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Peroxidase Activity in Traumatic Skin Lesions

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Summary. Peroxidase acitivity was determined in experimental compression-excoriation lesions and incision wounds of rat skin after different periods of vital time. The peroxidase enzyme was extracted from the tissues by homogenization in 0.5% cetyltrimethylammoniumbromide, and the enzyme activity was measured from the supernatant by o-dianisidine-H_2O_2 assay. In the blood of the rats a mean activity of approx. 5.26 ± 1.11 U/g dry weight was observed. In the control specimens of the skin the activity was very low and generally below the detection limit of the methods used. In 30-min-old compression-excoriation lesions the mean peroxidase activity was 0.38 ± 0.21 U/g dry weight. In lesions older than 30 min the activity started to increase rapidly. In 4-h-old compression-excoriation lesions it was 10 times higher than the 30-min level and was 40 times higher in 12-h-old lesions and 70–100 times higher in 1–3-day-old compression-excoriation lesions, respectively. In 30-min-old incision wounds the mean peroxidase activity was 0.65 ± 0.37 U/g dry weight. The increase of the activity compared with the 30-min level was even faster in the incision wounds: in 4-h-old wounds the mean activity was 50 times higher, in 12-h-old wounds 200 times higher and in those of 1–5 days it was several hundreds of times higher. Compression-excoriation lesions made after death showed activity similar to the control specimens. Postmortem autolysis at +22°C resulted in a loss of the enzyme activity in 1-day-old compression-excoriation lesions so that after 3 days approx. 80% remained, and after 5 and 7 days approx. 40% was present. After 3 days of autolysis at +4°C, nearly 100% of the activity remained and approx. 90% was present after 5 and 7 days of autolysis. Increased peroxidase activity was also detectable in human vital excoriations in the specimens which were taken in autopsies several days postmortem.

Key words: Skin lesions, peroxidase activity – Vital time

Zusammenfassung. Die Peroxidaseaktivität wurde in kombinierten Quetsch- und Abschürfungsverletzungen sowie in Schnittwunden der Haut von Rat-

ten nach verschieden langen vitalen Reaktionszeiten bestimmt. Das Enzym wurde durch Homogenisierung in 0,5%iger Cetyltrimethylammoniumbromidlösung aus dem Gewebe extrahiert. Aus dem Supernat wurde die Peroxydaseaktivität nach dem o-Dianisidin-Wasserstoffperoxydverfahren bestimmt. Für das Rattenblut ergab sich der Durchschnittswert 5,26 ± 1,11 Einh. Peroxydaseaktivität je g Trockengewicht. Dagegen lag die Aktivität von Kontrollproben der Rattenhaut mit den hier verwendeten Verfahren meistens unter der Bestimmungsgrenze. Die 30 min alten Quetsch- und Abschürfverletzungen wiesen sämtlich Enzymaktivität auf, Durchschnittswert 0,38 ± 0,21 Einh. je g Trockengewicht. Die Aktivität stieg im weiteren Zeitverlauf rasch an und betrug im Vergleich mit dem 30 min-Niveau etwa das 10fache bei den 4 h alten Verletzungen, etwa das 40fache bei den 12 Stunden alten sowie etwa das 70 bis 100fache bei den 1–3 Tage alten Verletzungen. In den 30 min alten offenen Schnittwunden erreichte die Aktivität im Durchschnitt 0,65 ± 0,37 Einh. je g Trockengewicht; anschließend daran nahm die Aktivität relativ schneller zu, und zwar war sie im Durchschnitt bei 4 h alten Schnittwunden etwa 50fach im Vergleich mit dem Niveau nach 30 min, bei den 12 h alten etwa 200fach und bei denjenigen mit einem Alter von 1 bis 5 Tage mehrere hundertfach. Bei den postmoralen Quetsch- und Schürfverletzungen wurden entsprechende Aktivitäten wie im Kontrollgewebe festgestellt. Autolyse bei +22°C verminderte die Peroxidaseaktivität der 1 Tage alten Quetsch- und Schürfverletzungen in der Weise, daß nach Verlauf von 3 Tagen noch etwa 80% sowie nach 5 und 7 Tagen etwa 40% der Ausgangsaktivität übrig waren. Bei +4°C dagegen waren nach 3 Tagen etwa 100% sowie nach 5 und 7 Tagen etwa 90% erhalten. Ein Anstieg der Peroxidaseaktivität war ebenfalls im Obduktionsmaterial von humanen vitalen Schürfverletzungen der Haut nachweisbar, die mehrere Tage postmortal entnommen waren.

