Influence of Vitamin A on Wound Healing in Rats with Femoral Fracture

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Groups of healthy wounded rats with and without comminuted femoral fractures, and maintained on nutritionally complete commercial rat chow with and without supplemental vitamin A, were studied. The test wounds were standard dorsal skin incisions and s.c. polyvinyl alcohol sponge implants. In some experiments the rats were pair-fed; the rats with femoral fracture not receiving supplemental vitamin A were the lead group for determining food allowances. In other experiments, the rats were allowed food *ad libitum*. We found that *wound healing of rats with femoral fracture was increased when* supplemental vitamin A was given, but the supplemental vitamin A did not completely obviate the adverse effects of fracture. The ratio of the breaking strengths of the skin incisions after formalin fixation to the breaking strengths of the incisions in the fresh state was higher in the unsupplemented rats, supporting the results of our earlier experiments that vitamin A increases the rate of collagen cross-linking.

The injured patient must heal his wounds for successful recovery, but it is the patient with extensive injury who may have the greatest difficulty in doing so. The experimental evidence is clear that wound healing is impaired in animals with severe injury.[11,12] It is well documented that disturbances in the metabolism of protein, certain hormones and certain water-soluble vitamins, expecially ascorbic acid, may occur after serious injury and thereby influence wound healing.[7,10–12,16,18] Relatively little information regarding the metabolism of the fat-soluble vitamins after injury, and thereby their effects on healing in injured animals or patients, is available. Earlier studies in our laboratory, undertaken in rats mildly depleted on vitamin A and subjected to a skin incision and subcutaneous implants of polyvinyl alcohol sponges, suggested an increased requirement for vitamin A by such rats, since administration of vitamin A had a salutory effect on their survival, weight gain and the healing of their incisions and the formation of the sponge granuloma reparative tissue.[14] We undertook the present study to determine whether this ameliorating effect of supplemental vitamin A on wound healing would be demonstrable in rats with an additional severe injury, namely, femoral fracture. An incidental aspect of the study was the determination of the effects of supplemental vitamin A on the liver and serum concentrations of vitamin A in injured rats.

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Methods

The animals were male Sprague-Dawley rats* of approximately 325 gm body weight. The rats were kept individually in two-mesh steel cages at a room temperature of 22-25 C, and fed Rockland Mouse/Rat Complete Ration (meal)† and tap water.

Two experiments were performed on four groups of 12 rats. On four successive days, operations were performed with aseptic precautions by the same surgical team on groups of rats anesthetized with sodium pentobarbital (i.p.) supplemented with light ether anesthesia. Each rat received a 7 cm right paravertebral incision through the skin and panniculus carnosus. The wound was then undermined at three points to approximately 5 cm lateral to the skin edge by inserting Metzenbaum

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scissors at the upper, middle and lower poles, spreading the blades and tearing pockets between the superficial and deep muscles. A soft, water-moistened, sterile, porous polyvinyl alcohol sponge‡ (preweighed 22.0-22.9 mg) was then inserted into each pocket. Rats not selected for treatment with vitamin A received sponges containing 0.2 ml ethyl alcohol, while rats selected for oral and topical treatments with vitamin A received sponges containing 1 mg vitamin A,§ in 0.2 ml ethyl alcohol. The wound was then closed with 6 interrupted, evenly spaced, No. 36 stainless steel sutures. While still under anesthesia, 24 of the rats, Groups A and B, were subjected to a comminuted fracture of the right femur. This was done by making a small incision over the lateral aspect of the right thigh, separating the thigh muscles by blunt dissection and then closing a crushing clamp around the femur. The incision was closed with one stainless steel suture. All rats received a skin wound and 3 sponge implants. Half the animals received an additional injury, uni-lateral fracture of the right femur, and hereafter are designated as fracture groups of rats. Sutures were removed on the seventh postoperative day.

The rats of Groups B and C were pair-fed postoperatively according to the food intakes of Group A rats and Group D rats were pair-fed the food intakes of Group C rats for the corresponding postoperative day. Food intakes were measured daily and body weights determined every other day. The rats of Groups A and C were given orally each day 1 drop of peanut oil and their dorsal skin wounds anointed with 0.2 ml peanut oil for 7 days. Rats of Groups B and D were given orally each day 1 mg of vitamin A in 1 drop of peanut oil and 2 mg vitamin A in 0.2 ml peanut oil applied to their dorsal skin wounds starting promptly after operation and continued for 7 days. All animals were sacrificed by ether anesthesia on the 14th postoperative day.

‡Unipoint Laboratories, High Point, North Carolina 27260.

