



Successful reduction in skin damage resulting from exposure to the normal-mode ruby laser in an animal model

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SUMMARY. Normal-mode ruby laser (NMRL) irradiation of skin has now become an acceptable method of producing depilation. However, side effects, which include superficial burning and changes in skin pigmentation, still occur and, although temporary, can be distressing to the patient.

This paper reports a method by which the skin can be protected (or preconditioned) from damage during NMRL treatment by pre-heating to a lower, non-damaging level prior to irradiation. Using the black-haired mouse (C57Bl/10) as an animal model, an appropriate 'preconditioning' temperature was established by exposing the mouse skin to a range of temperatures, taking biopsies and staining the skin immunohistochemically for heat shock protein 70 (HSP 70) expression within the keratinocyte cells. Increased HSP 70 expression is stimulated by exposure to environmental stressors such as heat, so it was hypothesised that its increased expression conveyed increased cellular protection. The appropriate temperature (45 °C for 15 min) allowed for the superficial skin cells to be protected (as assessed by maximal HSP 70 staining) but undamaged (as assessed by haematoxylin and eosin staining), leaving the target hair-producing cells unprotected. Eight mice (16 flanks) were then exposed to this preconditioning temperature (eight of the flanks being growing-hair sites and eight resting-hair sites) and 5 h later exposed to a laser fluence known to cause mild skin damage and depilation (6 J/cm²). This exposure was to both the preconditioned and the adjacent non-preconditioned sites.

A statistically significant reduction in skin damage ($P < 0.001$), as measured by the time taken to heal and noted both clinically and histologically, was seen in the preconditioned sites in resting-hair regions but not in growing-hair regions. Depilation, established over an 8 week period, was successful in growing-hair regions within both preconditioned and non-preconditioned sites, but complete hair regrowth had occurred in preconditioned and non-preconditioned sites within resting-hair regions by 5 weeks. The latter finding was consistent with work already reported suggesting that NMRL-assisted depilation in this animal model is not successful for hairs in the telogen phase.

Successful preconditioning of mouse skin prior to laser exposure appears to reduce NMRL-induced skin side effects. In addition, the technique does not appear to adversely affect successful depilation. © 2001 The British Association of Plastic Surgeons

Keywords: laser, depilation, preconditioning, protection, damage, hair, skin, regrowth.

The normal-mode ruby laser (NMRL) has become rapidly accepted as a method of inducing depilation, with its role being defined in the fields of both reconstructive and aesthetic surgery. The ability to transfer hair-bearing skin to a non-hair-bearing site and then remove the hairs would vastly increase the range of potential donor areas. For example, autologous ear reconstruction uses skin from the temporal region of the scalp to cover the cartilage framework.¹ Although this produces a new ear with a good skin-colour match, hair regrowth can spoil the final result. A further example is the creation of a urethra using tubed scrotal skin.² Unfortunately, the hairs can act as a nidus for calculus formation resulting in outflow obstruction. Therefore, removal of the hairs would undoubtedly improve outcome. In addition, it has been estimated that between 5 and 10% of the Caucasian population suffer from hirsutism.^{3,4} The majority (85%) of severe cases and 50% of mild cases result from hyperandrogenaemia,⁵ but even successful treatment of the hormonal imbalance can leave an excess of unwanted and unsightly terminal hair growth.

The NMRL^{6–9} and, more recently, the alexandrite¹⁰ and NdYAG¹¹ lasers, have been used as depilators with varying success. All function by conversion of photon (light) energy to heat by a target substance (chromophore) within the patient's skin. The chromophore for ruby and alexandrite lasers is melanin whilst for the NdYAG laser it is black pigmentation such as carbon. The melanin is concentrated in greater amounts within the hair shaft in Caucasian individuals making the lasers theoretically more specific for the hair follicles, but in darker-skinned individuals, melanin is prevalent within the skin too. This can result in greater side effects, such as superficial burning, requiring the treatment dose to be reduced to potentially sub-therapeutic levels. To minimise the side effects still further, various methods have been devised, which include the use of Aloe vera cream, cooling packs, a cooled convergent sapphire lens⁹ and the application of topical hydroquinone cream⁸ bleaching the skin before NMRL treatment. None of these methods have been specifically investigated in all the work published to date.