Schlüsselwörter: Hautverletzungen, Peroxidaseaktivität – Vitale Reaktionen, Zeitschätzung

Introduction

The peroxidase reaction was described by Schönbein in 1863 when he showed that guaiac tincture turned blue in the presence of hydrogen peroxide when animal or plant tissues were added [1]. The same reaction was also observed when pus was added [2]. In 1907 Winkler [3] reported that on staining with Nadi reagent (α-naphthol + diethylparaphenylenediamine) small blue grains were noticed in the cytoplasm of the myeloic cells, whereas lymphocytes for example were not stained. Later on it was noticed that the Nadi reagent also contained hydrogen peroxide formed in the reagent by auto-oxidation [4]. Based on these types of observations, the active substance in the myeloic leucocytes was described as a peroxidase. Myeloperoxidase was isolated from pus by Agner in 1941 [4]. However, it was not easy to extract the enzyme from the cells and tissues and get it into solution for activity measurements until 1972 when Desser

et al. [5] introduced a detergent, cetyltrimethyl-ammoniumbromide, suitable for the extraction of myeloperoxidase from the cells. After that, in several experimental studies of the inflammatory reaction it has been shown that myeloperoxidase is a good marker enzyme for polymorphonuclear leukocytes, and its activity in the tissues correlates well with the number of such cells [6–12].

In forensic medicine the appearance and quantity of inflammatory cells in an area of trauma has been an important finding in the evaluation of the age and the vitality of lesions [13, 14]. Based on the literature mentioned above, it was assumed that measurements of myeloperoxidase activity could be suitable for forensic medical purposes by allowing quantitation of the inflammatory reaction and correlation with the age and vitality of the lesions. In the present paper peroxidase activity has been studied in experimental excoriations and incision wounds of the skin, and the activity has been correlated with the age of the lesions. The resistance of the peroxidase to postmortem autolysis was also studied, and a small number of autopsy specimens of human traumatic skin lesions were analyzed for the same purposes.

Materials and Methods

Experimental excoriations were made on the right side of the dorsal skin of the rats under ether narcosis with forceps, which were always closed to the same clasp and pulled off without opening. Thus the lesions were a result of both compression and excoriation of the skin. In addition to the lesions described, 5-cm-long incision wounds perforating the skin were made in the other series of experiments on the right side of the dorsal skin of the rats. After different periods of vital time, the whole excoriated area of the skin was taken as a specimen. From the incision wounds about a 1-mm-thick zone of the edges was taken as a specimen. Excoriation lesions of the skin were also made 1 min and 5 min postmortem; then specimens were taken after 1 h. To test the effect of portmortem autolysis the rats with one-day-old vital excoriations were kept after death for different periods of time at +4°C and +22°C, after which the specimens were taken. In all cases, control specimens of normal skin were taken from the opposite side of the dorsal skin of the rats. To analyze the peroxidase activity in blood specimens were taken from six rats by heart puncture. All the specimens were stored at −70°C from some hours to several days, or, if analyzed directly, the specimens were first frozen.

Myeloperoxidase was extracted from the specimens by a method described by Lundberg et al. [11]. However, in these experiments one additional freezing and thawing were used in the extraction procedure, and the centrifugation time and G value were also different from the original method. The stored specimens were thawed to room temperature, minced with scissors in a 0.5% solution of cetyltrimethylammoniumbromide (Merck), and homogenized thoroughly at room temperature using usually about 3 ml of 0.5% cetyltrimethylammoniumbromide solution per 100 mg of tissue wet weight. The homogenate was deep frozen and thawed again until some remnants of the ice were still in the center of the homogenate tube. The homogenate tube was then kept for 15 min in ice bath and centrifuged in a cold room (+4°C) for 30 min at the maximum speed of the Sorvall table centrifuge with angle rotor (about 4900 g). Myeloperoxidase activity of this supernatant was measured with the o-dianisidine-hydrogen peroxide method.

