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Median Effective Dose Determination and Histologic Characterization of Porcine (*Sus scrofa domestica*) Dermal Lesions Induced by 1540-nm Laser Radiation Pulses

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Background and purpose: **Light amplification by stimulated emission of radiation (laser) systems operating in the so-called "eye safe" region are gaining widespread use in industry, medicine, and military applications. This research effort was geared to study the effects of laser tissue interaction on human skin by using in vivo porcine skin as an animal model. The goals of the study were to determine the median effective dose (ED_{50}) for 1540-nm laser exposures, to evaluate the Yorkshire pig and the Yucatan mini-pig as animal models for laser exposure, and to characterize laser-induced skin lesions histologically.**

Methods: **A 1540-nm wavelength laser was used to expose multiple sites on the flanks of 10 pigs, using 0.8-ms pulses, ranging from 7 to 96 joules (J)/cm^2. Single pulses were delivered to the flank of Yorkshire and Yucatan pigs in a grid pattern. Exposure sites were evaluated immediately after exposure and at 1 hour and 24 hours for presence of gross lesions. Representative biopsy specimens were collected from lesion sites for histologic evaluation at the 24-hour endpoint.**

Results: **The ED_{50} for the two breeds differed in the amount of energy required to induce dermal lesions. Grossly, lesions in each breed were well demarcated and pale gray to brightly erythematous. Microscopically, lesions had epidermal layer damage as cellular swelling and nuclear pyknosis, loss of cellular detail, and coagulation necrosis at the dermal layer.**

Conclusions: **Findings suggest the presence of a different mechanism of laser-tissue damage in these two breeds. Photo-thermal mechanism appears to induce the skin lesions in the Yorkshire pig, whereas photo-thermal and photochemical mechanisms appear to be involved in lesion formation in the Yucatan mini-pig. All data obtained in this study will become part of database used by the American National Standards Institute (ANSI) to recommend laser safety standards for the occupational health and safety programs (OHSP), which will be used by industry and the military to base and update their current OHSP.**

Laser systems operating in the 1540-nm wavelength region have been gaining widespread use in military and industrial applications. Our present research effort is geared to collect exposure data, characterize laser exposure lesions histologically, and determine threshold values (median effective dose [ED_{50}]) for human skin exposures, using the pig as an animal model. In addition, the mechanism of laser-tissue interaction for the infrared laser wavelength is under investigation. The 1540-nm wavelength is in the near-infrared region of electromagnetic spectrum and is often incorrectly referred to as the "eye safe" laser region (1). The United States Army uses this wavelength for their Hand Held Range Finder, and similar systems are commercially available. These commercial systems may operate in a continuous wave or pulse mode, with many joules of energy output (2). The low altitude navigation and targeting for infrared for night (LANTIRN) systems of the US Air Force and Navy use the same wavelength while in training mode. New applications for infrared lasers are in development by the Department of Defense (DoD) with emphasis on increasing energy to meet mission requirements. The telecommunication industry also makes widespread use of 1540-nm lasers to provide pulses of light in fiber optic systems. These systems have been in use for several years, and are operating at energies and pulse duration in an infrared wavelength region for which safety standards are based on only a few biological data points (3–7). Since the use of these systems is widespread, it is necessary to establish suitable safety standards to ensure the health and safety of workers and the public. Data collected on these laser exposures will serve to expand the database on which national laser safety standards, ANSI Z136.1, are based.

