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Effect of Topically Applied β-Aminopropionitrile on Granuloma Tissue Biochemistry (42686)

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Abstract. The effects of the percutaneous transport of vehicles and the transport of β-aminopropionitrile (βAPN) in vehicles were studied in rats. The bioavailability of topically administered βAPN was determined by measuring the degree of collagen cross-linking inhibition in the underlying granuloma tissue. Granulomas were induced by subcutaneous implantation of polyvinylalcohol sponges. From the 4th to 12th days postimplantation, a 20 mg/cm^2 dose of βAPN fumarate was applied. Vehicles employed included dimethylsulfoxide (DMSO), urea, and occlusion. DMSO significantly enhanced the effect of βAPN in reducing the cross-linking of collagen. βAPN administered onto urea-pretreated skin and followed by occlusion in the granuloma tissue was more effective than βAPN in 30% DMSO, but only in the parameter reflecting extractibility of collagen into urea or thiocyanate solutions. The results suggest that βAPN administered topically in an appropriate vehicle penetrates the granuloma tissue and affects collagen polymerization. Though βAPN was topically administered, a systemic effect from the drug was evident, as documented by lower body weight of treated rats.

The long-term goal of our investigations has been to limit pathologic fibrotic formation, related to collagen maturation, by topically administering drugs, such as β-aminopropionitrile, which inhibit collagen cross-linking. The major limitation of topical drug administration to the skin surface is the imposing impermeability of the skin. It is well documented that the rate of percutaneous drug transport is limited mainly by the stratum corneum of the epidermal layer. Modifications of the stratum corneum either mechanically (stripping) or chemically are some of the ways of increasing the transport of drugs into the dermis and into the site of the injury. Several studies demonstrated that both urea (1) and DMSO (2) facilitate drug penetration. Another well-documented factor controlling percutaneous transport is the degree of skin hydration, again involving the stratum corneum (3).

β-Aminopropionitrile (βAPN) prevents the abnormal polymerization of collagen in a variety of fibrotic lesions (4). Our preliminary work on topical βAPN has shown that the drug can penetrate the skin and inhibit collagen cross-linking formation in sponge-induced subcutaneous granuloma tissue (5). To optimize skin penetration and to reach the injury site, we needed to find a vehicle for βAPN that would facilitate its transport across the stratum corneum barrier. Therefore, three different application methods were studied for improving the percutaneous absorption of βAPN: (i) adding DMSO to the topical formulation and painting it onto the skin, (ii) occluding the site with an impermeable film of Johnson's plaster, and (iii) pretreating the skin with urea and occluding.

Materials and Methods. A total of 20 female albino Wistar rats (120–145 g) was anesthetized with diethylether. A 2.5-cm skin area was closely shaved and implanted subcutaneously with two sterilized polyvinylalcohol (PVA) sponges (0.7 × 2.5 cm) through an incision made distal to the sponge placement (PVA sponges, Ivalon, Unipoint, Inc. NC). The animals were divided into four groups, one control and three experimental. The animals were fed commercial rat feed and given water *ad libitum*. The control group had 30% DMSO applied on the intact skin over the PVA sponge placement. In the three experimental groups, βAPN fumarate (Sigma Chemical Co., St. Louis, MO) was prepared in a 30% DMSO solution (200 mg/ml), and 0.1 ml/cm^2 was applied to a 2.5 cm^2 area once daily. The application methods of βAPN included (i) painting directly onto the skin, (ii) soaking

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gauze in the same volume of βAPN and occluding the application site with an impermeable membrane (Johnsonplast, Johnson & Johnson, Ltd., (India), and (iii) pretreating the shaved skin area with 20% urea for 5 min, followed by a thorough cleansing of the skin and then applying gauze-soaked βAPN and occluding, as indicated above. The treatment was started on the 4th day of implantation and continued until the 12th day. The animals were sacrificed at the end of the experimental period and the dissected sponges were analyzed as follows:

Chemical analysis. The harvested granuloma tissue was homogenized in saline and extracted with 5% trichloroacetic acid at 90°C for 30 min then analyzed for DNA (6), protein (7), and hydroxyproline (8) using either the supernate or the pellet for the appropriate analysis.

