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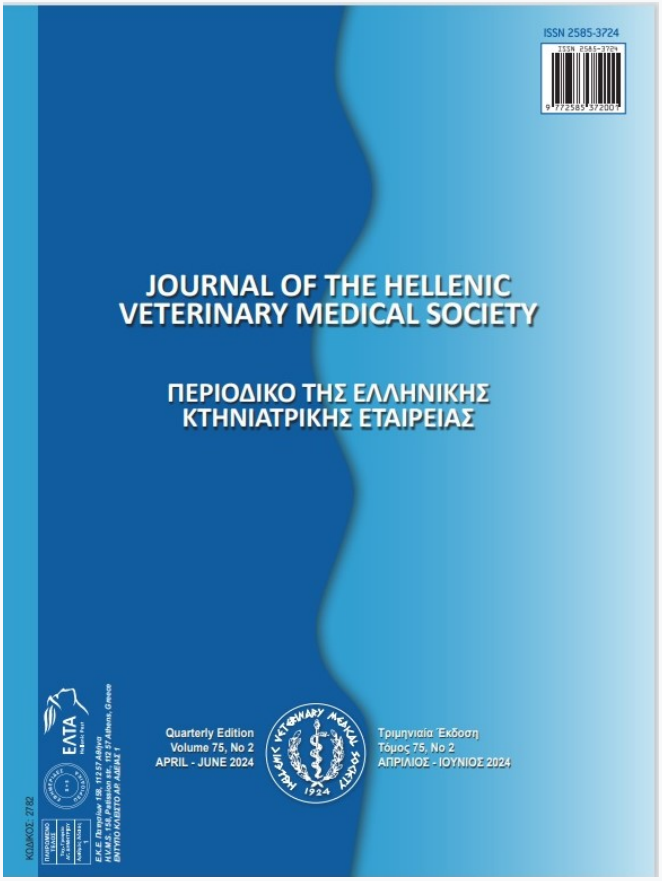

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Research article
Ερευνητικό άρθρο

Isolation and culture of feline keratinocytes and fibroblasts for the generation of artificial skin equivalent

S. Lavrentiadou[1], V. Angelou[2], K. Chatzimisios[2], L.G. Papazoglou[2]

[1]*Laboratory of Physiology, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54627 Thessaloniki, Greece*

[2]*Unit of Surgery and Obstetrics, Companion Animal Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54627 Thessaloniki, Greece*

ABSTRACT: This study aimed to isolate and culture keratinocytes and fibroblasts from feline skin to ultimately create an artificial engineered skin, including dermis and epidermis, that would be applied for the effective treatment of large cutaneous deficits in cats. To date, no data have been reported on the isolation and culture of feline skin cells. To exploit the potential to grow *in vitro* and cryopreserve epidermal keratinocytes and dermal fibroblasts, skin biopsies were obtained using a 6 mm biopsy punch, from 8 healthy cats that underwent ovariohysterectomy. Fibroblasts were isolated following collagenase digestion of the dermis and were grown in DMEM supplemented with 10% FBS. Keratinocytes were isolated from the epidermis following digestion with trypsin solution. Keratinocytes were grown on a collagen type I matrix, in a growth medium consisting of DMEM: F12 (3:1) medium supplemented with 10% FBS, 1 μM hydrocortisone, 1 μM isoproterenol, and 0.1 μM insulin. Both fibroblasts and keratinocytes were grown in a humidified atmosphere with 5% CO2 at 37^{O}C. The medium was changed twice a week and cells were grown up to 80-85% confluency for 4 passages. Cells at passages 1-2 were cryopreserved in a freezing medium at -80^{O}C. Fibroblast freezing medium consisted of DMEM supplemented with 30% FBS and 10% DMSO, whereas keratinocytes were cryopreserved in a keratinocyte growth medium supplemented with 10% DMSO. Both cell types were recovered following cryopreservation and cultured as described above until passage 4. Therefore, we can create a bank of fibroblasts and keratinocytes to recover cells for further culture and for the generation of autologous or heterologous skin equivalent *in vitro*. This is the first study reporting isolation and culture of keratinocytes and fibroblasts from feline skin, setting the grounds for the development of engineered feline skin.