Current national safety standards for near infrared (1400- to 2000-nm) wavelength laser exposures are based on exposures to various in vivo corneal and skin models (6–8). Yorkshire pig skin has been documented to share many of the anatomic, biochemical, and physiologic characteristics with the skin of humans, thus making it an excellent model for many research applications (8–11). The Yorkshire pig's skin has little melanin in the epidermal layers and the epidermis is thinner, compared with that of the human and Yucatan mini-pig (12, 13). These characteristics make the Yorkshire pig a less suitable model for

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the study the of infrared laser exposure effects. Recent studies, within our group, indicate the Yucatan mini-pig is a more applicable animal model for laser-induced skin injury investigations. The skin thickness of the Yucatan pig flank and that of the human arms, neck, and face is statistically identical (13). Skin thickness and the presence of melanin in the epidermis make the Yucatan mini-pig a more suitable model to study the effects of infrared laser exposures than does the Yorkshire pig (13–16). It is our hypothesis that epidermal pigment plays a role in the damage mechanism of infrared laser exposures. To the authors' knowledge, previous studies have not been conducted to characterize lesions histologically for 1540-nm wavelength laser exposures; however, Lukashev et al. (10) recently did ED_{50} studies using 1540 nm in a white "Krupnaya belaya" domestic pig (10). The objective of the study reported here was to perform a comparative study between Yorkshire and Yucatan mini-pigs laser exposures.

Materials and Methods

A total of five female Yorkshire pigs (Archer Farms, Belcamp, MD), each weighing 25 to 40 kg, and five female Yucatan mini-pigs (Charles River Laboratories, Wilmington, MA and Panepinto & Associates, Masonville, CO), each weighing 25 to 60 kg, were studied. The age of the animals of both breeds ranged from 5 to 9 months. All procedures performed were approved by the Uniformed Services University of the Health Sciences Animal Care and Use Committee. None of the animals were euthanized after skin sample collection as they were part of an animal- or tissue-sharing program. The pigs were housed in accordance with requirements listed in the *Guide for the Care and Use of Laboratory Animals* in a research facility approved by AAALAC, International. The animal rooms were environmentally controlled to provide a temperature between 22 and 25°C at 55 to 70% relative humidity, 100% fresh air exchange at a rate of 10 to 15/h, and a 12:12-hour light:dark cycle. Yucatan mini-pigs and Yorkshire pigs were fed standard commercially available diets (Miniswine Diet 8753, Harlan Tecklad Diets, Madison, WI and Hog Grower Chow OTC50, Purina Mills, Inc., St. Louis, MO, respectively) and had unlimited access to water.

Prior to laser exposure and biopsy specimen collection, all solid food was withheld for 24 hours. Pigs were pre-anesthetized with Tiletamine/Zolazepam (5 mg/kg of body weight) (Fort Dodge Animal Health, Ft. Dodge, IA) and Xylazine (1 mg/kg) (VEDCO, Inc., St. Joseph, MO) given IM. General anesthesia was induced and maintained with Propofol (0.17 mg/ kg/min) (Zeneca Pharmaceuticals, Wilmington, DE) given via an auricular vein catheter. The hair over the area of interest in these pigs was clipped prior to exposure. An endotracheal tube was used to ensure a patent airway. During the procedures, the animal physiologic parameters were monitored by use of a reflectance pulse oximeter (Vet Ox SDI 4402, Heska Corp., Ft. Collins, CO) and a digital thermometer (Temp Plus 2080, IVAC Crop., San Diego, CA). Animals were kept warm throughout the experimental procedure by use of a surgical table (Shortline, Kansas City, MO) with a thermal support system. Buprenorphine (Reckitt & Colman Pharmaceuticals, Inc., Richmond, VA) was given for analgesia at a dosage of 0.01 mg/kg prior to anesthesia recovery after laser exposure and biopsy procedures.

Laser delivery was accomplished, using an Er:Glass system (Laser Sight Technologies, Inc., Orlando, FL) producing 1540 nm of light with a 0.8 millisecond exposure time and in a variable range of energy densities from 7 to 90 J/cm^2. Divide the J/cm^2 value by the exposure time to obtain the W/cm^2 value, where W represents watts. This is a class-IV laser system with a maximal output of 1 J and a minimum pulse duration of 100 nanoseconds. The complete laser system set up is depicted in Figure 1. The energy probe (Molectron model J-25/700 and Molectron EPS 2000, Molectron, OR) and germanium detector (Thorlabs model PDA-25, Newton, OR) were located after the 90/10 beam splitter (CVI Instruments model BSI-1540-90- 1025-45UNP, Albuquerque, NM) and beside the beam splitter, respectively (16). The point for laser exposure was located 20.4 cm away from the focusing lens (Newport Optics model 103.OMMCX ER-G, Irvine, CA) for an elliptical (oval) spot size of 0.002982 ± 0.000269 cm^2 for the Yorkshire pigs and 0.002527 ± 0.000423 cm^2 for the Yucatan mini-pigs.

