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EFFECT OF GLUCOSE-1-PHOSPHATE ON CALLUS FORMATION

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The mechanism of calcification, and within it the origin and role of phosphates, have been studied extensively. The demonstration of the presence of glycogen in cartilage was a most important point in the elucidation of the mechanism [23, 10, 13]. The correlation between the presence of glycogen and the onset of calcification has been pointed out by HARRIS and others[27, 19, 25, 5]. The role of glycogen in calcification has been confirmed by the detection of the enzymes phophatase [26] and phosphorylase [16] in cartilage. The biochemical evidence was corroborated by histological studies demonstrating the presence of glycogen, phosphatase and phosphorylase and their correlation [12, 24, 15, 9, 18]. According to HARRIS, the phosphate esters formed in the course of glycogen breakdown serve as the substrate for phosphatase. The results obtained by ALBAUM et al. [1], also point in this sense. These authors have namely demonstrated several intermediates of glycolysis in cartilage and could accelerate calcification *in vitro* by the addition of glucose-1-phosphate (G—1—P) [21]. More recently, CARTIER and PICARD [3, 4, 5, 6, 7, 8] in their series of experiments have arrived at the conclusion that ATP is the main phosphate-donor. In contrast with this, DI STEFANO and NEUMAN [11] found ATP to inhibit calcification.

In the light of the above evidence we investigated *in vivo* the effect of the single phosphate esters on calcification. In the present paper the results obtained for glucose-1-phosphate (G—1—P) are discussed.

Materials and methods

A total of 36 albino rats, weighing about 100 g each, was used. Under ether anaesthesia the left femur was fractured and the fracture ends were fixed by means of a marrow nail. One group received no treatment after fracture, the other group was injected 0.5 ml of a 3 per cent G—1—P solution intramuscularly, above the site of the fracture, every day, from the day following operation until sacrifice. The G—1—P used was a potassium salt, prepared enzymatically, with a pH of 7.0. Three animals of each group were sacrificed 5, 8, 11, 14, 21 and 28 days after operation. The femurs were studied histologically (haematoxylin-eosin, Azan) and histochemically (toluidine blue, Hale, Hotchkiss and Ritter-Oleson). It has been found that callus formation was not absolutely identical in the 3 members of the same group. For this reason, average values were used in the comparative analyses.

Histological results

5-day control

Between the fracture ends a haematoma showing some organisation can be seen. Periosteally there are signs of increased activity. Subperiosteally, there is an islet of cartilage composed of a few cartilage cells. Between the fracture ends and periosteally, granulation tissue callus has appeared (Fig. 1).

On staining with toluidine blue there is slight metachromasia in the areas of the cartilage islets.

The connective tissue callus gives a mildly positive reaction to the Hale, Hotchkiss and Ritter-Oleson tests. Around the periosteal cartilage cells the ground substance, as well as the nuclei of cells, are Hotchkiss-positive.

5 days of treatment with G—1—P

Between the fracture ends there is an organised haematoma. Strong periosteal activity, and subperiosteally a big connective tissue callus of loose structure extending toward the fracture ends can be seen. Under the periosteum, islets of cartilage of various size are present (Fig. 2).

On staining with toluidine blue the periosteal islets of cartilage show marked metachromasia.

The connective tissue callus gives a positive reaction in the Hale, Hotchkiss and Ritter-Oleson tests. Around the periosteal cartilage cells it is mainly Hotchkiss' test which is positive.

8-day control

Between the fracture ends a strong granulation tissue callus is present, containing many islets of cartilage. Increased periosteal activity and periosteal cartilage formation are visible.

On staining with toluidine blue the islets of cartilage show increasing metachromasia.

Hale, Hotchkiss, Ritter-Oleson : Around the periosteal cartilage cells there is Hale and Hotchkiss positivity.

8 days of treatment with G—1—P

There is a preponderantly cartilaginous callus between the fracture ends, the islets of cartilage are separated from one another by granulation tissue. Intense periosteal activity with abundant cartilage formation can be seen.

