Distribution of esterase activity in porcine ear skin, and the effects of freezing and heat separation*

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Porcine ear skin is widely used to study skin permeation and absorption of ester compounds, whose permeation and absorption profiles may be directly influenced by in situ skin esterase activity. Importantly, esterase distribution and activity in porcine ear skin following common protocols of skin handling and storage have not been characterised. Thus, we have compared the distribution and hydrolytic activity of esterases in freshly excised, frozen, heated and explanted porcine ear skin. Using an esterase staining kit, esterase activity was found to be localised in the stratum corneum and viable epidermis. Under frozen storage and a common heating protocol of epidermal sheet separation, esterase staining in the skin visibly diminished. This was confirmed by a quantitative assay using HPLC to monitor the hydrolysis of aspirin, in freshly excised, frozen or heated porcine ear skin. Compared to vehicle-only control, the rate of aspirin hydrolysis was approximately three-fold higher in the presence of freshly excised skin, but no different in the presence of frozen or heated skin. Therefore, frozen and heat-separated porcine ear skin should not be used to study the permeation of ester-containing permeants, in particular co-drugs and pro-drugs, whose hydrolysis or degradation can be modulated by skin esterases.

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1 Introduction

Esterases are found in the endoplasmic reticulum and cytosol of many mammalian tissues [1–3]. In human skin, the distribution of esterases is inconsistent across the various skin layers [4–6]. The presence of esterases in the skin is important to the topical or transdermal delivery of drugs, because the enzymes may catalyse the hydrolysis of pharmaceuticals containing ester bonds. This can be disadvantageous in the case of ester derivatives that are intended to diffuse across the skin intact, as premature hydrolysis of the ester bonds in these drugs by skin esterases may greatly reduce their bioavailability [7]. However, topical ester pro-drugs or co-drugs, which are themselves inactive but designed to be metabolised in the skin to release the pharmacologically active moieties in situ, may benefit from the catalytic activity of skin esterases [8–12].

Excised porcine ear skin has been widely used to model human skin in a variety of pharmaceutical studies due to their structural and biochemical similarities [13, 14]. However, rarely is esterase activity in such applications given adequate emphasis, except perhaps where in situ metabolism is a desired outcome, such as in the case of topical pro-drugs [15] and co-drugs [16]. Since variations in esterase activity exists between species and tissues [4, 17], in order to effectively assess the in vivo pharmacokinetics for a topically applied drug using in vitro models, it is essential to match the levels of esterase activity as closely as possible to the in vivo setting, or at least account for any discrepancies between them. Esterase activity in human, rat and minipig skin has been characterised with a view to assess their suitability as ex vivo models of human dermatopharmacokinetics [4, 5, 18, 19]. To our knowledge, no such assessment has been reported for porcine ear skin. This is of particularly pressing concern considering the widespread use of this model to assess topical and transdermal drug delivery, and more so owing to the various modes of skin storage and manipulation prior to such assessments which may alter esterase activity. For example, a number of studies have shown, variously in human, rat and snake skin, that heating (at or above 60°C) generally reduces esterase activity, whereas esterase activity can be preserved following storage at or below −20°C [20–23]. However, the latter findings have been contradicted in our laboratories where frozen porcine ear skin previously demonstrated a reduced capacity to hydrolyse ethyl butyrate compared to fresh porcine ear skin [8]. We therefore considered it important to probe deeper into the distribution and activity of esterases in porcine ear skin.

The aim of this study was to qualitatively characterise the distribution of esterase activity in porcine ear skin at basal levels and following common protocols of skin storage and manipulation for permeation studies. The practical impact of differences in esterase activity between these tissues was assessed by examining the hydrolysis of aspirin (acetylsalicylic acid), a model ester drug, in fresh, frozen and heated porcine ear skin.

