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Effect of sample preservation on stress softening and permanent set of porcine skin

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ABSTRACT

Skin is a composite material with a complex structure which exhibits a wide range of behaviours such as anisotropy, viscoelasticity, hyperelasticity, plasticity etc. Indeed it remains a great challenge to understand its behaviour as it is involved in many consumer and medical applications. In most studies, experiments are performed in situ or in vitro on fresh tissues but most of the time samples are preserved before testing (fridge, freezer, saline solution etc.). In this paper, the impact of samples conservation on the softening behaviour and on the permanent set is studied in order to select the appropriate conservation protocol. Samples are extracted from several pigs' abdomens (direction parallel to spine) and the mechanical testing consists in loading–unloading uniaxial tension tests instrumented with digital image correlation inducing thus reliable strain measurements in a chosen region of interest. The results of this study revealed that preservation conditions must be carefully chosen; conservation in a saline solution and freezing without any caution alter the irreversible part of the global mechanical behaviour of the tissues.

1. Introduction

Studies of the mechanical behaviour of skin are of a great interest since skin strength is involved in many consumer and medical applications. The ultimate goal is to mimic in vivo mechanical behaviour of the skin via in vitro tests. In this context, fresh tissues should be used but in most cases, it is not possible to perform the test directly after the excision, tissues are therefore preserved either bathing in isotonic saline solution or frozen/cooled for various time periods. Many authors have raised the question of the influence of these methods of preservation on the mechanical properties of these tissues, concluding about tissues in vivo properties from tests performed in vitro. The literature is often contradictory concerning the effect of freezing on soft tissues (skin, tendons or ligaments) on mechanical properties. Most of the studies imply monotonic uniaxial tensile tests until breakage and concern freezing procedure with various storage times and temperatures on tendons and ligaments. Some authors assert that freezing at −20 °C, −70 °C or −80 °C with or without cryoprotectant for a short period have a moderate effect on their elastic properties (O'Leary et al., 2014; Ng and Chou, 2003; Woo et al., 1986; Foutz et al., 1992; Venkatasubramanian et al., 2006; Arnout et al., 2013) while in some others publications the contrary is observed: a decrease (Matthews and Ellis, 1968; Smith et al., 1996; Turner et al., 1988) or an increase (Dorlot et al., 1980; Ng et al., 2005) of mechanical properties. There were even some authors that promote an immediate mechanical testing as the microstructure is strongly affected by the storage (Viidik and Lewin, 1966). In Virues Delgadillo (2010) author perform a biaxial test on human arteries and conclude that freezing at −20 °C, −80 °C during 2 months with DMSO cryoprotectant is the better way to preserve biomechanical properties. From our knowledge there are very few studies on skin tissues; nevertheless the same kind of conclusions can be found in Haut (1989) and Kang and Wu (2011). From a mechanical point of view, some studies were carried out with uniaxial tensile tests and revealed skin anisotropy (Tong and Fung, 1976; Ridge and Wright, 1966) with a stiffer response along Langer's line (aligned with the collagen fibres network). As testing human skin is difficult, various skin specimens have been studied from cats (Veronda and Westmann, 1970), rats (Eshel and Lanir, 2001), mice (Del Prete et al., 2004) or pigs (Ehret et al., 2001). These studies were principally focused on elasticity/failure and viscoelastic properties. In Tong and Fung (1976), Ehret et al. (2001) and Muñoz et al. (2008) authors go further: the experimental protocol is completed via cyclic

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solicitations; as commonly observed for filled elastomers (Caro-Bretelle et al., 2013), skin exhibits a stress softening usually associated to the preconditioning. In the case of elastomeric materials, recovery of this effect is observed and this preconditioning is often named the Mullins effect. The present study focuses on the influence of porcine skin preservation on stress softening and permanent set. The chosen preservation procedure belongs to laboratories classical equipments such as freezers at −20 °C or −80 °C and saline solutions for several storage times. The tests were performed on skin samples extracted in parallel with tissue fibres. The softening effect and permanent set were investigated by cyclic loading with different load levels and for one loading rate. The tests are instrumented by photomechanics allowing overcoming experimental difficulties. The originality of the study is both the experimental procedure (cyclic tensile tests instrumented by photomechanics) and the required mechanical properties both in the reversible and irreversible range (elasticity, damage, permanent set etc.) in link with conservation procedure.

2. Materials and methods

2.1. Sample preparation

Skin tissue is mainly constituted (in term of dry weight) with a collagen fibres network (65–70%), some elastin fibres (5–10%) and proteoglycan (1.5–2%). Collagen is stiffer that elastin but absorbs more energy (Usyk and Mcculloch, 2002). As collagen structure in tissues changes with disease and ageing, the pigs (4) are chosen with approximatively the same weight and age. They are obtained within 24 h after the slaughtering from the abdominal region following Langer's line. The skins samples are obtained from a local slaughterhouse under the animals welfare regulations. Samples are punched out from the pressurized skin 30*100 mm^2 in size. Symmetrical notches were then perforated using a sharp punch cut in order to induce a strain localisation during mechanical testing (Fig. 1). This shape, named double-edge notched tensile (DENT), allows initiating necking between notches, in the area in front of which a camera is placed (Christmann et al., 2011). The radius curvature of 1 mm is used to keep the tensile load uniaxial: the stress heterogeneity remains inferior to 5%. The hypodermis was removed with a surgical scalpel. The average thickness (h) was measured with a calliper spanning the length of the sample in 4 different locations for all specimens (84); no significant difference was detected between them; the value of the thickness is evaluated around: 2 ± 0.3 mm.