On toluidine blue staining, both the cartilage between the fracture ends and the periosteal cartilage show marked metachromasia.

Hale, Hotchkiss, Ritter-Oleson : The cartilaginous ground substance in the callus exhibits Hale positivity.

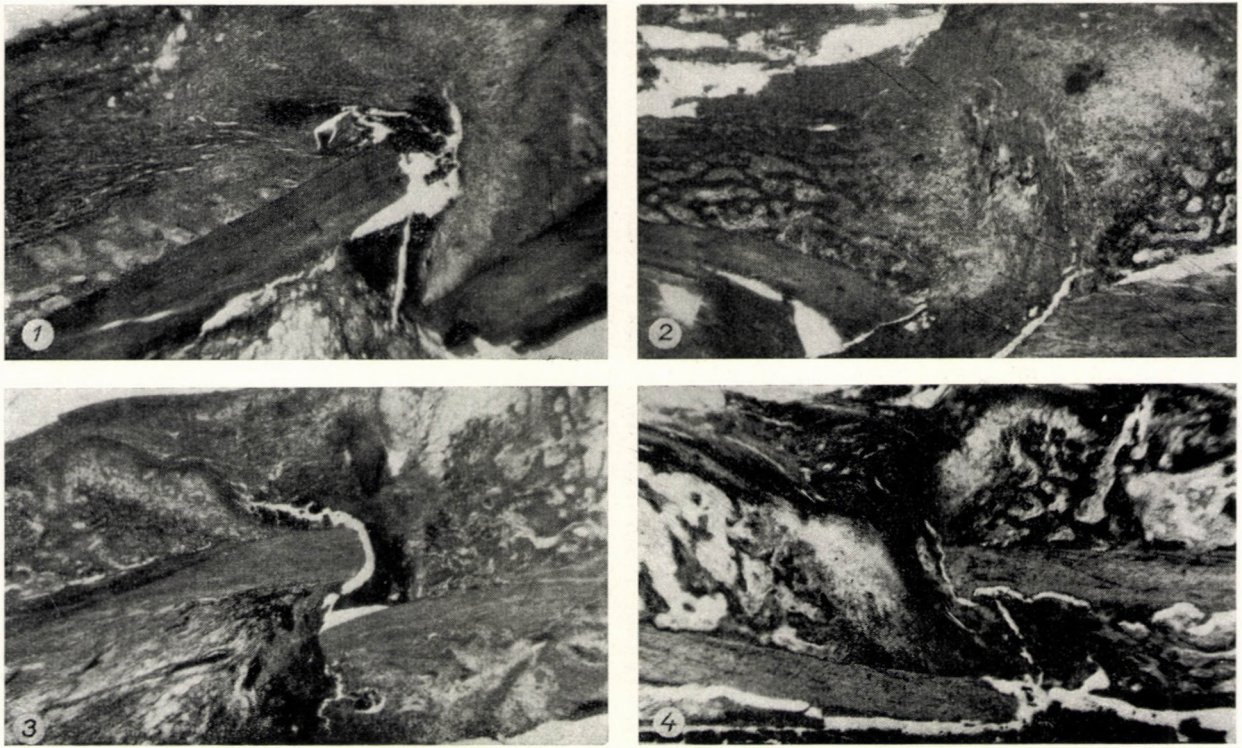

Fig. 1. 5-day control. Between the fracture ends a haematoma and signs of increased periosteal activity. Azan stain. Magnification, ×45. *Fig. 2.* 5 days of treatment with G—1—P. Increased periosteal activity, smaller and bigger islets of cartilage subperiosteally. Azan stain. Magnification, ×45. *Fig. 3.* 11-day control. Intensive periosteal activity, extensive periosteal cartilage formation extending toward the fracture ends. Azan stain. Magnification, ×45. *Fig. 4.* 11 day of treatment with G—1—P. Periosteally and between the fracture ends extensive periosteal cartilage is visible. Azan stain. Magnification, ×45

11-day control

Extensive islets of cartilage can be seen between the fracture ends, and among them a richly capillarized granulation tissue callus. Periosteal cartilage formation is excessive (Fig. 3).

