STUDIES ON GANGRENE FOLLOWING COLD INJURY. IX. THE EFFECT OF RUTIN AND OTHER CHEMICAL AGENTS ON THE COURSE OF EXPERIMENTAL FROSTBITE IN RABBITS [1, 2]

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Methods for the production of controlled cold injury and the course of events following untreated injury of this type in rabbits have been described earlier in this series of papers (1, 2). Such injury leads rapidly to massive edema of the frostbitten foot, drying and subsequent separation at the line to which the part was immersed in a freezing mixture. An invariable early feature of this controlled cold injury is loss into the injured region of large amounts of protein-containing fluid (3). The reversibility of this type of injury has been demonstrated by experiments in which the extent of tissue loss was greatly reduced or prevented by physical measures: rapid warming in water at + 42° C. (4), rigid plaster or plastic casts or elastic pressure dressings (5). This reversibility emphasizes the importance of events subsequent to thawing as causal factors in the production of gangrene and suggests that the possible beneficial effects of chemical agents should be considered.

Treatment of frostbite by means of drugs has been generally unsuccessful (6 to 8). Recently Lange and Boyd (9) reported that tissue loss following experimental frostbite was reduced by treatment with heparin intravenously, but Quintanilla, Krusen and Essex (10) were unable to demonstrate the effectiveness of this anticoagulant. This paper reports some attempts to influence the course of controlled experimental cold injury in rabbits by means of a number of chemical agents. The flavonol glycoside, rutin, was found to reduce the extent of tissue loss following frostbite of rabbit feet; the major part of this report is therefore devoted to this substance.

METHODS

Albino rabbits maintained on a diet of Albers' Family Ration, a dry commercial food in pellet form, were used in all experiments. The animals were anesthetized by the administration intraperitoneally of dial, 90 mgm. per kgm., supplemented with ether immediately before frostbite. The hair was removed from the ear or from one hind leg by close clipping. The distal 4 to 5 cm. of the ear, or the distal portion of the foot to the level of the tuberosity at the base of the fifth metatarsal, was frozen by immersion in a mixture of alcohol-water-ethylene glycol, cooled with solid CO_2 to − 55° C. The ears were immersed for 60 to 90 seconds, the feet for three minutes. Complete details of this procedure have been given previously (1).

EXPERIMENTAL RESULTS

1. Rutin.[3] Substances with Vitamin P activity have been reported to influence capillary permeability and fragility (11, 12). One of these substances, rutin, has become available recently in pure form. It has been reported to be effective in the treatment of capillary bleeding associated with hypertension in man (13, 14), and a recent report (15) indicates that it decreases the incidence of widespread petechiae and ecchymoses in dogs following X-irradiation. Because capillary injury is such a prominent feature in the lesions produced by exposure to severe cold, the effect of rutin was studied on standardized frostbite in rabbits in order to determine the possible prophylactic and/or therapeutic usefulness of this agent. In the following experiments rutin was adminis-

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[2] An abstract has appeared in Federation Proceedings, 1948, 7, 38.

[3] The rutin used in these experiments was supplied by the Western Regional Research Laboratory, United States Department of Agriculture, through the courtesy of Dr. Floyd DeEds.

TABLE I

Frostbite of rabbit feet

Closely clipped foot immersed to level of tuberosity at the base of fifth metatarsal in freezing mixture at −54 to −56° C. for three minutes. Foot thawed in air.

Rutin administered by stomach tube daily. Dose 100 mgm. per kgm. in rabbits No. 243, 244, 245 and 246. Dose 50 mgm. per kgm. in others.

Animal number	Doses of rutin		Appearance of gangrene, days after frostbite		Result
	Before injury	After injury	Wet	Dry	
222	5	17	4	7	Lost toes; dorsum scarred
224	10	6	3	6	Lost toes; dorsum scarred
225	6	6	3 (toes)	5	Lost toes only
230	1	5	5 (toes)	6	Lost toes only
229	1	5	—	—	Lost 2 phalanges of 1 toe
231	1	5	5 (toes)	6	Lost toes only
243	0	3	4 (toes)	4	Lost toes only
244	0	3	3	4	Lost toes and 1 cm. of foot
245	0	3	3 (toes)	5	Lost distal 2 phalanges
246	0	3	3 (toes)	4	Lost toes only
15 Controls*			2 (9)†	4(3)	Complete loss (11); loss of all but narrow plantar pad (4)
			3 (6)	5(9)	
				6(2)	
				7(1)	

* These animals are the same as those used in a previous study (1).

