The Effect of Tamoxifen on Healing of Skin Wounds in Senile, Adult Ovariectomized and Adult Non-ovariectomized Female Rats.

Thesis
Submitted for the partial fulfillment of M.Sc. degree in Anatomy

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2012
Acknowledgment

Frist of all I thank “ALLAH” for helping me to complete this work wishing it would be considered.

I would like to express my deep gratitude to **Prof. Dr. Emad Naguib Ghaly** Professor of Anatomy, Faculty of medicine, Cairo University, for his valuable advices, generous assistance and his marvelous effort to bring out this thesis in a scientific form.

I wish also to express thankfulness to **Prof. Dr. Mohamed Ehab Elden Mostafa** Professor of Anatomy, Faculty of medicine, Cairo University, for his support, generous help and his valuable guidance.

I owe my deep thanks to **Dr. Hanan Nabih Gad alla** Lecturer of Anatomy, Faculty of medicine, Cairo University, for her sincere effort, encouragement and her valuable advices.

Finally, I would like to express my gratefulness and respect to my family.
Abstract

The conditions of impaired wound healing in the elderly female due to estrogen deficiency are associated with substantial morbidity and mortality and impose a significant monetary burden upon the world’s health services. The present work demonstrated that selective estrogen receptor modulator (Tamoxifen) produced histological effects on skin wound healing. At the 5th day after wounding, tamoxifen was seemed to accelerate proliferative phase by prompting re-epithelialization, decreasing wound gap and early scab disappearance in the epidermis. In the dermis it early finished the inflammatory phase by decreasing the number of the inflammatory cells specially the macrophages, this might be due to tamoxifen inhibitory effect on wound expression of proinflammatory cytokines as macrophages migration inhibitory factor (MIF). It also prompted the proliferative phase in the dermis by stimulating fibroplasia, angiogenesis and wound contraction. Moreover, it encouraged remodeling phase by decreasing the area of the granulation tissue, enhancing formation of new skin appendages and stimulating collagen synthesis. Those effects might be due to tamoxifen ability to induce the activity of transformation growth factor β1, which is a chemotactic for macrophages, mitogenic for fibroblasts and stimulant for collagen synthesis and angiogenesis. Also, tamoxifen stimulated estrogen receptors (ERs) of the epidermis to produce its estrogenic effects. Moreover, tamoxifen induced keratinocytes expressing ERβ protein and decreased the number of the lymphocytes by its antiproliferative effect on lymphocytes and its inhibitory effect on P-glycoprotein which is responsible for transmembrane transport of cytokines in lymphocytes.

Key words:
Tamoxifen-aging-skin wound healing
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Insight has been gained in recent years into the role of estrogen in non-reproductive tissues including skin which appears to act as an end organ target for estrogenic action (Ashcroft et al., 2003a).

Human skin undergoes variety of changes that can potentially affect the process of wound healing. Each of the phases of healing demonstrates characteristic age-related changes as well. When taken as a whole, it is reasonable to conclude that there are global differences affecting wound healing between young and aged individuals (Gosain & DiPietro, 2004).

Estrogen is a major regulator of wound repair that can improve age related impaired wound healing in human and animal models (Ashcroft et al., 2003b).

Matrix metalloproteinase (MMP) enzyme may be an important factor during granulation tissue formation and early remodeling phases of healing. Estrogen has been shown to regulate MMP expression in skin. Absence of estrogen in ovariectomized rats decreases the activities of MMP during the healing process (Zecchin et al., 2004).

Selective estrogen receptor modulators (SERMs) are mixed agonists/antagonists i.e. able to apply tissue specific estrogenic or antiestrogenic activities (Hardman et al., 2007). One of them is tamoxifen (TAM), which acts as an estrogen agonist in uterus and an estrogen antagonist in breast. It has been used extensively for the treatment and
subsequent prevention of breast cancer due to its antagonistic activity in the breast (Jordan, 2003).

Hardman et al., 2007 demonstrated a beneficial effect of TAM on wound healing. They concluded that despite clearly different functions in some tissue (e.g. uterus), TAM acts as estrogen agonists i.e. potentially accelerates cutaneous wound healing in the skin.