On toluidine blue staining, the islets of cartilage between the fracture ends and the periosteal cartilage show strong metachromasia.

Hale, Hotchkiss, Ritter-Oleson : The islets of cartilage between the fracture ends give a mixed reaction. In the periosteal islets of cartilage two zones can be differentiated, a Hale positive outer one and a Hotchkiss positive inner one.

11 days of treatment with G—1—P

The area between the fracture ends is occupied by extensive islets of cartilage. There is abundant cartilage periosteally. Spongious bone is being formed at the site of the cartilagineous trabecules, from the marginal periosteum (Fig. 4).

The cartilage shows metachromasia on staining with toluidine blue.

Hale, Hotchkiss, Ritter-Oleson : The cartilage between the fracture ends shows a mixed reaction. Periosteally, two zones are visible, an outer Hotchkiss positive one and an inner, Hale positive one.

14-day control

The whole area between the fracture ends is filled by cartilage. Periosteal activity is decreasing, in the area between the periosteum and the compact layer an increasing amount of cartilage is replaced by bony trabecules similar to spongious bone (Fig. 5). A uniform disc of cartilage extends from the fracture line to the zone of increased periosteal activity.

The cartilage in the fracture line is strongly metachromatic on staining with toluidine blue.

Hale, Hotchkiss, Ritter-Oleson : There is no substantial difference from the 11-day specimen, except that in proportion to the rate of appearance of bony trabecules the extent of the reactive area decreases.

14 days of treatment with G—1—P

Cartilage is present between the fracture ends. The amount of periosteal cartilage is less, newly formed bony trabecules are visible between the periosteal cartilage and between the fracture ends. Hypertrophic osteoblasts can be found on the guiding cartilaginous trabecules, at the junction of the cartilage and the newly formed bony trabecules. The osteoblasts can be recognized by their big, light cytoplasm and marked nuclei (Fig. 6).