2 Materials and methods

2.1 Materials

Porcine ears were obtained from a local abattoir immediately post-mortem and prior to steam cleaning. Hanks balanced salt, ammonium thiocyanate (NH_4SCN) and the α-naphthyl acetate non-specific esterase kit were obtained from Sigma–Aldrich (Gillingham, UK). Dulbecco's modified Eagle medium (DMEM) and foetal bovine serum (FBS) were purchased from Invitrogen (Paisley, UK). The Optimum Cutting Temperature (OCT) embedding medium was obtained from Raymond A Lamb (Eastbourne, UK). All other reagents were obtained from Fisher Scientific (Loughborough, UK).

2.2 Skin preparation

Porcine ears were immersed in iced Hanks balanced salt solution (HBSS) for transportation to the laboratory. Following brief cleaning under running tap water, full-thickness skin was separated from the underlying cartilage by blunt dissection using a scalpel. Hairs were removed with an electric hair clipper.

2.3 Cross-sectional visualisation of skin esterase activity

Skin samples were cut into approximately 4 mm^2 pieces and embedded in OCT embedding medium for cryosectioning. To investigate the effect of explant conditions on skin esterases, the skin samples were placed in HBSS or DMEM supplemented with 10% FBS, and incubated at 32°C for 3, 6, 9 and 24 h before OCT embedding, alongside samples which were embedded immediately to establish baseline esterase levels. Cryosections (10 μm) were produced using the Leica CM3050 S cryomicrotome (Leica, Bensheim, Germany) and mounted on glass microscopy slides. Non-specific esterase staining was performed using the α-naphthyl acetate kit according to the supplier's protocol, with a slight modification. In brief, the cryosections were fixed in a citrate–acetone–formaldehyde solution for 30 s, with rigorous shaking for the last 5 s. The slides were thoroughly rinsed with deionised water and incubated for 10 min, at 37°C, in the dark, with the substrate solution. Other slides were frozen at −20°C for a week, or heated at 60°C for 15 min to denature enzymes, before staining with the α-naphthyl acetate kit. All slides were then rinsed with deionised water and counter-stained with Harris's haematoxylin. The specimens were examined by bright-field microscopy under the Olympus™ BH-2 microscope and representative micrographs taken with an Olympus™ DP-12 camera (both from Olympus, Oxford, UK).

2.4 En face visualisation of skin esterase activity

Full-thickness skin samples were used either without further treatment or subjected to the following treatments relating to common protocols used to prepare in vitro skin models for permeation studies: (a) explanted in DMEM supplemented with 10% FBS, for 24 h at 32°C; (b) frozen at −20°C for 1 week; (c) heated in tap water at 60°C for 1 min; (d) incubated with 3.8% ammonium thiocyanate solution in phosphate buffered saline (pH

7.4) for 30 min. Each skin sample was then tape-stripped with adhesive tape to extract cells for non-specific esterase staining. Seven successive tape strips were obtained for each skin sample. However, the loss of structural strength in heat- and chemical-treated skin samples rendered complete tape strips progressively more difficult to obtain. Non-specific esterase staining of the tape strips was as described for the cryosections above but without counter-staining with haematoxylin.

2.5 Hydrolysis of acetylsalicylic acid

Freshly excised, frozen (−20°C for 1 week) and heated (60°C for 1 min) porcine ear skin samples were used. Full-thickness skin samples, each weighing 3 g, were cut to 4 mm^2 (en face dimensions) pieces and placed in universal bottles containing 8 mL of 100 μg mL^{-1} aspirin solution in HBSS. At pre-defined time points, a sample of aspirin solution was removed from each bottle, diluted 1:1 with the mobile phase, and analysed by high performance liquid chromatography (HPLC).