This paper describes a technique to increase the ability of the viable keratinocytes in the epidermis of mouse skin to resist damage from a noxious stimulus, for instance heat, by prior exposure of those cells to a sub-lethal dose of the stimulus. Such preconditioning, as described by Murry et al,¹² has been shown to increase significantly the survival of cardiac and skeletal myocytes after normally lethal ischaemic events in animal models.^{13,14} The stimulus chosen for preconditioning in this experiment was heat due to its ease of application. Upon discovering an apparently appropriate temperature to potentially protect the mouse keratinocytes without protecting the hair-producing cells, we exposed preconditioned skin sites to irradiation at the lowest laser fluence known to produce skin damage and depilation in this model. The skin wounding and the depilatory effect were assessed over time and compared with non-preconditioned irradiated controls to discover whether preconditioning could reduce skin side effects whilst maintaining the overall depilatory success.

Materials and methods

Before discovering whether preconditioning could protect skin from NMRL exposure it was necessary to establish an appropriate animal model, a reproducible stimulus that could evoke a preconditioning response and a sensitive and specific marker for successful preconditioning that could determine whether preconditioning had only occurred in those cells requiring protection (the surface keratinocytes) and not in those to be destroyed (the stem cells of the hair bulge, residing at approximately two-thirds of the way down the hair shaft, and the hair bulb). It also had to be established that the preconditioning did not itself cause any skin damage. To answer these questions, preliminary experimentation was performed.

Preliminary experimentation

An appropriate animal model. The black-haired mouse (C57Bl/10 supplied by Harlan UK Ltd., Bicester, UK) was used as the animal model because the hair shafts contain the chromophore for the NMRL and the follicles exist in two demonstrably obvious hair growth phases discernable by the apparent skin pigmentation present after trimming (Fig. 1). This model has also been used previously by Lin et al to assess the effect of hair growth phase on the success of NMRL-assisted depilation. They discovered that depilation was achieved only in hair follicles in the growing or anagen phase of the hair cycle and not in the telogen or resting hair phase.¹⁵ Therefore, any variation in depilatory success in this experiment would have to be assessed for follicles that were in the growing phase when exposed to laser irradiation.

Preconditioning. Heat was chosen to precondition the mouse skin because of its ease of application. A heating apparatus was constructed in the Bioengineering Department, Mount Vernon Hospital, Northwood, consisting of a thermostatically controlled 15 mm diameter metal plate. This was applied to the resting hair sites alone on the trimmed flanks of the anaesthetised mice at a range of

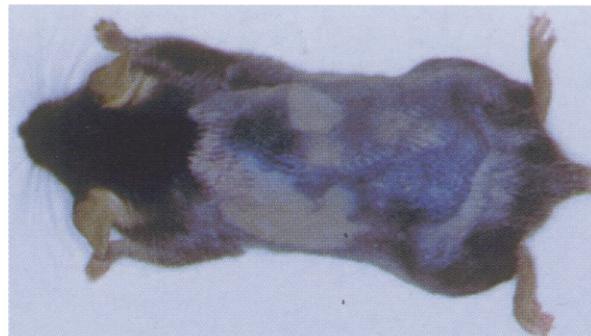


Figure 1—The back of a black-haired mouse after trimming. The hair follicles in the anagen (growing) phase are evident by the black patches on the skin whilst those in the telogen (resting) phase are indicated by the pink patches.



Figure 2—The application of the heating device to the anaesthetised mouse during preconditioning of the skin.

temperatures, which included 37 °C, 39 °C, 41 °C, 43 °C, 45 °C and 47 °C for a fixed duration of 15 min. Each temperature was used on six flanks (two flanks per mouse and three mice per temperature). The total number of mice used was 18 (Fig. 2).

