Acta orthop. scand. 44, 640–649, 1973

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DOES DIPHENYLHYDANTOIN ACCELERATE HEALING OF FRACTURES IN MICE?

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Accepted 27.ii.73

Administration of the anti-convulsant drug, diphenylhydantoin, frequently causes hyperplasia of the gingiva (Ziskin et al. 1941). In such hyperplastic tissue collagen synthesis is increased (Shapiro 1958, Stern et al. 1943), resulting in faster healing of gingival wounds (Shapiro 1958). Increased collagen synthesis has also been demonstrated in the skin of diphenylhydantoin-treated rats (Houck et al. 1960, Houck 1962); healing was accelerated (Kelln & Gorlin 1961, Shafer et al. 1958) as reflected by an increased tensile strength of the wound in early stages of healing. Furthermore, human and animal fibroblast-like cells in tissue cultures have shown an increased proliferation rate when diphenylhydantoin was added to the culture medium (Shafer 1960, Shafer et al. 1961).

The purpose of the present study was to find out whether stimulation of collagen synthesis by diphenylhydantoin has any effect on the rate of healing of fractures in the lower hind legs in young mice. The following variables were studied: (1) Tensile strength of the fracture callus after a healing period of 9 days and the breaking strength after 21 days. (2) Uptake of radioactive strontium (^{85}Sr) in the fracture callus after a healing period of 9 or 21 days. (3) Concentration and solubility of collagen in the fracture callus.

Financial support for this study was obtained from the Swedish Medical Research Council; Herman Järnhardts Stiftelse; Riksföreningen mot Reumatism, and Gustaf V:s 80-års fond.

MATERIAL AND METHODS

Eighty male albino mice about 4 weeks old and weighing 18–22 g were used. The animals were divided into 2 experimental and 2 control groups of 20 animals each. They were kept in separate cages and fed a standard laboratory diet and allowed water *ad libitum*. The experimental animals received a daily i.m. injection of diphenylhydantoin (Difydan, Leo Inc., Helsingborg, Sweden) 50 mg/kg body weight, dissolved in .02 ml of a solution composed of 20 per cent ethylene glycol and 10 per cent ethyl alcohol to which sodium hydroxide was added until the substance was dissolved at pH 11.8. The control animals received the solvent solution according to the same schedule. The drug and the solvent were stored frozen (–20° C) and the daily doses were thawed just before injection.

In all experimental and control animals the middle of the left tibial diaphysis was manually fractured under ether anaesthesia on the third day after the beginning of the treatment.

Determination of the tensile strength of fracture callus

Nine days after fracture one of the two experimental groups and one of the two control groups were weighed and sacrificed. Their fractured tibiae were immediately dissected free from the fibula and soft tissues and the tensile strength of the fracture callus was measured with a spring balance (Mess & Weg Technik, Wennigsen, West Germany) attached to the ankle joint by a thread sling, with the lower hind leg hanging from another thread sling through the knee joint (Figure 1). The balance was loaded in the direction of the long axis of the bone until the bone ends were separated at the fracture level. The force necessary to bring about this separation was recorded as "the tensile strength" of the fracture.

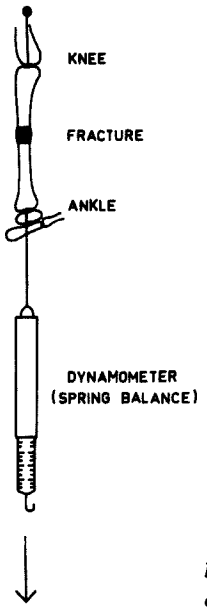

Figure 1. Experimental conditions for measuring tensile strength of healing fractures of the tibia in mice.

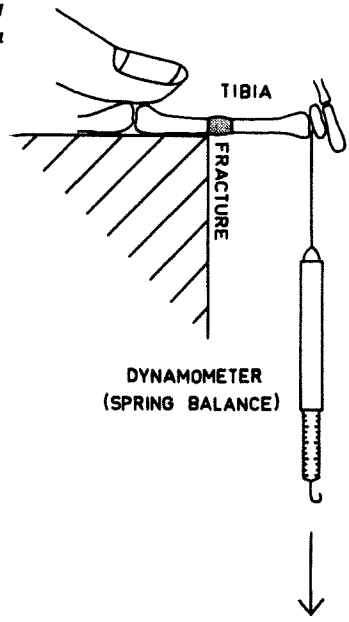

Figure 2. Experimental conditions for measuring breaking strength of healed fractures of the tibia in mice.