2.6 High performance liquid chromatography analysis

Hydrolysis of aspirin yields salicylic acid (SA) and acetic acid. A HPLC method was developed to simultaneously assay aspirin and SA. Samples were analysed at ambient temperature using an Agilent 1100 series automated HPLC system. The instrument was fitted with a Phenomenex C18, 5 μm, 250 mm × 4.6 mm column and a Phenomenex C18, 3 μm, 30 mm × 4.6 mm column (Phenomenex, Macclesfield, UK). Detection wavelength was set at 300 nm with a flow rate of 1 mL min^{-1}. The injection volume for both aspirin and SA was 100 μL. An isocratic elution programme was used based on the mobile phase of methanol (70%, v/v) and water (30%, v/v) at pH = 2. Calibration curves were constructed separately for aspirin and SA, each with a R^2 of >0.99.

2.7 Statistical analysis

One-way ANOVA and Tukey post hoc tests were performed using GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA). Statistical significance was defined as $p < 0.05$.

3 Results

3.1 Localisation of esterases in porcine ear skin

In cryosections of porcine ear skin, counter-staining of cell nuclei by haematoxylin enabled the stratum corneum, viable epidermis and dermis to be distinguished visually (Figure 1B). Black staining of esterase activity was observed in the epidermis of freshly excised skin (Figure 1A). The most intense staining, indicating the greatest amount of esterase activity, was observed proximal to the stratum corneum (SC). The SC was also stained in black, suggesting considerable esterase activity on the skin surface. This was verified by examining tape-strips of freshly excised skin in a separate experiment, whereby

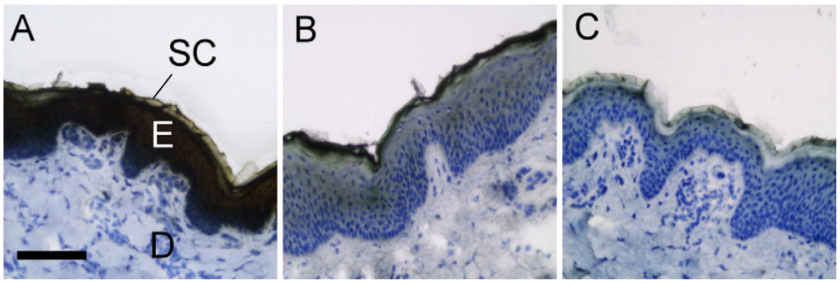

Figure 1: Representative images depicting the distribution of basal esterase activity in cryosections of porcine ear skin, visualised by histochemical staining. More intense black staining indicates a higher level of esterase activity. Skin pre-treatment: (A) freshly excised; (B) frozen at −20°C for a week; (C) heated at 60°C for 15 min. SC, stratum corneum; E, epidermis; D, Dermis. Images are at same magnification. Scale bar = 200 μm.

considerable esterase activity was detected using the same histochemical method, in up to the fourth tape-strip performed (Figure 2). Conversely, the dermis was entirely devoid of black staining, suggesting that no functional esterase was present within the dermis.

3.2 Esterase activity in frozen and heated skin

In frozen skin, epidermal staining for esterase activity was greatly reduced (Figure 1B). However, in the SC, the intensity of the staining was not noticeably different from freshly excised skin. In skin samples which had been heated at 60°C for 15 min, neither the epidermis nor the SC exhibited positive staining for esterase activity, suggesting obliteration of functional esterases by thermal treatment (Figure 1C). The first tape-strips of frozen and heated skin corroborate these observations for the SC (Figure 2). Consistent with observations made in freshly excised skin, no staining of esterase activity was observed in the dermis of either frozen or heated skin.

3.3 Esterase activity in skin explants

Similar to freshly excised skin, positive staining for esterase activity was observed in the viable epidermis and SC of skin explants (Figure 3). The presence of esterases in the SC was confirmed by the tape-strips of skin explants following 24-h incubation in FBS-supplemented DMEM (Figure 2). Between the explants incubated in HBSS and those incubated in FBS-supplemented DMEM, there was no discernible difference in esterase activity until 24 h post-incubation. At 24 h, esterase activity noticeably declined in those skin samples explanted in HBSS (Figure 3).