† The numbers in parentheses indicate the number of animals.

tered daily by stomach tube as a 10 mgm. per ml. suspension in 1 per cent gelatin.

The extent of tissue loss was studied following controlled cold injury of the feet of rabbits in six cases in which rutin, 50 mgm. per kgm. daily, was administered both before and after injury, and in four additional cases in which rutin was administered only during the period after frostbite. The results are given in Table I. Observations on the development of gangrene and the extent of tissue loss in 15 control animals are included for comparison. In untreated animals three-minute immersion of the foot into a freezing mixture at − 55° C. led to loss of the entire injured region in 11 of the animals and to loss of all but a small amount of tissue on the plantar surface of the foot in four animals. In none of the rutin-treated animals was the tissue loss as great as that observed in the controls. In most of the treated animals tissue loss was confined to the toes and in two instances involved only parts of the toes. The time of appearance of wet gangrene, although it occurred only in the toes of treated animals, was delayed as compared to the controls. The difference between the times required for the development of wet gangrene in the two series of animals was statistically significant ($P < 0.001$).

Doses of rutin similar to those used above failed to influence the course of cold injury to rabbit ears. Frostbite, produced by immersing the distal half of the ear in a freezing mixture at − 55° C. for one minute, led to gangrene and complete loss of the injured part in 12 control animals. Five animals treated with rutin both before and after injury developed gangrene and lost all of the frostbitten parts of the ears. In one animal which received rutin the appearance of wet and dry gangrene was delayed one and three days, respectively, as compared to control animals. No delay was observed, however, when the experiment was later repeated on the remaining ear of the same animal.

Direct observation of the circulation (16) in the ear of a rabbit treated with rutin before frostbite showed that the local circulatory changes were similar to those seen in frostbitten rabbit ears following procaine block of the stellate ganglion (*cf.* section 4 below). Stasis, ordinarily complete in true capillaries within 10 minutes after thawing, was delayed up to 80 minutes in animals treated with rutin. Carbon particles were seen moving in large vessels and thoroughfare channels, but none was observed in true capillaries. Subsequently carbon accumulated in true capillaries during the growth of massive edema, but the amount was less than in untreated ears at the same time after frostbite.

Since the process of stasis following cold injury appears to depend upon the loss of plasma from damaged capillaries (3) the effect of rutin upon loss of trypan blue from the blood was studied. Adult rats, anesthetized with pentobarbital (37.5 mgm. per kgm. intraperitoneally), were subjected to cold injury by placing upon the shaved abdomen 5-ml. beakers, 18 mm. in diameter, filled with solid carbon dioxide. Seven animals were used as controls and five were given 200 mgm. of rutin per kgm. body weight by stomach tube one hour before injury. Two beakers were applied to each animal; one was left in place for one minute and one was left two minutes. Ten minutes after application of the beakers 0.5 ml. per kgm. of 1.5 per cent trypan blue was injected into

the lateral tail vein. The interval between injection and the first appearance of blue in the injured areas was timed. Since the duration of injury did not alter the time of appearance of the dye in the injured areas the values have been grouped. The mean interval for appearance of dye in the controls was 32 seconds (S.E. $\pm$ 1.49), while that in animals treated with rutin was 68 seconds (S.E. $\pm$ 3.41). These times were significantly different ($P < 0.001$).

In order to determine the extent to which loss of trypan blue into frostbitten regions is influenced by vasoconstriction, Privine (naphazoline hydrochloride), 10 mgm. per kgm., was injected intraperitoneally into three rats 10 minutes before injury with solid carbon dioxide as described above.

The interval between trypan blue injection and appearance of blue in the injured regions was 35 seconds; this is not significantly different from the interval observed in control animals. It therefore seems unlikely that delayed penetration of trypan blue into frostbitten areas in rutin-treated animals is due to vasoconstriction of the type produced by Privine.