Extraction of collagen. The tissue was homogenized in 0.14 *M* NaCl at 4°C and extracted sequentially with 1.0 *M* NaCl in 0.05 *M* Tris–HCl buffer (pH 7.2) and 0.5 *M* acetic acid at 4°C for 72 hr, changing with fresh solution daily. An aliquot of each of the combined extracts was hydrolyzed with an equal volume of concentrated HCl and hydroxyproline content determined (8).

Solubility of insoluble residue in denaturing agents. The residue left after acid extraction was suspended in 6 *M* urea or 2 *M* KCNS at room temperature for 24 hr (9) and centrifuged at 20,000*g* for 30 min, and the amount of solubilized collagen was calculated by estimating the hydroxyproline content (8).

Estimation of aldehyde content. The collagen from neutral salt-soluble extract was precipitated with 15% NaCl (w/v) and purified according to the procedure of Piez *et al.* (10). The purified collagen was dissolved in dilute acetic acid and analyzed for aldehyde content (11).

Polyacrylamide gel electrophoresis. The subunit pattern of neutral salt-soluble collagen (NSC) was determined by subjecting denatured collagen samples to SDS–polyacrylamide gel electrophoresis using 5% gel (12). The amount of each component was calculated from the ratio of areas under each peak.

Results. The topical administration of βAPN onto intact skin may exert toxic effects, reflected in a significantly lower body weight and granuloma tissue weight (Table I). As shown by our previous investigations (13) only a small portion of topically administered βAPN fumarate (in 70% DMSO) will be resorbed within 24 hr, the amount not exceeding 10% of the administered dose. Thus, we assume that less than 5 mg βAPN was resorbed. While in this study the inhibition of body weight growth after topical βAPN was on average only 20%, parenteral administration of 40 mg βAPN per 100 g rat body weight for 14 to 21 days resulted in 60% inhibition of body weight (14).

The total content of granuloma tissue components, such as DNA, noncollagenous proteins, and collagen (hydroxyproline) (Table II) was not affected by the three βAPN modes of administration onto the intact skin. This finding agrees with studies of others using βAPN in systemic administration,

TABLE I. BODY WEIGHT GAIN OF RATS AND THE AMOUNT OF GRANULOMA WET TISSUE IN TOPICAL βAPN TREATMENT USING VEHICLES

Group[a]	Body weight (g)[c]		Granuloma tissue (g/pellet)[b,c]
	Initial	Final	
1. Control	135.0 ± 16.0	146.0 ± 17.0[d]	0.60 ± 0.13[d]
2. Painting	126.0 ± 7.0	134.0 ± 9.0	0.63 ± 0.08
3. Occlusion	133.0 ± 9.0	125.0 ± 10.0	0.54 ± 0.13
4. Urea + occlusion	133.0 ± 7.0	114.0 ± 9.0	0.44 ± 0.12

[a] Five rats in each.
[b] Granuloma tissue is the average of 10 sponge pellets.
[c] Variability is given as mean ± SD.
[d] Groups 1 and 2 are significantly higher than group 4 at $P < 0.05$.

TABLE II. CONTENT OF DNA, NONCOLLAGENOUS PROTEINS, AND TOTAL HYDROXYPROLINE IN GRANULOMA TISSUE TREATED BY TOPICAL βAPN ADMINISTERED THROUGH VARIOUS VEHICLES

Group	mg/g wet tissue		
	DNA	Noncollagenous protein	Hyp
Control	1.15 ± 0.21	57 ± 7.4	5.8 ± 0.77
βAPN			
Painted	1.18 ± 0.20	60 ± 5.3	5.9 ± 0.80
Gauze-occlusive	1.10 ± 0.21	62 ± 7.2	5.5 ± 0.62
Urea/gauze, occlusive	1.12 ± 0.22	58 ± 4.8	5.8 ± 0.85

Note. The data are given as $X \pm$ SD, based on five analyses per group.

where the drug effected only collagen polymerization (16–18). Thus, it appears that βAPN does not affect cell proliferation and protein synthesis by fibroblasts (19, 20).

The effect of the tested βAPN treatments on the extractibility of collagens into neutral salt (NSC) and acetic acid (ASC) is shown in Table III. A significant increase in NSC was observed equally in all βAPN-treated groups ($P < 0.005$) when compared to control. The extractibility of collagen into acetic acid was the same in all groups.