3.4 Hydrolysis of acetylsalicylic acid in the presence of porcine ear skin

Generally, with all skin treatments and in the vehicle-only control, the concentration of aspirin decreased over time, accompanied by a corresponding increase in SA concentration

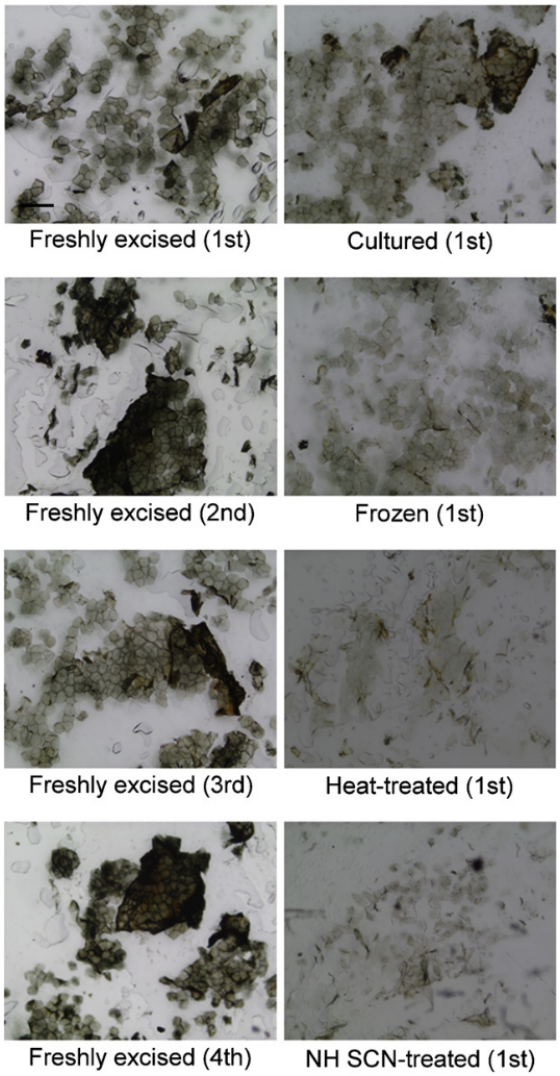

Figure 2: Esterase in the SC of porcine ear skin, visualised by histochemical staining of tape strips. Panel labels denote skin storage or handling conditions prior to histochemical staining, and the tissue depth shown is identified in parentheses as the tape strip number. Images are at same magnification. Scale bar = 100 μm.

