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Extractable Iron in Experimental Hematomas

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Summary. The extractable iron and total iron were determined in experimental, subcutaneous hematomas (5 ml blood) of rats after different periods of vital time. The extractable iron was determined from homogenate extract obtained after 24-h incubation with concentrated HCl diluted 1:3 and protein precipitation. For the determination of total iron, the specimens were wet-ashed. The same iron determinations were also made for the corresponding control specimens of the subcutaneous tissue. Extractable iron started to rise over the control values in 2-day-old hematomas, being about 5 times higher after 3 days, about 15 times higher after 7 days, and about 20 times higher after 14 days. The ratio of extractable iron and total iron expressed as percentages was about 1% in 1-h and in 1-day-old hematomas, about 20% in 3-day-old hematomas, about 50% in 7-day-old hematomas, and about 65% in 14-day-old or older hematomas. The effect of autolysis for 3 days at room temperature was studied on 1-h-old hematomas. Extractable iron values were slightly higher in the autolyzed specimens, but the difference was statistically insignificant as compared to specimens taken immediately after death.

Key words: Hematoma, extractable iron – Vital time

Zusammenfassung. Das Extraktionseisen und Gesamteisen wurden in experimentellen, subkutanen Hämatomen (5 ml Blut) bei Ratten nach verschiedenen Vitalzeitperioden bestimmt. Isolierung des Extraktionseisens erfolgte durch Extraktion während 24 h bei Zimmertemperatur mit 1:3 verdünnter Salzsäure und Proteinfällung. Der Gesamteisengehalt wurde aus der mittels Naßeinäscherung behandelten Probe bestimmt. Entsprechende Bestimmungen vom normalen subkutanen Gewebe der Ratte fanden ebenfalls statt. Bei den Hämatomen mit einem Alter von 2 Tagen und darüber überstiegen die Extraktionseisen-Mittelwerte die Werte vom Kontrollgewege, und zwar waren sie bei 3 Tage alten Hämatomen etwa 5mal so hoch, bei 7 Tage alten etwa 15fach und bei 14 Tage alten 20fach. Bei Berechnung mit Abzug der

Werte vom Normalgewebe ergab sich der Anteil des Extraktionseisens am Gesamteisen in den Hämatomen im Mittel etwa in der Größenordnung von 1% in 1 h alten und 1 Tag alten Hämatomen, etwa 20% in 3 Tage alten, etwa 50% in 7 Tage alten und etwa 65% in 14 Tage alten Hämatomen. Untersuchung des Einflusses, den 3tägige Autolyse bei 22°C auf die Eisenwerte von Hämatomen mit dem Alter 1 h hat, erbrachte, daß die Extraktionseisengehalte in den autolysierten Proben ein wenig höher waren, jedoch ohne statistisch signifikante Unterschiede.

Schlüsselwörter: Hämatome, Extraktion von Eisen – Vitalzeitperioden

Introduction

The Berlin blue reaction has been used for a long time in forensic medicine and pathology for the timing of hematomas. Pigments containing iron have been demonstrated in 24–48-h-old hematomas although a positive Berlin blue reaction is observed more regularly in hematomas 4–8 days old [1]. Hamdy et al. 1957 [2] tried to determine "the easily split iron" in cattle tissue bruises aged from 15 min to 9 days by incubating the bruised tissue in 0.4% HCl for 24 h at 37°C and then determining the iron as the ferrous compound of o-phenanthroline. They noticed only a very slight increase, from 0.30 mg iron/100 g tissue in 15-min-old hematomas to 0.57 mg iron/100 g tissue in 5-day-old hematomas.

According to the earlier literature, the iron liberated by the degradation of hemoglobin and stored in phagocytes and tissues as Berlin blue positive pigment is very poorly soluble. Virchow [3] used strong, warm sulfuric acid to remove this pigment from old hematomas. To remove the same pigment from histological tissue sections other authors, such as Lillie [4], Gedigk [5], and Gedigk and Strauss [6], used 5% oxalic acid, 10% sulfuric acid, and 10%–20% hydrochloric acid. The iron in hemoglobin is mentioned as being strongly bound and should not be liberated by acid extraction [7]. Trichloracetic acid precipitation of proteins has been used to determine "the non hemi iron", and then the iron has been analyzed from the extract obtained [8]. However, this is obviously too mild a procedure to dissolve the iron pigment in hematomas.

The present paper describes a simple acid extraction procedure for measuring the iron liberated by the degradation of hemoglobin. The concentration of "the extractable iron" and the ratio of the extractable iron and total iron in hematomas have been correlated with the age of the hematomas.