Cell migration is a key element of wound healing process. Recently it has been shown that tamoxifen induces cytoskeleton remodeling and cell migration (Acconcia et al., 2006).

Gragnani et al., 2009 reported that addition of tamoxifen to standard treatment of skin wounds, may lead to improved wound healing in keloids by decreasing the expression of transformation growth factor- β (TGF-β), with consequent inhibition of both fibroblast proliferation and collagen production. The same authors (2009) added that topical tamoxifen citrate chemical treatment has been shown to improve scarring.

The aim of the current work was to study, compare and contrast the effect of tamoxifen on wound healing process either with lacking estrogen as, in senile and adult ovariectomized rats, or with normal body estrogen as, in adult non-ovariectomized rats and assis its beneficial effect as a concomitant therapy line during wound treatment.
Skin is the largest organ in the body. It exerts multiple vital protective functions against environmental aggressions (Widelitz et al., 1997). It is formed of three layers which are the epidermis (and its appendages) superficially, the dermis and the hypodermis which is the deepest layer and is technically not officially skin, but rather connects the skin to everything beneath. It is formed of connective tissue layer underneath the dermis (kanitakis, 2002 and Luis & Jose, 2003).

Structurally, the skin consists of two layers (epidermis and dermis); each provides a distinct role in the overall function of the skin (Michael & Wojciech, 2006):

**Epidermis (Fig. 1)**

The epidermis is formed of a stratified squamous epithelium that provides the first barrier of protection from invasion by foreign substances into the body and retains the body fluids. Keratinocytes in the basal layer of the epidermis are epidermal precursor cells that undergo both continuous proliferations, to maintain their population and differentiation when they migrate in the direction of the skin surface (Fuchs & Raghavan, 2002).

The sequential differentiation of the keratinocytes results in the formation of several anatomically distinct layers, including the stratum basal, the stratum spinosum, the stratum granulosum, the stratum lucidum and the stratum corneum (Bouwstra et al., 2003).
Stratum basal is the deepest layer of the epidermis (closest to the dermis). It consists of a single layer of columnar or cuboidal cells called Keratinocytes which rest on the basement membrane. Basal cells are the stem cells of the epidermis. Their mitotic activity refills the cells in the more superficial layers as these are eventually shed from the epidermis. Keratinocytes in the stratum basal accumulate intracellular keratin and secrete a waxy material into the intercellular space (Krause, 2005).

Stratum spinosum is formed of 5-15 layers of cells. Their cells become irregularly polygonal (Krause, 2005).

Stratum granulosum in thick skin consists of few layers of flattened cells. Only one layer may be visible in thin skin. The cytoplasm of its cells contains numerous keratohyaline granules (Hugo & Bryant, 2005). The nuclei of the outer layer show degenerative changes.

Stratum lucidum consists of several layers of flattened dead cells. (Victor & Mariano, 2005).

Stratum corneum is formed of four layers of died cells. It is the outermost layer of the epidermis and serves as a barrier for transepidermal water loss that is essential to prevent dehydration (Robert & Howard, 2005). The cells are completely filled with keratin filaments (horny cells) which are embedded in a dense matrix of proteins (Victor & Mariano, 2005).
There are other types of cells in the epidermis. Five to ten per cent of epidermal cells are non-keratinocytes, including mainly Langerhans cells, melanocytes and Merkel cells (James et al. 2005).

The mechanism of stratification is poorly understood. Although studies in vitro have led to the view that stratification occurs through subsequent movement of epidermal cells (Vaezi et al., 2002). It was found that a new keratinocyte takes about 60 days to migrate to the surface and undergo shedding. So about a gram of the skin is lost from human body each day by shedding. Wrinkles occur due to slower skin regeneration that occurs naturally with aging (Christine & Maggie, 2003).

**Dermis (Fig. 1)**

The dermis is the thick layer of connective tissue to which the epidermis is attached. Its deepest part continues into the subcutaneous tissue without a sharply defined boundary (Bruse, 1994). The dermis is composed of a papillary layer and a reticular layer. Fibroblasts are found in the dermis and produce collagen (Luis & Jose, 2003).