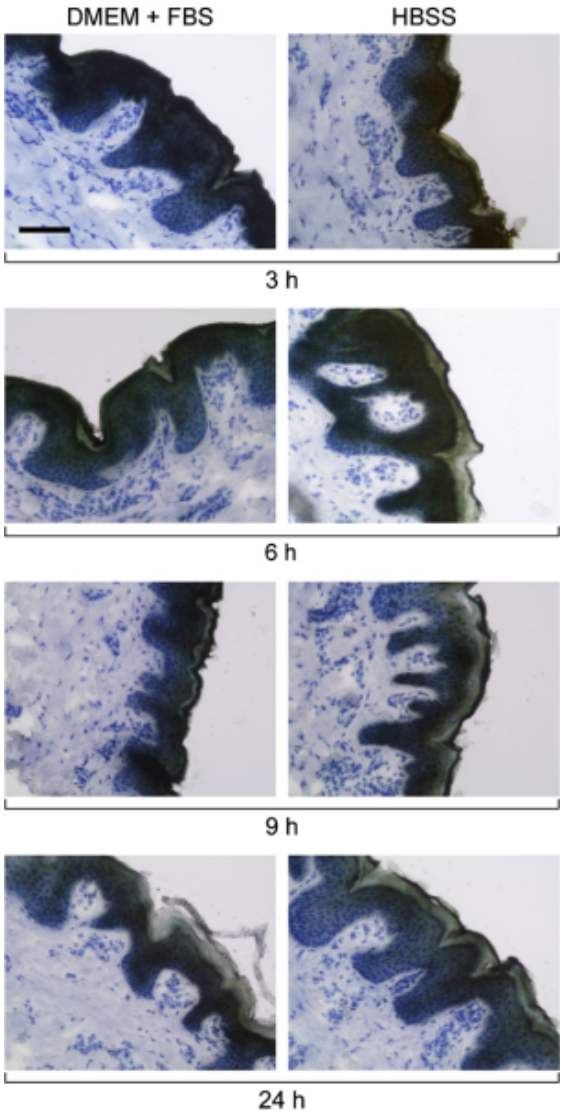

Figure 3: Representative images depicting the effect of explant condition on the level of esterase activity in porcine ear skin explants over time. Skin samples were explanted in HBSS or DMEM supplemented with 10% FBS for up to 24 h. The level of esterase activity in skin cryosections was visualised by histochemical staining. More intense black staining indicates a higher level of esterase activity. Images are at same magnification. Scale bar = 200 μm.

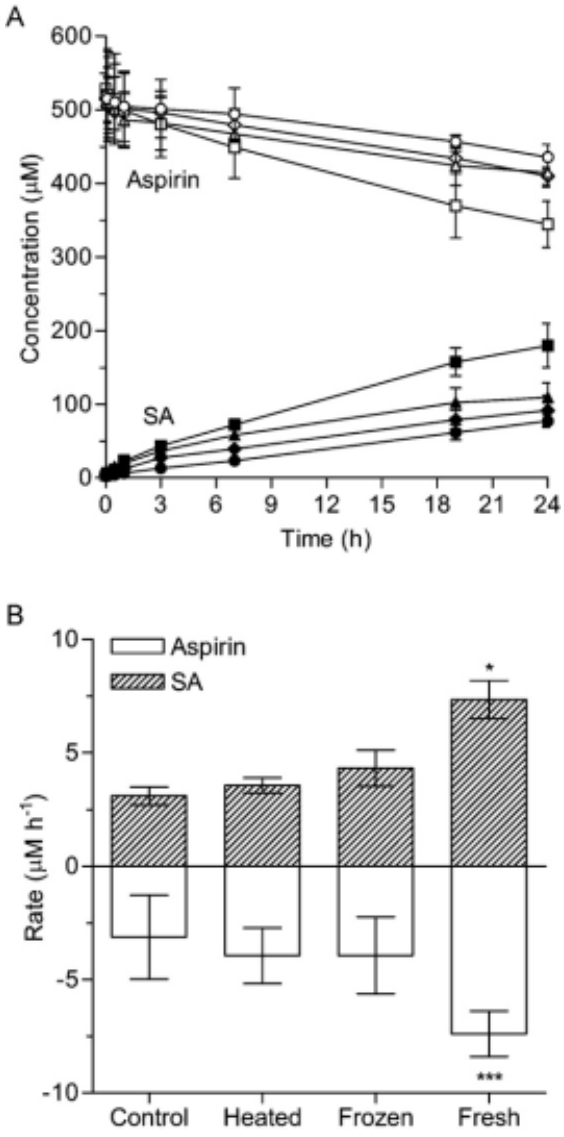

Figure 4: (A) Changes in aspirin and SA concentrations in the presence of vehicle-only (circles), freshly excised skin (squares), frozen skin (triangles) or heated skin (diamonds). (B) Comparison of the overall rates of aspirin hydrolysis under the aforementioned conditions. Data are mean ± standard deviation ($n = 3$; for vehicle-only control, $n = 2$). $^{***}p < 0.001$, $^{*}p < 0.05$ compared to vehicle-only control.

