including the enzyme reactions—demonstrated it clearly enough to be of practical value. The granulocytes were observable in all the stainings, but the alkaline phosphatase reaction was superb for visualizing them, since this enzyme is strong in the granulocytes of the guinea pig, and the background is negative. The other enzyme reactions tested, i.e., naphthylamidase, non-specific esterase, and lactic dehydrogenase proved to be of less value. No decrease or increase in the skin could be observed of the kind that has been demonstrated in burns several hours old (Pioch 1966). Unfortunately, the presence of granulocytes was not an "all or nothing" phenomenon, as a few of the exposed animals had no inflammation, i.e., "a false negative" result, and some controls had granulocytes, i.e., a "false positive" reaction. The latter probably represents a reaction to minor mechanical trauma to the paw during the normal life of the animal. No negative vital reactions were observed in the epidermis, and it seems that only vital thawing brings out the lesion in the form of a disappearance of the enzyme reaction within a few hours, since this was not seen in the present samples, which were thawed postmortem. Such a sequence of events would apply to cases who had lived a few hours after rewarming from hypothermia. The membrane permeability changes necessary for the change in the enzyme reaction probably develop after thawing.

The lesions in the skin were perhaps not real frostbites but at least partly lesions caused by above zero temperatures, thus resembling trench foot. A weak circulation may function up until the very last phase of hypothermia, preventing freezing of the skin. The lesion initiating the inflammatory reaction could not be detected histologically. It was either endothelial, as is assumed in cold injuries leading to occlusion of the circulation, or occurred in the dermal structures (Kreyberg 1949). The granulocytes were mostly located in the endothelium of the venules and invaded the dermis to a lesser extent, which speaks for an endothelial

lesion or an inflammation which had not proceeded far enough before death. There was no discoloration in the dermal fibers with pentachrome staining and no pyknosis or other signs of damage in the sweat or apocrine glands. It is probable that the lesion would have been seen within a few days if the animals had been rewarmed, as was shown in the experiments with rabbits (Kulka 1965).

Estimation of the exposure time from the degree of the inflammatory reaction, as can be done in the case of mechanical injuries (Hirvonen 1968), did not seem possible in our experiments. The progress of the reaction was much slower in these hypothermic guinea pigs than in local frostbites in anesthetized normothermic guinea pigs, the state of inflammation being about the same in animals having lived in the frost for about 6 h as in the normothermic animals 1–2 h after freezing and thawing of the skin (Laiho and Hirvonen 1971). Comparable observations have been made in experimental skin wounds of guinea pigs, in which the cellular and enzyme reactions were delayed by stress, such as immobilization and cold during the first 3 h. The delay was most distinct (about 20%) in those animals which died in the cold (Bode et al. 1980). The reason for the delay is probably a slowing down of the blood circulation in the skin since the same kind of delay has been observed after loss of blood and ethanol intoxication (Berg et al. 1977).

Determination of how the inflammatory reaction would develop in human victims in analogous situations would need careful methodological and comparative studies. Could an enzyme-histochemical method be found, for instance, which would demonstrate the few granulocytes at the early phase of inflammation substantially better than "routine" HE staining?

In conclusion, it is justified to say that mild changes could be observed in these cases of cold injuries occurring before death due to hypothermia. This mild inflammatory reaction is in fact a classic vital reaction and is worthy of further study, since one cannot expect to learn much more using the histological and histochemical methods routinely used in laboratories. If similar inflammation is also demonstrable in humans, it remains to be seen whether these can be detected in real cases of accidental hypothermia which have occurred in subzero temperature.

References

1. Armed Forces Institute of Pathology (1968) Manual of histologic and special staining technics, 3rd edn. Luna/McGraw-Hill, New York
2. Barka T, Andersson P (1965) Histochemistry. Hoeber Medical Division, New York Evanston London
3. Berg S, Bode G, Garbe G, Sillus U (1977) Der Einfluß von Blutverlust und Alkohol auf die frühen Wundreaktionen. Z Rechtsmed 80:39
4. Bode G, Garbe G, Ick D (1980) Der Einfluß von Kälte bzw. Tod durch Erfrieren auf die frühen Wundheilungsvorgänge an Hautschnittwunden. Beitr Gerichtl Med 38:119
5. Hirvonen J (1968) Comparative histochemical studies on the inflammatory and/or vital reaction after infliction of incised wounds in the skin and adipose tissue of guinea pigs. J Forensic Med 15:161
6. Kreyberg L (1949) Development of acute tissue damage due to cold. Physiol Rev 29:156
7. Kulka J (1965) Cold injury of the skin. Arch Environ Health 11:484

8. Laiho K, Hirvonen J (1971) Reactions of the guinea pig's skin and adipose tissue to experimental frostbite. Acta Pathol Microbiol Scand [A] 79:91
9. Penttilä A, Ahonen A, Laiho K (1974) The effect of freezing and thawing on histamine and 5-hydroxytryptamine of the rat skin. Zacchia 49:1
10. Pioch W (1966) Die histochemische Untersuchung thermischer Hautschäden und ihre Bedeutung für die forensische Praxis. Schmidt-Römhild, Lübeck

Received February 1, 1982