A sensitive and specific marker for successful preconditioning. Preconditioning is believed, in part, to work via the increased production of a group of intracellular proteins called heat shock proteins (HSPs), first described by Ritossa in 1962.¹⁶ Their task is to bind to other proteins within the cell, promoting the correct tertiary folding of those proteins and preventing them from becoming denatured by an adverse stimulus that would otherwise lead to cell death.^{17,18} The most commonly expressed protein in this family is HSP 70,¹⁹ a 70 kD protein, which can be detected by immunohistochemical techniques approximately 5 h after exposure to the stimulus.²⁰ Any increase in expression of HSP 70 could relate to an increased ability of the cell to withstand an adverse stimulus. Therefore, skin biopsies were taken from the heat-exposed sites on the mouse flanks 5 h after heating and fixed in formal saline before undergoing routine paraffin-wax embedding. Random transverse sections were taken in pairs and one of the pairs stained for HSP 70 expression using HSP 70 monoclonal primary antibody (supplied by Neomarkers Ltd., Stratech,

Luton, UK) and a detection system specifically designed to reduce the non-specific background staining obtained when using mouse monoclonal antibodies to stain mouse tissue (Ultravision mouse tissue detection system anti-mouse, HRP/DAB from Lab Vision Corporation, Fremont, CA, USA). The sections were then analysed for HSP 70 expression, its site and extent within the skin of the black-haired mouse.

Skin damage. The second histological section of the pair was stained with haematoxylin and eosin (H&E) to ascertain whether the temperature to which the mouse skin had been exposed produced skin damage. This could be assessed by checking the level of cell vacuolisation, epidermal loss and nuclear pyknosis seen in the sections.

Results of the preliminary experiments

Figure 3 shows a representative section from a specimen that was exposed to heat at 37 °C for 15 min, with minimal HSP 70 expression noted in the epidermal cells. Figure 4A shows a representative section from a specimen exposed to 45 °C for 15 min showing positive HSP 70 expression in the cells of the epidermis and extending approximately half-way down the follicle. The putative site of the hair bulge, below the sebaceous gland, has been identified and this shows minimal expression. Figure 4B shows a representative section from the same specimen that has been stained with H&E. Little cellular damage was noted in these specimens. Figure 5A shows a representative section taken from a specimen stained with HSP 70 and exposed to 47 °C. Greater expression of the protein is seen within the keratinocytes of the skin surface and also throughout the length of the hair follicle down to the bulb, suggesting that the bulge cells could also be protected. In addition, Figure 5B shows a section from a specimen also exposed to 47 °C, stained with H&E, manifesting increased cellular damage at the skin surface and within the hair follicle.

Conclusions of the preliminary experiments

These results implied that successful skin preconditioning of the black-haired mouse, as judged by an increase

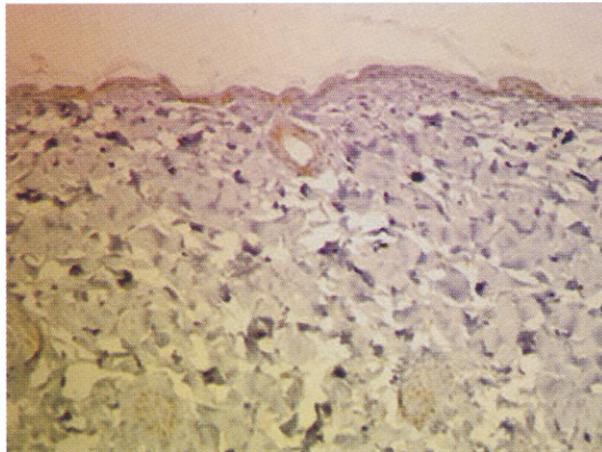


Figure 3—Representative transverse section of a specimen of mouse skin exposed to 37 °C for 15 min and stained for HSP 70 expression. No positive staining is seen in the epidermis ($\times 130$).

in HSP 70 expression within the keratinocytes of the skin but not the bulge region, with no apparent cellular damage, could be achieved and that the most appropriate temperature for this was 45 °C for 15 min.

