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EXPERIMENTAL STUDY OF THE EFFECT OF THE ADMINISTRATION OF HYALURONIDASE ON THE HEALING OF FRACTURES

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In recent years interest has centred more and more on connective tissue in the discussion of the callus problem (cf. *K. W. Block*), and the greatest attention has been paid to the collagen fibres and the intercellular substances. As to connective tissue in general, recent research has shown that the intercellular substance and its degree of permeability are of decisive importance in a number of physiological functions and that it plays an essential role in inflammatory processes (*Möller*). As the discovery of a new enzyme, hyaluronidase[1], has provided us with a tool with which it is possible to increase the permeability of connective tissue, I thought it might be of interest to study what effect increased permeability produced by injections of this enzyme might have on the formation of callus. Hyaluronidase is capable of increasing the permeability of the tissue by an enzymatic effect on the hyaluronic acid contained in the inter-cellular substance; this acid, like the chondroitin sulphuric acid contained in cartilage and spongy bone, is an acid muco-polysaccharide (*Pirie*). Hyaludonidase has been observed to cause a certain histological change in the collagen bundles while fibroblasts seem to be unaffected (*Möller*).

THE PROBLEM

It does not sound unreasonable to suppose that the resorption of the necrotic débris accumulated around a fresh fracture might be accelerated by increasing the local permeability owing to the admini-

[1] Hyaluronidase used in the present investigation was placed at my disposal by the courtesy of LEO, Manufacturers of pharmaceutical chemicals.

stration of hyaluronidase, and in this manner influence callus formation, but it is also possible that hyaluronidase might impede the accumulation of an adequate concentration of bone salts necessary for calcification and also affect the intercellular substance and the maturation of the collagen. The purpose of the experiments was to elucidate certain aspects of these problems.

EXPERIMENTS

A piece of bone about 3 mm long was resected from approximately the middle of either fibula of full-grown rabbits. This bone was thought to be very convenient for the study on account of its anatomy, and, secondly, because it is technically readily accessible. Further, as the lower fibula in rabbits fuses with the tibia, no fixation treatment is necessary, and the animals have full use of their limbs immediately after the operation, the fracture being well protected from mechanical and statical strain. Resection was carried out with the aid of a pair of small sharp forceps and, after a little practice, pieces could be removed which were almost completely uniform both with regard to size and site of excision. Three days after the resection 2 I.U. hyaluronidase, dissolved in 2 ml. normal saline, were injected into one leg at the site of the resection. Two ml. of normal saline were injected in a similar manner into the site of resection in the other leg which was used as a control. Injections were given daily for one week, i.e. 3rd to 9th day. The reason why no injections were given until the 3rd day was that it was desired to obviate infection via puncture holes; according to *Hechter, Dopkeen* a. *Yudell,* this is liable to occur if injections are given during the first two days i.e. before a wall of fibrinous tissue has had time to form.

After the tenth day healing of the resection was followed-up roentgenographically at intervals of 7 days. The results are given in table I. In order to form an idea as to whether and to what degree the injected saline had influenced healing, another series (8 animals) was subjected to bilateral resection, and local injections of 2 ml. saline were given unilaterally for a week, beginning the 3rd day after operation, in the same manner as with the control side in the series just described. The other side received no injections and thus served as a control. The animals were followed-up roentgenographically in the same manner as the others.

Histological examination and particularly chemical analysis of callus requires a quantity of tissue larger than that obtainable from the small specimens removed from the fibula. These examinations were

therefore complemented by 5-6 mm. specimens resected from the middle of the diaphysis of the radii of 9 other rabbits. In these series the specimens were likewise removed from both sides, hyaluronidase being injected into one leg as before, the other serving as a control. The animals were killed 6, 9, 12, and 15 days after the operation. The callus found in the area of the resection in four animals was then carefully removed and analysed chemically for calcium and phosphorus in the following manner. The specimen was subjected to a temperature of 450° C. in an electric oven, the ash dissolved in concentrated hydrochloric acid and the calcium content determined by means of a photometer, while the phosphorus content was assessed by the *Bolz-Mellon* method using sodium molybdate and hydrazine sulphate[1]. The results of these analyses are given in table II.

The specimens removed from the other five animals in this series were examined histologically. After decalcification in the usual manner the material removed was embedded in paraffin and sectioned, slices being cut both from the outer and from the middle parts of the callus. The sections, which were 10 μ thick, were stained with chromotrope and hemotoxylin or by the collagen staining method of *Hansen*. The results of these examinations will now be described.

