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Protective value of skin tanning induced by ultraviolet radiation plus a sunscreen containing bergamot oil

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Synopsis

Some sunscreen formulations utilize bergamot oil to enhance tanning induced by ultraviolet radiation. To determine the tanning capabilities of such a product, and to assess the protective value of the induced tan, we chose the skin of miniature pigs to test a sunscreen-psoralen product (5% ethyl hexyl cinnamate + bergapten 30 ppm, Laboratoires Goupil S.A.). Material was applied to the skin prior to ultraviolet radiation exposure during the induction phase of the study, and pigmentation was assessed visually. Subsequently, test sites were challenge-irradiated, and erythema production and sunburn cell formation were assessed. The product was effective in inducing tan without erythema, and the induced tan was effective in protecting the skin from subsequent exposure to ultraviolet radiation as assessed by sunburn cell formation.

INTRODUCTION

Sunscreen preparations containing bergamot oil are commercially available (1–3). This combination can be regarded as a sunscreen, protecting against acute ultraviolet radiation (UVR) damage, to which bergamot oil, as a source of bergapten (5-methoxypsoralen), is added to enhance melanin production (tanning). Theoretically, the level of furocoumarin is less than that required to elicit phototoxicity, but sufficient to induce tanning.

To evaluate a product of this type in an animal model, certain criteria have to be met. The model should have morphologic and physiologic characteristics similar to man, it should display tanning capability comparable to man, and there should be methodologies available to assess acute damage in non-tanned and tanned skin. Recently, the skin of miniature swine has been found to meet these criteria (4).

This study was designed to determine whether a bergamot oil-containing sunscreen product was effective in enhancing UVR-induced tanning of swine skin without provoking phototoxicity, and whether the induced tan afforded protection against subsequent exposure to UVR. For the latter determination both erythema induction and sunburn cell (SBC) production in the epidermis were used to assess acute damage.

MATERIALS AND METHODS

ANIMALS

Four closed colony Yucatan miniature pigs [Buk:(TRF)YS, Buckshire Corporation, Perkasie, PA] were used. The animals (3 females and 1 male) weighed about 15 Kg, were 4 to 5 months old, were fed commercial hog chow (Ralston Purina Co., St. Louis, MO) once a day, and had water available freely.

The normal skin of these animals has been described (4,5). Briefly, the skin was sparsely haired, and had areas which, to the eye, appeared white (i.e., non-pigmented), while other areas appeared naturally pigmented (Figure 1).

TREATMENT MATERIALS

Four lotions were received from Laboratoires Goupil S.A. (Cachan, France). "Vehicle," "Vehicle + Bergapten," "Vehicle + Bergapten + Sunscreen," or "Vehicle + Sunscreen." According to the supplier, the sunscreen ingredient was 5% ethyl hexyl cinnamate, and the source of bergapten (30 ppm) was bergamot oil. The dosage of bergapten in the finished product was determined with high performance liquid chromatography by the supplier (6).

TREATMENT AND IRRADIATION

The study was divided into two phases: an "induction phase" during which combinations of treatment materials and UVR were used in an attempt to induce pigmentation in non-pigmented skin, and a "challenge phase" during which the skin sites under investigation were challenged with a series of UVR exposures to determine the protective value of induced or natural pigmentation.

INDUCTION PHASE

On one of the female pigs, both sides were used; otherwise, only one side of each pig was used. On the first day of the "induction phase," each pig was anesthetized and its hair was clipped as described previously (4,5). Eight rectangular sites (2 × 6 cm) were demarcated on appropriate skin types (i.e., non-pigmented or pigmented) with adhesive tape according to the experimental protocol (Table I), labeled with a felt-tipped permanent marking pen, and treated with the appropriate test materials (2 $\mu l/cm^2$) using a Hamilton syringe (Hamilton Company Inc., Whittier, CA). Thirty minutes later irradiation was started with a long-arc 6 kW xenon lamp filtered with WG320 Schott glass (1-mm thick) to simulate approximately 30°N latitude mid-summer solar UVR spectrum (7). Induction sites III, IV, V, and VI were irradiated for approximately 40 minutes [accumulating 200 Robertson-Berger (RB) counts] and then covered with aluminum foil; irradiation of sites VII and VIII was continued to 400 RB counts (approximately 80 minutes). The 400-RB-count irradiation was approximately an exposure needed to produce barely perceptible erythema in the non-pigmented skin sites of these animals. The range of intensity, measured with a Sunburning Ultraviolet Meter (RB meter, Solar Light Co., Philadelphia, PA) was 0.68–0.73 sunburn units (SBU) per hour (8); the approximate incident doses (not biologically weighted) from 250 to 400 nm were 640 and 1280 J/m^2 for the incident dose analogs of 200 and 400 RB counts,