Material and Methods

The experimental hematomas were produced by injecting 5 ml blood (obtained by heart puncture) s.c. on the medial site of the left pelvic limb of the rats. The whole hematomas were removed as specimens after various periods of vital time from 1 h to 28 days. Hematomas 1 h old were taken as specimens also after 3-day autolysis at room temperature (+22°C). Control specimens of normal subcutaneous tissue were taken from the same place on the medial site of the right pelvic limb of the rats. At least six rats were used for each time period studied.

After the hematomas were minced with scissors in a small amount of distilled water, they were frozen and thawed twice and then thoroughly homogenized using 10 ml distilled water per gram of wet weight of hematomas.

For the determination of the extractable iron, 2 ml of homogenate plus 1 ml of concentrated hydrochloric acid were mixed and left to stand at room temperature for 24 h. Then 1 ml 20% trichloracetic acid – 5% Na-pyrophosphate was added, mixed, and the mixture was left to stand for 10 min at room temperature and then centrifuged for 10 min at the maximum speed of the Sorval table centrifuge with an angle rotor. The supernatant obtained was passed through filter paper and distilled water was added (10 ml). The iron determination was made with Perkin Elmer atomic absorption spectrophotometer using standards in concentrated hydrochloric acid diluted 1:10. The following zero specimens were used: (a) 2 ml aq. dest. + 1 ml concentrated HCl + (after 24 h) 1 ml of 20% TCA – 5% Na-pyrophosphate + aq. dest. ad 10 ml and (b) concentrated HCl diluted 1:10.

For the determination of the total iron content, 1 ml of the homogenate mentioned above was mixed with 3 ml concentrated nitric acid + 1 ml concentrated sulfuric acid + 1 ml concentrated perchloric acid [9]. Then the mixture was allowed to boil at a mild temperature for 3 h in a well ventilated space, although the mixture was not allowed to dry. After cooling, distilled water was added (10 ml). Standards were made in 3 N nitric acid. The following zero specimens in atomic absorption spectrophotometric determination were used: (a) 1 ml aq. dest. + 3 ml concentrated nitric acid + 1 ml concentrated sulfuric acid + 1 ml concentrated perchloric acid, after 3 h boiling aq. dest. ad 10 ml, and (b) 3 N nitric acid.

The dry weight of hematomas and tissues was used as a baseline of the iron concentrations obtained. One milliliter of the homogenate used for iron determinations was put in a dried and weighed glass tube and dried overnight at 120°C. The tube with the dried homogenate was weighed, and the weight of the tube was subtracted.

In addition to the hematomas studied, the corresponding iron determinations were also made for the control tissue specimens.

Statistical analysis was performed using Student's *t*-test.

Results

The mean values of the extractable iron in subcutaneous control specimens varied from 0.014 ± 0.010 to 0.055 ± 0.022 mg/g dry weight. In the 1-h-old and 1-day-old hematomas the extractable iron values were approximately at the control level, although they started to rise above it in 2-day-old hematomas. The mean extractable iron values were about 5 times higher than the controls in 3-day-old hematomas, about 15 times higher in 7-day-old hematomas, and about 20 times higher in 14-day-old or older hematomas. However, the standard deviations (SD) were rather high, especially in the older hematomas, and some irregularities in the curve appeared, as the site of 5-day-old hematomas (Fig. 1).

The mean values of the total iron in subcutaneous control specimens varied from 0.042 ± 0.015 to 0.135 ± 0.051 mg/g dry weight. The mean values of the total iron were approximately at the same level in 1-h-old hematomas and in hematomas 9 days old or older, the mean values being about 1.5–1.6 mg/g dry weight. The variation was rather high in these values, and a temporary decrease was observed in the values from 1 day to 7 days (Fig. 2).

When the total iron content in the whole hematomas was calculated after first subtracting the values for the control tissue from the analysis results for the hematomas, a mean value of 2.610 ± 0.106 mg was obtained for 1-h-old