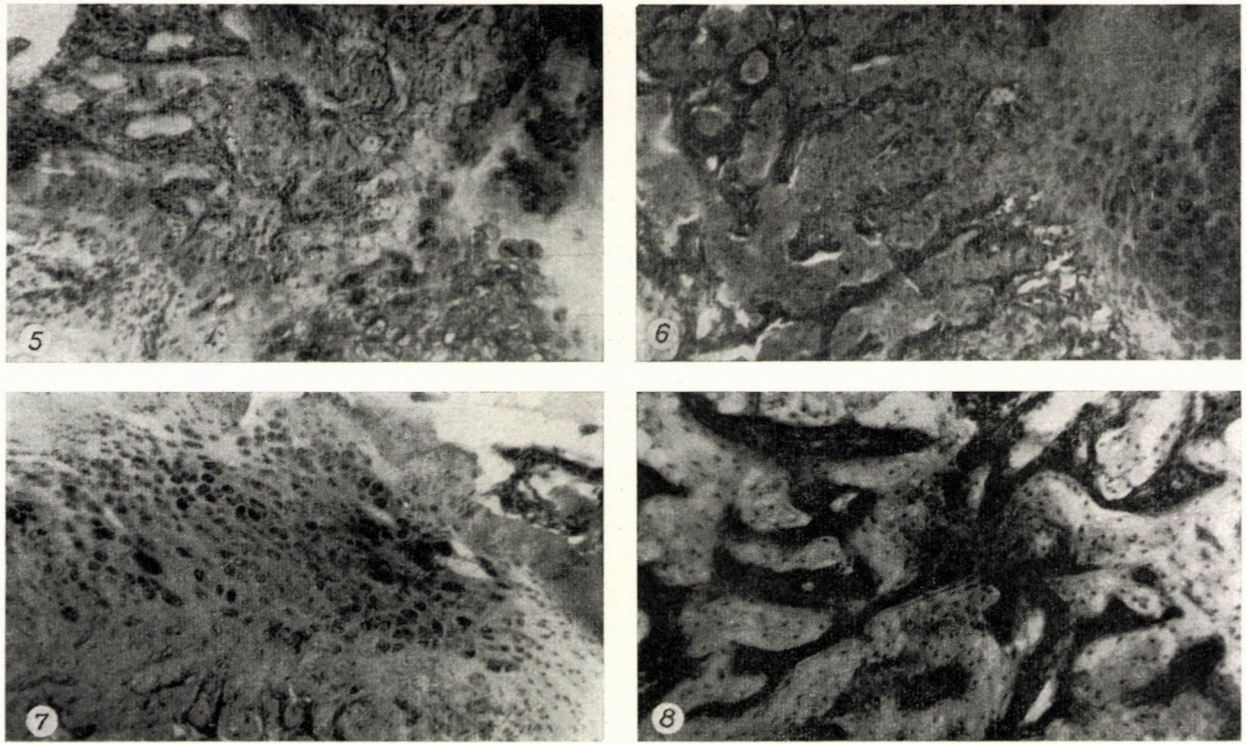

Fig. 5. 14-day control. At the cartilage-bone junction in the callus numerous dilated capillaries, young bony trabecules with occasional osteoblasts are seen. Haematoxylin-eosin stain. Magnification, ×150. *Fig. 6.* 14 days of treatment with G—1—P. In the callus, at the cartilage-bone junction elongated guiding trabecules with large numbers of hypertrophic osteoblasts. Haematoxylin-eosin stain. Magnification, ×150. *Fig. 7.* 28-day control. At the site of the callus, in the centre, the remaining islets of cartilage are still visible and around them occasional bony trabecules. Haematoxylin-eosin stain. Magnification, ×150. *Fig. 8.* 28 days of treatment with G—1—P. The callus is replaced throughout by newly formed bony trabecules. Haematoxylin-eosin stain. Magnification, ×150

The remaining cartilage shows very strong metachromasia on toluidine blue staining.

In the Hale, Hotchkiss and Ritter-Oleson tests, Hotchkiss positivity dominates in the callus, without, however, there being a significant difference between specimen and corresponding control.

21-day control

The area between the fracture ends is filled by cartilage. The periosteal cartilage is reduced in size, newly formed bony trabecules grow from the periosteum towards the fracture ends, extending also in between the latter. Osteoblasts appear along the guiding trabecules.

The remaining cartilage shows strong metachromasia with toluidine blue.

Hale, Hotchkiss, Ritter-Oleson : the Hotchkiss reaction preponderates in the callus.

21 days of treatment with G—1—P

In the area between the two fracture ends the amount of cartilage is markedly reduced while that of newly formed bony trabecules increases. These take the place of the periostal cartilage. Great numbers of hypertrophic osteoblasts are still found along the guiding trabecules.

The cartilage shows marked metachromasia on toluidine blue staining.

Hale, Hotchkiss, Ritter-Oleson : Hotchkiss positivity preponderates in the callus.

28-day control

A very small amount of cartilage has been left between the fracture ends, the fracture is almost completely repaired by newly formed bone (Fig. 7).

Hale, Hotchkiss, Ritter-Oleson : The picture is almost the same as after 21 days.

28 days of treatment with G—1—P

The healing of the fracture is nearly complete. The adjacent areas of the old compacta show resorptive phenomena, the site of the fracture is filled by spongious bone (Fig. 8). The newly formed bony trabecules in the periosteum are under resorption.

Hale, Hotchkiss, Ritter-Oleson : the result does differ significantly either from the control, or from the 21-day one.

Discussion

The results outlined above suggest that administration of G—1—P enhances fracture healing. In the treated animals the islets of cartilage appear sooner between the fracture ends, the increased periosteal activity ceases faster and, as a result, the processes of differentiation, consolidation and healing proceed at a quicker rate. The experimental evidence indicates that G—1—P accelerates formation of the cartilaginous callus and its ossification. In the animals treated with G—1—P, hyperthrophic osteoblasts appear along the guiding trabecules in 14 days already.

