Title: The Role of Estrogen in Cutaneous Ageing and Repair

Authors: Holly N. Wilkinson and Matthew J. Hardman

The School of Life Sciences, The University of Hull, Hull, HU6 7RX, United Kingdom.

H.wilkinson@2016.hull.ac.uk; M.hardman@hull.ac.uk

Corresponding author:

Matthew J. Hardman
School of Life Sciences
University of Hull
Hull
HU6 7RX
United Kingdom
Email: m.hardman@hull.ac.uk
Tel: +44 (0)1482 461879

Abstract

Combined advances in modern medical practice and increased human longevity are driving an ever-expanding elderly population. Females are particularly at risk of age-associated pathology, spending more of their lives in a post-menopausal state. Menopause, denoted by a rapid decline in serum sex steroid levels, accelerates biological ageing across our body's tissues. Post-menopause physiological changes are particularly noticeable in the skin, which loses structural architecture and becomes prone to damage. The sex steroid most widely discussed as an intrinsic contributor to skin ageing and pathological healing is 17β-estradiol (or estrogen), although many others are involved. Estrogen deficiency is detrimental to many wound healing processes, notably inflammation and re-granulation, while exogenous estrogen treatment widely reverses these effects. Over recent decades, many of the molecular and cellular correlates to estrogen's beneficial effect on normal skin homeostasis and wound healing have been reported. However, disparities still exist, particularly in the context of mechanistic studies investigating estrogen receptor signalling and its potential cellular effects. New molecular techniques, coupled with increased understanding of estrogen in skin biology, will provide further opportunities to develop estrogen receptor-targeted therapeutics.

Keywords: Estrogen; Ageing; Wound Healing; Skin; Endocrinology; Hormones.

Introduction

The “live fast, die young” adage is one of many theories proposed to explain the rarity of ageing and the menopause throughout the animal kingdom [1]. With the advent of modern medicine, human life expectancy has surpassed that of most other animals, and continues to rise [2]. Increasing longevity is implicated in many age-associated physiological changes, which are in-turn linked to significant endocrine system alterations. Increased longevity, combined with a stable age of menopause onset, means that women are spending more of their lives in an estrogen-deficient post-menopausal state [2]. While the immediate effects of the menopause-associated decrease in sex hormones are infertility and vasomotor symptoms, the long term effects include osteoporosis, cardiovascular disease and skin disease (reviewed in [3]). In the skin, accelerated ageing immediately follows the menopause, with by far the most discussed intrinsic contributor being the decline in serum levels of 17β- estradiol [4]. Reduced 17β-estradiol levels have been linked to the multiple metabolic mechanisms of ageing, including cellular senescence and oxidative stress [2], suggesting a high-level regulatory contribution. Here we will explore current knowledge on the role of estrogen in skin ageing and wound healing, with a particular focus on emerging opportunities for clinical intervention.

Mechanisms of Ageing

Cellular senescence is described as the termination of cellular proliferation with critical shortening of telomeres, and can be induced by a range of factors, including stress or oncogene expression [5]. Senescent cells are quiescent, unable to divide but metabolically active. They communicate within their cellular environments through the release of DNA-damage signals, collectively termed the senescence-associated secretory phenotype (SASP) [6]. These communicative features are now widely accepted to contribute to age-related disease [5]. In fact, Baker et al., [7] demonstrated that senescent cell depletion directly reduced age-associated pathology in a murine natural ageing model, while others have shown direct anti-senescent effects of 17β-estradiol supplementation *in vitro* [8] and *in vivo* [9].