The dermis assumes the important functions of thermoregulation and supports the vascular network to supply the avascular epidermis with nutrients. The dermis contains mostly fibroblasts which are responsible for secreting collagen, elastin and ground substance that give the support and elasticity of the skin. Immune cells are also present in the dermis; they are involved in the
defense mechanism against foreign invaders passing through the epidermis (Victor & Mariano, 2005).

Fig. (1): A photograph of a section in the scalp skin showing the layers that compose the skin. (Hx. & E.; X100) (Victor & Mariano, 2005)
Skin vascularity

The cutaneous vessels originate either directly from the source arteries (septocutaneous or fasciocutaneous perforators) or as terminal branches of muscular vessels (musculocutaneous perforators) (Taylor & Pan, 1998). They emerge from the deep fascia in the vicinity of the intramuscular septa or near tendons and travel toward the skin and then they form extensive subdermal and dermal plexuses. Cutaneous vessels ultimately anastomose with each other to form a continuous vascular network within the skin (Taylor & Pan, 1998).

Cutaneous blood flow is required for essential oxygenation and metabolism. Large amount of heat can be exchanged through the regulation of cutaneous blood flow (Charkoudian, 2003).

The papillary layer of the dermis is richly supplied with capillaries, while larger blood vessels may be found in deeper levels of the dermis (Arthur & Tony, 2004).

Histology of skin aging

The sum effect of intrinsic and extrinsic aging is a progressive loss of function, increased vulnerability to the environment and decreased homeostatic capability (Fisher et al., 1997).

Intrinsic aging is defined as the changes in the skin that occur in sun-protected areas, independent of environmental insults. Extrinsic aging is comprised of the cumulative changes of long-standing environmental exposure, most notably to UV radiation from sunlight (Uitto & Bernstein, 1998).
With age, there is a flattening of the dermal ridges that maintain contact with the epidermis, resulting in decreased surface contact between the dermis and epidermis giving the appearance of atrophy (Neerken et al., 2004). The cellular contents of the dermis, consisting of fibroblasts, mast cells, and macrophages, are decreased with age. Also there is marked thinning, skin laxity, fragility, and wrinkles (Swift et al., 2001&Jessica et al., 2006).

Age-associated skin changes include reduction in cutaneous blood flow and disordered elastin morphology, resulting in decreased elasticity of the skin (Waller & Maibach, 2005). Activation of matrix metalloproteinases (MMPs) by UV radiation results in disorganized collagen fibrils and the accumulation of abnormal elastin-containing materials (Fisher et al., 2008).

**Estrogen Receptors in Skin**

The estrogen receptors (ER) are reported to vary in different parts of the body. The numbers of receptors are higher in facial skin than in the skin of the thigh or breast (Hasselquist et al., 1980).

Estrogen receptors β (ERβ) appear to be more widely expressed and have shown to be present in both male and female reproductive tissues in addition to non-reproductive tissues including the lung, bladder, heart, adrenal, thymus, kidney, pituitary, hypothalamus and skin (Taylor & Al-Azzawi, 2000).
Immunohistochemical studies localized estrogen receptors type α in the dermal papilla of follicles of mouse skin. ERβ are not transcripted in mouse skin homogenates (Chanda et al., 2000).

Human estrogen receptors type α is generally restricted to fibroblasts whereas ERβ is expressed in fibroblasts and in keratinocytes throughout the epidermis. After menopause, estrogen receptors number decreases significantly (Hall & Phillips, 2005).

**Healing of skin wounds**

Wound healing is one of the most complex biological events after birth; it is a dynamic, interactive process involving soluble mediators, blood cells, extracellular matrix (ECM) and parenchymal cells (Adam & Richard, 1999).

Skin is a complex tissue (Kanitakis, 2002), and thus in addition to the damage at the level of individual cells, a full-thickness wound also results in damage to many other structures. Those structures include (from the outside in) the epidermal keratinocyte layer together with associated epidermal appendages, the basement membrane (BM) that underlies the dermis (McNeil & Kirchhausen, 2005).