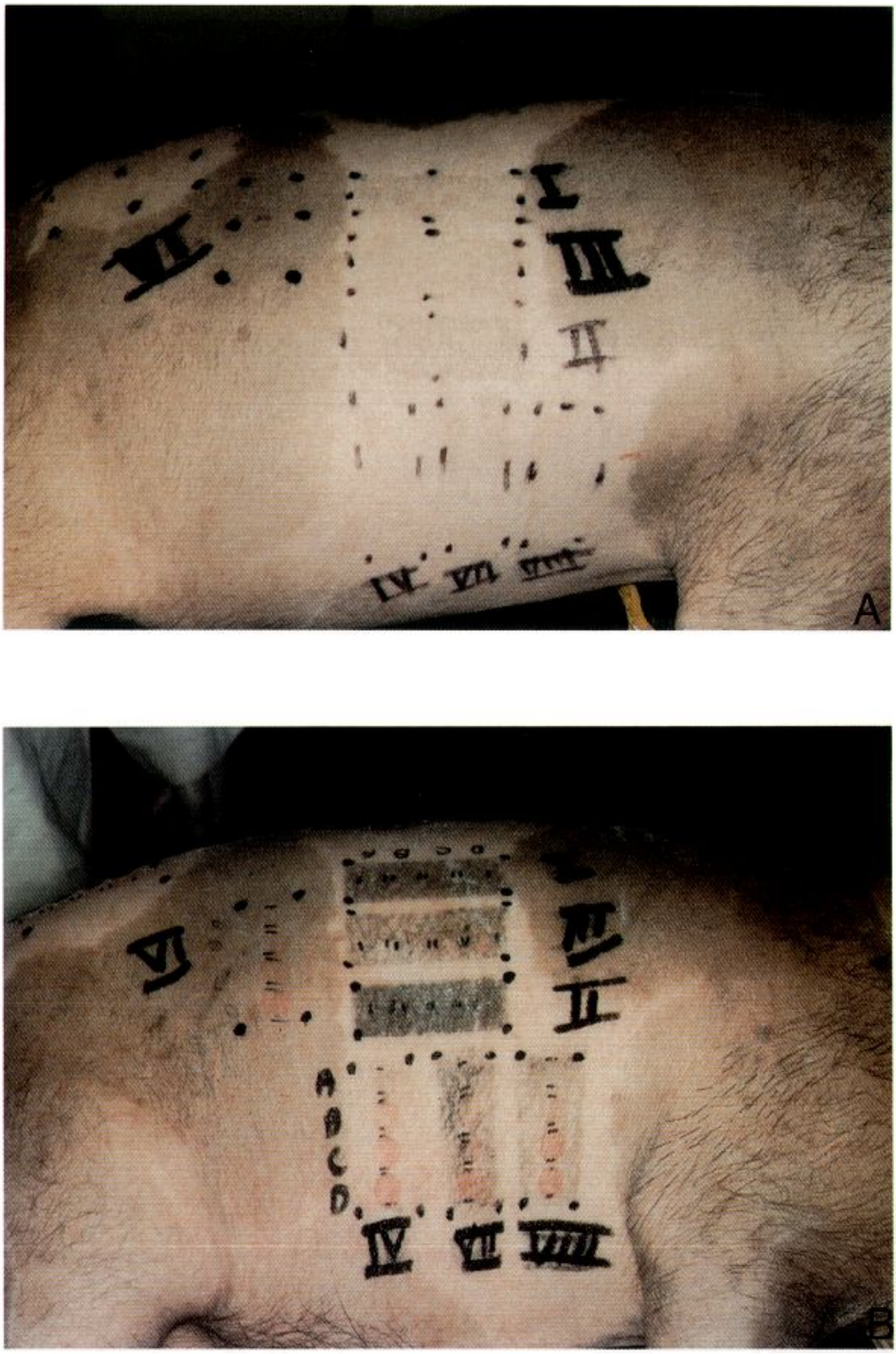


Figure 1. Photographs of the right side of pig #3 during the study. A. Photograph taken during the first week of the induction phase. Induced mild pigmentation can be seen in several induction sites. Note that the Roman numerals on the pig do not correspond to the induction groups in the study. B. Photograph taken 24 h after the challenge irradiations. Various degrees of pigmentation can be seen in the induction sites. The circular erythemic areas in the induction sites indicate the responses to the four exposures of challenge irradiation. Note that in the non-pigmented sites or the mildly pigmented sites, three affected areas can be seen corresponding to the 2, 3, and 4 min challenge irradiations. In the darker sites fewer erythemic areas can be seen.