RESULTS

1. It will be apparent from table I, which shows the course of healing as seen in the roentgenogram, that in all cases except two (Nrs. 9 and 14) the formation of bony callus was evidently slower in the leg treated with hyaluronidase than in the other leg, the control leg treated with saline. In most cases bony callus was seen two weeks earlier in the control side than in the other; in three cases (Nrs. 2, 5 and 11) no signs of healing could be detected in the roentgenogram during the experiments, and in these cases the fragmented ends seemed to be without reaction. Except for the slow rate of healing no roentgenological difference was observed between the callus in the side treated with hyaluronidase and the control side.

In an endeavour to find out whether and to what extent injections of normal saline had influenced the healing of the fracture, both fibulas of the rabbits were resected,—as already mentioned—and one side treated with saline and the other left untreated. In four of these animals no difference could be observed between the two sides, in

[1] The chemical analysis was carried out by Mjölkcentralens forskningslaboratorium to whom I feel indebted.

TABLE I

Roentgenologically visualizable development of callus in the leg treated locally with hyaluronidase and in the other leg treated locally with normal saline.

Animal Nr.	10 days		17 days		24 days		31 days		
	Hyaluronidase	contr.	Hyaluronidase	contr.	Hyaluronidase	contr.	Hyaluronidase	contr.	
1	0	0	0	0	0	+	?	+++	
2	0	0	0	+	0	+	0	+	No reaction in side treated with hyaluronidase.
3	0	0	0	+	0	++	?	+++	Slightly increased decalcification in the side treated with hyaluronidase.
4	0	0	0	+	+	++	++	+++	
5	0	0	0	+	0	++	0	+++	No reaction in side treated with hyaluronidase.
6	0	0	0	++	+	+++	+	+++	
9	0	0	0	0	++	++	+++	+++	
10	0	0	0	+	0	+++	+	+++	
11	0	0	0	0	0	+	0	++	No reaction in side treated with hyaluronidase.
13	0	0	0	0	?	++	+	+++	
14	0	0	++	+	+++	++	+++	+++	

three the leg treated with saline healed somewhat quicker, and in one case the reverse occurred.

2. Results of the chemical analysis for calcium and phosphorus are given in table II and show that the ash content, the calcium content and the phosphorus content of the callus in the leg treated with hyaluronidase were conspicuously low up to the 9th day, i.e. as long as the hyaluronidas injections were given. But on the 12th day no such difference could be observed. The assessment of the results will be discussed on page 10.

3. Histological examination showed a fairly well developed connective tissue callus with initial formation of collagen on the 6th day. Material removed on the 9th, 12th and 15th days showed progressive formation of normal callus rich in collagen fibres and enchondral ossification. No definite difference could be demonstrated between the callus of the side treated with hyaluronidase and the control side. Nor could any signs of local vascular injury or tissue edema be detected.

DISCUSSION

The experiments show that local injections of hyaluronidase in the operative defects of the fibula in rabbits retard the formation of osseous callus. The cause of this retardation may feasibly be attributed to various factors such as the pH of the hyaluronidas solution, nutritional disturbances, injury to the collagen fibres and intercellular substance, increased diffusion owing to enhanced permeability of the tissues and removal of the salts necessary for the precipitation of the calcium apatite, disturbed activity of the phosphatase or other enzymes. That local pH changes may impair the formation of callus is generally accepted (*K. W. Block, Häbler, Sterling & Stewart, Swenson & Claff*) and some researches (e.g. *Gradojevic, Kubányi, Selvaggi*) have produced varying effects on the callus formation in animals by means of alkaline or acidotic agents. In the present experiments, however, the pH level could not be held responsible for slow formation of callus because the pH level depends solely upon the solvent, which in this case was normal saline.

Some authors such as *Ridgon* a. *Hechter* claim that the injection of impure hyaluronidase may be followed by injury to the capillaries. The degree of purity of the enzyme used here was very high and the solutions were of low concentration, besides which no evidence of vascular injury or edema could be observed histologically.

The assumption that the hyaluronidas injections caused changes in the intercellular substance could not be confirmed by the experiments

from the means available to the writer at the time of the investigation. *Möller* claims that the collagen bundles may be acted upon by hyaluronidase and that as these bundles play an essential part in calcification,—as small apatitic crystals forming in a regular manner around the bundles (*Block, Henschen* and others)—it does not sound unreasonable to suppose imperfect maturity of the collagen (cf. *Hertz*) to be the cause of such changes in the intercellular substance. However, in the present investigation the histology of the collagen was found to be normal and to differ in no respect from the "control" side.