According to the free radical theory, ageing is a consequence of sustained increased ROS (reactive oxygen species). Here, normal cellular metabolism combined with extrinsic factors, such as UV radiation, leads to local production of ROS, including superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) [10]. In large quantities, ROS can overwhelm antioxidant defences, damaging DNA, proteins, lipids and carbohydrates [10]. ROS are

therefore detrimental to many important cellular functions, from proliferation and differentiation, to overall survival. Interestingly, estrogen is a potent antioxidant, able to reverse oxidative damage in ovariectomised (Ovx) rodent studies [11]. Ovariectomy (surgical removal of the ovaries) is a widely used experimental model of human menopause. Ovx animals show many of the hallmarks of oxidative stress responses, such as increased ROS production and DNA damage, while also exhibiting age-related pathologies, such as renal failure [12]. In almost every study, administering exogenous estrogen to Ovx animals attenuates disease and restores normal function [12]. The protective effects of estrogen have also been demonstrated at the cellular level. For example, in aged murine keratinocytes 17β-estradiol reduced age-related oxidative stress (lipid peroxidation) and apoptosis (shown by caspases 3 and 8; [13]). Additionally, estrogen protected against H_2O_2 oxidative damage in human dermal fibroblasts (HDFs) and immortalised keratinocytes (HaCaTs), allowing maintenance of normal collagen synthesis and cellular proliferation, respectively [10].

Skin Ageing

In the skin, intrinsic and extrinsic factors combine to impart age-associated loss of structure and function [14]. Physiologically, human skin is characterised by decreased epidermal and dermal thickness, reduced elasticity and collagen content, and increased susceptibility to age-associated dermatoses, such as dryness, wrinkling and infection (reviewed in [15]). Intrinsic skin ageing leads to decreased epidermal proliferation, less dermal vasculature, reduced collagen content and increased MMP (matrix metalloproteinase) production (reviewed in [16]). By contrast, extrinsic "photo-ageing" is accompanied by more severe changes to matrix structure, heightened MMP activity, melanocyte redistribution, reduced Langerhans cells and characteristic accumulation of non-functional dermal elastin (dermal elastosis) [16]. Overall, intrinsically aged skin appears thin with fine lines whereas photo-aged skin appears thickened with deep wrinkles and age spots [5].

Conserved molecular pathways govern the physiological effects of skin ageing. For example, ROS generation (via UV radiation and cellular metabolism) leads to the production of protein tyrosine phosphatases (PTPs), phosphorylation of tyrosine kinase receptors and activation of downstream pathways (e.g. MAPK) and transcription factors, such as AP-1 (activator protein-1). Interestingly, AP-1 directly induces MMPs -1, -3 and -9 while suppressing procollagen-1 expression [17]. Moreover, senescent cells accumulate throughout the dermis and epidermis in aged human skin [18], mirroring

observed effects *in vitro* in HDFs and human epidermal keratinocytes (HEKs). These mechanisms have been evaluated via profiling senescence markers (e.g. senescence-associated beta galactosidase, SA-βgal) and overall assessment of cellular function [5]. Thus, the next question to consider is how estrogen regulates skin cell behaviour and is able to prevent the age-associated decline in skin functionality.

The Link between Skin Ageing and Estrogen

Skin extracellular matrix is deposited early in life, and gradually deteriorates over time. Post-menopause, the rate of deterioration (i.e. overall structural skin ageing) correlates more convincingly with estrogen deficiency than with chronological ageing [19]. In fact, during early menopause (<5 years) a 30% reduction in the skin structural proteins, collagen types I and III, can be observed [20]. In later years, overall collagen abundance is rapidly diminished and linked directly to the decline in estrogen [3]. Further, the deleterious effects of estrogen deprivation have been proven experimentally in the context of UV exposure, where Ovx mice show enhanced skin damage and photo-ageing, measured as wrinkling, reduced skin elasticity and increased elastic fibre damage [21].

Interestingly, estrogen can be locally synthesised within the skin; a concept termed "intracrinology" [2]. Here, resident skin cells contain the enzymatic machinery necessary to synthesise estrogens from DHEA and even cholesterol [**Figure 1**; 2]. Crucially, regulation of cutaneous estrogen is dependent on local estrogen-metabolising enzymes that are produced by resident skin cells and hair follicles (16). These include: aromatase (ARO), catalysing the conversion of androgens to estrogens; and 17β-hydroxysteroid dehydrogenases 1 (17βHSD1) and 2 (17βHSD2), which convert E1 (estrone) to E2 (17β-estradiol) [4]. Estrogen then signals through two nuclear hormone receptors, ERα and ERβ, with skin expression of ERs dependent on body location and cell type. In humans, ERβ is more predominantly expressed in scalp keratinocytes than ERα, yet both receptor types have been identified in neonatal foreskin keratinocytes (reviewed in [22]). Interestingly, in both sexes, epidermal ERβ expression has been shown to decline with age [23].