Preconditioning experiment

Twelve mice were used for this experiment and all had their hair trimmed before experimentation. Four were anaesthetised and had a growing hair site on each flank exposed to the heating apparatus at 45 °C for 15 min. A further four were anaesthetised and had a resting hair site on each flank exposed to the heating apparatus at 45 °C for 15 min. The final four had the heating apparatus applied to both growing and resting hair sites at the aforementioned temperature for the same time. All animals were allowed to recover for 5 h in individual boxes before being removed in turn, anaesthetised a second time and exposed to a fluence of 6 J/cm² from a NMRL (pulse duration of 900 µs and spot size of 7 mm) (SLS Biophile Ltd., Llanelli, UK). Irradiation was to both the preconditioned and non-preconditioned sites in the growing hair regions in the first group, the resting hair regions

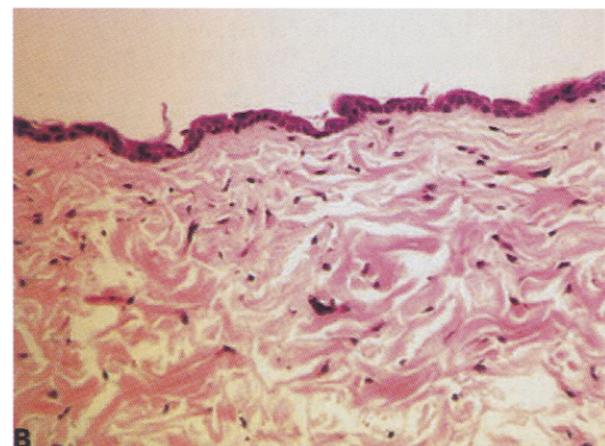
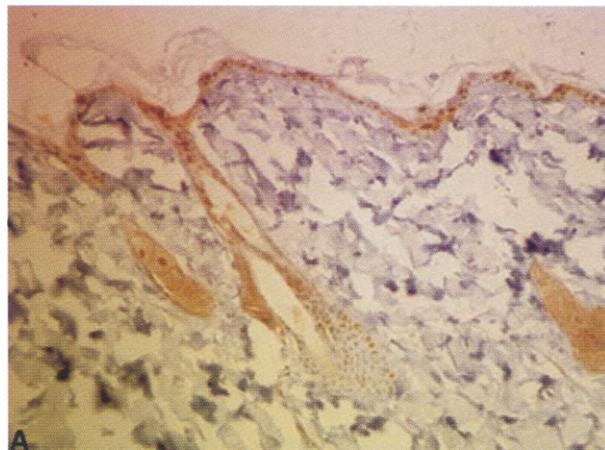


Figure 4—Representative transverse sections of specimens of mouse skin exposed to 45 °C for 15 min. (A) Section stained for HSP 70 shows increased expression within the nuclei of the cells of the epidermis and the cells lining the hair follicles to the extent of the sebaceous gland ($\times 130$). (B) Section stained with H&E shows no evidence of cellular damage within the epidermis ($\times 130$).

