

EFFECTS OF TWO CATIONIC TRIPHENYLMETHANE DYES ON THE HEALING OF SKIN INCISIONS

A Tensiometric and Histologic Study in the Rat

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Abstract. The strength of sutured skin incisions in rats was recorded 7 days after exposure to two different cationic triphenylmethane dyes, crystal violet and brilliant green, in aqueous solutions. Control wounds were treated with distilled water. The dyes significantly reduced the breaking load of the wounds. The development of strength in wounds treated once with crystal violet was followed for 21 days after surgery. The breaking load of dye-exposed wounds was significantly lowered at 7, 10, 14 and 21 days after wound infliction as compared with control wounds. The dye increased and prolonged the inflammatory reaction after the operative trauma, and delayed the onset of fibroplasia and collagen formation. The results obtained from incisional wounds are probably valid also for open wounds.

In a previous study in rats (14) crystal violet, a topical antimicrobial agent, in dilutions from 1:100 to 1:10 000 inhibited the growth of granulation tissue into subcutaneously implanted cellulose sponges. These findings correspond to results in tissue culture, where this and related triphenylmethane dyes significantly hampered the multiplication of fibroblasts and epithelial-like cells (16).

It has been stressed that antiseptics should be tested for possible tissue-damaging potency (21) as well as antibacterial efficacy (20) using methods as closely as possible reflecting the conditions under which they are clinically used. The results from in vitro and sponge implantation studies are not necessarily valid for the healing of wounds. Rydberg & Zederfeldt (22) have shown that there seems to be a certain tolerance or threshold to tissue injury in wounds, as measured by the tensile strength. The level of tissue regeneration at different time intervals after exposure to these

dyes, widely used in clinical practice, is not known.

The aim of the present investigation was to study to what extent crystal violet affects tissue regeneration in healing skin incisions as revealed by breaking load determination and histopathologic examination. For comparison, a minor study was made with brilliant green.

MATERIAL AND METHODS

Animals. Seventy male albino rats of the Sprague-Dawley strain with an average weight of 300-400 grams were used for tensiometric studies, divided into 9 groups (A-I) according to Tables I and II. Eight additional rats were used for histologic examinations. The animals were kept in separate cages at room temperature, fed a standard diet and given water *ad libitum*.

Infliction of wounds. Under ether anaesthesia, applied by the open mask method, the backs were shaved with electric clippers. The midline of the back and the positions of the incisions and stitches were drawn on the skin in order to achieve uniformity.

Two 6 cm longitudinal incisions were made symmetrically 1.5 cm from and parallel to the midline, one on each side (Fig. 1). The incisions were perpendicular to the skin surface and included the skin and the underlying subcutaneous muscle. While the wound margins were elevated with small hooks, the cavity of the wound was filled with 3 ml of the experimental solution or the same volume of sterile water. The incision was then closed by continuous through-and-through sutures with stitches 5 mm apart and 5 mm from the wound edge, taking in the skin and the subcutaneous muscle. Black braided silk (No. 000) and curved triangular needles (No. 18) were used. The fluid was left in the wound cavity for about 5 minutes while the wound was sutured. Before the sutures were tightened and tied the fluid was allowed to escape. The wound edges were meticulously apposed. No wound dressings were used.

Table I. Effect of triphenylmethane dyes on breaking load of wounds 7 days after wound infliction and dye instillation

Group	Dye	Concentration	No. of animals	Breaking load (newton) (mean $\pm$ S.E.)		Difference, % (Mean $\pm$ S.E.) ^a	Level of significance
				Test wounds	Control wounds		
A	Brilliant green	1:2 000	5	1.92 $\pm$ 0.20	3.65 $\pm$ 0.42	-43.8 $\pm$ 8.8	0.01 $> p >$ 0.001
B	Crystal violet	1:1 000	5	1.07 $\pm$ 0.25	3.82 $\pm$ 0.55	-70.4 $\pm$ 7.0	$p <$ 0.001
C	Crystal violet	1:2 000	10	1.62 $\pm$ 0.13	4.20 $\pm$ 0.22	-60.9 $\pm$ 3.3	$p <$ 0.001
D	Crystal violet	1:10 000	10	2.85 $\pm$ 0.32	3.75 $\pm$ 0.34	-24.1 $\pm$ 3.8	$p <$ 0.001
E	Crystal violet	1:20 000	5	3.38 $\pm$ 0.45	4.03 $\pm$ 0.39	-17.6 $\pm$ 5.8	0.05 $> p >$ 0.01
F	Crystal violet	1:50 000	9	2.94 $\pm$ 0.29	3.75 $\pm$ 0.31	-20.4 $\pm$ 7.0	0.05 $> p >$ 0.01

^a Calculated on values from symmetrical wound pairs. Mean = (test value - control value)/control value.