Consequently, estrogen receptor expression and localisation is crucial for maintaining normal skin function, especially post-menopause, with ERβ signalling emerging as a key suppressor of skin ageing [22]. For example, Chang et al., [24] showed treatment with ERβ-selective agonists (WAY-200070 and ERB-041) significantly dampened pro-inflammatory marker expression (e.g. MMP-1 and IL-6) in primary keratinocytes and fibroblasts, whereas ERα-selective agonist treatment had no effect. In the same study, a

topical ERβ agonist (ERB-041) protected against photo-ageing, reducing wrinkling in UV exposed murine skin [24]. Clinically, topical 17β-estradiol and/or oral Hormone Replacement Therapy (HRT) can ameliorate many hallmark features of skin ageing, increasing skin elasticity and hydration, reducing wrinkles and augmenting collagen synthesis (reviewed in [22]).

Despite the observed protective effects of estrogens against a wide range of age-associated diseases, there remains controversy over the long-term health risks of HRT. Large epidemiological studies have shown correlations between HRT and the development of reproductive cancers [e.g. 25] although elevated DHEA-derived sex steroids have also been postulated to protect against reproductive cancers [2]. Intriguingly, many authors have established that ERα activation is a main driver of reproductive cancers, while ERβ provides anti-tumorogenic effects in this context [26]. In colon cancer, controversy around the effects of ERβ signalling remains, with both pro- [27] and anti-tumorigenic [28] effects of ERβ reported. Therefore, discriminative targeting of ERβ via selective agonists, such as phytoestrogens (genistein and S-equol) and selective estrogen receptor modulators (SERMs), may be a viable option for anti-ageing treatments [29].

Cutaneous Wound Healing and Ageing

The most important function of the skin is to act as a barrier to the outside world, preventing infection whilst maintaining and regulating thermal stability [30]. The skin has evolved crucial mechanisms to repair breaches to this barrier. In young, healthy individuals, this repair process occurs rapidly via a series of complex cellular "wound healing" processes (summarised in **Figure 2**) [31]. Here, keratinocytes migrate to close the epidermal barrier (re-epithelialisation), fibroblasts reform the damaged dermis (matrix deposition), and endothelial cells provide new vasculature. Late-stage healing then ensues, where fibroblasts remodel the extracellular matrix (ECM), thus leading to the formation of a collagen-rich scar [31].

The skin's ability to heal injuries steadily diminishes with age, correlating with increased cellular senescence. Indeed, age is a major risk factor for the development of chronic, non-healing wounds, skin fragility and associated chronic healing risk factors (e.g. diabetes mellitus) [31]. Age-associated immunoscenescence inhibits haemostasis via impaired platelet function and PDGF expression in both humans [32] and mice [33]. The aged wound milieu becomes flooded with pro-inflammatory cytokines and MMPS, while expression of protease inhibitors (e.g. TIMPs, [33]) is dampened. The local

inflammatory profile is altered with delayed, excessive, yet functionally impaired local immune cell recruitment (reviewed in [14]). Subsequently, excessive proteolysis combined with local fibroblast senescence limits adequate matrix deposition. Specifically, aged fibroblasts lose their responsiveness to TGFβ-1 (transforming growth factor beta 1) and CTGF (connective tissue growth factor), leading to reduced collagen expression, and increased AP-1 mediated MMP expression [17]. Senescence reduces alpha smooth muscle actin (α-SMA) expression, denoting perturbed myofibroblast differentiation, altered matrix remodelling, and impaired wound contraction [31]. Finally, aging impairs re-epithelialisation [32] with reduced keratinocyte proliferation, incomplete terminal differentiation, and impaired response to environmental stimuli [34]. Curiously, murine age-associated keratinocyte defects (reduced proliferative capacity, impaired migration and a less dynamic transcriptional response) are only revealed upon injury [35].