Healing by primary intention, in which wound edges are drawn together. It is characterized by the absence of major loss of tissues and occurs quickly, with wound closure in three to seven days (Darlene & Randolph, 2006). Secondary intention, or healing by scar tissue formation, is the chosen method
of healing when large amount of tissues are lost as the wound margins are nonviable or cannot be approximated, or when there is a high bacterial bioburden (Darlene & Randolph, 2006). Repair begins immediately following injury by attempting to reestablish continuity (Darlene & Randolph, 2006) and restoring the full function and a normal appearance to the injured tissue (Shaw & Martin, 2009).

**Stages of wound healing**

**Homeostasis:** it includes vasoconstriction, platelet aggregation and fibrin deposition which occur to control local bleeding (Hari & Micheal, 2000). Initially, there is neural reflex to cellular injury that causes transient vasoconstriction. This neurogenic response is quickly followed by hyperemic response in which blood flows into the wound. Coagulation seals in injured blood vessels and temporally closes the wound space (Mulder et al., 2002). Platelets not only facilitate the formation of a haemostatic plug but also secrete several mediators of wound healing, such as platelet-derived growth factor (that attract and activates macrophages and fibroblasts), basic fibroblast growth factor, transforming growth factor-β (TGF-β) and vascular endothelial growth factor (Bahou & Gnatenko, 2004).

The scab is formed 48 hours after wounding which is formed from exudates, necrotic cells and blood clots. The keratinocytes migrates beneath the scab to form the four keratin layers (Vidinsky et al., 2006).
**Inflammatory phase**

Inflammation is a series of reactions by vascularized tissue in response to an injury. The purpose of inflammatory reaction is to remove all foreign debris along with the dead and drying tissue. The inflammatory stage was traditionally studied by separating the tissue events into acute and chronic components; acute inflammation is the immediate and early microvascular response to an injurious agent, but chronic inflammation is longer-lasting response (Darlene & Randolph, 2006). The initial phase of acute inflammation is of short duration; it is completed 72 hours after wounding and is regressed after 96 hours (Vidinsky et al., 2006).

The process of cell migration along a chemically-mediated concentration gradient is called chemotaxis. The arrival of leucocytes and tissue macrophages to the site of injury is a critical part of the inflammatory process because phagocytosis of cellular debris and foreign antigen is necessary to assure proper wound healing (Darlene & Randolph, 2006). Inflammatory stage includes accumulation of neutrophils followed by macrophages infiltration. Neutrophils are activated and arrived to a wound within minutes. They have an important cleaning role and kill the invading microorganisms through several strategies (Kim et al., 2008).

**Proliferative phase (Granulation phase)**

It is the process of replacing the dead cells by exactly cell population. It begins at the first day of healing and reaches the peak between the fifth and
sixth day. It lasts approximately three weeks. Three major components occur simultaneously in this phase: re-epithelialization, fibroplasias (fibroblasts migrate to the injured area), with angiogenesis and wound contraction (kumar et al., 2003).

Formation of granulation tissue is the central pathological process in healing by repair. It is the new stroma that replaces the fibrin clot. Granulation tissue is composed of small blood vessels, proliferative fibroblasts (kumar et al., 2003), myofibroblasts which have contractile abilities to reduce the wound size and inflammatory cells (neutrophiles, monocytes, leukocytes) (Edward, 2004). Granulation tissue is contributed to by fibroblasts that are drawn from several sources: primarily the healthy dermis at the wound margins, from which fibroblasts can divide and produce collagen and other components in the extracellular matrix. First the fibroblasts have a vertical orientation then after six days of wounding they become parallel to the basement membrane (Hinz, 2007).

Factors affect tissue repair and granulation tissue formation are the epidermal growth factor (produced by epidermal cells around the injured area), endothelial growth factor, fibroblast growth factor, which are produced from macrophages and the transformation growth factor β which mediates fibroblast activity (Ruth et al., 2009).
Angiogenesis, which is integral to successful wound repair, involves sprouting of wound-edge capillaries followed by their invasion into the site of damage. After a few days, a microvascular network is apparent throughout the wound (Ruth et al., 2009).