Solutions. Aqueous solution of crystal violet (C1 42555) in dilutions of 1:1 000, 1:2 000, 1:10 000, 1:20 000 and 1:50 000 (in per cent: 0.1, 0.05, 0.01, 0.005 and 0.002 respectively, w/v). Brilliant green (C1 42040) 1:2 000 (0.05%) in water. For the control wounds distilled water was used. All solutions were sterile.

Crystal violet is a constituent of gentian violet (13), and brilliant green is part of the "triple dye" solution.

Tensiometry. The healing periods were 7, 10, 14 and 21 days (Tables I and II). The sutures were removed immediately before tensiometry in the 7- and 10-day-old wounds, and in those tested at 14 and 21 days on day 10. Animals with stitch abscesses were discarded. The breaking load (in newton units¹) of each wound was measured *in situ* with a special type of tensiometer originally described by Sandblom, Petersen & Muren (25) and as modified by Holm-Pedersen & Zederfeldt (10).

¹ N = newton, international force unit; 1 N = 0.102 Kp (kilogram-force) = 0.225 lb_f.

Three segments of each wound were examined and the mean of these measurements was taken as the breaking load value of that wound.

The difference in breaking load between the dye-exposed and the control wound of each animal is given as a percentage of the value for the control wound according to principles discussed by Zederfeldt (30). Generally accepted methods were used for statistical analysis (28). Estimations of significance of differences were made using Student's *t*-test.

Histology. In the 8 additional rats used for histologic study, the dye-exposed wounds were treated with crystal violet 1:2 000. Seven days after wound infliction, specimens from 4 rats were excised from symmetrical parts of control and dye-treated wounds and 3 weeks after surgery from the other 4 rats. The specimens were stretched to the *in vivo* size and shape, mounted on cork-plates and fixed for 3 hours in Bouin's solution. They were further processed in the conventional way for paraffin embedment. The sections were stained with

Table II. Effect of crystal violet 1:2 000 on breaking load of wounds after 7-21 days of healing

Group	Healing period (days)	No. of animals	Breaking load, newton (mean $\pm$ S.E.)		Difference, % (mean $\pm$ S.E.) ^a	Level of significance
			Test wounds	Control wounds		
C	7	10	1.62 $\pm$ 0.13	4.20 $\pm$ 0.22	-60.9 $\pm$ 3.3	$p <$ 0.001
G	10	5	4.98 $\pm$ 0.39	6.52 $\pm$ 0.55	-22.2 $\pm$ 6.4	0.05 $> p >$ 0.01
H	14	10	9.82 $\pm$ 0.38	11.74 $\pm$ 0.41	-16.2 $\pm$ 1.7	$p <$ 0.001
I	21	8	17.10 $\pm$ 0.60	21.29 $\pm$ 1.25	-17.5 $\pm$ 6.0	0.05 $> p >$ 0.01

^a Calculated on values from symmetrical wound pairs. Mean = (test value - control value)/control value.

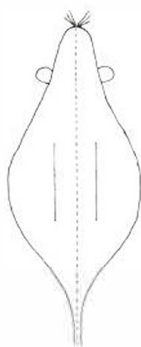


Fig. 1. Location of skin incisions.

haematoxylin-eosin, according to Van Gieson, McManus' periodic acid method and with toluidine blue at pH 0.5 and pH 4.0. The evaluation was performed on coded slides. The experimental and the control wound in each animal were compared with regard to degree of inflammatory reaction, fibroplasia (amount of fibroblasts), tissue damage and formation of collagen fibres and ground substance.

RESULTS

Breaking load. The results of the breaking load measurements 7 days after wound infliction are shown in Table I. Differences in strength between test and control wounds are expressed as percentages of the control wound's breaking load. The breaking load of wounds exposed to crystal violet was lower for all concentrations of the dye tested than for control wounds, as well as for wounds treated with brilliant green (1:2 000). The differences in breaking load between test and control wounds are significant.

For healing periods of 10, 14 and 21 days the results are shown in Table II and Fig. 2, which also includes the standard error at each point. The breaking load of wounds treated with crystal violet (1:2 000) increased during the entire period but was significantly lower for all post-surgical periods than the breaking load in control wounds. Between 7 and 21 days postoperatively, the slopes of the regression line of strength of control wound to this period of healing ($y = 1.25x - 5.31$) and of the regression line of strength of test wound to the same healing period ($y = 1.11x - 6.04$) are almost equal.

