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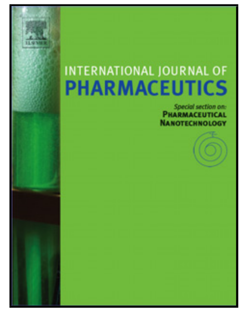

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Transcutaneous immunisation assisted by low-frequency ultrasound.

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Abstract

Low-frequency ultrasound application is known to increase the skin's permeability to large molecules such as vaccines, and to enable transcutaneous immunisation. Sodium dodecyl sulphate (SDS) – a skin irritant - is often included in the coupling medium at 1% w/v, as this has been found to enhance skin permeability. In this paper we show, for the first time, the feasibility of low-frequency ultrasound-assisted transcutaneous immunisation in the absence of SDS. Antibody titres were strongly influenced by experimental conditions. SDS presence in the coupling medium increased antibody titres, though a lower concentration of 0.5% w/v generated much higher titres than the commonly used 1%w/v, despite causing less skin damage. A lower ultrasound duty cycle of 10% generated higher antibody titres than a duty cycle of 20%, also despite causing lower skin damage. Such lack of correlation between skin damage and immune responses indicates that enhancement of skin permeability to topically-applied antigen (as indicated by changes in skin integrity) was not the main mechanism of low-frequency ultrasound-assisted skin immunisation.

Keywords: low frequency ultrasound, immunisation, transcutaneous, duty cycle, SDS concentration, skin damage

1. Introduction

Immunisation by topical application of vaccine or antigen on to the skin has the advantages of avoiding needle-associated problems such as risk of blood-borne transmission, needle-related pain, phobia and other injuries, which is expected to increase patient compliance and vaccine coverage. However, because the skin is a good barrier, its permeability to topically applied substances may need to be increased to obtain optimal immune responses by this route, and various techniques are being investigated. One such method is the application of low frequency ultrasound to the skin via a liquid coupling medium (Mitragotri & Kost 2004; Machet & Boucaud 2002).

While the literature on ultrasound-induced transdermal drug delivery is substantial, that on ultrasound-assisted transcutaneous immunisation is scant and includes a few reports on ultrasound-assisted vaccination in fish, for application in fish farming (Fernandez-Alonso et al., 2001; Zhou et al., 2002a-b; Navot et al., 2004), a report on the role of the macrophage system in the mediation of the immunostimulating effects of low frequency ultrasound (Khodareva & Sizyakina 1991) and a paper on transcutaneous vaccination in mice (Tezel et al., 2005). In the latter study, the authors showed enhanced levels of serum antibody titres, following topical application of antigen on to skin pre-treated with low-frequency ultrasound in the presence of sodium dodecyl sulphate (SDS). The anionic surfactant SDS has been shown to increase skin permeabilisation and enhance ultrasound-assisted transcutaneous delivery; in *in vitro* studies using pig skin, permeability to mannitol was increased 200-fold when SDS was included in the coupling medium compared to an 8-fold increase for ultrasound only (Mitragotri et al., 2000). This study also showed that ultrasound enhanced SDS permeation into the skin and it has been suggested that ultrasound application increased SDS dispersion in the stratum corneum (as opposed to its localisation in aggregates), which would result in a greater fraction of the stratum corneum exposed to SDS (Mitragotri et al., 2000; Tezel et al., 2002). More recently, Lavon et al., (2005) showed that when skin was concomitantly exposed to ultrasound and SDS, its pH decreased. This led the authors to propose that at the lower skin pH, SDS can permeate to a greater extent into the skin and thereby exert a greater disrupting effect, due to its existence as the free fatty acid at low pH and thus greater solubility in the stratum corneum lipids.

The scant literature on low-frequency ultrasound-assisted skin vaccination means that the influence of many experimental parameters, such as animal species, the nature and volume of coupling medium, ultrasound duty cycle and sonication time, on the immune responses is poorly understood. In this paper, we have investigated the influence of i) ultrasound duty cycle, ii) SDS presence and iii) its concentration in the coupling medium, on immune responses in mice following transcutaneous immunisation with tetanus toxoid (TTxd). We have also explored relationships between the extent of ultrasound-induced skin damage and anti-tetanus immune responses. Pre-treatment of skin with ultrasound prior to antigen application, as opposed to concurrent antigen and ultrasound application (when the antigen is present in the coupling medium), is possible due to the fact that the skin remains highly permeabilised for a number of hours following ultrasound application (Mitragotri et al., 1996) and has the advantage of avoiding exposure of antigen to potentially damaging ultrasound waves. Control experiments where the antigen was administered intramuscularly (i.m.) or topically to intact skin (in the absence of ultrasound) were also performed. In a third control group, skin was pre-treated with a solution of SDS only prior to topical antigen administration. The presence and extent of skin damage was assessed by *in vivo* measurement of trans-epidermal water loss (TEWL) – an indicator of skin integrity (Nangia et al., 2005) – and/or by histological examination of skin biopsies following immunisation using the various ultrasound protocols. Systemic immune responses were analysed by ELISA to detect antigen-specific IgG and by *in vivo* toxin neutralisation assays in mice to determine levels of functional tetanus toxin-neutralising antibodies.