§Crystalline vitamin A acetate, Eastman Kodak Co., Rochester, New York 14650.

In both experiments, the dorsal skin pelt was excised, restored as nearly as possible to its *in situ* dimensions on a multi-bladed cutter and then sectioned into seven 0.6 by 6.0 cm strips of skin. Cephalad strips 1, 2, and 4 were tested on a tensionometer for breaking strength in the fresh state and caudal strips 5, 6, and 7 were tested after 72 hours fixation in 10% buffered formalin. Strip 3 and the left sponge after excision were saved for histology. One of the sponges on the right side of the animals was analyzed for total hydroxyproline content by the method of Woessner[19] and the remaining right sponge stored at 20 C. On the 11 day study of experiment 2, sections of the skin incisions and sponge granulomas were stained with hematoxylin-eosin as well as Mowry's colloidal iron stain for histologic study (including collagen and acid mucopolysaccharides) in a semi-quantitative manner.[15] The slides were labeled by a code not known to the pathologist at the time of his examination.

The second experiment was a replicate of the first but all the rats were subjected to the additional injury of uni-lateral fracture of the right femur, were fed *ad libitum* postoperatively and sacrificed a few days earlier. Previous to sacrifice, blood was withdrawn from the heart while the rats were under ether anesthesia and sera were prepared. The livers were excised, weighed and wrapped in aluminum foil, and stored frozen at −20 C as were the sera, until analyzed for vitamin A by a modified Carr-Price method.[1]

Results

Experiment 1

All the rats withstood the wounding and additional femoral fracture procedure except for one rat with fracture found dead 5 days after injury. The mean body weights of the pair-fed rat groups at operation and when sacrificed two weeks later are summarized in Table 2 along with the food intake data. The first two days after operation both fracture groups (A and B) lost about 6% of their body weight. This loss was substantially the same as

TABLE 1. *Recapitulation*

Group*	Injury	Oral	Topical	Postop. Day Sacrificed
		Experiment 1: Pair-fed		
A	Incision/Fracture	Peanut oil	Peanut oil	14
B	Incision/Fracture	Vitamin A†	Vitamin A§	14
C	Incision	Peanut oil	Peanut oil	14
D	Incision	Vitamin A†	Vitamin A§	14
		Experiment 2: Ad libitum-fed		
A_2	Incision/Fracture	Peanut oil	Peanut oil	11
B_2	Incision/Fracture	Vitamin A†	Vitamin A§	11
C_2	Incision/Fracture	Peanut oil	Peanut oil	10
D_2	Incision/Fracture	Vitamin A†	Vitamin A§	10

*12 rats per group.

†1 mg vitamin A acetate.

§2 mg vitamin A acetate.

TABLE 2. *Body Weights and Food Intakes of Injured Rats, Some Given Oral and Topical Vitamin A*

Experiment 1: Pair-Fed						
				Postop. Intake†		
Group	Day	Wt. gm.*	Days	Food	Prot.	Cals.
A. Peanut Oil Incision/Fracture	Operated	365 ± 3	2	5.9	1.5	20
	Sacrificed	386 ± 4	14	7.3***	1.8	25
B. Vitamin A Incision/Fracture	Operated	369 ± 3	2	5.5	1.4	19
	Sacrificed	397 ± 3§	14	6.9**	1.7	24
C. Peanut Oil Incision	Operated	371 ± 3	2	5.5	1.4	19
	Sacrificed	390 ± 3	14	7.2	1.8	25
D. Vitamin A Incision	Operated	369 ± 3	2	5.4	1.4	19
	Sacrificed	408 ± 3§	14	7.0‡	1.7	24

*Mean ± S. E. †Average food intake (gm/100 gm body wt) for first two days and entire postoperative period. Protein (gm/100gm body wt) equals T N determined by micro-Kjeldahl × 6.25. Calories/100 gm body wt estimated from manufacturer's ration composition. §Gained more weight than peanut oil group (P<0.01). **Lower food intake than peanut oil group (P = 0.05). ‡Lower food intake than peanut oil group (P<0.001). ***Pair-fed fracture rats given peanut oil ate more food than wounded rats given vitamin A (P<0.02).

found for wounded rats without fracture given peanut oil, whereas similar rats given vitamin A lost slightly less weight. Two weeks after operation fracture rats given peanut oil had gained 21 gm and those given vitamin A 28 gm. This 33% difference in weight gained by the vitamin A supplemented rats was significant (P<0.01). Wounded rats given peanut oil gained 19 gm for the same period, while those given vitamin A gained 39 gm. This two-fold difference in weight gain was likewise significant (p<0.01).