A skin exposure grid pattern was used to deliver the laser pulses to the flank of the animal. The grid pattern was composed of 3 to 4 rows of 7 to 15 squares measuring approximately 2.5 cm^2 each. Square boundaries were marked with black ink (Sharpie marker, Sanford, CT). Prior to each individual laser exposure, the distance between the focusing lens and the flank skin was measured. The animal was positioned by moving the surgical table 2.5 cm horizontally to expose the next square. After exposing all the squares in the row, the surgical table was raised 2.5 cm and the same procedure was repeated. Energy delivered was systematically varied for each exposure. Areas exposed with given energy density for Yorkshire pigs (N = 6) and for Yucatan mini-pigs (N = 7). Three independent evaluations were performed for each exposure site for the presence of laser-induced skin lesions at 0, 1, and 24 hours. At 24 hours after exposure and with the animal under anesthesia, biopsy specimens were collected, using a 6-mm biopsy punch. All samples were fixed in 10% formalin, blocked in paraffin, cut to 5-μm thickness, stained with hematoxylin and eosin (H&E), and mounted for histologic evaluation.

The first step in the evaluation process was determination of the presence of skin lesions at 0, 1, and 24 hours after laser exposure in the Yorkshire and Yucatan mini-pigs. Skin lesions, recorded by three independent graders, were determined by use of a single blind process requiring all graders to agree on the presence of damage before a site of exposure was counted as a lesion. If any one grader disagreed, the exposure site was counted as a site of exposure without the presence of a lesion. The 24-

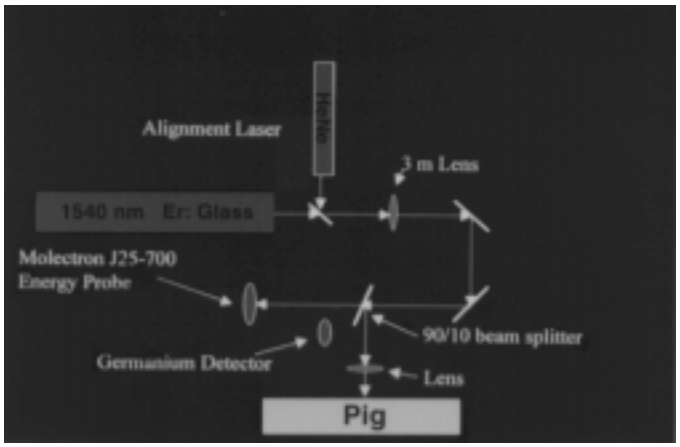

Figure 1. Simple schematic of current set up for 1540-nm laser delivery to in vivo skin models.

hour lesion data were evaluated, using SAS (Version 6.4, SAS Institute, Inc., Cary, NC) Probit analysis to obtain ED_{50} information at the 95% fiducial limits, with a *P*-value set at < 0.005 (17).

The second step included histologic evaluation of H&E-stained slides to determine epidermal lesion extent and to characterize the type of tissue and cellular damage induced by the laser exposure. Special stains, Fontana and Masson-Trichrome, were used to further characterize the tissue damage at the basal and dermal layers. Histologic evaluation findings of skin biopsy specimens collected at 24 hours after exposure were used to correlate observations between microscopic and gross lesions.