Keywords: feline; artificial skin; skin equivalent; keratinocytes; fibroblasts;

Corresponding Author:
Lavrentiadou S, BSc, PhD, Laboratory of Physiology, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 54627 Thessaloniki, Greece
E-mail address: vasso_1991@hotmail.com

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INTRODUCTION

Feline skin is comprised of two layers, epidermis, and dermis, separated by a basal membrane, and each layer is subdivided into other layers. Namely, the epidermis includes stratum basale, stratum spinosum, and stratum corneum and the dermis includes stratum papillare and stratum reticulare (Pavletic, 2018). The main cells of the epidermis and dermis are the keratinocytes and fibroblasts, respectively. Artificial skin equivalents consist mainly of keratinocytes and fibroblasts and rarely they also include melanocytes (Roger et al., 2019). Application of skin equivalents provides the advantage of faster *in vitro* proliferation compared to a second-intention healing process (Llames et al., 2006). Human skin equivalents have been used extensively in pharmacology and cosmetology, especially after the limitation of the use of animal testing, for research in wound healing, skin development, alopecia disease, stem cell renewal, and toxicology screening (Souci and Denesvre, 2021; Sanchez et al., 2022). Applications of biosynthetic skin have been described less frequently in veterinary medicine, mainly in dogs (Serra et al., 2007; Cerrato et al., 2016a; Bauhammer et al., 2019), horses (Cerrato et al., 2014), rabbits (Kondo et al., 1990), pigs (Dame et al., 2008) and mice (Ikuta et al., 2006). However, to date, no work has been reported with feline skin.

The advantage of biosynthetic skin over other custom wound treatment methods is the protection from dehydration, the oxygen supply, the low incidence of infection or rejection, and the promotion of the healing process. Moreover, growing cells *in vitro* can produce adequate amounts of skin equivalents from small skin samples or stocks of cells in a relatively short time (Llames et al., 2006; Przekora, 2020). Biosynthetic skin has been created from canine skin biopsies and has been applied in large wounds with encouraging results (Serra et al., 2007; Cerrato et al., 2014; Ramió-Lluch et al., 2017). However, the number of dogs in which the biosynthetic skin was applied is small, thus further investigation is required in order to assess the efficacy of bioengineered skin in healing large defects. In cats, there are no reported cases of keratinocyte or fibroblast isolation or attempts to generate skin equivalents to facilitate faster wound healing.

The aim of the present study was to develop for the first time a protocol for the isolation, culture, and cryopreservation of feline keratinocytes and fibroblasts as a first step in the development of artificially engineered skin equivalent in cats. Our hypothesis was that 70 isolation, culture, and cryopreservation of feline keratinocytes and fibroblasts could be feasible.

MATERIALS AND METHODS

Materials

Collagenase type I (SCR103), Millipore, USA; High Glucose DMEM (LM-D1111), Ham's F12 (LM-H1235), Ca^{2+}, Mg^{2+}-free PBS (LM-S2041), Amphotericin B (LM- A4109) and 100X Penicillin-Streptomycin solution (XC-A4122) were purchased from Biosera, France; FBS, Standard, South America origin (P30-3306), DMSO for cell culture (P60-36720100) and Trypsin 0.05%/EDTA 0.02% (P10-023100) were from Pan-Biotec, Germany; Collagen Type I, F12, Human Recombinant Insulin (41-975), Biological Industries; Hydrocortisone (20739) and Isoproterenol (15592) were purchased from Cayman Chemical, USA; all cell culture plasticware were from SPL Life Sciences. Microscopy was performed on an inverted optical microscope (Euromex, Holland).

Feline skin biopsy collection

In the present study, 8 female, intact, domestic shorthaired cats were used. The cats were admitted to the clinic for ovariohysterectomy. Informed consent was obtained from the owner of each cat preoperatively. All cats had a complete blood count, and a serum biochemical analysis and were screened for feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) preoperatively. Skin samples were obtained from the abdominal area of cats undergoing ovariohysterectomy. A 6 mm biopsy punch was used for the biopsy and 2 samples were obtained from each cat. Following the harvest of the skin, the subcutaneous tissue, and the vessels were removed, and the remaining tissue was washed with phosphate buffer saline (PBS) and stored in cold Dulbecco's Modified Eagle's Medium (DMEM) to be transferred to the laboratory.