The first two days after operation all the injured rats ate 20-23% less food than during the remainder of the postoperative period. Thereafter, the food intake rose, reaching preoperative levels by the end of first week. Pair-feeding resulted in slightly lower food intakes (3-5%) for rats supplemented with vitamin A (Table 2).

Skin incisions of rats with fracture two weeks after injury were found significantly stronger (19%, P<0.02) for rats given vitamin A than for rats given peanut oil when tested in the fresh state (Table 3). Among rats given vitamin A the skin wounds of rats without fracture were significantly stronger (25%) than wounds of fracture rats (P<0.001) and stronger (50%) than wounds of fracture rats given only peanut oil. The ratio of the formalin-fixed to fresh breaking strength of skin wounds was significantly lower for the vitamin A groups (P<0.02) suggesting an *in vivo* increase in cross-linking of the reparative collagen in the vitamin A supplemented rats.[13]

No difference in hydroxyproline content was found for the sponge granulomas of rats with or without fracture whether given peanut oil or vitamin A. However, among vitamin A treated rats, rats with fracture had significantly higher hydroxyproline contents (10%) in sponge-induced granulomas than wounded rats (P=0.02).

TABLE 3. *Wound Strength and Sponge Granuloma Hydroxyproline of Injured Rats Given Oral and Topical Vitamin A: (Mean ± S.E.)*

Experiment 1: Pair-Fed					
		Breaking Strength, Gm.			
Group		Fresh	Formalin	Formalin / Fresh	OHP (μg/100 mg sponge)
	14 Days Postop				
Incision/Fracture		577 ± 38	2073 ± 75	4.0 ± 0.2	3215 ± 199
B. Vitamin A Incision/Fracture		688 ± 36[a]	2254 ± 82	3.5 ± 0.2[c]	3315 ± 140[d]
	14 Days Postop				
C. Peanut Oil		671 ± 47	2273 ± 91	3.9 ± 0.3	2923 ± 116
D. Incision		866 ± 35[b]	2440 ± 106	3.0 ± 0.2[c]	3004 ± 124

[a]Significantly increased over fracture rats given peanut oil (P<0.02).
[b]Significantly increased over fracture rats given vitamin A and over peanut oil groups (P<0.001).
[c]Significantly lower than peanut oil group (P<0.02).
[d]Significantly increased over wounded rats given vitamin A (P = 0.02).

TABLE 4. *Body Weights and Food Intakes of Injured Rats, Some Given Oral and Topical Vitamin A*

Experiment 2 Ad libitum Fed						
Group		Wt, gm†	Days	Postop. Intake* Food	Prot	Cals
A_2 Peanut oil Incision/Fracture	Operated Sacrificed	357 ± 2 371 ± 4	2 11	4.0 6.9	1.0 1.7	14 23
B_2 Vitamin A Incision/Fracture	Operated Sacrificed	365 ± 2 380 ± 3	2 11	4.8 7.2	1.2 1.8	16 25
C_2 Peanut oil Incision/Fracture	Operated Sacrificed	364 ± 2 371 ± 3	2 10	4.5 6.9	1.1 1.7	15 24
D_2 Vitamin A Incision/Fracture	Operated Sacrificed	361 ± 2 369 ± 3	2 10	4.7 7.2§	1.2 1.8	16 25

*Average food intake (gm/100 gm body wt) for first two days and entire postoperative period. Protein (gm/100 gm body wt) equals TN determined by micro-Kjeldahl × 6.25. Calories/100 gm body wt estimated from manufacturer's ration composition. †Mean ± S.E. §*Ad libitum* fed rats given vitamin A ate more food than rats given peanut oil (P = 0.05) in 10 day study.

Experiment 2

Ad libitum fed rats with fracture given peanut oil (groups A_2 and C_2) or vitamin A (groups B_2 and D_2) lost about 5% of their body weight the first two days after injury. By the sixth postoperative day, all the rats had begun to gain weight and when sacrificed, the vitamin A groups had regained only one more gram than the peanut oil groups as compared with their weights at operation. The vitamin A and peanut oil groups had a reduced food intake of 35 and 38% respectively during the first two postoperative days (Table 4). Food intake reached preoperative levels after 6 days matching preoperative intakes the remaining postoperative period. When the postoperative food and nutrient intakes of the *ad libitum* fed rat groups were compared, the vitamin A groups ate slightly more (4%) than the peanut oil groups.

When tested for breaking strength, skin wounds of rats given vitamin A compared with peanut oil groups were found significantly stronger (31%, P=0.01) at 11 days postoperatively. The hydroxyproline contents of the sponge granulomas of the vitamin A groups were higher than for the peanut oil groups (49%, P=0.01). The hydroxyproline contents of sponge-induced granulomas were also significantly higher (36%, P = 0.01) for the vitamin A group at 10 days postoperatively but the wound strengths were not significantly different in the two groups of rats.