Determination of the breaking strength of fracture callus

The animals in the remaining experimental group and control group were weighed and sacrificed on the 21st day after fracture. The left hind legs were X-rayed and dissected free from soft tissues and from the fibula at the level of the fracture. The breaking strength of the fracture calluses were immediately determined with the same balance (Figure 2), which was now fastened by a thread sling over the frontal side of the ligaments of the ankle joint, with the tibia firmly pressed against a tabletop with the fracture callus at the edge. The balance was loaded perpendicular to the long axis of the bone until it fractured the bony callus. The load necessary to produce this fracture was recorded as the "breaking strength".

Determination of the 24-hour uptake of ^{85}Sr

Twenty-four hours before sacrifice every experimental and control animal received an equal dose of about .1 μCi ^{85}Sr in an i.m. injection of .1 ml aqueous solution of strontium-nitrate. After determination of the tensile or breaking strength the fracture callus was dissected under a dissection microscope, weighed (wet weight), and 10 calluses from each set were used for measuring the uptake of ^{85}Sr. The activity was determined with a Well type scintillation detector and compared with the simultaneously registrated activity of a standard solution of 1 per mille of the injected doses. Afterwards the calluses were ashed for 24 hours at 560° C and the ash was again weighed (ash weight). The "specific activity" of the individual calluses was calculated as cpm of the sample per mg ash as per mille of injected dose and corrected for background activity.

Determination of collagen concentration and solubility in fracture callus

In connection with the dissection the remaining 10 calluses from each set were transferred to acetone and dehydrated in this liquid for 7 days with daily changes of the acetone. The material was then weighed (dry weight), and homogenized in 6 ml of .5 M NaCl solution in an ultra turrax homogenizer (Janke & Kunkel KG, Staufen i.Br. West Germany). The homogenized tissue was then extracted in a shaking machine, centrifuged and washed twice with 2 ml .5 M NaCl. The pooled supernatant obtained by this procedure was called the salt-soluble fraction. The precipitate was afterwards homogenized in 6 ml cold .5 M citric acid buffer (pH 3.6), extracted for another 24 hours, centrifuged and washed in the same way. The combined supernatants and the precipitate after these extractions were called the acid soluble and insoluble fractions, respectively. The different soluble and insoluble fractions were then hydrolyzed in 10 ml 6 M HCl by autoclaving in 130° C for 4 hours and the hydroxyproline content was determined according to Woessner, and used as an expression of the collagen content. The total hydroxyproline concentration was calculated as the sum of the content of hydroxyproline in the fractions of different solubility. In previous experiments this calculation had been found to include an error of less than ± 5 per cent.

Statistics

Standard statistical methods were used. Probability levels exceeding 5 per cent are referred to as significant.

RESULTS

There was no difference in weight gain between the diphenylhydantoin-treated and the control animals (Table 4).

After the healing period of 9 days all fracture calluses were still unstable and the fracture ends were held firmly together by callus consisting mainly of fibrous tissue and cartilage. After 21 days the fractures were clinically stable, the stabilizing callus consisting mainly of mineralized osteoid trabeculae with marrow cells scattered among the trabeculae. The macroscopic, histologic and X-ray appearance of the healing fractures was independent of the treatment given.

The tensile strength of the fracture calluses 9 days after fracture was markedly increased on the experimental animals, treated with diphenylhydantoin (Table 1). The breaking strength of the fractures after a healing period of 21 days was also significantly increased in the experimental group (Table 1).

Between wet weight and tensile strength of the fracture calluses there was a significant positive correlation ($.01>p>.001$). There was, however, no correlation between animal weight and tensile strength of the fracture, whether the animal had been treated with diphenyl-

hydantoin or with the solvent. Neither was there any correlation between the tensile strength and the ash weight of the sample.

The breaking strength was correlated with the size of the callus (ash or wet weight) but not with the body weight of the animals.