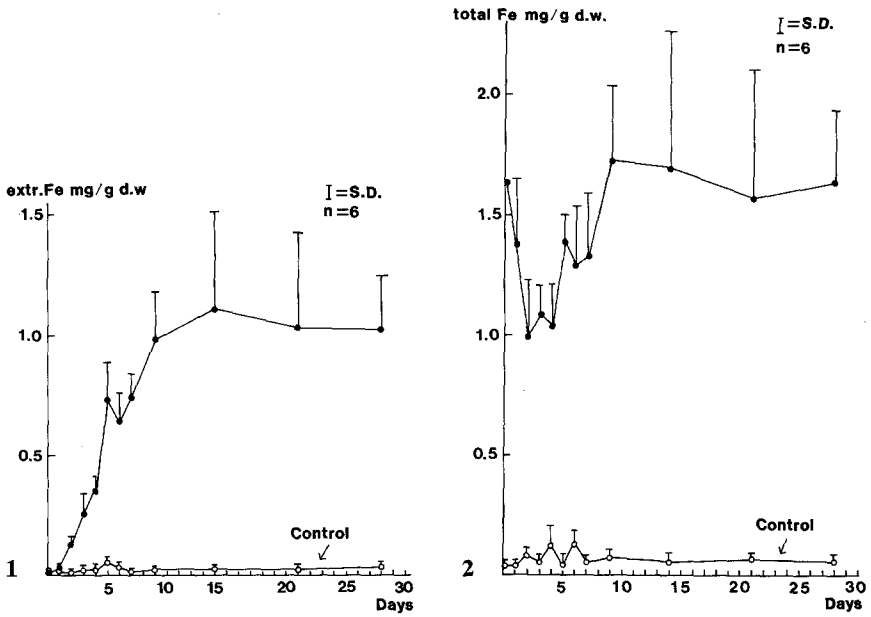

Fig. 1. The extractable iron mg/g dry weight in subcutaneous hematomas of different ages from 1 h to 28 days and in the corresponding specimens of the subcutaneous control tissue. The mean values of the hematomas of 2–28 days are statistically different from those of the controls ($P < 0.001$)

Fig. 2. The total iron mg/g dry weight in the same hematomas and control specimens as in Fig. 1

hematomas. The iron content of each whole hematoma decreasedd over 1 week to about half the 1-h level. After that a slight decrease was observed so that after 28 days about 30% of the starting level of iron was present (Fig. 3).

The corresponding control values were subtracted from the values of the extractable iron and total iron of the hematomas, and the "cleaned" values of the extractable iron were expressed as percentages from the "cleaned" values of the total iron. The mean values of percentages were about 1% in 1-h-old and 1-day-old hematomas, about 20% in 3-day-old hematomas, about 50% in 7-day-old, and about 65% in 14-day-old or older hematomas (Fig. 4).

The mean values of the extractable iron per dry weight or as percentages from the total iron were a little higher in autolyzed, 1-h-old hematomas than in those from which the specimens were taken immediately after death. On the contrary, the mean values of the total iron per dry weight and the total iron in the whole hematoma were a little lower in the autolyzed hematomas. However, these differences were not statistically significant (Table 1).

Discussion

The method for the determination of the extractable iron used in these studies was developed on the basis of some pilot experiments in which the iron extrac-

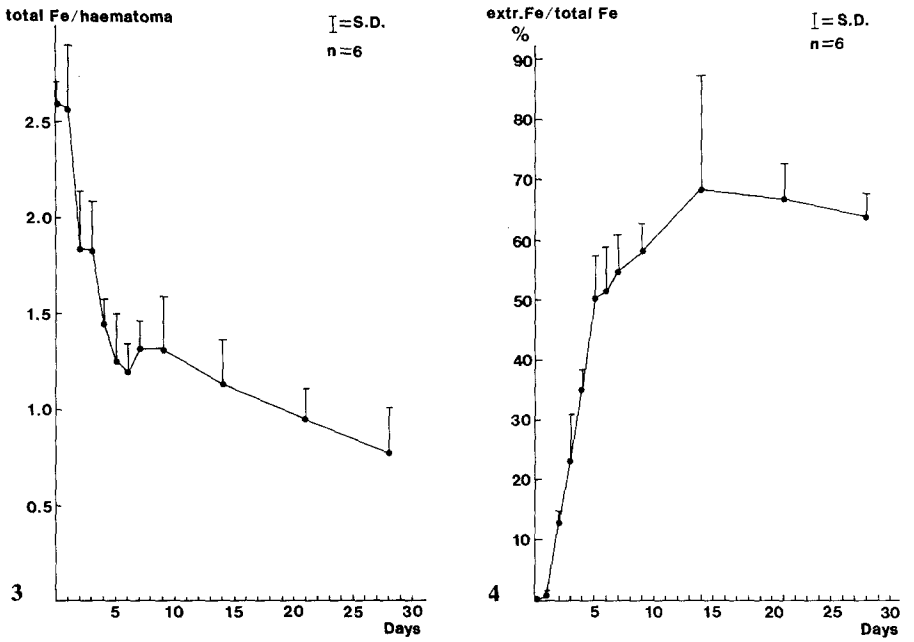

Fig. 3. The total iron content mg of the whole hematomas after the subtraction of the corresponding control values. The hematomas are the same as in Figs. 1 and 2

Fig. 4. The extractable iron expressed as percentages of the total iron in the same hematomas as in Figs. 1–3. Before the calculation of percentages the corresponding control values of the extractable iron and of the total iron have been subtracted from those of the hematomas. The mean values of the hematomas of 2–28 days are statistically different from those of 1 h and 1 day ($P < 0.001$)

Table 1. The effect of autolysis for 3 days (temperature + 22°C) on the iron (Fe) values of 1-h-old hematomas, as compared to the values of 1-h-old hematomas taken as specimens immediately after death. Means and SD are given. The differences are not statistically significant