An increased peripheral vasoconstriction associated with fatal muscle trauma in dogs has been shown by Wang *et al.* (17) to result in prolongation of fluorescein circulation time. It was considered possible that rutin produces a type of vasoconstriction which may prolong the fluorescein circulation time. Consequently the circulation time from lateral tail vein to eyes and oral mucosa was measured with this dye in five control rats and in five rats given 200 mgm. per kgm. of rutin by stomach tube one hour before the fluorescein. The mean circulation times were 5.18 and 5.41 seconds, respectively. These times are not significantly different. Failure to observe a prolonged fluorescein circulation time in rutin-treated rats is further evidence against the view that increased peripheral vasoconstriction occurs after rutin administration. These observations do not exclude the possibility that redistribution of local blood flow by closure of precapillary sphincters without major effect upon the arterioles may account for the results obtained with rutin.

2. Vasodilator drugs. Carbon dioxide, nitroglycerine and acetyl-β-methyl choline chloride (Mecholyl) were administered to a limited number of animals in order to observe the effect of vasodilatation on the course of severe cold injury.

Carbon dioxide, 7 per cent or 10 per cent in oxygen, was administered by inhalation to four animals after injury of the ear at $-$ 55° C. for 90 seconds (three animals) or 60 seconds (one animal). Inhalation of the CO_2 for 15 minutes following thawing resulted in an increase in respiratory rate and hyperemia of the uninjured ear. This treatment was repeated two or three times at 30-minute intervals. Complete loss of the part of the ear immersed in the freezing liquid occurred as it did in controls. Comparison of the time of appearance of gangrene in the frostbitten animals treated with CO_2 with the time of appearance of gangrene in control animals (1) indicates that gangrene was delayed about one day in the treated animals. Carbon dioxide administered by means of iontophoresis (one hour daily for four days) to the injured ear of two animals frostbitten for 90 seconds at $-$ 52° C. was not effective in accomplishing retention of frostbitten tissue. Wet gangrene appeared on the fourth and fifth days and dry gangrene on the fifth and sixth days after injury; these times are about one day later than in control animals.

Nitroglycerine was administered to two animals following frostbite of the ears. After 90-second immersion at $-$ 52° C., there were given intravenously 0.15 mgm. per kgm. nitroglycerine, 14 and 42 minutes after injury. After 60-second immerson at $-$ 55° C. this dose of nitroglycerine was given six times during the first hour after thawing. In these animals wet gangrene appeared on the fifth and fourth days and dry gangrene on the seventh and fifth days, respectively, after injury. Complete loss of the frostbitten portion of the ears occurred.

Mecholyl was administered by iontophoresis to one animal following frostbite of the foot for three minutes at $-$ 54° C. The treatment was repeated on the second day. Wet and dry gangrene appeared on the third and fifth days after injury. Tissue loss involved the entire injured portion of the foot except for a small amount of tissue on the plantar surface; such tissue loss is not different from that in untreated animals (1).

3. Anticoagulants. Dicumarol (3,3'-methylenebis [4-hydroxycoumarin]) was used in order to ascertain the effect of prolonged clotting time upon

the course of experimental frostbite. It is unsuitable as a possible therapeutic agent because of the slow onset of action. Dicumarol was administered by stomach tube in doses of 5 or 10 mgm. per kgm. (18). The mean prothrombin time, measured by the one-stage method of Quick with brain thromboplastin (19), was 17 seconds before treatment and 87 seconds at the time of frostbite. In four of six animals treated with dicumarol, fatal hemorrhages occurred on the day of frostbite or on the following day so that results could not be determined. In two animals, following frostbite of one foot at − 55° C. for three minutes, tissue loss involved the entire injured part of the foot with the exception of a tongue of tissue in the plantar surface; similar results were obtained in four of 15 control animals (1).