Twenty lesions were characterized for size by taking one horizontal (width) and ten vertical (depth) measurements per specimen. The vertical measurements of skin lesions were taken approximately every 0.08 mm, using a light microscope (Microstar IV, Reichert Scientific Instruments, Buffalo, NY) with an intra-ocular micrometer (Reichert Scientific Instruments, Buffalo, NY). This acted as a random method of selecting measurement sites to prevent reader bias. The measurements were taken at a magnification of 20X, and values were reported in microns (μm) to describe the mean depth of the lesions in both types of pig. The penetrability of the laser beam into skin tissue, lesion formation, and gross appearance were correlated with these data. In addition, the structural damage in the various epidermal layers (strata corneum, lucidum, granulosum, spinosum, and basale), basal membrane, and dermal layer were examined.

Results

Of the 10 pigs studied, 5 had pigmented skin (Yucatan) and the rest had non-pigmented skin (Yorkshire). The original number of exposures per individual pig and the gross lesion characterization are summarized in Table 1.

The greatest readily apparent difference between the two breeds was the appearance of gray to pale erythematous skin lesions after laser exposures in the Yucatan mini-pigs, in contrast to pale to bright erythematous skin lesions in Yorkshire pigs. In both breeds of pig, we observed well demarcated, elliptical (oval) and macular lesions < 1 mm wide. A detailed breakdown of lesions observed at 0, 1, and 24 hours after exposure is summarized in Table 2.

Histologically (Figures 2 and 3), we see well demarcated foci of epidermal necrosis and lesion development at or near the basal layer, with underlying dermal tissue damage in both breeds of pig. The strata corneum and lucidum appear unaffected by laser exposure, whereas the strata granulosum, spinosum, and basale have nuclear pyknosis, cellular swelling, cytoplasmic vacuolation, and loss of cellular detail (18–22). Laser exposure appears to affect the tissues of the dermal layer near the stratum basale of the epidermis by inducing coagulation necrosis and connective tissue fiber degeneration.

Histologic and ED_{50} data suggest a difference in lesion formation mechanisms. Compared with Yorkshire pigs, Yucatan mini-pigs require increased energy density (J/cm^2) to form 1540-nm laser-induced lesions. A summary of the statistics, energy exposures, and data points are presented in Table 3.

The mean depth ($\bar{X}$) values of laser-induced lesions was determined for the Yucatan and Yorkshire pigs. In addition, the effective absorption coefficient (μeff) for 1540-nm laser light in the skin was calculated by using the following Beer's Law formula: $\mu_{eff} = - 1/ \bar{X} \ln (T)$, where ($T$) represents the relative transmission of 1540-nm laser radiation for lightly (0.888) and heavily pigmented (0.905) human skin (23). These results are summarized in Table 4.

Table 1. Summary of gross observation of laser-induced skin lesions

Breed	Pig No.	Total Exposures[b]	Time lesion observed (h) 0 hr	1hr	24hr
Yorkshire	1	36	0	0	23
Yorkshire	2	41	0	0	29
Yorkshire[a]	3	21	0	0	0
Yorkshire	4	43	26	28	29
Yorkshire	5	44	25	28	28
Yucatan	1	110	18	27	37
Yucatan	2	60	0	15	30
Yucatan	3	21	4	6	14
Yucatan	4	30	9	28	30
Yucatan	5	26	2	14	20

[a] Exposure at low energy, lesion formation not expected.
[b] Indicates total number of individual exposures per pig.

Table 2. Gross appearance of laser-induced skin lesions

Breed	Lesion appearance[a]	Total No. of lesions 0 hr	1 hr	24 hr	Total exposures
Yorkshire	Oval, macular, pale to bright erythema	51	56	109	185
Yucatan[b]	Oval, macular, gray to bright erythema	33	90	131	247

[a]Describes the gross appearance of the hyperimic areas (lesions), oval (elliptical) lesions.
[b]Gray coloration at the lesion is related to skin pigmentation not to necrosis