The myeloperoxidase assay was done as described by Lundberg and Arfors [10], except that amounts of the supernatants and reagents suitable for normal cyvettes were used: 0.3 ml of the supernatant was mixed with 1.8 ml of 0.01 *M* phosphate buffer, pH 6.5, containing 0.0005% hydrogen peroxide (Merck) and 0.223 mg/ml o-dianisidine dihydrochloride (Sigma). The change in absorbance at 460 nm (+25°C) was measured with a spectrophotometer with a recorder (Beckman). In the zero determinations the same amounts of supernatant and rea-

gents were used, but without hydrogen peroxide. The zero values obtained were subtracted from the values of the enzyme assay. One unit of myeloperoxidase activity was defined as the amount of enzyme decomposing 1 μmol peroxide/min at 25°C [15]. The relationship between oxidized dye and moles of hydrogen peroxide decomposed was calculated by using the molar absorbancy $1.13 \times 10^4 \text{cm}^{-1}$ at 460 nm [15]. The enzyme activity was expressed as units per g dry weight of the tissue, and for this purpose a sufficient aliquot of the homogenate was dried in an oven at 120°C for at least 24 h and weighed. The statistical analysis of the results was performed using Student's *t*-test.

Results

In most of the control specimens of the normal skin of the rats the peroxidase activity remained under the detection limit of the methods used. In about 24% of the control specimens a mean activity of about 0.16 ± 0.14 U/g dry weight was observed. The blood of the rats had an average activity of 5.26 ± 1.11 U/g dry weight.

In the specimens of the 15 min-old vital compression-excoriation lesions the peroxidase activity was nearly the same as in the control specimens. In the 30-min-old lesions an average activity of 0.38 ± 0.21 U/g dry weight was observed. In the older vital compression-excoriation lesions the average activity started to rise rapidly. For example, in 4-h-old lesions it was about 10 times higher than in the 30-min-old lesions, and was about 40 times higher in 12-h-old ones and

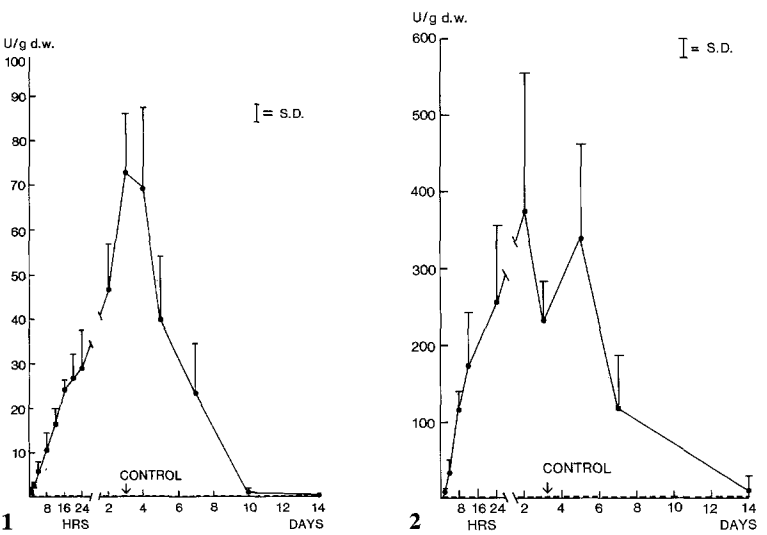

Fig. 1. Peroxidase activity units/g dry weight in experimental compression-excoriation lesions of different ages from 30 min to 14 days and in the corresponding specimens of the control skin. $n = 6$, except in 30-min-old lesions $n = 18$ and in 1-day-old lesions $n = 24$. The mean values of the lesions of 2 h to 7 days are statistically different from the mean of 30-min-old lesions ($P < 0.001$)