Isolation and culture of fibroblasts

All processes in the cell culture laboratory were performed in a vertical laminar airflow cabinet (Telstar) under aseptic conditions. Each sample was further cleaned from remaining blood vessels and fat tissue and was minced with a scalpel in small fragments (app. $1mm^3$). For the isolation of fibroblasts, tissues were incubated in the presence of 3 volumes of collagenase type I (2mg/ml). Incubations were carried out with continuous rotation at 37^o C for 5-6 hours. Following digestion, the supernatant (dermis digestion

fraction) was passed through a 100 μm cell strainer and was centrifuged at 1200 rpm for 5 min, at RT. Fibroblasts were then washed with 2 ml DMEM and were finally resuspended in Fibroblast Growth Medium (FGM): DMEM high glucose, 10%FBS, 1X PS, 1X amphotericin (2.5 μg/ml). The concentration and the total number of fibroblasts were determined using a Neubauer slide. The cells were seeded in a T25 cell culture flask at app 10,000 cells/cm^2 and cultured in a humidified atmosphere with 5% CO_2 at 37°C. The medium was changed twice a week and cells were passaged following detachment with Trypsin 0.05%/EDTA 0.02%.

Isolation and culture of keratinocytes

The undigested fraction of the collagenase digestion included the epidermis and was further treated with 2 volumes of 0.05%Trypsin-0.02%EDTA (TE) solution for 1 h at 37° C with continuous rotation. The undigested tissue was allowed to settle, and the keratinocyte-containing supernatant was removed in a new tube with FBS (final concentration 10%) to stop the trypsinization. Two ml of TE solution were added to the undigested fraction and the reactions were further incubated at 37° C for an additional 30 minutes. This procedure was repeated once more, and the pooled keratinocyte fractions were passed sequentially through a 100 μm and a 40 μm cell strainer. The flowthrough was centrifuged at 1200 rpm, RT, for 5 minutes and the supernatant was discarded. The keratinocytes in the pellet were washed with DMEM and were finally resuspended in 2 ml Keratinocyte Growth Medium (KGM): DMEM: F12 (3:1), 10%FBS, 1 μM hydrocortisone, 1 μM isoproterenol, 0,1 μM insulin, 1X PS and 1X amphotericin. A collagen-coated T75 cell culture flask was seeded with the cell suspension. The cells were grown in a humidified atmosphere with 5% CO_2 at 37°C and the medium was changed twice a week. Cells were passaged following detachment with Trypsin 0.05%/EDTA 0.02%.

Cryopreservation of primary cells

In order to cryopreserve the isolated fibroblast and keratinocytes, cells were grown to 80% confluency washed with Ca^{2+}, Mg^{2+}-free PBS, and detached from the culture flask with TE solution. FBS was added to a final concentration of 10% to inhibit trypsin and cells were washed with DMEM. Following washing with DMEM, fibroblasts were resuspended in DMEM supplemented with 30% FBS and 10% DMSO, while keratinocytes were resuspended in KGM supplemented with 10% DMSO. All cells were stored in 2-ml cryovials at -20°C for 24 hours and finally were transferred at - 80°C.

RESULTS

***In vitro* culture of keratinocytes and fibroblasts**

Isolation of fibroblasts from feline skin samples was achieved by digesting the dermis in 0.2% collagenase solution. Following digestion, we recovered approximately $5x10^5$ fibroblasts/g of tissue. Cells were seeded in a T25 flask at a density of 10,000 cells/cm^2 and were cultured for approximately 10 days or until they reached an 80% confluency before they were passaged to a T75 flask (Figure 1). Fibroblasts were cultured for up to 4 passages, by which time the growth rate was dramatically reduced and the cell morphology changed.

Figure 1. Isolated fibroblasts, day 7 in culture (magnification x100).

Isolation of keratinocytes was achieved by treating the epidermis fraction with 0,05% trypsin-0,02% EDTA. Keratinocytes reached an 80% confluency after about 7-8 days in culture and were kept in culture for up to 4 passages (Figure 2).

Post-thawing recovery of primary cells

Both keratinocytes and fibroblasts were recovered in the appropriate medium after their cryopreservation at -80°C. Cells were quickly defrosted in a 37°C water bath and washed with DMEM. Fibroblasts were resuspended in FGM and seeded in a T25 flask. Keratinocytes were resuspended in KGM and seeded in a collagen-coated T75 flask. Cells were alive after thawing and were successfully cultured (Figure 3). Both cell types had normal morphology and growth rate post-thawing, reaching confluency within 7- 10 days in culture.