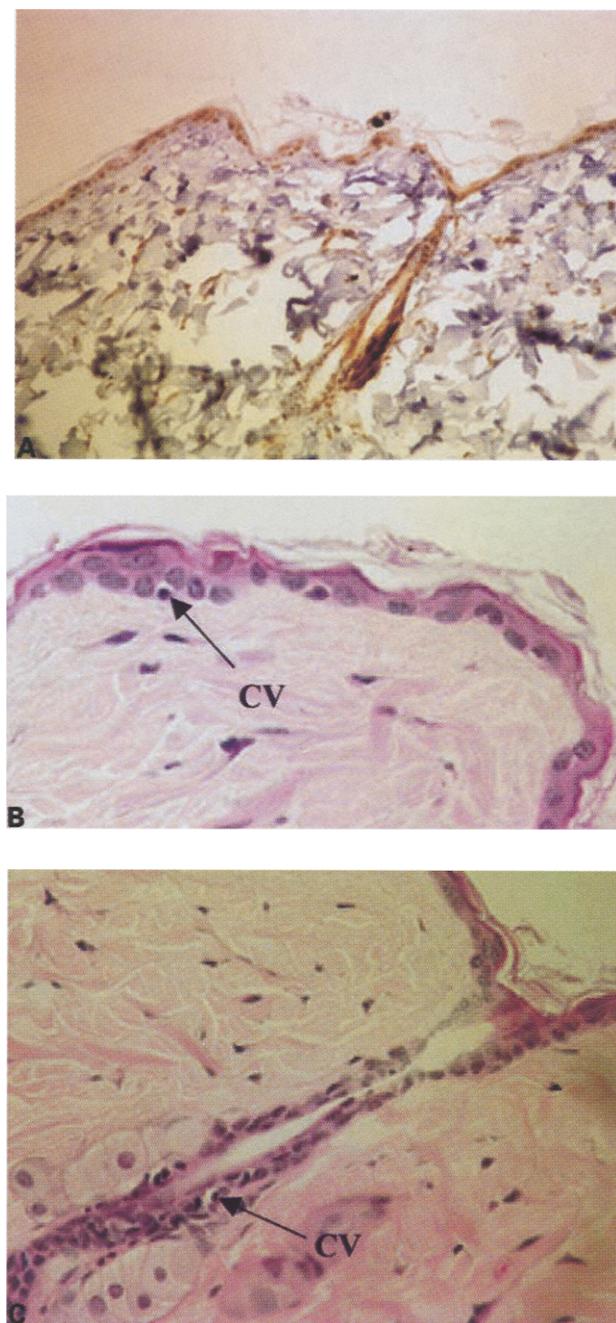


Figure 5—Representative transverse sections of specimens of mouse skin exposed to 47 °C for 15 min. Section stained for HSP 70 (A) shows increased expression within the nuclei of the cells of the epidermis and throughout the cells of the hair follicle to the hair bulb ($\times 130$). Sections stained with H&E show damage to the epidermal cells (B $\times 400$) and to the cells lining the follicular canal (C $\times 200$). CV: cell vacuolisation.

in the second group and both in the third group. We had previously identified the fluence used as being the lowest to produce both mild skin damage and depilation in the black-haired mouse. The animals in the first two groups were then replaced in their boxes whilst the final four mice were sacrificed and biopsies taken from all laser-exposed sites and negative controls.

The remaining eight mice were then observed on a daily basis to assess skin damage and on a weekly basis to assess hair regrowth. Skin damage was scored according

Table 1 Skin-wound scoring system adapted from Lin et al¹⁵ with a modification to include the area of damage as a percentage of the whole area exposed

0	no skin damage
1	erythema
2	blistering/clear exudate or crust
3	blood-stained exudate or crust 0–30% of the treatment area
4	blood-stained exudate or crust 30–60% of the treatment area
5	blood-stained exudate or crust 60–100% of the treatment area
6	ulceration

Table 2 Hair-regrowth scoring system adapted from Lin et al¹⁵

0	none (0% regrowth)
1	sparse (0–33% regrowth)
2	moderate (33–66% regrowth)
3	full (66–100% regrowth)

to the system devised by Lin et al¹⁵ with a slight modification to include estimated percentage area of burn compared to the whole irradiated field (the spot size) as shown in Table 1. This continued until full wound healing had occurred. Hair regrowth was also scored according to the system used by Lin et al¹⁵ (Table 2) and this continued over an 8 week period. The time scale chosen was longer than that established by Dry²¹ as the longest for follicles in the skin of the black-haired mouse to complete one hair cycle. This helped ensure that an accurate hair-regrowth score was achieved.

The biopsies taken from the preconditioned and non-preconditioned sites exposed to laser irradiation in both growing and resting hair regions and the negative controls were processed for routine paraffin-wax histology. Random transverse sections were taken and stained with H&E to assess the extent of cellular damage occurring at the different sites.

Results

All the mice followed over the 8 week period survived both anaesthetics with no apparent systemic ill effects.

Skin damage

Figure 6 shows the mean time (\pm s.d.) taken for preconditioned and non-preconditioned sites exposed to 6 J/cm² to heal in resting hair regions and growing hair regions. The mean time to heal for preconditioned sites in resting hair regions was 8 days (± 0.75) whilst that for non-preconditioned sites was 9.5 days (± 1.2). When the median values were analysed using the Mann-Whitney rank sum test, a statistically significant difference ($P < 0.001$) was noted between the two groups. The mean time to heal for preconditioned sites in growing hair regions was 11.4 days (± 1) and for non-preconditioned sites was 12.1 days (± 1.25); these values showed no statistical difference on analysis. Figure 7 shows two of the eight exposed flanks in resting hair regions 3 days after laser irradiation. A clinical difference in the extent of skin damage between the preconditioned and the non-preconditioned sites can be seen; this was noted in five of the