Discussion

Anatomically, the skin of the Yucatan and the Yorkshire pigs is similar, with a variable degree of epidermal pegs and dermal papillae depending upon location (13). The decreased amount of hair, increased concentration of melanin granules, and increased epidermal thickness of the Yucatan mini-pig represents the greatest anatomic difference when comparison is made with the Yorkshire pig and has the greatest similarity to human skin (24). We postulate that skin response to laser light exposure increases as the degree of pigmentation decreases, and that epidermal chromophores are somehow protective. The method of laser tissue interaction for 1540-nm wavelength laser-induced skin lesions is still under investigation. Our group is conducting 1540-nm wavelength laser exposure research in Yorkshire and Yucatan pigs to further characterize differences in lesion formation, extent of tissue damage, and amount of energy needed to induce a visible skin lesion. The current hypothesis presented by our group is that melanin plays a role in the 1540-nm laser lesion formation.

There is a statistically significant difference at the 95% fiducial limits between the ED_{50} values found for these two breeds. As seen in Table 3, the lower 95% fiducial limit (50.72) for the Yucatan mini-pig does not encompass the upper 95% fiducial limit (44.81) for the Yorkshire pig. Also, the narrow bands of response for the two breeds serve to document the highly predictable nature of the skin injury on exposure to 1540-nm laser wavelength. The ED_{50} values and their associated fiducial limits were calculated using the probit function of SAS software. SAS Probit analysis is a statistical method used to determine the relationship between the strength of laser exposure and the proportion of cases that develop lesion formation. The program is useful to evaluate a dichotomous output (lesion/no lesion data) that is considered to be influenced or produced by the laser exposure energy levels (variable). It provides an excellent estimation of the effects of various laser exposure energies and has the