The effect on the ground substance is difficult to evaluate. There were differences in the results of the Hale and Hotschkiss reactions between the G—1—P treated animals and the controls ; the difference was most marked at 11 days. It is known that COBB believes that Hotchkiss positive granules to be glycogen. HELLER—STEINBERG arrived at the same conclusion, because the Hotchkiss reaction turns negative after digestion with saliva. If these assumption are accepted, our results would suggest that glycogen formation is increased by treatment with G—1—P, confirming the histological evidence obtained. It should be pointed out, however, that the differences in the results of the Hotchkiss, Hale and Ritter-Oleson tests between the G—1—P treated animals and the controls were not so marked during the course of healing as to allow unequivocal conclusions. The less so, as the biochemical processes involved have not been fully elucidated and the Hotchkiss reagent is known to react with different compounds (polysaccharides, glycoproteins).

It may be also asked why it was necessary to use marrow-nailing? The difficulties involved (BÖHLER, MATZEN), for example that the size of the nail distinctly influences callus formation, etc., are well-known. Still, we have chosen this method in view of the following. (I) The fracture ends could thus be held in place. This was controlled by rays and only the animals showing satisfactory reposition were used. In this way standard conditions could be ensured in the experiments. A slight degree of dislocation still resulted, but much less than without the use of nailing. (II) The limb was forced to function, a significant factor in healing (KROMPECHER). On the other hand, this gave rise to periosteal callus formation ; it is known that when the marrow nail injures the endosteum, periosteal callus formation predominates (MCLEAN, URIST).

Finally, we wish to emphasize that the present results do not yet prove the specific action of G—1—P. It could not be decided whether G—1—P exerted a specific effect or its role in enhancing fracture healing was only that of a general phosphate donor. It appears, however, certain that G—1—P has a favourable influence on the process of healing. The mechanism of this influence will be investigated in experiments involving the use of different phosphate esters.

Summary

Healing of fractures has been investigated in albino rats, comparing untreated controls with animals treated with 0,5 ml of a 3 per cent glucose—1—phosphate solution intramuscularly. The animals were examined 5, 8, 11, 14, 21 and 28 days after inducing the fracture and marrow nailing. The evaluation was based on histological (haematoxylin-eosin, Azan) and histochemical (toluidine-blue, Hale, Hotchkiss, Rittel—Oleson) studies of the femurs of the experimental animals.

It has been found that G—1—P seems to accelerate the healing of fractures. This conclusion was based on the following.

(I) In the animals treated with G—1—P the phase of increased periosteal activity and periosteal cartilage formation developed earlier (in 5 days) than in the controls.

(II) In the animals treated with G—1—P the periosteal cartilage was more extensive and the cartilaginous callus developed in 6 to 14 days, as compared to 14 to 21 days in the controls.

(III) In the animals treated with G—1—P increased periosteal activity ceased earlier and new bone formation, both periosteally and between the fracture ends, began after 14 to 21 days, as compared to 21 to 28 days in the controls.

During that period hypertrophic osteoblasts appeared along the guiding trabecules.

(IV) In the animals treated with G—1—P, consolidation was complete in 21 to 28 days, while in the controls reparation resulted only after 28 days.

(V) No unequivocal interpretation can be given of certain differences in histochemical reaction.

(VI) Investigations are in progress to elucidate whether G—1—P acts on the process of healing as a specific factor or only as a general phosphate-donor.