Estrogen and Wound Healing

Despite chronological ageing being a major risk factor for poor healing, a picture is emerging where estrogen deficiency, rather than known gerontogenes, primarily contributes to delayed healing ontogenesis. For example, microarray-based profiling of genes differentially expressed in wounds from young and elderly men, identified 78% as estrogen-regulated, while only 3% were age-associated [36]. This correlates with marked pleiotropic restorative effects of estrogen treatment (summarised in **Figure 3**) in aged cells *in vitro*, during *in vivo* wound repair [29, 37, 38], and in clinical acute wound studies of post-menopausal women [32] and elderly subjects of both genders [39]. Others have even demonstrated a preventative role for HRT, protecting post-menopausal women from developing chronic leg ulcers [40].

Direct evidence shows manipulation of inflammatory cell function by estrogens through cytokine modulation. One example, the cytokine Macrophage Migration Inhibitory Factor (MIF), known to delay healing [41], is downregulated with 17β-estradiol treatment [42]. Similarly, serum levels of MIF strongly correlate with menopause and estrogen treatment (HRT) [43]. Topically applying estrogen to elderly acute wounds also dampens inflammation, decreasing neutrophil numbers and elastase production, therefore aiding wound closure and collagen deposition 7 days post-injury [39]. The effects of estrogen on neutrophils can be attributed in part to reduction of the neutrophil adhesion molecule L-selectin, leading to diminished neutrophil localisation at sites of inflammation [22]. Similarly, estrogen has been shown to reduce expression of

pro-inflammatory cytokines, such as TNFα, and promote a shift from M1 to M2 macrophage polarisation [44]. Estrogen also imparts potent mitogenic effects on keratinocytes and fibroblasts, promoting *in vitro* [37] and *in vivo* migration [29, 38]. Most recently, 17β-estradiol has been shown to protect against bacterial-mediated inhibition of healing in young mice [45]. These new data support reports of a beneficial role for estrogen treatment in reversing healing delay in young diabetic mice [46]. Indeed, these age-independent effects suggest an even wider role for estrogen-mediated promotion of healing.

Curiously, there remains some discrepancy in regard of estrogen's role in male wound healing. Gilliver et al., [47] report substantially delayed wound repair in 17β-estradiol-treated male mice via ERα-mediated inhibition of re-epithelialisation and increased proteolysis. In the same year, Campbell et al., [37] reported that signalling via ERα in the absence of ERβ in female mice also delayed healing. However, in human studies, topical 17β-estradiol was able to promote healing in elderly subjects of both genders [39]. These data, which may reflect species-specific differences in local aromatase levels [48], highlight limitations in the use of animal models for skin endocrine research.

Full elucidation of cutaneous estrogen synthesis and signalling is essential for future ER-based pharmacological manipulation of wound repair. Our current knowledge is that treatment with the ERβ agonist, DPN, effectively restores wound repair in Ovx mice, while the ERα agonist, PPT, does not. Similarly, wound repair rates are significantly delayed in mice lacking ERβ, accompanied by impaired re-epithelialisation, excessive inflammation and reduced collagen deposition [37]. We are only just starting to explore the potential role of ER gene polymorphisms, splice variants and co-activators/co-repressors [49, 50]. Pre-clinical studies suggest that harnessing ER-mediated signalling, using a compound repurposing approach, could be clinically appealing. Existing clinical SERMs (tamoxifen and raloxifene) [29] and phytoestrogens (genistein) are known to promote healing in aged models of wound repair [29, 38]. Translation of these ERβ-targeted studies into the clinic remains a promising proposition.

Conclusion

It is clear that post-menopause, the rate of skin ageing increases. However, the link between estrogen-deficiency and skin ageing in peripheral tissues remains largely unresolved. Recent studies now reveal crucial insight into estrogenic changes with age, accounting for many of the physiological and architectural features associated with pathological cutaneous healing. From a clinical perspective, our understanding of

estrogen's beneficial effects on wound repair, particularly the benefits of SERMs, continues to open new avenues for targeted intervention. For example, it now appears that estrogen may directly protect against the effects of pathogenic wound infection. Further molecular and mechanistic studies are now essential to fully understand how signalling through ERs can modulate the complex processes of skin ageing, infection and wound repair.

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Author Contributions

Both authors wrote the paper.

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