DISCUSSION

To the authors' knowledge, the present study is the first report on the isolation, culture, and cryopreservation of primary fibroblasts and keratinocytes from feline skin biopsies. The isolation and culture of fibroblasts and keratinocytes, and the development of skin equivalent have been previously reported mainly in humans (Black et al., 2010; Bauhammer et al., 2019; Roger et al., 2019; Donato et al., 2022), but also in several species, other than cats (Kondo et al., 1990; Ikuta et al., 2006; Serra et al., 2007; Dame et al.,

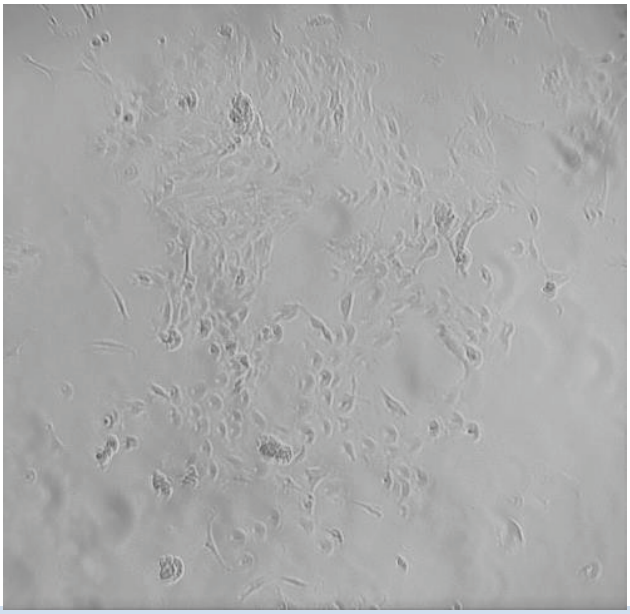

Figure 2. Isolated keratinocytes, day 4 in culture (magnification x200)

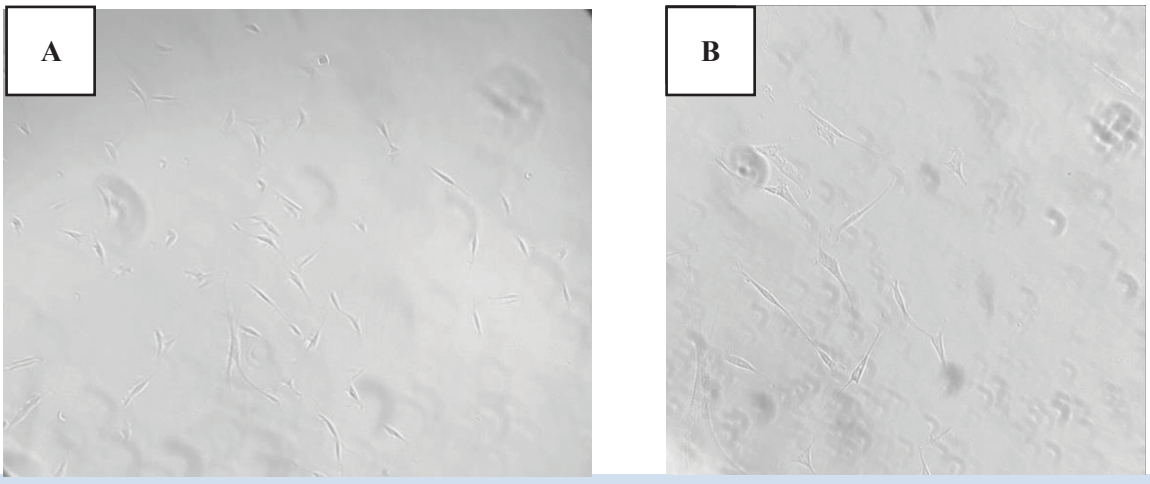

Figure 3. Culture of frozen-thawed fibroblasts, day 4 post-thawing. A. Magnification x100, B. Magnification x200