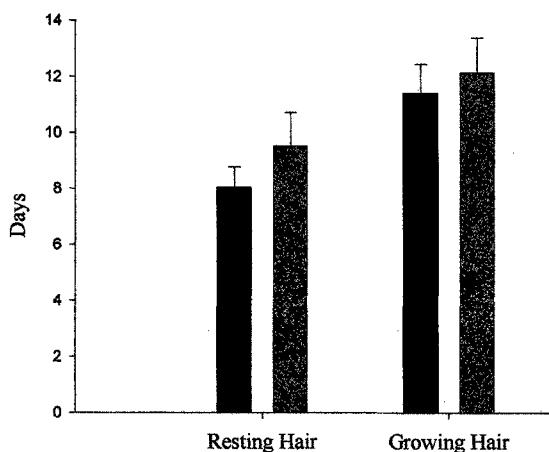


Figure 6—Mean (\pm s.d.) time for the skin damage resulting from NMRL irradiation at 6 J/cm^2 to heal in both resting and growing hair sites that had (black bars) and had not (grey bars) been preconditioned prior to laser exposure.

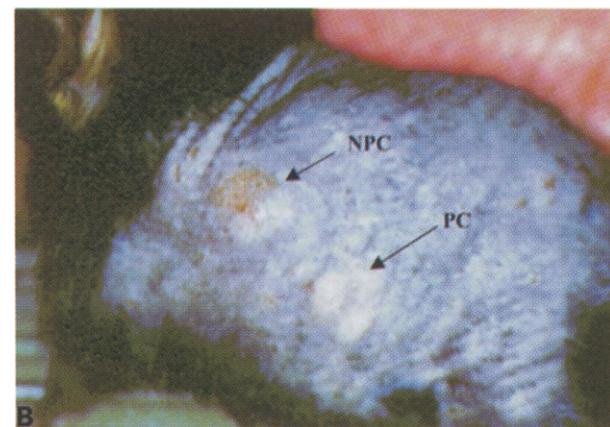
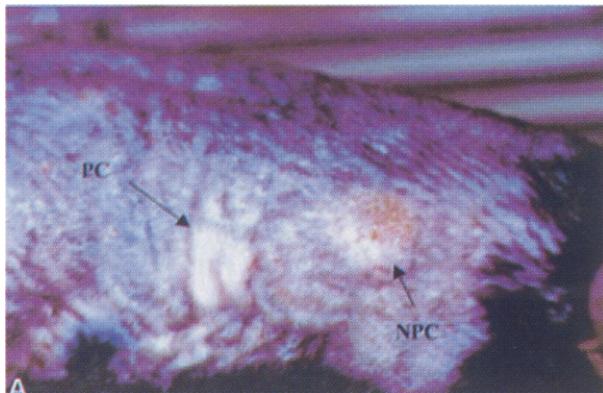


Figure 7—(A,B) Two of the eight flanks 3 days after exposure to NMRL irradiation at 6 J/cm^2 showing the clinical difference in skin damage between the preconditioned (PC) and the non-preconditioned (NPC) sites.

eight flanks irradiated in resting regions in this experiment. No such difference, though, was seen between the preconditioned and the non-preconditioned sites in the growing hair regions.

Representative transverse sections taken from a non-preconditioned (Fig. 8A) and a preconditioned (Fig. 8B)