2 Materials and Methods

2.1 Animals

Adult female Balb/C and NIH mice used for immunisation at The School of Pharmacy and for the *in vivo* challenge studies at the National Institute for Biological Standards and Control (NIBSC) respectively were purchased from Harlan (Oxon, U.K.). Where necessary, all animal procedures were approved by The School of Pharmacy's or the NIBSC's Ethical Review Committee and were performed in accordance with the Animals (Scientific Procedures) Act 1986. The animals were given food and water *ad libitum* during the course of the experiments.

2.2 Materials

Tesa® economy double-sided tape was obtained from RS Ltd. (Northants, UK). Ketaset® injection (containing 100 mg/mL of ketamine hydrochloride with benzethonium chloride 0.01% as a preservative) was obtained from Fort Dodge Animal Health Ltd. (Southampton, UK) and Xylazine 2% was purchased from Milipledge Veterinary (Nottinghamshire, UK). Sodium dodecyl sulphate, Tween® 20, 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablets (diammonium salt) and hydrogen peroxide (30 % v/v) were purchased from Sigma-Aldrich Company Ltd (Poole, UK). Disodium hydrogen orthophosphate, potassium dihydrogen orthophosphate and sodium chloride, used to prepare phosphate buffered saline (PBS), were all analytical grade and purchased from VWR International Ltd. (Poole, Dorset, UK). PBS tablets were purchased from Oxoid Ltd, Basingstoke. Citric acid was from Fisher, UK, and the rabbit anti-mouse IgG-horseradish peroxidise conjugate was obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Nunc™ Maxisorb Immuno ELISA plates were obtained from Thermo Fisher Scientific (Loughborough, UK). For immunisation and coating of ELISA plates, purified non-adsorbed tetanus toxoid (NIBSC code 02/126, 12.5 mg/ml, 5000 flocculating units [Lf] per ml) was used. For ELISA, an in-house reference serum with a tetanus antitoxin potency of 1.68 IU/ml was used. For the *in vivo* toxin neutralisation test, the International Standard for Tetanus Anti-toxin, Equine (NIBSC catalogue number TE-3, 120 IU/ampoule) was used as the reference.

2.3 Assessment of ultrasound-induced skin damage

Balb/c mice (6-8 weeks old, whose abdominal fur had been removed using electric clippers 24h previously), in groups of 5, were anaesthetised using an intra-peritoneal injection of a mixture of 0.9 ml/kg of ketamine

HCl solution (100 mg/ml) and 0.5 ml/kg of xylazine solution (20 mg/ml), and were placed on their back. The baseline pre-sonication TEWL was measured using the condenser-chamber evaporimeter, AquaFlux^{TM} (BIOX Ltd, UK). Subsequently, a custom-built flanged cylinder was attached to the shaved abdomen using double-sided tape (Tesa, UK) and firmly held by a clamp fixed to a retort stand. The sonicator probe was lowered into the flanged cylinder and placed at 7.5mm from the skin. The flanged cylinder was then filled with 20 mL of the coupling medium (water or SDS aqueous solution at concentrations 0.5 or 1 % w/v), and pulses of ultrasound waves (20 kHz, generated by VCX500 at 20% machine amplitude, Sonics & Material Inc, USA) were applied for a total sonication period of 45 s. Ultrasound was applied with a duty cycle of 10 or 20% (i.e. 0.1s on 0.9s off or 0.2s on 0.8s off respectively). The ultrasound protocols 1-4 described in Table 1 were used. After sonication, the ultrasound probe, coupling medium and the flanged cylinder were removed, and the abdominal skin was washed with 500 mL of water and wiped dry with tissue paper. For the three groups exposed to an ultrasound duty cycle of 20%, the skin TEWL was measured at 5, 15, 30, 45 and 60 minutes post-sonication, to determine the effect of sonication on TEWL and the post-sonication recovery. At the end of the experiment, animals were killed by exposure to carbon dioxide gas and skin biopsies were taken. The biopsies were treated with formalin and alcohol, embedded in paraffin wax, sectioned, stained with haematoxylin and eosin and examined with light microscopy to determine the effect of ultrasound and SDS concentration on skin damage. For the group exposed to an ultrasound duty cycle of 10%, skin damage was only evaluated by examination of skin biopsies prepared as described above.