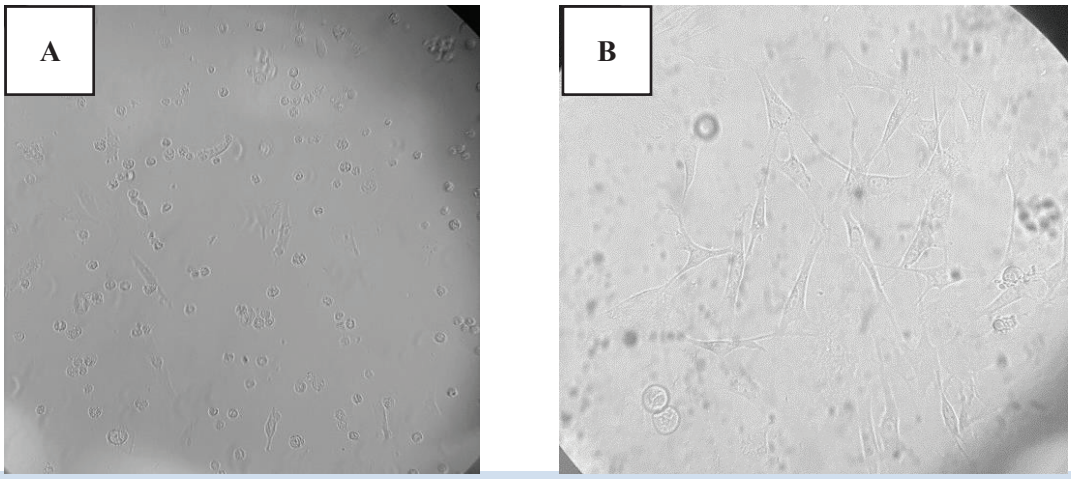

Figure 4. Culture of frozen-thawed keratinocytes. A. Day 1 post-thawing. B. Day 7 post-thawing (magnification x200

2008; Cerrato et al., 2012, 2014; Abramo et al., 2016; Ramió-Lluch et al., 2017; Souci and Denesvre, 2021). The initial phases for isolation and culture of keratinocytes and fibroblasts differ among the different protocols but are similar to a great extent in the majority of protocols in dogs, with biopsies obtained usually from the abdominal skin (Cerrato et al., 2013, 2014).

In the present study, we attempted to follow other published protocols, to eventually develop one for the culture of fibroblasts and keratinocytes from skin biopsies from the abdominal area of female cats. The protocol that resulted in the best yield of primary cells and their optimal growth *in vitro* was the canine protocol with slight modifications. Isolation of keratinocytes can be achieved by enzymatic disintegration of the epidermis by digesting the samples with trypsin (Cerrato et al., 2012, 2013, 2016b) or through cultures of explants as described in horses (Cerrato et al., 2014). In the present study, the enzymatic digestion was the one with an adequate yield of cells to initiate an *in vitro* culture. Similarly, fibroblasts were isolated following a collagenase-mediated disintegration of the dermis. Isolated keratinocytes seemed to reach high confluency in about 7-8 days, which is in agreement with the results in dogs (Serra et al., 2007; Cerrato et al., 2012, 2013). However, feline fibroblasts required more time to proliferate *in vitro*. The use of collagen type I scaffold enhances keratinocyte proliferation (Ścieżyńska et al., 2019), as it was also recorded in the present study, where keratinocytes could not survive and proliferate in collagen-free culture plates.

This study further investigated the potential to cryopreserve feline primary cells. Fibroblasts and keratinocytes at passage 1 or 2 were frozen at -80^{o}C for at least 1 week. Cells were allowed to defrost and extend their culture. Both fibroblasts and keratinocytes survived freezing and continued growing under optimal conditions. This finding demonstrates the potential to freeze primary cells from healthy cat donors, thus creating a type of feline primary cell bank that will be readily available upon request. Nevertheless, this is a preliminary study that is ultimately aiming to the generation of an engineered feline skin equivalent to be applied to large or chronic skin defects or injuries in cats.

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REFERENCES

Abramo F, Pirone A, Lenzi C, et al (2016) Establishment of a 2-week canine skin organ culture model and its pharmacological modulation by epidermal growth factor and dexamethasone. Annals of Anatomy - Anatomischer Anzeiger 207:109-117. https://doi.org/10.1016/J.AANAT.2016.03.009.

Bauhammer I, Sacha M, Haltner E (2019) Establishment of an in vitro model of cultured viable human, porcine and canine skin and comparison of different media supplements. Peer J e7811.

Black AT, Hayden PJ, Casillas RP, et al (2010) Expression of proliferative and inflammatory markers in a full-thickness human skin equivalent following exposure to the model sulfur mustard vesicant, 2-chloroethyl ethyl sulfide. Toxicol Appl Pharmacol 249:178- 187. https://doi.org/10.1016/j.taap.2010.09.005.