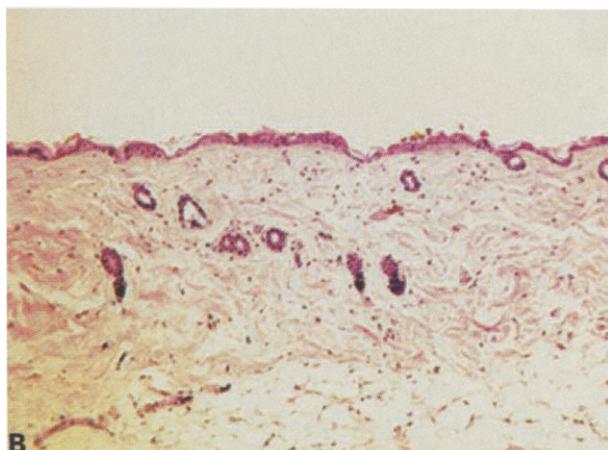
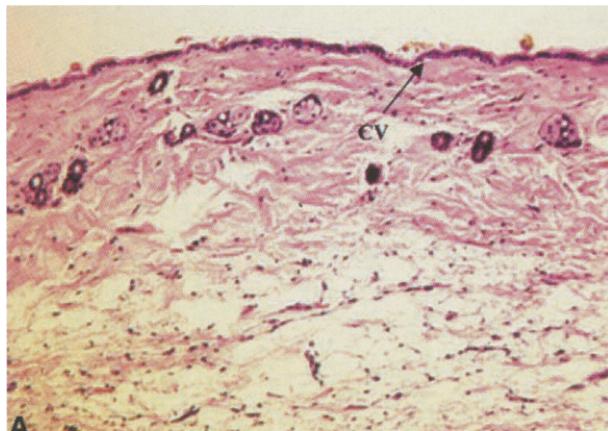


Figure 8—Representative transverse sections of specimens of mouse skin after undergoing NMRL exposure at 6 J/cm^2 (H&E stain; $\times 65$). (A) Non-preconditioned skin; note the extent of the epidermal damage in terms of cell vacuolisation. (B) Skin preconditioned at 45°C for 15 min 5 h before NMRL exposure; note minimal epithelial damage.

laser-exposed site in a resting hair region, stained with H&E, show that the epidermal damage, as evidenced by nuclear pyknosis and cell vacuolisation, appears to be greater in the non-preconditioned section, which supports the clinical findings.

Depilation

Figure 9A shows the mean weekly hair-regrowth scores (\pm s.d.) in the laser-exposed preconditioned and non-preconditioned sites in resting hair regions. Full hair regrowth was noted at all sites by 5 weeks after laser irradiation. No statistical difference was found between the weekly scores for hair regrowth at the two sites.

Figure 9B shows the results for laser-exposed preconditioned and non-preconditioned sites in growing hair regions. The hair-regrowth scores were significantly less at both sites ($P=0.038$) than for the trimmed non-irradiated control sites, which had fully regrown (median values analysed by the Mann-Whitney rank sum test). In addition, no statistically significant difference was found between the median values of the hair regrowth scores achieved on a weekly basis at the preconditioned and non-preconditioned sites (also assessed by the Mann-Whitney rank sum test).

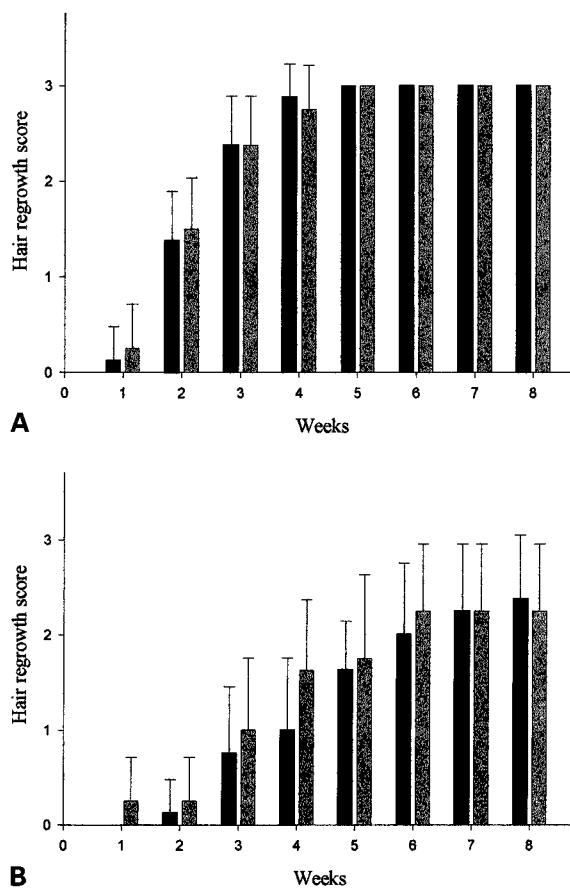


Figure 9—Weekly hair regrowth scores in preconditioned (black bars) and non-preconditioned (grey bars) sites irradiated by a NMRL at 6 J/cm^2 in (A) resting hair regions and (B) growing hair regions.