Fig. 2. Peroxidase activity units/g dry weight in experimental incision wounds of different ages from 30 min to 14 days and in the corresponding specimens of the control skin. $n = 6$, except in 30-min-old and 1-day-old wounds where $n = 12$. The mean values of the wounds of 2 h to 7 days are statistically different from the mean of 30-min-old wounds ($P < 0.001$)

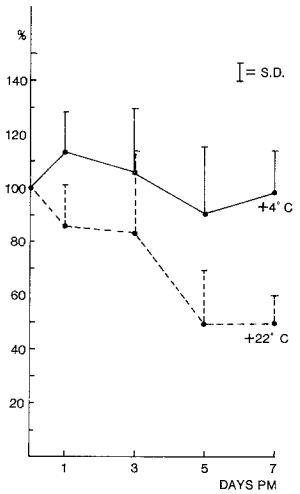

Fig. 3. Peroxidase activity % in 1-day-old vital compression-excoriation lesions when the rats were kept 1–7 days after death at temperature of +4°C and +22°C before the specimens were taken. 100% is the mean value of the 1-day-old vital lesions when the specimens were taken immediately after death

about 70–100 times higher in 1–3-day-old excoriation-compression lesions. In the 5–7-day-old lesions the peroxidase activity began to decline slowly and was at the control level in the 2-week-old lesions (Fig. 1). The peroxidase activity of the lesions made 1 min and 5 min postmortem did not differ from those of the controls.

In the 15-min-old experimental incision wounds the peroxidase activity determined from the edges of the wounds was 0.23 ± 0.12 U/g dry weight, and in 30-min-old incision wounds the mean activity was 0.65 ± 0.37 U/g dry weight. After that, however, the activity increased more rapidly and to higher levels than in the compression-excoriation lesions. For example, in the 4-h-old wounds the mean activity was about 50 times higher as compared with the mean level of the 30-min-old wounds. In the 12-h-old wounds the mean activity was about 200 times higher and in those of 1–5 days it was several hundreds of times higher. In the older incision wounds the activity began to decline slowly. However, the variation of the values was clearly higher in the specimens of the incision wounds than in those of the compression-excoriation lesions (Fig. 2).

The postmortem autolysis at +22°C resulted in a loss of the enzyme activity so that after 3 days about 80% of the activity remained and after 5 and 7 days about 40%. At +4°C, nearly 100% of the activity was present after 3 days of autolysis and about 90% after 5 and 7 days (Fig. 3).

In the few autopsy specimens analyzed the control skin gave nearly similar results as the control skin of the rats. In the lesions with an age under 15 min the activity values were, however, higher than in the experimental excoriations. As vital time increased, the peroxidase activity seemed to also increase in the human excoriations (Table 1).

Discussion

Extraction procedures other than that used in these experiments have given higher values of myeloperoxidase for normal rat skin [7]. Those extraction pro-

Table 1. Peroxidase activity in human traumatic excoriations in 12 autopsy cases. (P.M. = postmortem

Autopsy specimen	Vital time	Autopsy days P.M.	Activity U/g dry wt.	
			Excoriation	Control
1	< 1 min	5	0.16	0.12
2	< 5 min	2	2.28	0.31
3	< 5 min	2	0.45	0.21
4	< 15 min	3	0.99	0
5	< 30 min	6	0	0.08
6 a	35 min	6	0.13	0
6 b	35 min	6	0.55	0.11
6 c	35 min	6	0.27	0
7 a	45 min	3	1.14	0
7 b	45 min	3	0.59	0.39
8	2 h	6	10.27	0
9	23 h	4	74.04	0
10	24 h	4	103.80	0.32
11 a	36 h	3	216.45	0
11 b	36 h	3	168.93	0
12	11 days	2	65.82	0

cedures have, however, been more complicated, containing several sonifications and several freezings and thawings of the homogenate and generally needing high-speed centrifugation to remove the turbidity of the supernatant. The method used in these experiments [11] is a rather simple one and usually gave a clear supernatant from the experimental samples which was useful in the spectrophotometric work. However, in the autopsy specimens turbidity of the supernatants was more common than in the experimental ones.