(Figure 4A). The rate of aspirin consumption also mirrored the rate of SA production (Figure 4B). In increasing order, the rate of aspirin hydrolysis was: freshly excised skin > frozen skin > heated skin > vehicle-only control. The maximal rate of hydrolysis, obtained with freshly excised skin, was approximately 200% higher than that of the vehicle-only control ($p < 0.001$ according to aspirin consumption; $p < 0.05$ according to SA production). Compared to in vehicle-only control, the rate of aspirin hydrolysis was not statistically significant in the presence of frozen or heated skin. The rate of SA production was significantly higher in the presence of freshly excised skin compared to frozen ($p < 0.01$) and heated ($p < 0.001$) skin. Although statistical significance was not demonstrated ($p > 0.05$) in the rates of aspirin consumption between freshly excised, frozen and heated skin, by 24 h, significantly lower levels of aspirin remained in the presence of freshly excised skin compared to frozen and heated skin ($p < 0.05$ in both cases).

4 Discussion

Although the distribution of esterases has been described in human skin and that of other animals, similar data are lacking for porcine ear skin despite its prolific use in pharmaceutical studies. Earlier studies have concluded that skin esterase distribution and activity may vary from one species to another, and indeed between different sites in the same organism [5, 18, 19]. Therefore, characterisation of esterase distribution and activity in porcine ear skin is paramount to validate this model for pharmaceutical investigations. An attempt by Meyer and Neurand [24] to characterise enzyme distribution in the skin of the domestic pig unfortunately did not include an analysis of the porcine ear, hence the need for the present study, which characterises the distribution and activity of porcine ear skin esterases not only under basal conditions, but also following common methods of culture, storage and handling in the course of pharmaceutical studies.

Our results show that the epidermis was most intensely stained with the esterase staining kit, and is thus the major source of esterases in porcine ear skin. The localisation of esterases in the epidermis proximal to the SC is in agreement with a previous report on esterase activity in human skin [5]. This perhaps relates to the functional role of the epidermis as a metabolically active tissue. We were initially surprised at the finding of esterase activity in the SC, since the SC is composed of non-viable corneocytes, and an early study has reported no esterase activity in human SC [5]. However, a more recent study has demonstrated that the human SC is in fact metabolically active, and the authors were able to quantify the hydrolysis of a methylumbelliferone acyl ester in human SC using an in vivo tape-stripping method [25]. Furthermore, considering that the skin surface is a natural host to many microorganisms, some esterase activity detected in the SC is likely to be of microbial origin. This is probable as many microorganisms found on mammalian skin are known to produce esterases [26]. Regardless of the origin of the esterases, the implications of their distribution in porcine ear skin need to be considered carefully when designing permeation experiments for ester-containing permeants utilising this skin model. The abundance of esterases on the skin surface may predispose these permeants

to hydrolysis before skin penetration occurs and consequently result in inaccurate skin permeability data for the ester.

The staining results also demonstrate greatly diminished porcine ear skin esterase activity following frozen storage at −20°C. Additionally, porcine ear skin esterase activity was virtually obliterated following heating at 60°C for 1 min (Figure 1, Figure 2). These were corroborated by quantitative data comparing the rate of aspirin hydrolysis, which produced SA in a 1:1 molar ratio, in the presence of freshly excised, frozen, heated skin or vehicle only (Figure 4). These observations strongly suggest that, firstly, aspirin hydrolysis was catalysed by porcine ear skin esterases, and secondly, the skin esterases were denatured or otherwise inactivated to different extents by the freezing process and thermal treatment. These are important observations because freezing and heating are commonly employed for porcine ear skin storage and manipulation (i.e. heat-separation of epidermal sheets) for permeation experiments [27, 28], but their effects on porcine ear skin esterases have not been elucidated. For topical ester pro-drugs and co-drugs, where in situ hydrolysis of the parent ester compounds is anticipated, it is especially important to consider the effect of freezing or heating skin samples on the release of therapeutically active constituents and thus efficacy of the pro-drugs or pro-drugs.