Discussion

The protection of tissues from a potentially lethal stressor by prior exposure to a sub-lethal stressor, not necessarily of the same type,²² has been the subject of extensive research since 1986 when Murry et al¹² described its effect in reducing cell death in the heart of the dog. Since then, the concept of preconditioning has emerged.²³ Further work by other groups has shown that both heat stress^{13,24} and ischaemic preconditioning^{14,25,26} can protect skin flaps and muscle flaps from ischaemic damage. Polla and Anderson reported an in vitro study showing that heat preconditioning of cultured human fibroblasts 2 h prior to CO_2 laser exposure significantly increased their subsequent survival when compared with non-preconditioned irradiated controls.²⁷

This study used an accepted *in vivo* animal model to assess the depilatory effect of the NMRL. We first showed that increased synthesis of HSP 70 could be induced preferentially in keratinocytes of the black-haired mouse by exposure to heat stress and that an apparently ideal temperature for preconditioning the skin could be found that was also associated with minimal keratinocyte damage. The 15 min exposure time was chosen as the heat needed to be given over a short enough time to maintain a large enough heat gradient through the skin. This would stimulate the superficial keratinocytes to produce HSP 70 whilst preventing the cells of the bulge

region from doing so. Resting hair sites were used for this part of the experiment as the sections were easier to cut and the position of the bulge region within the dermis is fixed regardless of the phase of the hair cycle.

Heat preconditioning of resting hair sites 5 h prior to laser exposure at 6 J/cm^2 significantly reduced skin damage compared to non-preconditioned irradiated controls in terms of the median time to heal, as evidenced histologically and photographically. However, no such difference was noted in the growing hair sites. The anatomies of these two regions are markedly different. Although the density of hair follicles remains the same, the hair shafts are wider and longer in the growing hair regions, extending to the subcutaneous fat up to $500 \mu\text{m}$ below the skin surface.²⁸ The melanin is also heavily concentrated throughout the length of the hair shaft in contrast to hair shafts in the resting regions, which are thinner and shorter (up to $250 \mu\text{m}$ in length²⁸), extending within the dermis alone with pigmentation present only in the shaft above skin level. Therefore, it could be that the greater quantity of melanin in the growing hair regions converted a larger amount of photon energy to heat and so effectively 'swamped' the preconditioning response. It is important to note, though, that only eight flanks were used per growing region so a better response could possibly have been achieved if the study numbers had been greater.

The influence of preconditioning on depilation in this experiment could only be assessed indirectly. At 6 J/cm^2 , the depilatory response in the preconditioned resting hair regions was the same as that in the non-preconditioned resting hair regions: namely all hair had regrown by 5 weeks after laser exposure. In the growing hair regions, the depilatory response was again the same in both preconditioned and non-preconditioned sites, both having achieved depilation at the end of the 8 week study period. Although this could imply that preconditioning did not adversely affect depilatory success, since no effect on wound healing was seen in the growing hair sites and no depilatory response was seen in the resting hair sites, this cannot be assured.

No study to date, to our knowledge, has assessed the current methods in use to protect the skin from laser-induced side effects. A protective effect was seen in the skin of the black-haired mouse in this experiment when preconditioned prior to exposure if the hair follicles were in the resting phase. Mouse skin, however, differs from human skin since the epidermis of the mouse is only two cells thick whereas that of the human contains a greater number depending on the site. In theory this could mean that mouse skin is more susceptible to variations in laser fluence than human skin as the distance over which the heat has to diffuse to injure the viable cells of the skin is much less. Nevertheless, a threshold is bound to exist beyond which no amount of preconditioning can protect the keratinocytes and the question remains at what level this is and is it great enough to provide protection for human skin? With this in mind it is our opinion that heat preconditioning prior to laser irradiation of the skin may help to reduce the side effects currently seen in clinical practice and would be worth investigating further in a clinical trial.

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Paper received 1 February 2000.

Accepted 2 October 2000, after revision.