Liver vitamin A was significantly increased (5-14 fold) among animals given vitamin A as compared with those given peanut oil (P<0.001). No differences in serum vitamin A concentrations were found among the vitamin A and peanut oil groups (Table 6).

For histologic evaluation of the skin incisions and sponge granulomas of the rats sacrificed at 11 days, a quantitation for each category described was based on arbitrary scales of 1 to 3 and the ratios of ground substance to collagen calculated (Table 7). No statistically significant differences in collagen and ground substance were found by the examination of skin wounds. Ground

TABLE 5. *Wound Strength and Sponge Granuloma Hydroxyproline of Injured Rats Given Oral and Topical Vitamin A: (Mean ± S. E.)*

Experiment 2: Ad libitum fed					
Group		Breaking Strength, gm Fresh	Formalin	Formalin/Fresh	OHP μg/100 mg sponge)
	11 Days Postop				
A_2 Peanut Oil Incision/Fracture		461 ± 21	1594 ± 59	3.7 ± 0.2	1993 ± 45
B_2 Vitamin A Incision/Fracture		606 ± 36*	1838 ± 63*	3.3 ± 0.2	2975 ± 158*
	10 Days Postop				
C_2 Peanut Oil Incision/Fracture		410 ± 27	1230 ± 52	3.3 ± 0.2	1864 ± 107
D_2 Vitamin A Incision/Fracture		399 ± 26	1276 ± 49	3.5 ± 0.2	2524 ± 134*

*Significantly increased over peanut oil group (P = 0.01)

TABLE 6. *Liver and Serum Vitamin A Levels of Injured Ad libitum Fed Rats Given Oral and Topical Peanut Oil or Vitamin A: (12 Rats/Group)*

	11 Days Postoperatively					
	Liver Wt, gm			Liver Vit. A		Serum Vit. A
Group*	Wet	Per Kg Body Wt	Dry	μg/Gm	μg	μg/100 ml
Peanut Oil	12.8 ± 0.5	34.6 ± 1.2	3.6 ± 0.1	20 ± 1	70 ± 5	62 ± 3
Vitamin A	12.7 ± 0.3	33.3 ± 0.6	3.6 ± 0.1	100 ± 19†	372 ± 78†	69 ± 2
			10 Days Postoperatively			
Peanut Oil	11.9 ± 0.3	32.1 ± 0.6	3.4 ± 0.1	17 ± 1	57 ± 5	73 ± 4
Vitamin A	12.2 ± 0.3	32.8 ± 0.6	3.6 ± 0.1	231 ± 14†	835 ± 47†	64 ± 3

(Mean ± S. E.)
*With skin incision and unilateral femoral fracture.
†Significantly increased over peanut oil group (P<0.001).

substance of sponge granulomas of vitamin A fractured rats compared with the peanut oil group was significantly increased (P = 0.02) as was the ratio of ground substance to collagen (P = 0.001) at 11 days postoperative. No differences were apparent in the numbers of fibroblasts in the wounds or sponge granulomas of the peanut oil and vitamin A groups.

Discussion

At present the physiological action of vitamin A in wound healing remains unclear, but some previous studies have been made on the relationships between vitamin A and the biological control of wound healing.[17] The protective effect of the vitamin in reversing the inhibitory effect of anti-inflammatory steroids such as cortisone[8] and other anti-inflammatory agents[9] such as aspirin has attracted a great deal of attention. In this regard Howes et al.[6] reporting on the retardation of wound healing by cortisone observed that a hormone-like substance which can completely stop such profound defense mechanisms as the sprouting of new blood vessels and fibroplasia is not only significant in itself, but conversely, the finding suggests that an offsetting mechanism must likewise exist whenever a wound heals.

There is now general agreement that vitamin A has hormone-like properties and yet functions as a vitamin.[20] Ehrlich and Hunt[3] have shown that vitamin A overcame this inhibitory effect of cortisone on the rate of gain in tensile strength in early stages of healing of primarily closed wounds in rats, yet vitamin A alone had no effect on wound healing in normal animals. Hunt *et al.*[8] later reported that vitamin A stimulated healing of cortisone-retarded open wounds in rabbits and had some benefit in the healing of open wounds in some patients. In these experiments vitamin A again did not significantly enhance healing beyond the normal rate when cortisone was not given. Lee and Tong[9] also demonstrated that the healing retardation of salicylates, hydrocortisone and prednisone can be reversed by applying retinoic acid on the wound. Recently, Chernov et al.[2] reported that the administration of vitamin A reduces the risk of stress ulcers in severely stressed patients.