Histologic examination. The results are shown in Table III. The dye-treated wounds disclosed at 7 days a more pronounced inflammatory reaction and a less pronounced fibroplasia and collagen formation. All dye-treated wounds showed a

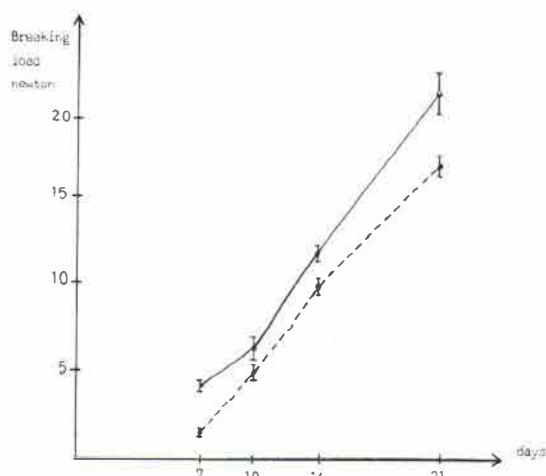


Fig. 2. Effect of crystal violet 1:2 000 on breaking load of wounds after 7–21 days of healing. ---, Test; —, control. Range bars indicate S.E.M.

cleft in the subpannicular layer lined by a thin layer of fibroblasts and inflammatory cells, and the cut ends of the pannicular muscle showed basophilia and shrinkage of the muscle bundles, a sign of tissue damage. Only one water-treated wound had a small subpannicular cleft and signs of moderate tissue damage. The pattern was about equal in the dermis and in the subpannicular layer. There were no obvious differences in formation of ground substance.

In the 21 day wounds the inflammatory reaction was, on the whole, mild and remained mostly as a foreign body reaction around debris in the wound cleft or remnants of a subpannicular cleft, which explains the two water-treated wounds with more pronounced reaction. The degree of fibroplasia seemed about equal but the fibroblasts of

Table III. Wounds treated with crystal violet 1:2 000 as compared with control wounds treated with water

± = more pronounced, 0 = no difference, — = less pronounced reaction in crystal violet treated wounds

Age of wound	No. of pairs of wounds	Inflammatory reaction			Fibroplasia			Collagen		
		+	0	—	+	0	—	+	0	—
7 days	4 subcutis	3	1	0	0	1	3	1	0	3
		3	1	0	0	0	4	0	1	3
21 days	4 subcutis	2	1	1	1	3	0	0	2	2
		0	2	2	0	4	0	0	4	0

the dye-treated wounds were more immature. The collagen formation also seemed to be about equal but again the fibres of the dye-treated wounds were more immature, e.g. the water-treated wounds disclosed more closely packed and coarser collagen fibres.

A rough estimation of the width of the wound cleft showed that the dye-treated wounds, with exception of one 7 day wound, had a slightly broader cleft than the water-treated control wound. This was more marked in the 21 day wounds, where the sutures had been removed at 10 days of healing.

DISCUSSION

In the present study the strength of wounds at different post-surgical intervals has been determined *in situ*. This method has been used extensively for the study of systemic and local factors influencing the healing process (cf. 4, 17, 22–24, 30). The development of strength in healing skin incisions is functionally important, and the method is sensitive enough to record even minor variations of local factors in the wound region (26). As the present study is concerned with the effects of a local factor, the experimental design made it possible to compare the healing of the test and control wounds—symmetrically positioned and inflicted by a carefully standardized technique—in the same animal at the same time. In this way, both wounds were influenced by similar internal and environmental conditions as recommended by Sandblom (24). One drawback of this method is, however, that a wound cannot be examined on more than one occasion. Therefore if there is more than one point of observation, each is represented by its own animal group.

With the experimental design used, the breaking load should reflect the healing capacity of tissues. The breaking strength (breaking load value per square cm wound area) has not been determined, because skin thickness can be expected to be equal on both sides of the back of the rat as well as in the group of animals as a whole, all rats being about the same age and weight.

The determinants of the strength of an incised wound are different after various healing periods. The wound-healing process has been divided into three phases from tensiometric aspects: the lag or

latent phase, the period of fibroplasia, and the period of maturation (cicatrizization) (11). These phases can be correlated with histological observations. During the lag phase, which lasts 2–4 days in the rat (12), the breaking load is low and based mainly on the strength of the fibrin clot in the wound. Microscopically there is an inflammatory reaction with dilated blood vessels, exudation of plasma proteins and emigration of polymorphonuclear leukocytes and monocytes. Epithelialization is usually completed within 3–5 days and, if the wound margins are carefully apposed, as early as 24 hours (18). Then comes the period of fibroplasia. Capillaries and fibroblasts proliferate followed by an increase of the collagen concentration. At the end of this period, 2–3 weeks after wound infliction, the strength of the wound is about 20% of its ultimate level. Later, during the period of maturation, the breaking load increases slowly but without any increase of the collagen content of the wound (23, 29). This further increase of strength is probably caused by an increasing number of inter- and intramolecular bonds and remodelling of collagen fibres (19).