2.4 Transcutaneous immunisation

For transcutaneous immunisation, Balb/c mice were shaved, anaesthetised and exposed to ultrasound as described in Section 2.3. After sonication, the ultrasound probe, coupling medium and the flanged cylinder were removed, and the abdominal skin was washed and wiped dry as described in Section 2.3. Mice (in groups of 5) were then immunised by pipetting one hundred µl of purified non-adsorbed tetanus toxoid (containing 30 Lf, 75 µg of TTxd in PBS per dose) on to the prepared skin surface. The antigen solution was spread over the prepared skin area using a gloved finger, and left in place for one hour. Subsequently, the mouse abdominal skin was washed with lukewarm tap water, dried and the mouse was returned to its cage. The procedure was repeated with the same antigen dose on days 15 and 46 to administer the first and second booster antigen doses respectively and the animals were bled on days 14, 45 and 60 via the tail vein to determine antibody levels. Blood samples were allowed to coagulate at 5°C overnight before separating the serum by centrifugation at 5000rpm for 10 minutes. The serum was collected and stored at -20ºC until assayed for antibody levels by enzyme linked immunosorbent assay (ELISA) or *in vivo* toxin neutralisation test as described in Sections 2.5-2.6 below.

In control experiments, 2 groups of mice (n=5 in each group) were treated as above, except that ultrasound was not applied. Instead, mouse skin was exposed to 20mL of either PBS or 1% w/v SDS solution for 5 min prior to antigen application for 1h. As a positive control, one group of mice was immunised i.m. with 50 µL of the antigen solution (containing 2 Lf, 5 µg of TTxd in PBS) in the hind leg. Animals were bled and received booster antigen doses as described above. The different immunisation protocols are summarised in Table 1.

2.5 Determination of anti-tetanus toxoid IgG titres using ELISA

To measure the total anti-TTxd IgG responses an equal volume of serum was pooled for each of the 5 animals in the group and analysed by ELISA. 96-well ELISA microplates were coated with 100 µl per well of purified TTxd (0.5 Lf/ml in 0.05M carbonate buffer pH 9.6) and incubated at 4°C overnight. The ELISA plates were then washed three times in PBS containing 0.05% v/v Tween-20 (PBS-T) and dried by blotting onto absorbent paper towel. The plates were then blocked with 150 µl of PBS-T containing 5% w/v skimmed milk powder (Marvel) for 1 h at 37°C. The plates were washed as described previously and serial two-fold dilutions of test and reference serum (diluted in PBS-T containing 1% skimmed milk powder) were prepared across the plate. The final volume of serum in each well was 100 µl. The plates were then incubated for 2 h at 37°C. After 2 h, plates were washed as described previously and antigen specific IgG antibodies were detected using a horseradish peroxidase-conjugated rabbit anti-mouse IgG diluted 1/2000 in PBS-T containing 1% w/v skimmed milk powder (100 µl per well). After a further incubation at 37°C for 1 h, and a final wash, the chromogen solution (prepared immediately before use by dissolving 1 ABTS tablet in 20 ml 0.05 M citric acid buffer, pH 4.0 and adding 5 µl of 30% v/v hydrogen peroxide solution) was added and the reaction was allowed to develop for 30 min. The optical density was then measured at 405 nm (A_{405}) using a Multiscan ELISA plate reader (Thermo

Life Sciences, UK). Antibody responses were analysed by an in-house parallel line bioassay program using a minimum of 3 points for test and reference samples. ANOVA was used to test the significance of departures from linearity and parallelism. The anti-TTxd IgG titres were expressed in IU/ml against the in-house reference mouse serum.