Table I
Experimental Protocol and Results of Induction Phase

Induction group	Initial skin type	Treatment material	Irradiation (RB counts)	Summation of pigmentation scores* (Range of scores)
I	Non-pigmented	Vehicle	0	0
II	Pigmented	Vehicle	0	15 (2–5)
III	Non-pigmented	Vehicle	200	15 (2–5)
IV	Non-pigmented	Vehicle + Bergapten	200	15 (2–5)
V	Non-pigmented	Vehicle + Sunscreen	200	4 (0–1)
VI	Non-pigmented	Vehicle + Bergapten + Sunscreen	200	13 (2–4)
VII	Non-pigmented	Vehicle + Sunscreen	400	7 (1–2)
VIII	Non-pigmented	Vehicle + Bergapten + Sunscreen	400	11 (1–4)

* Pigmentation scores: 0 = no pigmentation (i.e., skin looked like naive non-pigmented skin); 1 = the lowest degree of pigmentation; 2 to 5 = increasing intensities of pigmentation.
During the induction phase, treatment materials were applied to selected skin types (i.e., non-pigmented or pigmented) on miniature pigs and subsequently exposed to one of three levels of irradiation in an attempt to elicit pigmentation in non-pigmented skin. After completion of the induction phase, induction sites were scored for pigmentation. The scores for the five sets of induction groups were summed, and summations and ranges were tabulated.

respectively. During the irradiation the rest of the pig, along with sites I and II, was covered with aluminum foil. Subsequently, all felt-tipped pen skin markings were examined and renewed as needed to ensure relocation of the sites.

On the eighth and fifteenth day of the induction phase for each pig, the above treatment and irradiation procedures were repeated. Therefore, each pig was treated and irradiated three times during the induction phase. During the induction phase the pigs also were monitored for changes in gross appearance of the skin and photographs were taken.

Seven days after the last treatment and irradiation in the induction phase, the eight induction sites were arbitrarily rated with respect to their degree of pigmentation. Sites which appeared non-pigmented were assigned a score of 0, sites which displayed the least degree of pigmentation were assigned the score of 1, and sites which displayed more intense pigmentation were scored from 2 to 5, depending on the number of distinguishable grades of pigmentation on each pig. No attempt was made to assign equal pigmentation ratings between pigs, so that a 2 rating for one pig may not have been comparable to that rating in another pig with respect to the absolute degree of pigmentation. The pigmentation scores for the five sites were summed, and the summations for each induction group were tabulated along with their ranges.

CHALLENGE PHASE

Immediately after rating the degree of pigmentation, each pig was anesthetized and

induction sites were challenge irradiated. Four circular areas (1 cm in diameter) were irradiated in each of the eight induction sites with a xenon short-arc solar simulator with a WG320 Schott glass filter (1-mm thick). The spectral distribution of the emission of this source is such that approximately 70% of it is between 290 and 400 nm (9). The output was checked for consistency at regular intervals with the RB meter. Each circular area was irradiated for one of the following durations (min): 1, 2, 3, 4. The exposure needed to elicit a barely perceptible erythema in naive non-pigmented skin was approximately 1.7 min (0.92 SBU). The four areas within each induction site were demarcated with aluminium foil and white adhesive labeling. After completion of the irradiation, the areas were marked with a felt-tipped pen and labeled for future identification.

Twenty-four hours after the challenge irradiations, each pig was anesthetized and all challenge areas were observed for erythema. For each induction site on each pig, the lowest irradiation exposure which produced erythema at 24 h post-irradiation was tabulated. The erythema scores in the tabulation represented the shortest duration of irradiation exposure (1, 2, 3, or 4 min) which resulted in perceptible erythema. If none of the exposures resulted in erythema, an erythema score of 5 was assigned to the induction group for that challenge site on the pig. These scores were ranked and compared statistically via the Friedman Test (10).