Time PM (days)	Extractable Fe (mg/g d.w.)	Total Fe (mg/g d.w.)	Total Fe per hematoma (mg)	Extractable Fe/ total Fe (%)	*n*
3	0.030 ± 0.019	1.591 ± 0.212	2.379 ± 0.169	1.015 ± 1.867	10
0	0.005 ± 0.011	1.643 ± 0.355	2.610 ± 0.106	0.175 ± 0.428	6

tion ability of 5% oxalic acid (5 g/100 ml), concentrated sulfuric acid diluted 1:10 and 1:5, and concentrated hydrochloric acid diluted 1:5 and 1:3 were compared using as material 1-h-old hematomas and, on the other hand, hematomas 1 week old and 9 days old. After 24 h of acid digestion at room temperature, protein precipitation was made. In these preliminary experiments, it appeared that the highest concentrations of each acid used resulted in rather similar iron extraction from the older hematomas. The highest iron extraction from 1-h-old hematomas was obtained with 5% oxalic acid. Thus, sulfuric and

hydrochloric acid seemed to result in the better differentiation between the iron extraction values obtained from 1-h-old hematomas and 7–9-day-old hematomas. Since sulfuric acid is known to contain more metallic impurities than hydrochloric acid, the latter was chosen for these studies. Pyrophosphate was used together with trichloracetic acid because of the belief that it formed some soluble complexes with iron and possibly hindered iron from attaching to precipitated proteins [10]. Compared with that from trichloracetic acid only the precipitation from trichloracetic acid plus pyrophosphate gave only a slightly higher iron extraction in acid digested 1-week-old hematomas, but the difference was not significant. Thus, in practice the addition of pyrophosphate to trichloracetic acid may be useless. The trichloracetic acid concentration and the precipitation time of the proteins were the same as recommended by Massey in 1957 [8] for "the non hemi iron" determination from enzymes and other materials.

The extractable iron started to increase in the 2-day-old hematomas. This finding is in accordance with the observations that the first Berlin blut positive macrophages can be seen in 24–48-h-old hematomas [1]. Also the activity of heme oxygenase, which is the rate-limiting enzyme in hemoglobin degradation, has been shown to be increased in 2-day-old hematomas [11]. Since the extractable iron values were very low in 1-h-old hematomas, it seems possible that its increased values in older hematomas represent the iron liberated in connection with the degradation of hemoglobin in phagocytes.

In 2-week-old or older hematomas the extractable iron values were about 65% of the total iron determined by wet ashing. At this time, most of the hemoglobin has already been degraded in hematomas of the size used in these experiments [12]. Thus, the rather large difference between the values of extractable iron and total iron may mean that the method used here cannot extract all the iron stored in tissue after the degradation of hemoglobin in old hematomas. This is in accordance with the earlier literature which mentions that this iron pigment is poorly soluble [4].

The concentration of extractable iron alone cannot be used to estimate the age of the hematomas in forensic medicine because its concentration obviously depends on the amount of hemoglobin present and degraded in tissue. According to these experimental results the ratio of the extractable iron and total iron, expressed as percentages, has a good correlation with the age of hematomas during the 1st week. This ratio also partly solves the problem encountered with the extractable iron alone, because the total iron concentration, minus the control values, is related to the concentration of hemoglobin present before in the tissue. To use this ratio for the timing of hematomas, the concentrations of extractable iron and total iron of the control tissue must be subtracted from the same values of the hematomas to eliminate the ratios resulting from the tissue iron and not derived from the hematoma. In fact, the iron values of hematomas obtained from the chemical analysis are partly from normal tissue and partly from the hemoglobin in the hematoma. However, it is very difficult, if not impossible, to evaluate the amount of normal tissue connected with each hematoma. It has thus been easier to subtract most of the tissue iron by assuming that the whole weight of the hematoma represents control tissue. This error may not be too great if the control tissue values are very low compared to the

value of the hematoma. However, when the hemorrhage in the tissue is very slight and the values of both extractable iron and total iron are nearly the same as in the control tissue, such calculations could give very unreliable results. This is because of the error discussed above and because of the variation and inaccuracy of the determination methods.

The values of the percentage ratio of the extractable iron and total iron presented in this paper naturally are not applicable to the practical work in the autopsies because of the possible differences in hematomas between rats and human beings. Also the changes in the size of the hematomas and their existence in different tissues could result in great changes in the speed of the degradation of hemoglobin. Consequently, there could be a great variation in the concentration of extractable iron and its ratio to total iron. The effects of the autolysis should be studied more thoroughly, although the few experiments performed with young hematomas in this paper seem to indicate that not very much extractable iron is liberated during autolysis for 3 days at room temperature.

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