2.6 Protection against tetanus following *in vivo* challenge

In order to confirm whether the serum IgG from immunized mice was protective against tetanus toxin, passive challenge studies were performed in groups of female NIH mice using the onset of paralysis as the end point. The potency of tetanus antitoxin in the test serum sample was determined by comparing the dose necessary to protect mice against the paralytic effects of a fixed dose of tetanus toxin with the quantity of a reference tetanus antitoxin required to give the same protection. A series of dilutions of the reference antitoxin (International Standard for Tetanus Anti-toxin, Equine TE-3) and of pooled serum samples from each group were prepared in gelatin-phosphate buffered saline (GPBS) at concentrations of 0.0007 – 0.002 IU/ml. A fixed volume of purified tetanus toxin solution (containing approximately 50 mouse paralytic doses (50 x PD_{50}) was then added to all samples and the mixtures were allowed to stand for 30 minutes at room temperature to allow toxin neutralisation to occur. Mice (in groups of 4) were then injected subcutaneously in the hind leg with 0.5 ml of toxin/antitoxin mixture and observed over a 96 h period for signs of paralysis. The protective capacity of the test mouse serum samples was compared against the ability of the reference antitoxin to confer protection against the paralytic effects of the tetanus toxin in 50% of the animals. Tetanus antitoxin concentrations were expressed in IU/mL.

2.7 Statistical analyses

Statistical analyses were performed using SPSS software version 15 (SPSS Inc.) to determine the influence of ultrasound application on TEWL. Paired t-tests between TEWL at 0 and 5 minutes were conducted to determine the immediate change in TEWL upon sonication. To statistically assess the influence of SDS presence and its concentration on TEWL recovery with time post-sonication, repeated measures ANOVA was conducted on TEWL values for 5-60 minutes. Results were considered statistically significant when $p<0.05$.

3. Results and Discussion

3.1 Influence of SDS concentration and duty cycle on ultrasound-induced changes to skin integrity in vivo

In order to identify possible relationships between ultrasound-induced changes to the skin's structural integrity (which is expected to be related to antigen flux into the skin) and immune responses, the impact of SDS presence and concentration in the coupling medium and ultrasound duty cycle on *in vivo* skin integrity (indicated by TEWL and histological examination of excised skin) was determined.

In the absence of SDS in the coupling medium, the mild Protocol 1 (<1min of sonication applied in brief (0.2s) pulses separated by fairly long intervals (4x pulse duration) using a large volume (20mL) of coupling medium) caused a small (less than 2x) but statistically significant increase in TEWL ($p<0.05$, paired t test between time 0 and 5 min, Figure 1) which returned to baseline levels after 1h, and histological examination showed no obvious disruption of the skin barrier (Figure 2b compared to Figure 2a). Inclusion of SDS in the coupling medium caused a larger (4-5x) increase in TEWL, whose magnitude and recovery was related to SDS concentration (Figure 1, repeated measures ANOVA, $p<0.05$). The greater increase in post-sonication TEWL and lack of recovery after 1h by the higher (1%w/v) SDS concentration was reflected in histological assessment, where vertical channels in the skin were revealed (Figures 2d). Increased in vivo skin damage in the presence of SDS reflects previous in vitro reports on enhanced skin conductivity by ultrasound/SDS combinations (Mitragotri et al., 2000). The negative effect of 1% SDS on skin integrity could be reduced by a milder (10% versus 20% duty cycle) ultrasound protocol, as shown in Figure 2e. The stratum corneum was visible, without any loss, there were no vertical ‘channels’ that were observed in skin sonicated with a 20% cycle, and the micrograph was similar to that of control non-sonicated skin (Figure 2a). Lesser skin damage with the lower duty cycle could be due to some skin recovery during the longer (0.9 vs 0.8s) pulse interval from any damage incurred during the much shorter (0.1 vs 0.2s) pulse and a lower rate of increase in the coupling medium temperature (Dahlan et al., 2005) which would limit any heat-associated adverse effects on skin integrity.

3.2 Anti-tetanus toxoid IgG and neutralising antibody responses following parenteral or topical antigen administration in mice

3.2.1 Topical antigen application to intact skin in the absence of ultrasound

The primary responses were below the limit of detection, and the secondary responses are shown in Figure 3 and in Table 2. In the absence of ultrasound (protocols 5-6), IgG titres remained low or undetectable (Figure 3), correlating with previous reports on antigen application to intact skin, in the absence of adjuvants (Tierney et al., 2003, Glenn et al., 1998; Strid et al., 2004; Godefroy et al., 2005). Interestingly, the observed failure of SDS skin pre-treatment to enable transcutaneous immunisation is in contrast to a previous report that showed antibody titres to hen lysozyme antigen when the latter was applied to shaved mouse skin pre-treated with 100 µL of SDS solution for 10 minutes (Huang et al., 2006). The positive results by Huang et al. could have been due to much higher SDS concentrations used (though the concentration is unclear, being reported as 0.1-5.0% v/v - without an indication of the SDS concentration in the stock solution), the smaller hen eggwhite lysozyme antigen (~10x smaller than tetanus toxoid), and the use of a patch over the antigen formulation. Patch application could have increased skin hydration, known to increase skin permeability (Zhai & Maibach 2005). Other studies in mice have shown protective response to tetanus merely by prolonging the duration of antigen contact with skin by use of a patch (Naito et al., 2007).