Immediately after the erythema scoring, biopsy specimens (6 mm diameter disposable punch; Chester A Baker Laboratories, Inc. Miami, FL) were taken from all challenge areas. Biopsies were processed and SBC indices were determined as described previously (4,5,11). Briefly, the number of epidermal cells per millimeter of interfollicular epidermis (IFE) and the number of SBC per millimeter of IFE were determined, and the SBC index was determined from the formula:

$$\frac{\text{Number of SBC/mm}}{\text{total number of epidermal cells/mm}} \times 100.$$

Each mean SBC index was determined from fifty estimates (10 from each challenge site on each animal side) except for one mean which was determined from 40 estimates because one biopsy specimen was accidentally destroyed (induction group III exposed for 4 minutes). SBC indices were plotted against the exposures to challenge irradiation. Slopes of the regression lines, residual variance, and standard error of estimate of these slopes were calculated. To compare these slopes statistically, a two-tailed Student's t test was used (12).

RESULTS

During the induction phase of the study some of the initially non-pigmented induction sites tanned gradually, and they were markedly pigmented at the end of this phase (Figure 1). Occasionally, uneven erythema without distinct borders was seen in induction sites VII and VIII, where exposures to 400 RB counts were used. None of the induction sites displayed erythema at the end of the induction phase (i.e., at the start of challenge), and none of the test articles elicited responses indicative of phototoxicity where suberythemic exposures to UVR were used.

No change in the appearance of skin was seen in sites treated only with the vehicle

Table II
Results of the Challenge Phase in Terms of the Erythema Scores

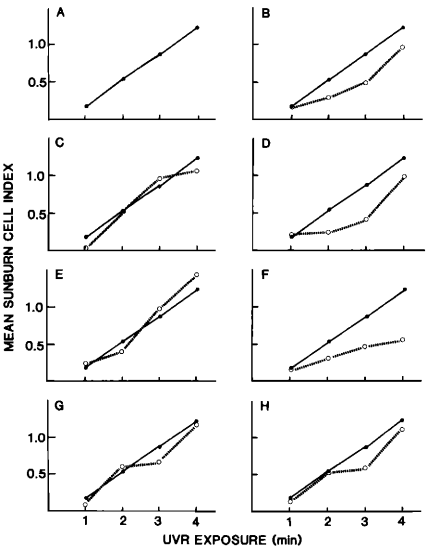
Induction group	Initial skin type	Treatment material	Irradiation (RB counts)	Results of challenge phase Erythema score* (Pig number) 1	2(1s)	3	2(rs)	4
I	Non-pigmented	Vehicle	0	2	2	2	2	2
II	Pigmented	Vehicle	0	2	3	3	2	3
III	Non-pigmented	Vehicle	200	2	2	5	2	4
IV	Non-pigmented	Vehicle + Bergapten	200	2	3	5	2	4
V	Non-pigmented	Vehicle + Sunscreen	200	2	2	2	2	3
VI	Non-pigmented	Vehicle + Bergapten + Sunscreen	200	2	2	4	2	4
VII	Non-pigmented	Vehicle + Sunscreen	400	3	2	3	2	2
VIII	Non-pigmented	Vehicle + Bergapten + Sunscreen	400	2	4	3	2	4

* Erythema score represented the exposure time (i.e., 1, 2, 3, or 4 min) needed to elicit a barely perceptible erythema in each of the induction sites. A score of 5 indicated that none of the challenge exposures of UVR produced erythema.
After completion of the induction phase, all induction sites were challenged with exposures to radiation from a xenon short-arc solar simulator with a WG320 Schott glass filter. Twenty-four hours later the challenged sites were assessed for erythema.

during the induction phase of the study (i.e., induction groups I and II). The summation of pigmentation scores for the induction groups indicated that induction sites III, IV, VI, and VIII were pigmented to a degree equal to or comparable to that of the normal pigmented skin of the pig (i.e., induction group II).

After challenging the induction sites with increasing exposures to UVR in the challenge phase, the erythema scores indicated that there was some apparent relationship between the degree of pigmentation and subsequent susceptibility to UVR (Tables I and II). The challenge exposures of 2, 3, and 4 minutes often resulted in perceptible erythema in non-pigmented induction sites and mildly pigmented sites, while induction sites with moderate or marked pigmentation either displayed erythema after longer challenge exposures (e.g., 3 and 4 minutes) or did not display erythema after any of the challenge exposures. However, erythema scores were variable within induction groups, and statistical analysis indicated that there was no significant difference among the induction groups with respect to this endpoint.