Although freezing serves to preserve proteinaceous enzymes by arresting enzymatic proteolysis, it also introduces mechanical stress which leads to protein degradation in the process [29]. Freezing at −20°C for 1 month has been reported to effectively inactivate β-glucuronidase and sulfatase in porcine ear skin [30]. However, different enzymes may be differentially susceptible to this mode of inactivation. In the case of esterases, we are not aware of any pre-existing report about the effect of freezing on porcine ear esterase activity and its impact on cutaneous drug metabolism. Indeed, Abla et al. [15] used porcine ear skin which had been frozen at −20°C for up to 2 months and still were able to verify hydrolysis of an ester pro-drug in the skin, although it is not clear what proportion of the hydrolysis was enzymatic or non-enzymatic. On the other hand, the heat-separation procedure (60°C for 1 min) described in this study is a common protocol for epidermal membrane isolation, although variations in incubation time ranging from 45 s to 2 min exist between different protocols [27, 28, 31]. Heat-separated epidermal membranes thus derived have been used to evaluate skin permeation of pro-drugs [32, 33]. For cryosections in this study, the incubation time was extended to 15 min to ensure complete thermal denaturation of skin esterases to provide sound negative controls for the histochemical assessment of esterase activity. Interestingly, others who used heat-separated (60°C for 2 min) human epidermal membranes to evaluate skin permeation of pro-drugs have reported a lack of hydrolysis of the pro-drugs in the skin, due probably to thermal denaturation of skin esterases [32, 33]. These results highlight the value of using freshly excised skin samples in investigations where skin metabolism of the drug under investigation is of utmost importance.

The NH_4SCN method of epidermal separation produced no detectable esterase activity in the SC (Figure 3). Thiocyanate (SCN^-) has been shown to inhibit certain enzymes, including prostatic acid phosphatase [34] and carbonic anhydrase [35]. Whether it inhibits esterases is not certain but conceivable. Nevertheless, this is an important observation because some researchers may be compelled to use NH_4SCN to obtain

epidermal membranes for permeation studies now that the heat-separation protocol has been shown to inactivate skin esterases. Chemical separation with NH_4SCN has the advantage of being quick and simple, but its effects on esterases need first to be ascertained before it can be used as a sound replacement for heat separation of epidermal membranes.

Furthermore, serum-supplemented DMEM is better able to preserve skin esterase activity than HBSS, especially for durations beyond 24 h. This may be related to the pH-buffering capacity of each culture medium, or it may reflect the viability of the skin explants. Enzyme activity is pH-dependent, and a decline in culture may indicate a deviation from the optimal pH as a result of acidification of the culture medium by metabolic waste products. Due to its lower bicarbonate and phosphate content, and the absence of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), HBSS (4.2 mM bicarbonate; 0.78 mM phosphate) has a lower pH-buffering capacity than serum-supplemented DMEM (44 mM bicarbonate; 0.91 mM phosphate; 25 mM HEPES) [36, 37]. This, in addition to the absence of essential nutrients such as amino acids and vitamins, may make tissue deterioration more likely to occur in skin explanted in HBSS. Consequently, net degradation of esterases may be more likely to occur in skin explanted in HBSS due to the gradual loss of cell viability and the inability of non-viable skin explants to synthesise and replenish functional esterases. This is unlikely to impact on tissue viability in the short term, and HBSS should provide a suitable medium for the transport of excised skin from the abattoir to the laboratory within the time frame of a typical experiment. For longer explants, the use of FBS-supplemented DMEM is warranted.

5 Conclusion

In conclusion, like in human skin, esterases in porcine ear skin is localised in the epidermis, and appears concentrated just below the SC. The SC of porcine ear skin also exhibits considerable esterase activity. These esterases can metabolise topically administered ester drugs, as demonstrated by the hydrolysis of aspirin in this study. Furthermore, esterase activity in excised porcine ear skin is significantly reduced by freezing and heating. For porcine ear skin explants, the choice of culture medium may have an impact on the viability of the tissue, which will also affect the performance of skin esterases. Since skin esterases are crucial for in situ activation of topical co-drugs and pro-drugs, whereas in other applications avoidance of drug metabolism by skin esterases may be needed to ensure system bioavailability, due consideration should be given to the storage and handling conditions skin samples are subjected to prior to experimentation.

Conflicts of interest

All authors declare no conflicts of interest.

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