For each porcine, 21 skin specimens were preserved before testing following several procedures, as it is mentioned in Table 1:

- 9 were bathed in a normal saline solution and kept in fridge (4 °C) during 4, 24 or 72 h.
- 3 of them were fresh frozen at −20 °C for one week with a decrease in temperature of about 1 °C per minute,
- 3 were fresh frozen at −80 °C for one week with a decrease in temperature of about 1 °C per minute,
- 3 were fresh frozen in freezing medium (Phosphate Buffered Saline (PBS) plus cryoprotectant) for one week. The cryoprotectants used alone were glycerol (10%), or trehalose (60 and 100 mM). The cryovials (25 ml) containing the samples (which were no folded) were placed in an isopropanol chamber and store them at −80 °C for one week in order to allow a decrease in temperature of about 1 °C per minute.
- 3 were preserved in fridge (4 °C) and tested within the 2 first hours of conservation. They constitute our reference.

Frozen specimens are thawed at controlled temperature and humidity during 1 hour prior to testing. The samples bathed in a saline solution were blotted with absorbent paper to avoid presence of any excess saline solution.

2.2. Instrumented tensile test and digital image correlation

The uniaxial tensile tests were performed using a Dartec universal test setup at ambient temperature. Because some studies refer to mechanical and environmental change as testing temperature or loading rate due to a collagen denaturation (Zhou et al., 2010; Kang and Wu, 2011), tests conducted in the present study were performed with a constant crosshead speed equal to 0.5 mm/min. The material and geometrical heterogeneities lead to variable loading rate which can induce damage in the extra-cellular matrix. The ends of the specimens (sections 40*30 mm^2) were gripped between specially adapted jaws of the tensile test device (see Fig. 1). They were subjected to non-monotonic loadings (Fig. 2):

- two loading–unloading cycles at 10 kN, 20 kN and 50 kN; the unloading procedure being performed until the load reached zero.
- The last cycle at 50 kN is followed by a loading until breakage.

The nominal stress (σ) is defined as the ratio between the uniaxial force recorded by the test equipment ($\boldsymbol{F}$) and the initial area at the minimal cross-sectional of the sample ($\boldsymbol{A_0}=\boldsymbol{L_0 h}$, $\boldsymbol{L_0}$ is the initial width at the minimal cross section) as described in Eq. (1):

$$\sigma = F/A_0 \tag{1}$$

Table 1
Conservation protocol.

Tests	*Conservation methods*	*#Specimens (#samples per specimen)*
4 pigs (c_1, c_2, c_3, c_4) 3 samples by tests 84 tests	+4 °C during 2 h (Ref)	4 (3,3,3,3)
	−20 °C during 7 days	4 (3,3,3,3)
	−80 °C during 7 days	4 (3,3,3,3)
	−80 °C (cryopreserved) during 7 days	4 (3,3,3,3)
	Saline solution during 4 h	4 (3,3,3,3)
	Saline solution during 24 h	4 (3,3,3,3)
	Saline solution during 72 h	4 (3,3,3,3)

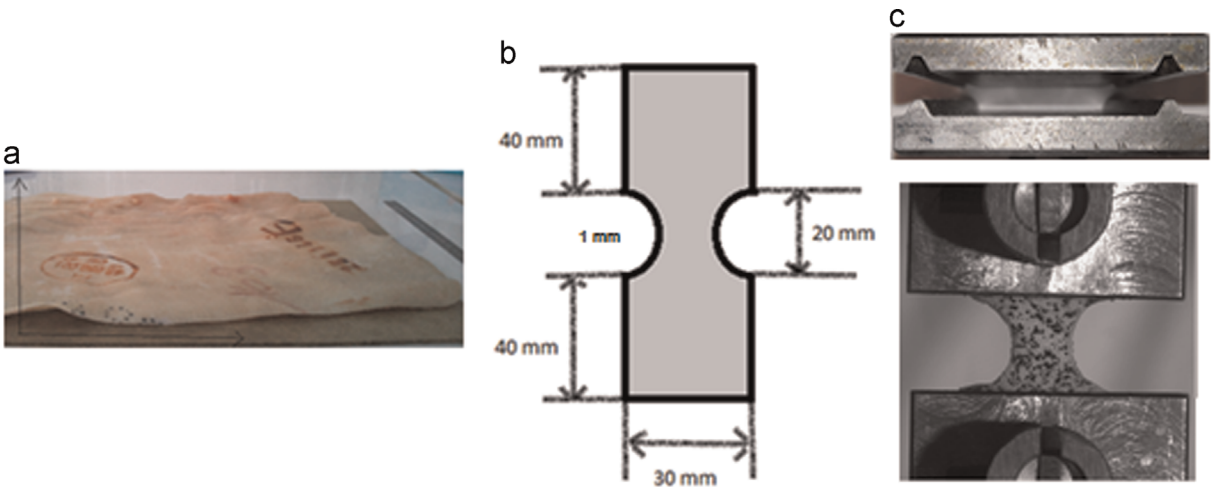

Fig. 1. Native porcine skin before punching (a) useful dimension of specimens (thickness 2 mm) (b) sample gripped in its initial state (c).

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