Insoluble collagen residues, left after sequential extraction of the granuloma tissue with 1 *M* NaCl and 0.5 *M* acetic acid and then extracted into 2 *M* KCNS, showed significantly elevated solubilities in βAPN-treated groups ($P < 0.01$–0.001) when compared to control (Table IV). The effectiveness of βAPN was also significantly enhanced by pretreatment of skin with urea. Similarly insoluble collagen showed significantly greater solubility in 6 *M* urea in βAPN-treated groups ($P < 0.001$) (Table IV).

TABLE III. SOLUBILITY PATTERN OF GRANULOMA COLLAGEN IN TOPICAL βAPN TREATMENT USING VEHICLES

Group	NSC % of total Hyp	ASC % of total Hyp
1. Control	32.57 ± 6.96	5.78 ± 2.16
2. Painting	67.24 ± 7.43[a]	5.35 ± 2.29
3. Occlusion	71.36 ± 9.09[a]	6.27 ± 2.33
4. Urea + occlusion	74.72 ± 10.76[a]	6.27 ± 2.64

Note. Variability is given as means ± SD; $N = 5$.
[a] $P < 0.005$ vs control.

Among the three experimental groups the effectiveness of the urea–gauze–βAPN treatment method with occlusion significantly increased ($P < 0.05$) the solubility of residual (insoluble) collagen, by either thiocyanate or by urea, than did the direct treatment method of βAPN topical administration in 30% DMSO, with or without occlusive dressing. The results indicate that topical administration of βAPN by the methods tested significantly interferes with intramolecular cross-linking within the collagen molecule (NSC, aldehyde content) and also with larger aggregates of collagen, which are cleaved by the action of urea or thiocyanate. Both agents are known to dissociate hydrophobic contacts and ionic bonds.

The aldehyde content of NSC was significantly less in all βAPN applications, as shown in Table V. This finding shows the indirect evidence of inhibition of lysyl oxidase activity with lower production of alde-

TABLE IV. SOLUBILITY OF INSOLUBLE COLLAGEN IN DENATURING AGENTS IN TOPICAL βAPN TREATMENT USING VEHICLES

Group	Liberated Hyp (μg/ml)	
	2 *M* KCNS	6 *M* urea
1. Control	3.78 ± 0.80	4.62 ± 0.76
2. Painting	7.35 ± 1.03[a]	8.40 ± 0.46[b]
3. Occlusion	8.78 ± 1.37[a]	9.25 ± 0.75[b]
4. Urea + occlusion	9.86 ± 0.76[b,c]	10.08 ± 0.88[b,c]

Note. Variability is given as mean ± SD; $N = 3$.
[a] $P < 0.01$ vs control.
[b] $P < 0.001$ vs control.
[c] $P < 1.05$ group 2 vs group 4.

TABLE V. ALDEHYDE CONTENT OF GRANULOMA NEUTRAL SALT-SOLUBLE COLLAGEN IN TOPICAL βAPN TREATMENT USING VEHICLES

Group	Aldehyde content (μmole/100 mg)
1. Control	3.12 ± 0.33
2. Painting	1.72 ± 0.38[a]
3. Occlusion	1.52 ± 0.53[b]
4. Urea + occlusion	1.46 ± 0.41[a]

Note. Variability is given as mean ± SD; $N = 3$.
[a] $P < 0.01$ vs control.
[b] $P < 0.02$ vs control.

hydes derived from oxidative deamination of ε amino groups of some lysyl or hydroxylysyl residues in the collagen polypeptide chain.

The densitometric pattern of polyacrylamide gel electrophoresis of NSC subunits is given in Fig. 1. Table VI presents the statistical evaluation of the pattern. The data show that treatment with βAPN resulted in a significant increase of α_1, α_2, β and α/β ratio in all treatment methods. The α/β ratio data indicates that the combination of all treatment methods (Group 4) resulted in the significantly highest βAPN effect.