Table 1. Mechanical properties of fracture callus in diphenylhydantoin-treated (experimental) and control mice.

Period of healing Tested property	9 days tensile strength (g)	21 days breaking strength (g)
Experimental fracture callus Mean ± SE	323.5±28.7	1123±113
Control fracture callus Mean ± SE	239.4±26.5	721± 145
Calculated significance of the difference between experimental and control samples (Student's t-test)	.05>P>.01[x]	.05≥P>.01[x]

Table 2. Concentration of total and extractable collagen in fracture callus of diphenylhydantoin-treated (experimental) and control mice.

Period of healing	Hydroxyproline mg/g ± SE					
	9 days			21 days		
Solubility	.5 M NaCl soluble	.2 M citrate soluble	Total content	.5 M NaCl soluble	.2 M citrate soluble	Total content
Experimental fracture callus SE	.028 ±.010	.020 ±.011	20.84±1.00	.068 ±.014	.030 ±.010	22.18±1.05
Control fracture callus	.00	.00	20.80±1.00	.00	.00	21.23±.92

The size of the callus (ash and wet weight) was increased in the diphenylhydantoin-treated mice although not significantly (Table 4).

There was no significant difference in the content of total collagen in the fracture calluses in the experimental or control sets (Table 2). The concentration of collagen extractable in neutral salt- and citrate buffer

solutions was very low and not measureable (below .01 mg hydroxyproline per g callus) in the control samples. In the experimental samples, however, there were low but measureable quantities of both salt- and acid-soluble collagen (Table 2).

The 24-hour uptake of ^{85}Sr (specific activity) was about the same in all calluses irrespective of healing time or treatment given (Table 3). The wet weight/ash weight ratio was also independent of the treatment given, but was significantly higher after a healing period of 9 than after 21 days (Table 4).

Table 3. "Specific activity" $\frac{cpm\ (sample)}{cpm\ (standard=1\ ‰\ of\ dose) \times mg\ ash\ (sample)}$ *of fracture callus in diphenylhydantoin-treated (experimental) and control mice.*

Period of healing Days		9	21
Specific activity of fracture callus ± SE	Experimental specimens	2.34±.39	2.84±.38
	Control specimens	3.14±.42	2.94±.46
Significance of difference (student's t-test)		$P>5\%^{-1}$	$P>5\%^{-1}$

Table 4. Weights of experimental (diphenylhydantoin-treated) and control specimens. On no occasion was there any significant weight difference between experimental animals or samples.

Period of healing Days	0	9	21
Body weight of experimental animals. Mean (g) ±SE	18.9±.49	21.7±1.38	24.5±.74
Body weight of control animals. Mean (g) ±SE	18.5±.51	22.2±1.32	25.3±.78
Wet weight of fracture callus. Mean (mg) ±SE	Experimental samples	42.1±5.8	45.5±4.0
	Control samples	34.8±3.2	37.8±3.0
Ash weight of fracture callus. Mean (mg) ±SE	Experimental samples	4.28±.63	9.09±1.76
	Control samples	4.08±.65	7.95±1.85
Wet weight/ash weight	Experimental samples	9.8	5.0
	Control samples	8.5	4.3

DISCUSSION

The improved fracture healing in animals treated with diphenylhydantoin, as documented by the higher mechanical strength of the callus of healing and healed fractures in mice, is in agreement with previous findings of earlier organized fracture haematomas in the mandible of rabbits, treated with diphenylhydantoin (Sklans et al. 1967). The organic matrix of bone is to more than 90 per cent composed of collagen (Eastoe & Eastoe 1954, Mills & Bavetta 1968). Diphenylhydantoin has been shown to stimulate the synthesis of collagen in various tissues (Houck et al. 1960, Shafer et al. 1961). This higher rate of synthesis of collagen may therefore contribute to the better healing of fractures in animals treated with this drug. The amount of collagen extracted from the fracture callus with a neutral salt or dilute acid buffer solution was larger in the animals treated with diphenylhydantoin. This observation, indicating a lesser degree of cross-linking in this material, may be related to an increased synthesis of collagen in the healing bone tissue of the diphenylhydantoin-treated animals (Jackson & Bentley 1960). Readily extractable collagen includes recently synthesized collagen and degradation products (Jackson & Bentley 1960, Laitinen 1967). Future investigations with the aid of radioisotopes may perhaps produce more convincing evidence of increased collagen synthesis in the healing bones of diphenylhydantoin-treated animals.