REFERENCES

1. Albaum, H. G., Hirschfeld, A., Sobel, A. E.: (1952) Calcification VIII. Glycolytic Enzymes and Phosphorylated Intermediates in Preosseous Cartilage. Proc. Soc. Exper. Biol. *79*, 682. — 2. Böhler, L.: (1944) Die Technik der Knochenbruchbehandlung im Frieden und im Kriege. Maudrich, Wien. — 3. Cartier, P., Picard, J.: (1955) La minéralisation du cartilage ossifiable. I. La minéralisation du cartilage in vitro. Bull. Soc. Chim. Biol. *37*, 485. — 4. Cartier, P., Picard, J.: (1955) La minéralisation du cartilage ossifiable. II. Le système atpasique du cartilage. Bull. Soc. Chim. Biol. *37*, 661. — 5. Cartier, P., Picard, J.: (1955) La minéralisation du cartilage ossifiable III. Le mécanisme de la réaction atpasique du cartilage. Bull. Soc. Chim. Biol. *37*, 1159. — 6. Cartier, P., Picard, J.: (1955) La minéralisation du cartilage ossifiable. IV. La signification de la réaction atpasique. Bull. Soc. Chim. Biol. *37*, 1169. — 7. Picard, J., Cartier, P.: (1956) La minéralisation du cartilage ossifiable. V. Glycolyse et glycogénolyse du cartilage. Bull. Soc. Chim. Biol. *38*, 697. — 8. Picard, J., Cartier, P.: (1956) La minéralisation du cartilage ossifiable. VI. Influence des phosphodérivés du catabolisme glucidique sur le métabolisme et la minéralisation du cartilage. Bull. Soc. Chim. Biol. *38*, 707. — 9. Cobb, J. D.: (1953) Relation of Glycogen, Phosphorylase and Ground Substance to Calcification of Bone. Arch. Path. *55*, 496. — 10. Creighton, C.: cit. Glock. — 11. Di Stefano, V., Neumann, W. F.: (1953) Calcium Complexes of Adenosinetriphosphate and Adenosinediphosphate and their Significance in Calcification in Vitro. J. Biol. Chem. *220*, 759. — 12. Follis, R. H., Jr., Berthrong, M.: (1949) Histochemical Studies on Cartilage and Bone. I. The Normal Pattern. John Hopkins Hosp. Bull. *85*, 281. — 13. Gendre, H.: cit. Glock. — 14. Glock, G. E.: (1940) Glycogen and Calcification. J. Physiol. *98*, 1. — 15. Gomori, G.: (1943) Calcification and Phosphatase. Amer. J. Path. *19*, 197. — 16. Gutman, A. B., Gutman, E. B.: (1941) Phosphorylase in Calcifying Cartilage. Proc. Soc. Exper. Biol. *48*, 687. — 17. Harris, H. A.: (1932) Glycogen in Cartilage. Nature *130*, 996. — 18. Heller—Steinberg, M.: (1951) Ground Substance, Bone Salts and Cellular Activity in Bone Formation and Destruction. Amer. J. Anat. *89*, 347. — 19. Hoffmann, A., Lehman, G., Wertheimer, R.: (1928) Der Glykogenbestand des Knorpels und seine Bedeutung. Pflügers Arch. *220*, 183. — 20. Krompecher, St.: (1937) Die Knochenbildung. Fischer, Jena. — 21. Marks, P. A., Shorr, E.: (1950) A Method for Evaluating the Relation of Glycogen to Inorganic Salt Deposition in Surviving Cartilage Slices in Vitro. Science. *112*, 752. — 22. Matzen, P. F.: (1955) Lässt sich der physiologische Heilablauf des Knochenbruchs beschleunigen? Wiss. Z. Univ. Halle Math. Nat. *4*, 1111. — 23. Neumann, E.: cit. Glock. — 24. Pritchard, J. J.: (1952) A Cytological and Histochemical Study of Bone and Cartilage Formation in the Rat. J. Anat. *86*, 259. — 25. Robison, R.: (1923) The Possible Significance of Hexosephosphoric Esters in Ossification. Biochem. J. *17*, 286. — 26. Robison, R., Rosenheim, A.: (1934) Calcification of Hypertrophic Cartilage in Vitro. Biochem. J. *28*, 684. — 27. Sundberg, C.: (1924) Das Glykogen in menschlichen Embryonen von 15, 27 und 40 mm. Z. Anat. *73*, 168.