Heparin was administered intravenously to five animals after frostbite of one foot at − 55° C. for three minutes (three animals) or two minutes (two animals). The treatment with heparin, 10 mgm. per kgm. intravenously, was continued for three days; at this time wet gangrene had appeared in all animals and further administration of heparin was deemed useless. This dose of heparin was given to one animal daily, to a second every 12 hours and to a third (two-minute frostbite) every eight hours. In two animals (one frostbitten three minutes and one two minutes) external pressure dressings were applied two hours after injury and left in place for six days; heparin, 10 mgm. per kgm., was administered to these animals every 12 hours for three days. The tissue loss in the last animal mentioned above involved the toes only; this was the result to be expected from this duration of injury without treatment (1). All other animals treated with heparin, or with heparin plus pressure dressing, lost the entire frostbitten portion of the foot to the level of immersion. In these experiments heparin appeared to be completely without benefit, and the addition of heparin administration appeared to vitiate completely the beneficial results ordinarily obtained with pressure dressings alone (5).

4. Procaine block of the stellate ganglion. Interruption of the sympathetic nerve supply to extremities injured by cold has been described as an important aid in the treatment of frostbite (20, 21). An experimental approach to this problem was reported by Orlov (22). Rat tails exposed to ethyl chloride spray for two minutes became necrotic if left untreated, but if nerve block was produced by procaine infiltration of the tissues at the base of the tail during the first few hours after injury necrosis was prevented. The procedure employed by Orlov does not permit clear-cut distinction between effects which may be the result of sensory paralysis and those which are the result of interfering with the sympathetic supply.

In the present study both ears of six rabbits were frostbitten by immersion for one minute at − 55° C. This degree of injury produced a uniform series of changes followed by gangrene and loss of tissue in control animals (1). Procaine block of the stellate ganglion on one side was produced from three to 125 minutes after freezing, while the animal was still under the anesthetic (Dial). In four of these animals a second procaine injection was made on the day following injury. Usually 1.0 ml. of 2 per cent procaine solution with 1:30,000 epinephrine was introduced, but in some cases as much as 2.0 ml. were used because of failure to obtain prompt vasodilation. The preparation was judged to be a satisfactory one only if a marked and persistent increase of 4° to 5° C. in skin temperature could be demonstrated in the ear on the blocked side. The effect of procaine block of the stellate ganglion on the local circulation of the ear following cold injury has already been presented (16). Complete loss of the distal part of the ear to the level of immersion occurred. The only difference which appeared between the ears subjected to procaine block and those which were untreated was a delay of about one day in the development of wet and dry stages of gangrene. Wet gangrene appeared on the second day in one animal, on the third day in two and on the fourth day in three. Dry gangrene appeared on the fourth day in two animals, on the fifth in three and on the sixth day in one.

5. Miscellaneous. The possible usefulness of a number of other drugs and hormones in the treatment of experimental frostbite, with the aim of prevention of gangrene, was briefly explored in rabbits after injury of the ear for one minute at − 55° C. or the foot for three minutes at − 55° C. Vasoconstrictors (epinephrine and/or synephrine), suggested by the work of Lake (23),

did not influence the development of gangrene. Desoxycorticosterone and progesterone, selected for their possible effect on capillary permeability (*cf.* 24) and water retention (25), were also without benefit. In addition a limited number of experiments were carried out to test the effect of increasing blood volume by whole blood transfusion, or increasing the colloidal osmotic pressure of the plasma by the administration of 25 per cent human plasma albumin [4] and of increasing the sodium ion concentration in the extracellular phase. Because of the small number of experiments and the uniform failure of any of these agents to delay or prevent gangrene following standard cold injury, these results are not presented in detail.

DISCUSSION

In view of the generally unfavorable results obtained in the experimental treatment of severe cold injury by means of chemical agents, the response of such injuries to treatment with rutin represents a singularly interesting exception. It is of interest that during the last war Greene noted that the incidence of "shelter foot" was high among those deficient in Vitamins C and P (26) and Lake (7) has recommended the use of these vitamins in the treatment of cold injury. Not only is the favorable response of frostbite to treatment with rutin of therapeutic interest but it may serve also as a possible means of elucidating the mechanism of action of substances having Vitamin P activity as well as the nature of cold injury itself.