3.3.2 Ultrasound-assisted transcutaneous immunisation

Pre-treatment of mouse skin with ultrasound (with PBS as the coupling medium, protocol 1) prior to topical antigen application induced significant levels of anti-TTxd IgG and protective toxin-neutralising antibodies, although responses were still more than 30-fold lower when compared to those induced by conventional parenteral immunization (Figure 3, Table 2). However, the levels of protective toxin-neutralising antibodies were above those considered necessary for protection against tetanus in humans (Balmer et al., 2007). Achieving protective levels of anti-tetanus immune responses following antigen application to ultrasound-pretreated skin, in the absence of SDS in the coupling medium, or adjuvant, shows the promise of ultrasound for skin immunisation, and further study with other antigens and in other species is merited. For ultrasound-assisted skin permeabilisation, SDS is often included in the coupling medium at 1% w/v, (Tezel et al., 2004; Mitragotri & Kost 2001; Tezel et al., 2005), despite the fact that it is a skin irritant (Agner & Serup 1990), especially when applied concomitantly with other irritants (Fluhr et al., 2005). However, we show for the first time the feasibility of using low-frequency ultrasound for skin immunisation, in the absence of SDS. It seems that application of low-frequency ultrasound, even in the absence of SDS in the coupling medium, enables sufficient antigen to permeate into the skin to be taken up by the cells of the skin immune system. Alternatively, it may be that topical ultrasound application, which is known to increase epidermal Langerhans cell density (Tezel et al., 2005), stimulated the skin immune system sufficiently such that a relatively low permeation of the antigen into the skin could result in considerable antibody titres.

When SDS was included in the ultrasound coupling medium (20% duty cycle, protocols 2-3), the anti-tetanus IgG and neutralising antibody titres were increased further, reflecting the synergistic effects of ultrasound and SDS on skin permeability to topically applied drugs (Mitragotri et al., 2000; Tezel et al., 2002). However, the increase in antibody titres was inversely proportional to the SDS concentration in the medium (Figure 3, Table 2), and did not correlate with the extent of skin damage observed in the presence of SDS (Figures 1, 2c-d). The highest immune responses were seen in the presence of 0.5% w/v SDS and, after 3 antigen doses, IgG and neutralising antibody titres were 12-fold higher than those obtained with ultrasound alone and only 3-4fold lower than those induced by conventional i.m. injection of antigen. Doubling the SDS concentration in the coupling medium (to 1% w/v) had a negative effect on antibody titres, with a 4-5 fold reduction in anti-TTxd IgG and neutralising antibody titres. This suggests a possible detrimental effect of SDS presence in the coupling medium on antigen integrity. Ultrasound application in the presence of SDS is known to reduce the pH of the deeper skin layers (Lavon et al., 2005), and low pH reduces the structural integrity and antigenicity of tetanus toxoid (Xing et al., 1996).

The lack of correlation between observed changes in the structural integrity of the skin barrier and immune responses was again seen in mice immunised in the presence of a reduced ultrasound duty cycle of 10% (protocol 4). In the presence of 1% SDS, the lower duty cycle of 10% generated two-fold higher IgG and neutralising antibody titres than those obtained with the higher ultrasound duty cycle at the same SDS concentration (Figure 3, Table 2). This lower ultrasound duty cycle did not induce the structural changes in the skin barrier observed with the higher cycle and the same SDS concentration (Figure 2d-e) providing further evidence that the antibody responses may not be related solely to the permeability and hence antigen dose in the skin. Other factors such as the extent of stimulation of the skin immune system are likely to play an important role in the response to skin immunisation. It was mentioned earlier that ultrasound application on its own, in the absence of antigen, increases epidermal Langerhans cell density (Tezel et al., 2005). What is not known is the influence of ultrasound protocols on such stimulation of Langerhans cells. A greater stimulation of Langerhans cells could have occurred by the 10% duty cycle ultrasound due to the longer duration over which ultrasound was applied - a period of 450 seconds - in contrast to half that duration for the 20% duty cycle to obtain a total sonication time of 45s in both cases. Greater stimulation of the Langerhans cells could compensate for lower antigen presence in the skin. This requires further investigation.