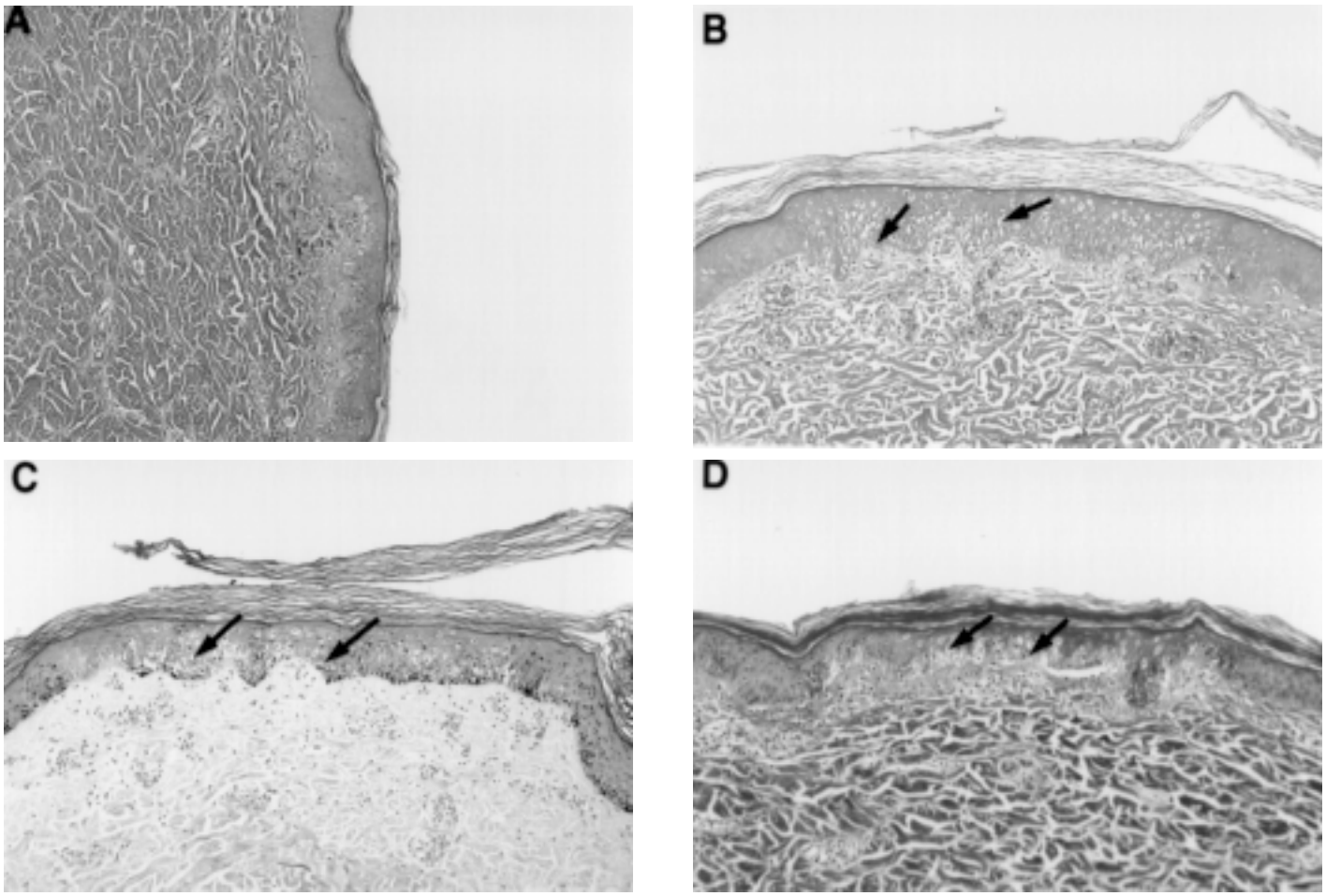

Figure 2. Photomicrographs of cross sections of the flank skin from Yucatan mini-pig. **(A)** Normal skin, H&E stain. **(B–D)** Laser-induced skin lesions. The tissue damage to epidermal layers and the underlining dermal layer seen as nuclear piknosis, cell vacuolation, and loss of cellular detail (black arrows). **(B)** H&E stain. **(C)** Notice dark melanin granules in the epidermal layer. Fontana stain. **(D)** Notice the damage to the basal and dermal layers. Masson trichrome stain. 50x magnification.

advantage of reducing the number of animals and the number of data points necessary to reach statistical significance (17, 25).

Our findings indicate three major differences in lesion formation after laser exposure in these breeds of pigs. Grossly, skin lesion formation was seen more acutely in the Yorkshire, compared with the Yucatan mini-pig. Also, the ED_{50} value in the Yucatan mini-pig requires higher energy pulses than it does in the Yorkshire pig. In addition, the μ_{eff} for the Yucatan mini-pig (8.18 cm^{-1}) differs from that for the Yorkshire pig (12.4 cm^{-1}). The value of 12.4 cm^{-1} calculated for the Yorkshire pig was identical to the attenuation coefficient of water for 1540 nm (26). Histologic evaluation of 50 H&E-stained slides documented similarities in cellular pathologic changes. These findings, together with the existing knowledge of differences in epidermal melanin concentration, as indicated by Eggleston et al. (13), and tissue thickness between the breeds, can be used to speculate that two laser-tissue damage mechanism are in operation. Current theory places water absorption with an increase in temperature as the sole mechanism for dermal tissue damage, using this exposure duration and 1540-nm light (5, 27). Our data support the theory that water absorption is not the sole mechanism of dermal tissue damage observed from 1540-nm laser exposures. Skin melanin appears to play a significant role in lesion development.

Some of the problems associated with this study include: the small size of laser-induced injuries and the blanching effect of formalin made the process of tissue sectioning for histologic examination difficult; the sequence of the pathologic process from injury to resolution was not addressed in this part of the study; representative skin lesion biopsy specimens were collected rather than every lesion, to correlate gross and histologic observations; microscopic measurements of the lesions were completed for 20 lesions to estimate the penetrability of the 1540-nm laser photons and the depth at which the lesions originate; the strata corneum and lucidum are considered translucent to laser light due to minimal water content, and differences in water content percentages between the epidermal layers for the two breeds have not been reported; differences in protein concentrations in the epidermal layers of the two breeds were not addressed in the study; and species and age differences in response to injury cannot definitively be answered with our data because our research only represents the first study trying to define the best animal model.