Most of the literature on rutin and related substances has thus far consisted of clinical reports and has emphasized the use of flavonol glycosides in conditions characterized by spontaneous capillary bleeding, believed to be the consequence of increased capillary fragility. Nearly all of the tests used as a basis for judging responses to treatment have involved some measure of the tendency of capillaries to rupture under increased internal pressure. This preoccupation with reduced incidence of capillary bleeding as a manifestation of rutin action has directed attention toward a phenomenon which is relatively non-specific. Capillary rupture may be the consequence of a wide variety of injurious influences, only some of which are similar to those encountered in scurvy and related deficiency states.

Among those reports in which Vitamin P-like substances are said to influence the permeability of capillaries as well as their tendency to rupture, we are aware of only one which presents evidence indicating specific study of this point (11). While injuries produced by cold have been reported to be accompanied by extravasation of blood, presumably from capillaries (8), the progress of severe cold injury to gangrene and loss of tissue is not always associated with rupture of small blood vessels. On the other hand, massive edema produced by loss of protein-rich fluid from the blood stream is an invariable consequence of such injuries. To the extent that the natural history of capillary hemorrhage may be described correctly in terms of a process having increased permeability as an early feature and complete loss of continuity as a late manifestation, the favorable action of rutin in reducing the amount of tissue lost from gangrene after cold injury and in reducing the incidence of capillary bleeding in other conditions (*cf.* 15) may be fundamentally similar.

A recent paper by Lee and Lee (27) presents excellent evidence that the stage of bleeding in experimental scurvy is preceded by marked engorgement of the true capillaries and venules. These authors have shown also that this engorgement is associated with a remarkable decrease in the sensitivity of metarterioles and precapillary sphincters to the application of epinephrine.

The extent of gangrene after cold injury has been reduced greatly by measures designed to control the amount of edema (5). Padded nondistensible dressings applied before swelling or pressure bandages applied after maximal swelling, were effective in saving about the same amount of tissue saved by treatment with rutin. Since neither of the physical methods of treatment prevented stasis in true capillaries or early loss of plasma from these damaged vessels, the preservation of injured tissue appeared to depend upon other factors.

Stasis in true capillaries of the ear was delayed, but not completely prevented, after frostbite in an animal treated with rutin. Gangrene and loss of tissue occurred in these frostbitten ears as well as in those of animals receiving no rutin. Prevention of gangrene by rutin was observed only in

[4] The human plasma albumin was kindly supplied by Cutter Laboratories, Berkeley, California.

rabbit feet injured by cold. The successful results obtained following the use of rutin by mouth as late as five minutes after injury suggest that the mechanism of its action is not the prevention of primary leakage and stasis in the true capillaries. It is more likely that rutin delays or prevents the gradual extension of vascular occlusion, ordinarily not complete in untreated frostbite in rabbits until about 50 hours after injury (2).

In any capillary system responding to injury by loss of the usual ability to retain plasma protein, leakage of protein continues only as long as blood flows through injured capillaries. Accumulation of packed red blood cells or occlusion of capillaries by contraction of precapillary sphincters (28) halts the flow of blood into these vessels and therefore stops the loss of protein-containing fluid. It is probable that closure of true capillaries by packed red cells after frostbite and possibly after prolonged application of tourniquets (29, 30) is responsible for progressive reduction in the rate of protein loss into the injured region with time after these injuries. In both these types of injury the protein concentration of edema fluid is high initially and is later progressively reduced. The data of Glenn *et al.* (31) show that the application of plaster casts to burned feet of animals sharply reduces the rate of loss of protein from the blood in the injured region.

The examples cited in the preceding paragraph present some circumstances in which there is reduction in rate of protein loss from injured capillaries, yet for none of these examples is there any convincing evidence that the reduced rate of loss is the result of decreased capillary permeability. In the case of frostbite, direct observations of capillaries (16), as well as indirect evidence based upon tests with fluorescein (32), indicate that the slowed rate of protein loss in the later stages of injury depends upon reduction of total area available for filtration of material from the capillaries.