4. Conclusions

We have shown that application of tetanus toxoid to skin pre-treated with low-frequency ultrasound resulted in anti-tetanus toxoid IgG and neutralising antibody titres that were above those required for protection against tetanus, even in the absence of SDS in the coupling medium. This indicates that SDS – a skin irritant - may not be required for low-frequency ultrasound assisted transcutaneous immunisation. SDS presence in the coupling medium at 0.5 or 1% w/v increased antibody titres, though the increase was inversely related to SDS concentration, despite lower skin damage caused by the lower SDS concentration. Ultrasound duty cycle (10 or 20%) was also found to influence antibody titres, the lower duty cycle generating two-fold higher IgG and neutralising antibody titres, again despite lower skin damage. This indicates that enhancement of skin permeability to topically-applied antigen (as indicated by changes in skin integrity) was not the main mechanism of low-frequency ultrasound-assisted skin immunisation.

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Figure legends and Table headings

Figure 1: *In vivo* TEWL from mouse skin when different coupling media were used. PBS (×); 0.5% w/v SDS solution (■) and 1% w/v SDS solution (▲). Time 0 shows baseline (before sonication) TEWL values. Means and standard deviation bars are shown, n=5.

Figure 2: Micrographs showing the influence of SDS concentration in the coupling medium and ultrasound duty cycle on skin integrity; A) control (non-sonicated skin), B-D) skin pre-treated with ultrasound 20% duty cycle using as coupling medium: B) PBS, C) 0.5%w/v SDS, D) 1%w/v SDS, and E) skin pre-treated with 10% duty cycle ultrasound using 1%w/v SDS as coupling medium. Bar represents 100μm.

Figure 3. Total anti-TTxd IgG responses in mice following immunisation via parenteral and transcutaneous routes (see Table 1 for full details). Immune responses were measured by ELISA using pooled serum from all animals in each group. Data are anti-TTxd IgG (with 95% confidence limits) calculated by parallel line analysis and expressed in IU/ml against an in-house mouse reference serum.

Table 1. Immunisation protocols for parenteral and transcutaneous immunisation of mice. Ultrasound was applied with a duty cycle of 20% (0.2s on 0.8s off) or 10% (0.1s on 0.9 s off) for a total sonication time of 45s, using 20ml of coupling medium (PBS or 0.5 or 1% w/v SDS solution), with the ultrasound probe at 7.5mm from the skin.

Table 2. Day 60 neutralising antibody responses in mice following immunisation via parenteral and transcutaneous routes (see Table 1 for full details). Immune responses were measured by ELISA using pooled serum from all animals in each group. Data are in IU/ml against a reference tetanus antitoxin. N.D= not determined.