Cerrato S, Brazís P, Meana A, et al (2012) In vitro development and characterization of canine epidermis on a porcine acellular dermal matrix. The Veterinary Journal 193:503- 507. https://doi.org/10.1016/J.TVJL.2012.01.031.

Cerrato S, Ramió-Lluch L, Brazís P, et al (2016a) Effects of sphingolipid extracts on the morphological structure and lipid profile in an in vitro model of canine skin. The Veterinary Journal 212:58-64. https://doi.org/10.1016/J.TVJL.2016.03.020.

Cerrato S, Ramió-Lluch L, Brazís P, et al (2014) Development and characterization of an equine skin-equivalent model. Vet Dermatol 25:475-e77. https://doi.org/https://doi.org/10.1111/vde.12134.

Cerrato S, Ramió-Lluch L, Brazís P, et al (2016b) Effects of sphingolipid extracts on the morphological structure and lipid profile in an in vitro model of canine skin. The Veterinary Journal 212:58-64. https://doi.org/10.1016/J.TVJL.2016.03.020.

Cerrato S, Ramió-Lluch L, Fondevila D, et al (2013) Effects of Essential Oils and Polyunsaturated Fatty Acids on Canine Skin Equivalents: Skin Lipid Assessment and Morphological Evaluation. J Vet Med 2013:. https://doi.org/10.1155/2013/231526.

Dame MK, Spahlinger DM, DaSilva M, et al (2008) Establishment and characteristics of Gottingen minipig skin in organ culture and monolayer cell culture: Relevance to drug safety testing. In Vitro Cell Dev Biol Anim 44:245-252. https://doi.org/10.1007/s11626- 008-9091-3.

Donato RF, Sanchez MM, Tonmoy I, et al (2022) Development of a Vascularized Human Skin Equivalent with Hypodermis for Photoaging Studies. https://doi.org/10.3390/biom12121828.

Ikuta S, Sekino N, Hara T, et al (2006) Mouse epidermal keratinocytes in three-dimensional organotypic coculture with dermal fibroblasts form a stratified sheet resembling skin. Biosci Biotechnol Biochem 70:2669-2675. https://doi.org/10.1271/bbb.60266

Kondo S, Hozumi Y, Aso K (1990) Long-term organ culture of rabbit skin: Effect of EGF on epidermal structure in vitro. Journal of Investigative Dermatology 95:397-402. https://doi.org/10.1111/1523-1747.ep12555492

Llames S, Garcia E, Garcia V, del Rio M, Larcher F, Jorcano JL, et al (2006) Clinical results of an autologous engineered skin. Cell Tissue Bank. (2006) 7:47-53. http://doi.org/10.1007/s10561-004-7253-4.

Pavletic MM (2018) The Skin. In: Atlas of Small Animal Wound Management and Reconstructive Surgery. John Wiley & Sons, Ltd, pp 1-15.

Przekora A (2020) A Concise Review on Tissue Engineered Artificial Skin Grafts for Chronic Wound Treatment: Can We Reconstruct Functional Skin Tissue In Vitro? Cells 9:1622. https://doi.org/10.3390/cells9071622.

Ramió-Lluch L, Cerrato S, Brazis P, et al (2017) Proof of concept of a new autologous skin substitute for the treatment of deep wounds in dogs. Veterinary Journal 230:36-40. https://doi.org/10.1016/j.tvjl.2017.11.003.

Roger M, Fullard N, Costello L, et al (2019) Bioengineering the microanatomy of human skin. https://doi.org/10.1111/joa.12942.

Sanchez MM, Tonmoy TI, Park BH, Morgan JT (2022) Development of a Vascularized Human Skin Equivalent with Hypodermis for Photoaging Studies. Biomolecules 12:1828. https://doi.org/10.3390/biom12121828.

Ścieżyńska A, Nogowska A, Sikorska M, et al (2019) Isolation and culture of human primary keratinocytes—a methods review. Exp Dermatol 28:107-112. https://doi.org/10.1111/exd.13860

Serra M, Brazís P, Puigdemont A, et al (2007) Development and characterization of a canine skin equivalent. Exp Dermatol 16:135-142. https://doi.org/https://doi.org/10.1111/j.1600-0625.2006.00525.x.

Souci L, Denesvre C (2021) 3D skin models in domestic animals. Vet Res 52:21. https://doi.org/10.1186/s13567-020-00888-5.