There was no difference in the mineral uptake by these calluses, as measured by the 24-hour uptake of ^{85}Sr. Neither was there any difference in the concentration of mineral (ash weight/wet weight ratio) in the fracture calluses of different maturity between the diphenylhydantoin-treated and control animals. With these parameters there was thus no measureable difference in the mineralization pattern of the experimental and control samples. In our estimation of "the specific activity" of the calluses, the uptake of ^{85}Sr was recorded in relation to the ash weight of the sample. The higher ash content of both the experimental and control specimens taken 21 days after fracture (Table 4) implies about a twofold increase in the uptake of radioactivity after 21 days than after 9 days. This result is in accordance with that of Lemaire (1966) and of Stacher & Firschein (1967).

There was a non-significant difference in the mean wet weight and ash weight of the calluses, which were heavier in the experimental animals both 9 and 21 days after fracture. Dissection of these small

samples gave rather large individual differences (Table 4) despite the use of the dissection microscope. The weight differences were not statistically significant, but the fact that the mean weight of the experimental specimens was higher on all four occasions suggested that the difference found was a true difference. If it was, diphenylhydantoin may act on bone healing by increasing the size of the fracture callus, the mechanical strength being a measure of callus mass rather than quality.

Diphenylhydantoin is a well-known drug that has been used in the treatment of epileptics for more than 30 years (Kelln & Gorlin 1961). Its toxicity is low (Geever et al. 1967). The dosage used was about 4 times higher than recommended for humans. Considering the higher metabolic rate in mice, this dose may be comparable with that used in antiepileptic treatment of human beings. No toxic effects of the drug were observed. Furthermore, the animal weight gain was normal and equal in the experimental and control groups (Table 4).

Diphenylhydantoin, however, has other effects on skeleton and metabolic reactions. It is known to depress the secretory activity in a number of endocrine glands (Pento et al. 1972) including inhibition of insulin release (Levin et al. 1970, Malherbe et al. 1972), and calcitonine release (Pento et al. 1972). Recent investigations have also shown that diphenylhydantoin, as well as other anticonvulsant drugs, may cause an alteration of the calcium metabolism in the skeleton resulting in osteomalacia after long-term therapy (Dent et al. 1970). Subnormal serum calcium levels and raised serum alkaline phosphatases have been observed (Richens & Rowe 1970). These effects have been attributed to liver enzyme activation causing accelerated breakdown of vitamin D (Richens & Rowe 1970, Hunter et al. 1971, Wright 1965). A therapeutic agent which also causes an increased metabolism of collagen in the skeleton may cooperate with the disturbed calcium metabolism, resulting in even faster decalcification of osteomalaic bone. As a matter of fact, osteomalacia, induced by anticonvulsant drugs, has mainly been described in connection with treatment by diphenylhydantoin and chemically related compounds.

The stimulating effect of diphenylhydantoin on the synthesis of collagen and its stimulating effect on healing of connective tissues has been demonstrated in different species (Houck 1962, Kelln & Gorlin 1961, Kolbert 1968), including man (Simpson et al. 1965). Thus, there is reason to believe that diphenylhydantoin may improve fracture healing also in other vertebrates. In fact, diphenylhydantoin has been reported to increase the healing rate of chronic leg ulcers in humans (Simpson et al. 1965).

SUMMARY

The antiepileptic drug diphenylhydantoin accelerates healing of experimental fractures in mice. The tensile strength of healing unstable fractures and the breaking strength of healed fractures were significantly greater in animals treated with diphenylhydantoin than in controls.

In the diphenylhydantoin-treated animals the fracture callus contained a larger amount of extractable collagen hinting to increased collagen synthesis, which in previous studies in other tissues has been shown to be an effect of the drug.

The uptake of tracer doses of ^{85}Sr per mg ash weight in fracture callus was not measurably affected by diphenylhydantoin-treatment. The mean weight of the fracture calluses of the experimental animals was higher on four different occasions, but on none were these differences statistically significant.

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