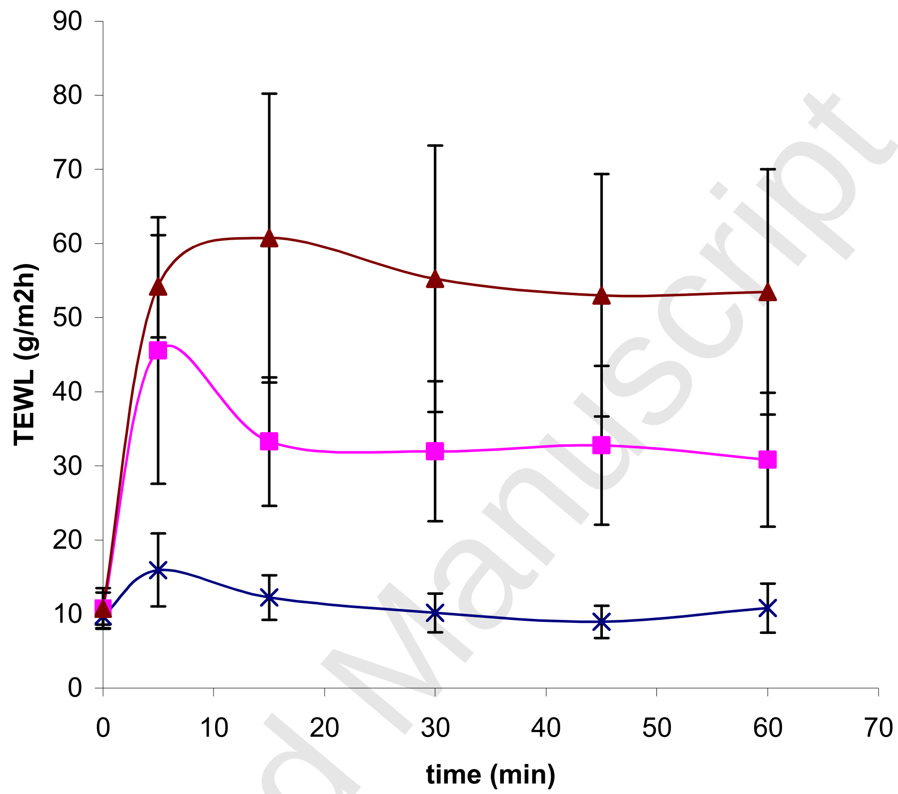


Figure 1: *In vivo* TEWL from mouse skin when different coupling media were used. PBS (×); 0.5% w/v SDS solution (■) and 1% w/v SDS solution (▲). Time 0 shows baseline (before sonication) TEWL values. Means and standard deviation bars are shown, n=5.

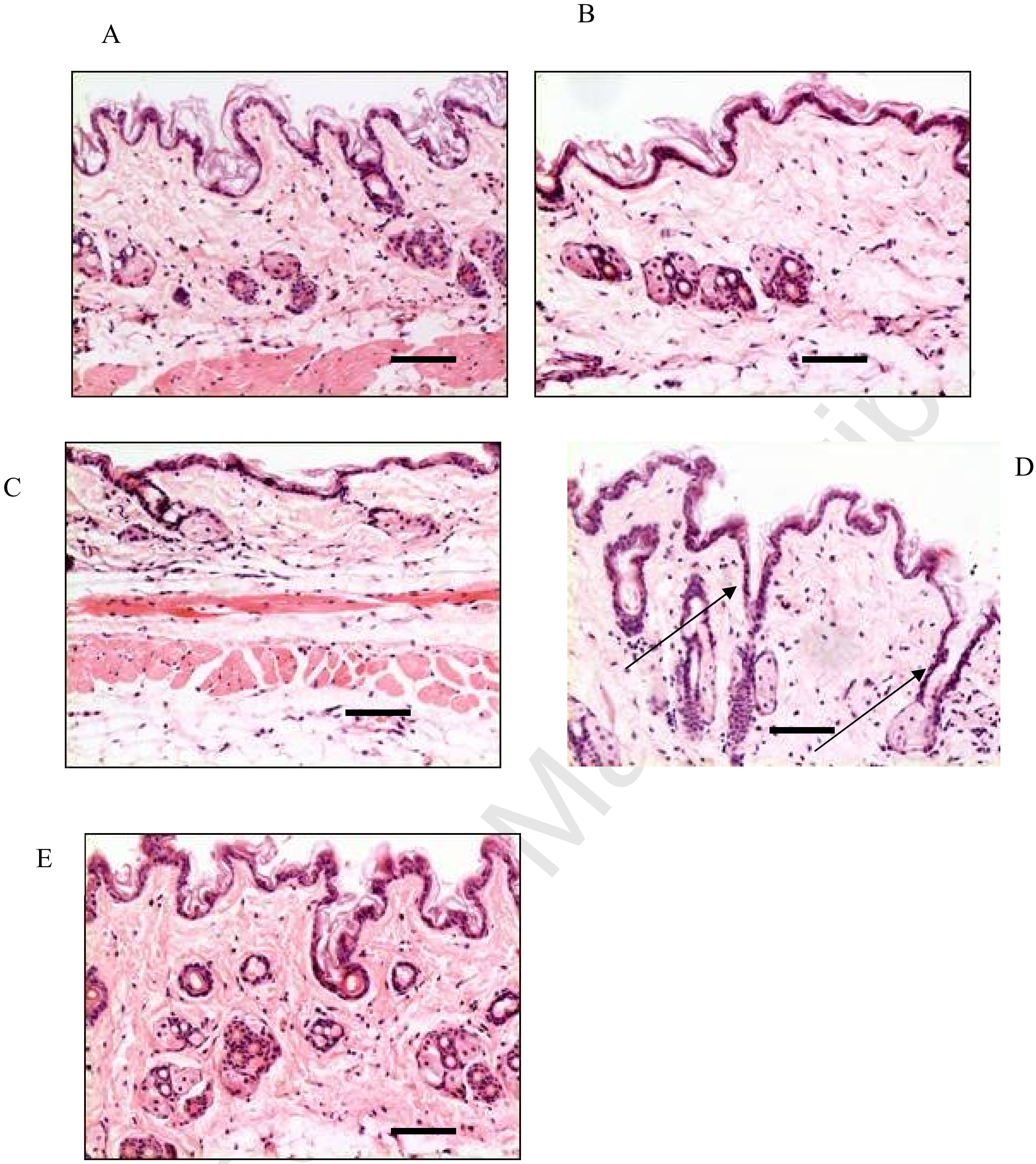
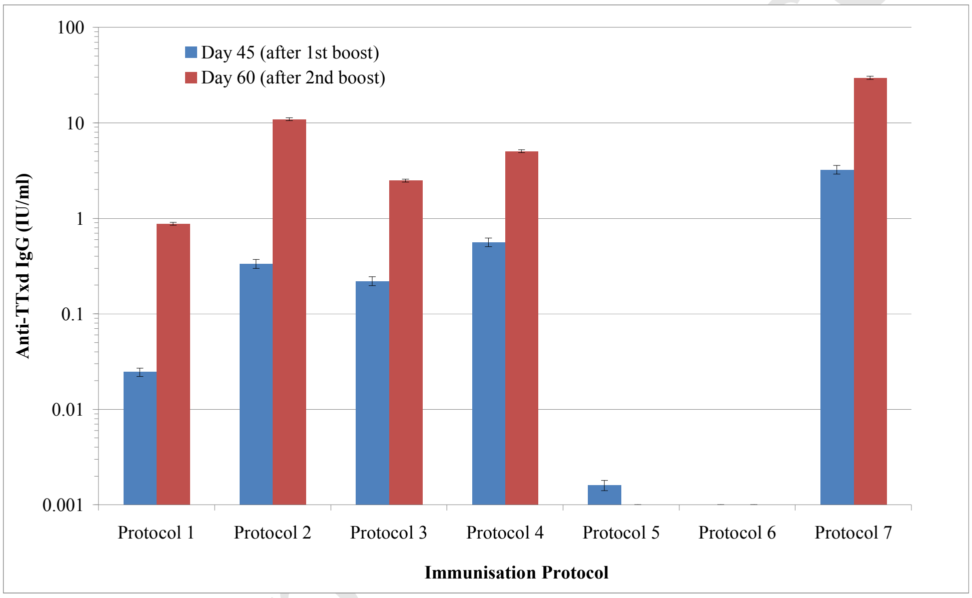