ДЕЙСТВИЕ ГЛЮКОЗЫ-1-ФОСФАТА НА ОБРАЗОВАНИЕ КОСТНОЙ МОЗОЛИ

И. ФЕЛДЕШ, Л. МЕСАРОШ и ДЬ. БОТ

Авторы проводили сравнение заживления переломов у белых крыс, леченных внутримышечным введением 0,5 см³ 3% глюкозы-1-фосфата (Г-1-Ф), как и у необработанных крыс. Они исследовали животных на 5, 8, 11, 14, 21 и 28 день после экспериментального перелома и после фиксацией отлома вбиванием гвоздей в мозговую полость кости. Оценка проводилась на бедренной кости животных на основании гистологической (гематоксилин-эозин, азан) и гистохимической (толуидиновая синька Геле, Хотшкисс, Риттер—Олесон) обработки. Оценка результатов экспериментального материала, повидимому, говорит за то, что лечение введением Г-1-Ф ускоряет заживление переломов. Это установление авторы сделали после оценки различных фаз на основании следующих наблюдений :

1. У животных, обработанных Г-1-Ф, стадия периостального возбуждения и периостально образующийся хрящ проявляются раньше чем у контрольных животных (уже на 5 день).

2. Наблюдается также и большое расширение периостального хряща, и раннее образование хрящевой мозоли у животных, обработанных Г-1-Ф (6—14 дней, а у контролей 14—21 день).

3. У животных, обработанных Г-1-Ф стадия периостального возбуждения прекращается раньше, а новообразование кости также происходит скорее, как периостально, так и между концами отломков (14—21 день. У контролей на 21—28 день). За это время проявляются вдоль направляющих шин гипертрофические остеобласты.

4. Процесс заживления, консолидация заканчивается раньше у животных, обработанных Г-1-Ф (21—28 дней). У контрольных животных после 28 дня.

5. Проявляющиеся в гистохимических реакциях отклонения авторы покамест еще не могут однозначно объяснить.

6. Проводимые в настоящее время дальнейшие исследования имеют целью выяснить вопрос : является ли данное действие Г-1-Ф специфическим, или же следует его оценивать как общее фосфато-донорное воздействие.

WIRKUNG VON GLUKOSE—1—PHOSPHAT AUF DIE KALLUSBILDUNG

I. FÖLDES, L. MÉSZÁROS und GY. BOT

Die Heilung von Knochenbrüchen wurde bei mit 0,5 ml 3%-igem Glukose—1—Phosphat (G—1—P) intramuskulär behandelten Albinoratten und bei unbehandelten Kontrolltieren verglichen. Nach Knochenbruch und Marknagelung wurde die Heilung am 5., 8., 11., 14., 21. und 28. Tage untersucht. Die Auswertung erfolgte am Femur der Tiere auf Grund histologischer (Haematoxylin-Eosin, Azan) und histochemischer (Toluidinblau, Hale, Hotchkiss, Ritter—Oleson) Aufarbeitung. Die Resultate scheinen dafür zu sprechen, dass die Behandlung mit G—1—P die Heilung von Knochenbrüchen beschleunigt. Diese Feststellung erfolgte nach Bewertung der verschiedenen Stadien an Hand folgender Beobachtungen :

1. Bei den mit G—1—P behandelten Tieren tritt das periostale Erregungsstadium und der sich periostal bildende Knorpel früher in Erscheinung (bereits am 5. Tage) als bei den Kontrolltieren.

2. Bei den mit G—1—P behandelten Tieren wurde eine grössere Ausdehnung des periostalen Knorpels und die raschere Entwicklung des knorpeligen Kallus beobachtet (6—14 Tage) als bei den Kontrollen (14—21 Tage).

3. Das periostale Reizstadium hörte bei den mit G—1—P behandelten Tieren früher auf und die neue Knochenbildung kam schneller zustande, sowohl periostal, als auch zwischen den Bruchenden (14—21 Tage ; bei den Kontrollen 21—28 Tage). Während dieser Zeit erscheinen den Stützschienen entlang hypertrophische Osteoblasten.

4. Bei den mit G—1—P behandelten Tieren wird der Heilungsprozesses die Konsolidation früher beendet (21—28 Tage) als bei den Kontrollen (28 Tage).

5. Die histochemischen Unterschiede können noch nicht eindeutig erklärt werden.

6. Untersuchungen im Gange sollen die Frage klären, ob G—1—P spezifisch, oder aber als ein allgemeiner Phosphatdonor wirkt.

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