Induction group I may be considered as baseline responsiveness for SBC indices, since these sites represented non-pigmented skin which was treated with the vehicle and not irradiated during the induction phase and had a summation of pigmentation score of 0. For induction site I the means and 95% confidence intervals, in parenthesis, for the endpoint of SBC index were 0.18 (0.07), 0.54 (0.10), 0.87 (0.14), and 1.21 (0.17) for the four challenge exposures of 1, 2, 3, and 4 min, respectively (Figure 2). Induction group II represented naturally pigmented skin which was treated with the vehicle and



Graph	Symbol	Induction group	Summation of pigmentation scores
A–H	●—●	I	0
B	0 ▮▮▮ 0	II	15
C	0 ▮▮▮ 0	III	15
D	0 ▮▮▮ 0	IV	15
E	0 ▮▮▮ 0	V	4
F	0 ▮▮▮ 0	VI	13
G	0 ▮▮▮ 0	VII	7
H	0 ▮▮▮ 0	VIII	11

Figure 2. Graphs with mean sunburn cell indices plotted against challenge-UVR exposure. While there was a trend toward a reduction in SBC production in induction groups II, IV, and VI, only induction group VI differed significantly from induction group I when slopes of regression lines were compared.

not irradiated during the induction phase. In this group there was a trend for SBC indices to be less than they were in induction group I, but the slopes of the regression lines for these two groups were not significantly different.

There was a similar trend in induction group IV for this endpoint, but the slope of the regression line was not statistically different from the slope for group I. In induction group VI, SBC indices were consistently lower than in induction group I, and the slopes of the regression lines were significantly different when these two groups were compared. In induction groups III, V, VII, and VIII, there was no indication that SBC indices differed from those seen in induction group I, and none of these slopes differed significantly from the slope in group I. Therefore, there was a trend for SBC indices to be lower in skin which was naturally pigmented and in skin which was tanned after pretreatment with either Vehicle + Bergapten or Vehicle + Bergapten + Sunscreen prior to the 200 RB count exposure to UVR. However, a significant decrease in SBC production was only seen in skin tanned with the Vehicle + Bergapten + Sunscreen pretreatment.

DISCUSSION

Quantification of changes in skin pigmentation is particularly difficult when invasive techniques must be avoided. In this study we arbitrarily assessed skin pigmentation by visually inspecting the skin and assigning numerical scores to the degree of pigmentation. Since this method was more qualitative than quantitative, no attempt was made to analyze the data statistically. However, the non-pigmented skin of these pigs was tanned by several treatment combinations, and this induced tan was often comparable to the pigmentation of the natural pigmented skin of these animals. Pretreatment, prior to irradiation, with either Vehicle or Vehicle + Bergapten permitted pigment induction in non-pigmented skin. Vehicle + Sunscreen pretreatment virtually abolished pigment induction due to UVR, while Vehicle + Bergapten + Sunscreen pretreatment permitted pigment induction. Therefore, while the sunscreen ingredient virtually negated the induction of tan, the addition of bergapten to the product re-established the tanning capability of UVR.

Even though there was some indication, within individual pigs, that induced pigmentation decreased susceptibility to erythema upon subsequent UVR, the differences were not statistically significant when the results from all subjects were pooled. This lack of statistical significance may be attributed to several factors, including between-pig variability with respect to the degree of induced pigmentation for each induction site, variability with respect to UV senstivity attributable to the anatomical location of the irradiation sites, the increment of challenge exposures, the relatively small number of experimental subjects, and difficulties associated with assessing both pigmentation and erythema qualitatively and quantitatively. Of course, one or more of these factors would also be expected to impact on the SBC data.

Epidermal cell damage was quantified by determining SBC indices after challenge irradiation. SBC production, in general, indicated a positive role for tan in providing protection against further UVR exposure. This is consistent with earlier works done with miniature swine (4,5).

However, certain anomalies in the study warrant consideration. The first involves induction group III which developed definitive tan that did not provide protection from subsequent UVR as assessed by SBC production. This may be attributed to one or more of the factors given above concerning statistical analyses. Alternatively, this may indicate mechanistic differences among the treatment groups with respect to tan induction and/or protection. The second anomaly involves the 4-minute challenge exposures and SBC production. In all except one group (i.e., induction group VI), SBC indices were very similar after 4 minutes of UVR exposure, and this similarity in responsiveness certainly contributed to the lack of statistical significance observed when induction groups II and IV were compared to group I. Again, this may be related to the factors given above. Alternatively, it may indicate that induced tan in the normally non-pigmented skin of miniature pigs is often less likely to offer protection against large UVR exposure.

In summary, the naturally white (i.e., non-pigmented) skin of miniature swine was successfully tanned by certain treatment regimens in combination with UVR, and a positive role for the protective value of tan was found when epidermal cell damage was assessed by SBC production after challenge irradiation. A bergamot oil-containing sunscreen product was effective in enhancing UVR-induced tan, and the induced tan of this

product afforded protection against subsequent exposure to UVR when epidermal cell damage was assessed.

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