The results of the chemical analyses (table II) show that in rabbits Nrs. 22 and 25 the callus on the side treated with hyaluronidase was much less than that on the control side, the ash content being 6.5 per cent and 3.5 per cent of the total dry content as compared with 16.9 per cent and 17.9 per cent respectively on the control side. In the cases treated with hyaluronidase the callus was thus poorer in salts during the period daily injections were given i.e. until the 10th day. This difference will also be apparent on direct comparison of the side treated with hyaluronidase and the control side as regards calcium content and phosphorus content calculated as a percentage of the dry content. This shows that the calcium content was more than ten times greater, and the phosphorus content two-three times greater in the control side than in the side treated with hyaluronidase. The Ca:P ratio which, according to *Annersten,* is low in the initial stage of callus formation and usually shows an increase up to about 1.6 on the loth day, was relatively low (0.41 and 0.71) as compared with the figures of the control side (1.91 and 1.83): this also suggests a slight retardation of the ossification. Three days after discontinuation of the daily injections of hyaluronidase the percentage of bone salts had already increased, so that there was then no longer any difference between the two sides (see table II).

The results of the above analyses suggest that local administration of hyaluronidase impairs the deposition of bone salts in callus tissue. At this point it should be remembered that the histological examination showed that the connective-tissue callus developed normally, and it should perhaps also be mentioned that *Levander* and *Urist* found that the development and differentiation of connective-tissue callus do not require the presence of bone salts. Whether the lack of calcium and phosphorus salts observed in the present investigation is due to direct prevention of a local accumulation of salts by a removal or diffusion of the salts, for example, through the tissue whose permeability is enhanced by the hyaluronidase: whether hyaluronidase directly inhibits fixation of the bone salts by the cells, or whether opti-

TABLE II

Calcium, phosphorus and ash content of soft tissue callus from the side treated with hyaluronidase and from the control side.

Animal Nr.		Duration of experiment in days	Dry content (dc) in mg.	Ash		Calcium		Phosphorus		Ca : P
				mg.	% of dc	mg.	% of dc	mg.	% of dc	
22	Hyaluronidase	6	97.5	6.3	6.5	0.38	0.39	0.92	0.94	0.41
	Control		71.6	12.1	16.9	3.21	4.48	1.68	2.35	1.91
25	Hyaluronidase	9	36.8	1.3	3.5	0.17	0.46	0.24	0.65	0.71
	Control		27.9	5.0	17.9	1.59	5.70	0.87	3.12	1.83
26	Hyaluronidase	12	45.8	7.3	15.9	2.05	4.48	1.33	2.90	1.54
	Control		58.4	9.8	16.8	2.65	4.54	1.55	2.65	1.71
28	Hyaluronidase	15	34.6	5.2	15.0	1.60	4.62	0.99	2.86	1.62
	Control		20.0	3.1	15.5	1.28	6.40	0.73	3.65	1.75

mal conditions for precipitation and phosphatase activity are changed, are questions that require further study.

The examination thus showed that local injections of hyaluronidase into the site of operatively produced defects of the fibula in rabbits retard bone repair and that this retardation is probably due to the enzyme that decreases the accumulation of bone salts in the callus tissue.

SUMMARY

A small piece of the fibula was resected from either leg of a series of rabbits. The site of resection of one side was then treated locally every third day with an injection of 2 I.U. hyaluronidase. The leg of the other side was used as a control. The leg treated with hyaluronidase was found to heal more slowly than the other leg. Chemical analysis also revealed a lower concentration of bone salts in the callus tissue treated with hyaluronidase. Histological examination of the soft tissue callus presented a normal picture.

RESUME

L'auteur a pratiqué une résection d'une partie des deux fibulae chez des lapins. A partier du 3ème jour et durant une semaine, il a injecté 2 R de hyaluronide à l'endroit de la résection. L'autre patte a servi de contrôle. Il a obtenu une ossification consolidée plus longue

dans le côté traité par l'hyaluronide. L'analyse chimique a démontré une diminution de la teneur en substances minérales dans le tissu du cal. L'essai a été complété par un examen histologique des parties molles de la formation calleuse, mais celui-ci n'a pas permis de constater de divergence avec la formation calleuse normale.

ZUSAMMENFASSUNG

Der Verfasser hat ein Stück jeder Fibula von Kaninchen reseziert und vom dritten Tage nach der Operation eine Woche hindurch 2 R Hyaluronidase in die Resektionsstelle injiziert. Gleichzeitig wurde die andre Seite zur Kontrolle verwendet. Als Resultat erhielt er eine verlangsamte Ossifikation auf der mit Hyaluronidase behandelten Seite. Durch chemische Analyse konnte man auch eine Verminderung von Knochensalzen in dem mit Hyaluronidase behandelten Kallusgewebe feststellen. Der Versuch wurde ausserdem vervolständigt durch eine histologische Untersuchung des Weichteilkallus, wobei es jedoch nicht möglich war Abweichungen von normalen Kallusbild nachzuweisen.

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