Microscopic evaluation and histologic characterization of the lesions in each breed of pig at various energy exposures (J/cm^2) were correlated to observed lesions. Further evaluation of tissue samples stained with Fontana and Masson-trichrome stains was necessary to correlate microscopic findings at the dermal

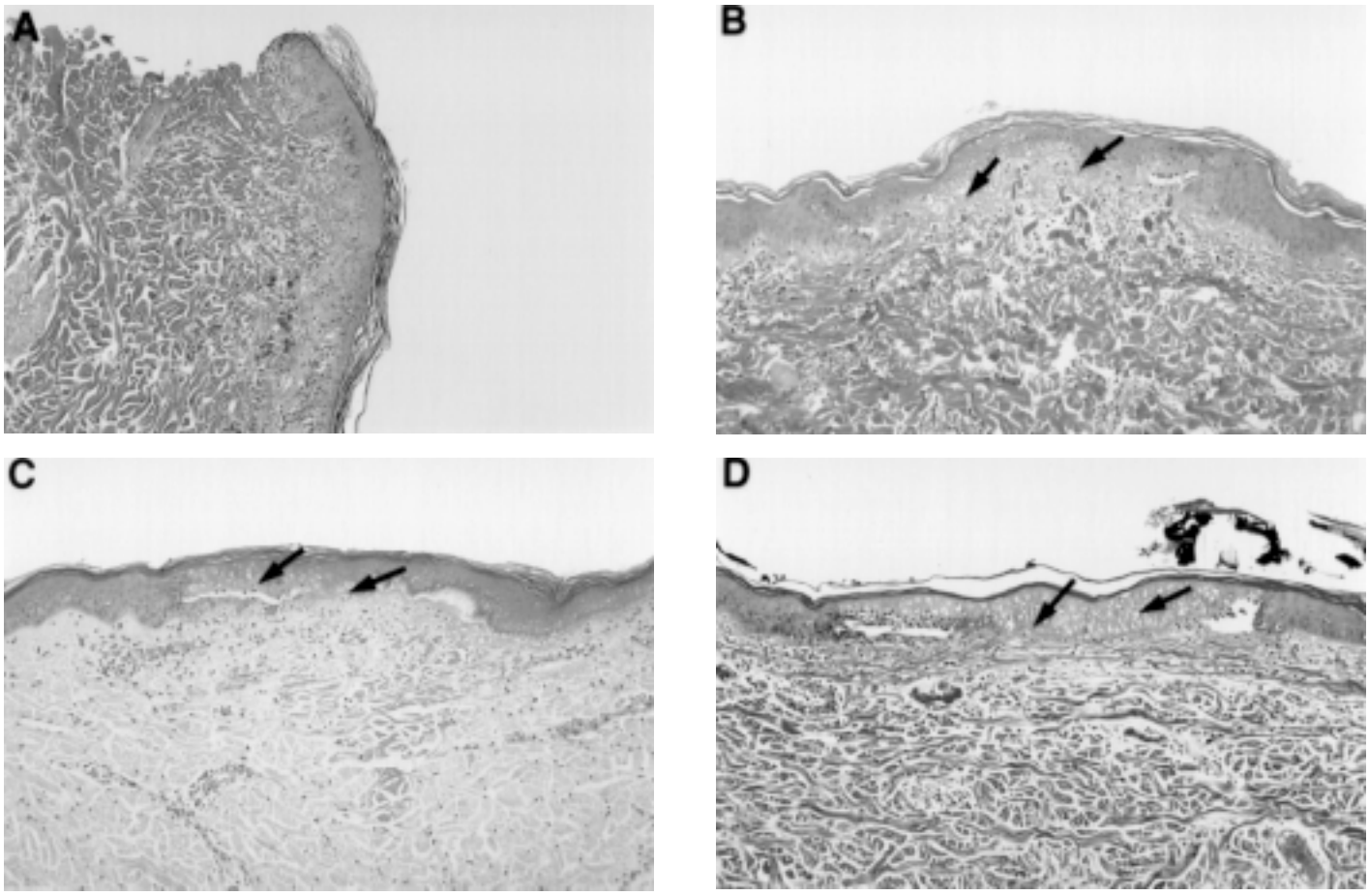

Figure 3. Photomicrographs of cross sections of the flank skin from Yorkshire pig. **(A)** Normal skin, H&E stain. **(B–D)** Laser-induced skin lesions. The tissue damage to epidermal layers and the underlining dermal layer seen as nuclear piknosis, cell vacuolation, and loss of cellular detail (black arrows). **(B)** H&E stain. **(C)** Notice lack of melanin granules in the epidermal layer. Fontana stain. **(D)** Notice the damage to the basal and dermal layers. Masson trichrome stain. 50x magnification.

Table 3. Summary of all statistics for pig skin laser exposure at 24 hours

	Exp.energy[a]	ED_{50}	95%LL	95%UL	Pearson'sX^2	X^2	Slope	Data points[b]
Yorkshire								
Energy $ED_{50,}$ mJ	35 to 255	128.30	123.20	133.64	38.82	1	28.10	180
$1/e^2$ ED_{50} spot avg[cd]	12 to 85	43.05	41.31	44.81	38.001	1	28.09	180[e]
Yucatan								
Energy ED_{50}, mJ	17 to 244	135.20	126.10	142.10	175.93	0	10.49	242
$1/e^2$ ED_{50} spot avg[cd]	7 to 96	53.53	50.76	56.24	175.94	0	10.49	242[f]

[a]Exposure energy represents the 90% energy delivered to the skin.
[b]Represents the total number of exposures done in 5 pigs per breed, 5 data points removed as outliers from the raw data for each breed.
[c]$1/e^2$ ED_{50} values given as J/cm^2. Divide this value by the exposure time to obtain the W/cm^2 value.
[d]$P < 0.01$ (χ^2 Test).
[e]N = 6 areas exposed with given energy density.
[f]N = 7 areas exposed with given energy density.

layer with macroscopic observations. This information was then used to postulate a possible laser tissue damage induction mechanism that involves melanin—a skin chromophore. The findings of this study will be valuable for further research into the pathologic changes in infrared laser-induced skin lesions, the subsequent healing process, and ultimately, the protection of all DoD personnel (28–30). This research compliments the directives of the Defense Science and Technology Strategy, Army Vision 2010 and the emerging concepts for Army modernization.