Figure 2: Micrographs showing the influence of SDS concentration in the coupling medium and ultrasound duty cycle on skin integrity; A) control (non-sonicated skin), B-D) skin pre-treated with ultrasound 20% duty cycle using as coupling medium: B) PBS, C) 0.5%w/v SDS, D) 1%w/v SDS, and E) skin pre-treated with 10% duty cycle ultrasound using 1%w/v SDS as coupling medium. Bar represents 100µm.



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Immunisation protocol	**Immunisation route**	**Skin Pre-treatment Prior to application of antigen**
1	Transcutaneous	Ultrasound 20% duty cycle + PBS
2	Transcutaneous	Ultrasound 20% duty cycle + SDS 0.5% w/v
3	Transcutaneous	Ultrasound 20% duty cycle + SDS 1.0% w/v
4	Transcutaneous	Ultrasound 10% duty cycle + SDS 1.0% w/v
5	Transcutaneous	PBS
6	Transcutaneous	SDS 1.0% w/v
7	i.m.	N/A

Table 2. Day 60 neutralising antibody responses in mice following immunisation via parenteral and transcutaneous routes (see Table 1 for full details). Immune responses were measured by ELISA using pooled serum from all animals in each group. Data are in IU/ml against a reference tetanus antitoxin. N.D= not determined.

Immunisation protocol		**In vivo anti-tetanus neutralising antibody titres (IU/mL)**
1	Ultrasound 20% duty cycle + PBS	0.62
2	Ultrasound 20% duty cycle + SDS 0.5% w/v	7.75
3	Ultrasound 20% duty cycle + SDS 1.0% w/v	1.54
4	Ultrasound 10% duty cycle + SDS 1.0% w/v	3.58
5	PBS	N.D
6	SDS 1.0% w/v	<0.08
7	Intramuscular	29.69