The rats in the studies reported in this paper were nourished normally up to operation and injury, and supplemental oral and topical vitamin A treatments were initiated postoperatively. This is in contrast to an earlier study[4] in our laboratory on the influence of vitamin A on wound healing in which the rats were deprived of vitamin A for varying periods preoperatively, as well as postoperatively. Vitamin A deficient rats had slower growth rates, weaker wounds, made less sponge granuloma reparative tissue than control rats, the reparative wound collagen was less cross-linked, and many died.

Our arbitrary choice to allow the group of rats with fracture given peanut oil in the first experiment to select their food voluntarily and then to give the same food allowances to their pairs in the other groups resulted in slightly lower (3-5%) food intakes for vitamin A treated animals with and without fracture. These latter groups of rats nevertheless gained more weight postoperatively than the rats with higher food intakes given peanut oil. In the second experiment in which all rats ate *ad libitum*, there was no apparent effect of vitamin A on food intake or body weight.

TABLE 7. *Comparison of the Histologic Evaluation of Wound Healing, Skin Incision Breaking Strength and Sponge Granuloma Hydroxyproline Content of Rats at 11 Days Postoper.*

Group Femoral Fracture	A—Peanut Oil Unilateral	B—Vitamin A Unilateral
	Skin Incision	
Collagen (Coll)	1.6 ± 0.1	1.4 ± 0.1
Ground Substance (G S)	1.3 ± 0.2	1.3 ± 0.2
Ratio $\frac{G\ S}{Coll}$	0.88 ± 0.09	1.03 ± 0.18
No. Fibroblasts	1.1 ± 0.1	1.1 ± 0.1
Breaking Strength	461 ± 21	606 ± 36†
	Sponge Granuloma	
Collagen (Coll)	2.5 ± 0.1	2.1 ± 0.2
Ground Substance (G S)	1.1 ± 0.1	1.5 ± 0.2*
Ratio $\frac{G\ S}{Coll}$	0.44 ± 0.03	0.74 ± 0.08§
No. Fibroblasts	2.2 ± 0.1	2.3 ± 0.2
Hydroxyproline	1993 ± 45	2975 ± 58†

(Mean ± S. E.)
Significantly increased over peanut oil group *P=0.02; †P=0.01; §P=0.001

In our experiments, after 2 weeks, oral vitamin A and the topical application of vitamin A to the skin incisions resulted in improved wound healing among rats with skin incisions only and also among rats with skin incisions and femoral fracture after 11 days (but not in the 10 day group) compared with similar rats given peanut oil. The lower formalin fixed: fresh breaking strength ratios found in experiment 1 for the vitamin A treated rats suggest the presence of greater cross-linking of reparative collagen in the wounds of rats given vitamin A. The increased hydroxyproline content of the granulomas of *ad libitum* fed rats in experiment 2 given vitamin A, particularly rats with fractures, indicates increased reparative collagen formation under the influence of vitamin A. This was supported by the histological appearance, i.e., slightly more collagen and a significantly lower ratio of G.S.: collagen of the sponge granulomas. Our findings are in agreement with those of Herrmann and Woodward[5] who reported an increase in tissue ingrowth and collagen formation into PVA sponge implants and a concomitant increase in wound strength in rats with dorsal skin incisions given local and systemic vitamin A.

These findings are at variance with those of Ehrlich and Hunt who reported that vitamin A, 1500 I.U. (0.5 mg) in peanut oil given intraperitoneally on the day of wounding, two and four days later had no effect on the breaking strength of dorsal incisions measured two days after the last vitamin A injection. Their controls received no injections. Our studies were undertaken with rats of the same strain and sex as those of Ehrlich and Hunt. In both cases, the rats were housed individually and ingested a commercial rat chow, but from different suppliers. In our experiments, oral (1 mg) and topical (2 mg) vitamin A were given for one week starting right after operation and the wound healing was evaluated 10-14 days postoperatively.

Summary

Oral and topical vitamin A given to rats with a skin wound with and without femoral fracture resulted in increased wound strength in pair-fed rats and *ad libitum* fed rats as compared to controls given peanut oil. Increased tissue ingrowth and collagen formation in sponge granuloma were found for *ad libitum* fed rats with fracture given vitamin A but was unchanged among pair-fed wounded rats with and without fracture. Histologic examination of the skin wound and sponge granuloma showed increased fibroplasia and collagen elaboration in *ad libitum* fed rats given vitamin A compared with similar rats given peanut oil.

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