The process by which laser radiation safety standards are updated and integrated into the occupational health and safety programs is summarized as follows. The ANSI issues safety standards on a five-year cycle. Board members representing experts from academia, industry, medical community and government agencies review current laser safety issues and literature. They, as a committee, evaluate any relevant new information impacting current standards and make recommendations to modify the standards. The committee then votes on changes to the standards. These changes are then adopted and promulgated as new standards. Generally, government agencies and industry adopt and incorporate the new standards into their standard operating procedures and in their Occupational Health and Safety Program. This serves to address the requirements for periodic revisions and upgrades to documents like Air Force

Table 4. Tissue damage penetration at 24 hours after laser exposure

	Yucatan	Yorkshire
Lesion sites evaluated	84 [a]	100 [b]
Mean lesion depth	122 μm	95.5 μm
Standard deviation	±32 μm	±27 μm
Normal skin depth	68±34 μm [c]	47±19 μm
Effective absorption coefficient	8.18 cm^{-1}	12.4 cm^{-1} [d]

[a] Obtained from measurements of 9 lesions.
[b] Obtained from measurements from 11 lesions.
[c] Flank skin statistically similar to human face, neck and arm skin.
[d] Identical to the attenuation coefficient of water at 1540 nm.

Occupational Safety and Health Standard (31) and revisions to the Medical NBC Battlebook, Chapter 6 (32), both of which deal with laser radiation protection. Information derived from this study will serve to establish maximal permissible exposure levels for the protection of workers and the general public.

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