The above considerations serve to emphasize the need for recognizing in phenomena, such as increased loss of protein from capillaries and perhaps even increased capillary fragility, the possible role of changes in the pattern of local blood flow. The experiments presented in this report on rats injected with trypan blue showed that the dye was slower to appear in frostbitten skin areas of rutin-treated animals than in controls. A similar effect of rutin in delaying the loss of dye following other types of injury has been reported by Ambrose and DeEds (33). Such differences could be produced either by decrease in the permeability of individual capillaries or by reduction of the total capillary area available for exchange in treated rats. Direct evidence permitting a decision between these two mechanisms is now available. Soluble preparations of various materials having Vitamin P activity have been reported to produce closure of precapillary sphincters and metarterioles upon topical application to the exposed rat mesoappendix (34). Intravenously administered rutin causes a 2.5-fold increase in sensitivity of vessels in the rat mesoappendix to topically applied epinephrine (35). This evidence, together with the response of cold injury to various forms of treatment (5), suggests that reduction of the size of the capillary bed is the dominant factor.

In the experiments reported here, heparin was ineffective in preventing tissue loss following frostbite of rabbit feet at − 55° C. Our data are not directly comparable to those of Lange *et al.* (9, 36), who reported beneficial effects of heparin therapy in experimental frostbite, since they used temperatures of −12° to − 20° C. and studied abdominal skin as well as rabbit feet. Frozen areas of abdominal skin overlie muscle and viscera through which blood flow continues during freezing, and the pattern of local blood flow and vertical temperature gradients in the abdomen are dissimilar to those in the extremities. Furthermore, in our experience (1), the temperature range of − 12° to − 20° C. used by Lange is one in which freezing of rabbit feet does not invariably occur even after exposure for periods as long as one hour. Gangrene does not occur without actual freezing if the period of exposure is as brief as one hour. The recent experiments of Quintanilla *et al.* (10), who were also unable to demonstrate the effectiveness of heparin in the treatment of frostbite of rabbit feet, illustrate the variability in the severity of injury in this temperature range.

SUMMARY

The effectiveness of a number of chemical agents in preventing gangrene following standardized severe cold injury to the feet and ears of rabbits has been studied. Rutin, 50 to 100 mgm. per kgm. per day by stomach tube, was effective in

restricting the loss of tissue from gangrene following frostbite of rabbit feet. In 15 control animals, immersion of the foot to the level of the tuberosity on the fifth metatarsal in liquid at − 55° C. resulted in complete loss of the exposed part in 11 cases and loss of all but a narrow portion of the plantar pad in four cases. Of ten animals similarly exposed but treated with rutin, nine lost only toes and one lost toes plus about 1 cm. of foot. Rutin was ineffective in preventing loss of tissue following frostbite of rabbit ears.

Rutin-treated animals did not develop stasis in the true capillaries of frostbitten ears as early as did untreated animals. The delay in onset of stasis was similar to that observed after procaine block of the stellate ganglion or rapid thawing of the frozen ear in water at + 42° C.

Rutin was found to delay the escape of trypan blue dye from the blood stream into frozen areas of abdominal skin in rats. The time from injection of the dye to first appearance of blue in the frostbitten areas of animals receiving rutin was approximately twice as long as that in controls. Vasoconstriction induced by intraperitoneal injection of Privine did not delay the appearance of trypan blue in frostbitten areas in spite of the marked blanching produced in normal skin areas, and the circulation time as measured by fluorescein was not increased by treatment with rutin. It therefore seems doubtful that rutin exerts its effect through peripheral vasoconstriction involving arterioles. Evidence is presented for the view that alteration in the pattern of blood flow through the capillary bed is a consequence of the administration of rutin.

Procaine block of the stellate ganglion delayed but did not prevent the development of gangrene in frostbitten ears.

The following drugs and hormones were found to be ineffective in the prevention of gangrene following frostbite.

1. Vasodilator agents: carbon dioxide, nitroglycerine and acetyl-β-methyl choline chloride (Mecholyl).

2. Anticoagulants: heparin and dicumarol.

3. Vasoconstrictors: epinephrine and/or synephrine.

4. Steroid hormones: desoxycorticosterone and progesterone.

Alterations of blood volume, plasma colloidal osmotic pressure and extracellular phase volume by the use of whole blood transfusion, administration of concentrated human plasma albumin and intravenous injection of sodium chloride solutions, all failed to prevent gangrene following standard cold injury.

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