Discussion. The use of DMSO, urea, and occlusion to increase the magnitude of percutaneous drug transport has been well documented. DMSO dissociates the lipid–lipid and lipid–protein interaction of the stratum corneum, which possibly enhances penetration (21). An occlusive covering on the skin reduces vapor loss from the skin, thus causing hydration of the stratum corneum (22). The keratolytic effect and increased water binding mediated by urea in the horny layer enhances drug permeation (1). Thus, vehicles form an integral part of the topical therapy and exert a profound effect on the penetration of the active drug across the stratum corneum barrier (3).

Of the three vehicle methods of percutaneous administration of βAPN under study, DMSO alone was found to profoundly increase the penetration of βAPN when compared to βAPN in saline (21). Occlusion alone did not improve penetration of βAPN when compared to βAPN in a 30% DMSO solution. A possible explanation is that (a) βAPN, being a small molecule, is readily diffusible (24) and (b) its high solubility in both water and DMSO favors easy penetration through the lipid-rich stratum corneum and water-rich dermis. Several reports have suggested that DMSO alone can alter collagen metabolism (23–25) and, therefore, in this study DMSO may be enhancing the effect of βAPN. However, in our recent publication (21) we found no difference between saline and 30% DMSO in the effect on the extractibility of collagen into neutral or acid solutions. The percentage of β-collagen subunits was significantly lower and α-collagen significantly higher in neutral salt-soluble collagen extracted from the granuloma tissue. We believe that DMSO at 30% concentration is not strong enough to elicit the reported effects on collagen structural stability. However, there is some evidence (Tables I and IV) indicating that pretreatment of the intact skin with urea renders the skin more permeable to the medication. This is shown by a significantly lower body weight, the granuloma tissue weight and increased extractibility of insoluble collagen by thiocyanate and urea, as well as by a significantly higher α/β collagen chain ratio of skins exposed first to urea and then treated with βAPN in 30% DMSO. Possibly, the urea solution alone administered onto the intact skin over the subcutaneously inplanted Ivalon sponges contributes to the dissociation of cohesive forces stabilizing collagen structure. Unfortunately, in our experimental design we did not study the effect of the urea vehicle on the tested parameters.

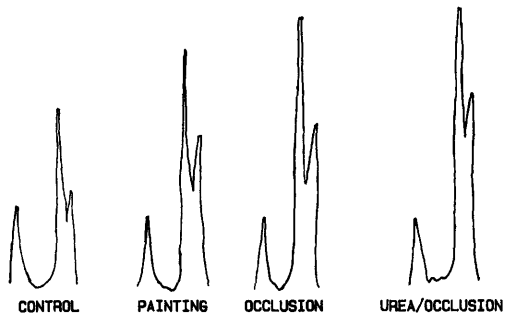

FIG. 1. Densitometer tracing of disc gel patterns obtained from neutral salt-soluble collagen extracted from granuloma tissue of control and experimental groups (βAPN administered in DMSO, with and without occlusion, and urea/occlusion).

TABLE VI. SUBUNIT COMPOSITION OF GRANULOMA NEUTRAL SALT-SOLUBLE COLLAGEN IN TOPICAL βAPN TREATMENT USING VEHICLES

Group	Percentages of subunit			
	α_1	α_2	β	α/β
1. Control	41.03 ± 1.05	20.51 ± 0.53	38.44 ± 1.58	1.60 ± 0.10
2. Painting	52.83 ± 0.52	26.44 ± 0.06	20.72 ± 0.45	3.82 ± 0.10[a]
3. Occlusion	58.53 ± 1.20	27.03 ± 0.67	19.43 ± 1.86	4.17 ± 0.52[a]
4. Urea + occlusion	54.73 ± 0.74	26.85 ± 0.13	18.41 ± 0.77	4.43 ± 0.28[a]

Note. Variability is given as mean ± SD; $N = 3$.
[a] $P < 0.001$ vs control.

This study shows that βAPN delivered locally to the site of the fibrotic injury by percutaneous absorption interferes with collagen polymerization. The results indicate the effectiveness of topical βAPN administered on the skin adjacent to the underlying fibrotic lesion. Even at these low dosages of topically administered βAPN in the vehicles shown, there is an indication of a systemic toxic effect as reflected the animal weight gain. Interestingly, in a similar study (21) no toxic effect was observed when βAPN was administered only in 30% DMSO. We believe that clinical pathologies, such as peritendinous adhesion, perineural adhesion, or scar contractures may be affected by the above treatment.

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