The results of the present investigation show that wounds treated once with crystal violet in concentrations much lower than used in practice have an impaired strength development after healing periods of 7–21 days. The difference in breaking load as compared with controls is greatest 7 days after wounding (–60.9%), and then diminishes for healing periods of 10, 14 and 21 days (day 10 –22.2%; day 14 –16.2%, day 21 –17.5%). This is compatible with an influence of crystal violet on the lag phase, which gets prolonged, thus causing delayed onset of the period of fibroplasia. When fibroblast proliferation and collagen synthesis have begun, the rate of gain of breaking load is almost as rapid in the dye-treated wounds as in control wounds. This is evident from the nearly identical slopes of the strength increment curves in Fig. 2.

The dynamic events of tissue inflammation dominate the lag phase. The operative trauma initiates an inflammatory reaction to which is added the foreign-body reaction from silk sutures (5). Water (hypotonic) instilled in wounds may also exert adverse effects on the tissues, even if it is not revealed by determinations of wound strength (22). According to Carrel (7), a certain

degree of inflammation is necessary for optimal repair. Abolition as well as increase of the inflammatory reaction will retard healing. It is still not known what is the optimal degree of inflammation for ideal wound healing. The control wound is certainly too traumatized to represent optimal healing conditions. Anti-inflammatory treatment or removal of the sutures after 3 days in comparable wounds increases the rate of healing, as measured by breaking load (3, 5). Any factor that further increases the inflammatory reaction or impairs the microcirculation in the wound area will have negative effects on the healing process.

The histologic studies of wounds 7 and 21 days after infliction confirmed that the cause of the lengthened lag phase in dye-treated wounds was an increase and prolongation of the acute inflammatory reaction. This is in agreement with our results of tissue growth into subcutaneously implanted dye-soaked sponges (14).

It is at present not known which tissue structures crystal violet interferes with in vivo during the lag phase. A pronounced cytotoxicity against fibroblasts and epithelial-like cells has been demonstrated in tissue culture (16). In the wound margins there are cells with reduced viability that should survive unless they are further damaged. These cells are probable targets for crystal violet, and the dye may also diffuse into the tissues, even injuring cells with full vitality. It is not known at present whether the dye also interferes with capillary function.

Plasma-proteins and polyanionic tissue compounds such as heparin and chondroitin sulphate may reduce the cytotoxicity of crystal violet solutions, probably by combining with the cationic dye (16). In the present wound healing study, the tissues were exposed to the dye too early after traumatization for any substantial neutralization of the dye to occur in this way.

In the literature there are only a few reports that similar dyes in therapeutically used concentrations may be toxic to exposed tissues. Cannon & Cope (6) painted donor skin areas of 3 patients with a mixture of gentian violet 3%, brilliant green 2% and acriflavine 2% ("triple dye"), and of 4 patients with gentian violet 2%. These wounds healed more slowly than control wounds treated with boric acid ointment. Dingwall & Andrus (8) obtained similar results in 4 humans

with skin burns treated with "triple dye" (gentian violet 2%, brilliant green 1% and acriflavine 0.1%). Baker (2) removed skin and subcutaneous tissues over thorax and abdomen in rats. The deep fascia and striated muscles were painted with "triple dye" (1.5% methylosaniline chloride, 1.5% brilliant green and 0.75% acriflavine). Of 10 rats treated this way, all developed severe muscle necrosis and 7 died. Microscopic changes in internal organs could not be demonstrated in the dead rats.

In clinical practice, wounds treated with gentian violet are mostly open, healing by second intention until they are infected and a need for antimicrobial therapy arises. In secondary healing wounds, wound contraction and epithelialization are of greater importance than in incisional wounds healing by primary intention. The results of this study should in principle, however, be valid even in open wound healing, as the healing process is regarded as qualitatively the same for wounds healing by first and second intention (1, 9, 27). There are only quantitative differences in the amount of newly formed tissue and healing time. The delayed formation of granulation tissue after exposure to crystal violet could indirectly affect the growth and function of the epidermis, the dermis and epidermis being an integrated system (15).

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