The peroxidase activity observed in the specimens of blood may be based partly on the leukocyte content of the blood and may represent myeloperoxidase activity. On the other hand, hemoglobin is known to have so-called pseudoperoxidase activity [16] which may cause part of the activity observed in the blood. In the measurements of the blood specimens it was observed that the activity increased somewhat with dilution of the specimens. This may mean that certain hemoglobin concentrations could be inhibitory for the assay reaction [17]. In the 15-min-old compression-excoriation lesions the peroxidase activity was at the control level in the experimental rat material. In the autopsy excoriations in the cases where the time alive after injury had been very short, for example under 15 min slightly higher activity was observed. These autopsy specimens were usually taken from cases of traffic accidents and generally contained more blood than the experimental ones from the rats. The larger amount of blood in the specimens could increase the peroxidase activity significantly. This is important to notice if the values are tried to correlate with the vital time and if the activity level in the specimens is rather low. The autopsy specimens

also produced a turbid supernatant in the extraction more often which could have resulted in more methodologic errors.

In the lesions over the age of 30 min the peroxidase activity started to increase in the compression-excoriation lesions as well as in the wounds. In the incision wounds the increase of activity was more rapid, and higher levels of activity were observed. This difference between the lesions mentioned may have several causes. In the wounds the lesions were deeper and more open than in the excoriations in which the lesions were rather superficial. In open wounds it is easier for bacteria to penetrate deeply which might result in a more rapid purulent inflammation. The variation of the values was also greater in the wounds than in the excoriations, possibly based on a greater variation of infection in the wounds of the different animals. On the other hand, in the wounds only a narrow zone of the edge was taken as a specimen, representing possibly the most inflamed area of the tissue. In the excoriations the whole area of the excoriated skin was taken as a specimen, not only the most infected superficial excoriated zone. The results were expressed per dry weight of the tissue analyzed, and the specimens from the excoriations might have contained more uninflamed tissue than the specimens from the wounds. One reason for the greater variation in the values of the wounds could be that it was difficult to cut zones with equal breadth from the edges of the wounds after scab formation.

In the experimental excoriations and wounds as well as in the few autopsy specimens analyzed, the peroxidase activity increased to levels clearly higher than those observed in the blood. Thus, the high activity in the lesions must be based on reasons other than the presence of blood in the specimens. In the experimental lesions the activity also increased as the vital time increased. Because the acute inflammatory reaction in the traumatic lesions occurs during the same period of time [13, 14] as the increase in activity it can be assumed that most of the peroxidase activity observed in these traumatic lesions was based on myeloperoxidase activity, which is located in the inflammatory cells, mainly in polymorphonuclear leukocytes [4, 18, 19] but also partly in monocytes [20]. Because the acute inflammatory reaction occurs in different types of traumatic lesions in all types of tissues, it can also be assumed that the increase of peroxidase activity is a general phenomenon which might be observed in all types of traumas in different tissues.

The peroxidase enzyme showed rather good resistance to postmortem autolysis in these experiments. This accords with the earlier literature, in which it is mentioned that in extracts in cetyltrimethylammoniumbromide the enzyme was stable for several days even at room temperature [9]. Also, the few autopsy specimens studied in this work showed rather high activity although the autopsies were done several days postmortem.

In the experimental studies the increase of peroxidase activity correlated rather well with the vital time during the first day. In adddition the enzyme was rather resistant to autolysis, and the postmortem lesions did not show any increase in the peroxidase activity. These findings could indicate that peroxidase activity measurements might also be used in forensic medical autopsies to determine the timing of trauma and to evaluate its vitality. To confirm this assumption, however, considerably more autopsy materials are needed than the few

cases shown in this paper. In addition, the enzyme assay values of the autopsy specimens would be necessary to correct for the peroxidase activity which resulted from different amounts of blood in the lesions.

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