Epithelialization is a primary response in burns, aberration and partial thickness wound when only the epithelium of the superficial dermis (in the hair follicles and sweat glands) is missing. Re-epithelialization of wounds begins within hours after injury. Epidermal and dermal cells no longer adhere to one another, because of the dissolution of hemidesmosomal links between the epidermis and the basement membrane, which allows the lateral movement of the epidermal cells (Adam & Richard, 1999).

In full-thickness open wounds, epithelialization comes only from the wound edges and occurs at the rate of one to two mm/day. Epithelial cells move quickly out from hair follicles and sweat glands left in the remaining dermis as well as from the leading wound edges (Mogford & Mustoe, 2001).

The complete regeneration of the epidermis was finished in the fifth day after wounding (Kumar et al., 2003).

**Remodeling**

It starts at the sixth day and continues for years after injury due to repeated synthesis and degeneration of collagen (Hari & Micheal, 2000). The dense
extracellular matrix that is randomly deposited early in repair is remodeled (Arthur & Tony, 2004).

Collagen remodeling during the transition from granulation tissue to scar is dependent on continued synthesis and catabolism of collagen at a low rate. The degradation of collagen in the wound is controlled by several proteolytic enzymes termed matrix metalloproteinases (Arthur & Tony, 2004). These enzymes are secreted by macrophages, epidermal cells, and endothelial cells, as well as fibroblasts. Metalloproteinases (collagenases) replace type III collagen with type I collagen, increasing tensile strength to approximately 80% of the original (Edward, 2004).

**Effect of Estrogen on Stages of wounds healing**

The sex hormones estrogen and testosterone are important mediators of wound repair (Strudwick et al., 2006). Differences in circulating sex steroid levels may underscore gender and age-related disparities in wound healing progression (Gilliver et al., 2006).

**Inflammatory phase**

Estrogen impairs neutrophil chemotaxis, reducing the rate of their migration to the wound site while increasing phagocytic function. The net result is more efficient clearance of debris with a net reduction in inflammatory cell-derived protease activity, which indirectly leads to enhanced matrix deposition (Margolis et al., 2002).
Elastase and metalloproteinase are mainly products of neutrophils. Elastase is capable of degrading a wide variety of structural and functional proteins deposited in wounds such as proteoglycans, collagen, and fibronectin (Kafienah et al., 1998). Fibronectin is an essential component of wound repair, influencing re-epithelialization, collagen deposition, and wound contraction. (Ashcroft et al., 1999). Estrogen has been shown to decrease neutrophils number and activity, thus decreasing the levels of elastase and metalloproteinase which lead to the improvement in matrix formation, retaining skin thickness and enhancing wound healing (Pirilä et al., 2002).

**Proliferative (Granulation) phase**

The cytokine Transforming growth factor (TGF-β) and its isomers are produced by several cell types at the wound site. They have been shown to play a role in the modulatory effects of estrogen on wound healing. TGF-β and its isomers are potent stimulators of fibroblast-driven gel contraction and probably stimulate granulation tissue contraction in vivo (Montesano & Orci, 1988). They are multifunctional, inducing angiogenesis, fibrosis, growth inhibition, apoptosis, differentiation and proliferation. They also contribute to Immune-regulation. They are up-regulated after skin wounding in mice. TGF-β1 is the most abundant isoform in all tissues and the only isoform stored in human platelets (Shen & Falanga, 2003). Exogenous addition of oestrogen accelerates wound healing and involves estrogen-induced increases in TGF-β1 secretion by dermal fibroblasts, leading to increased scarring (Ashcroft et al., 2003b).
In vitro, experiments suggest the potential of estrogen to accelerate angiogenesis via increased expression of vascular endothelial growth factor (VEGF) and other factors (Matthew & Gillian, 2005). Vascular endothelial growth factor (VEGF) enhances vascular permeability, induces chemotaxis, causes activation of monocytes/macrophages, and promotes growth of vascular endothelial cells. VEGF is produced by inflammatory cells to induce vascularization in the early stage of the wound healing process (Nogami et al., 2007).

Estradiol enhances (VEGF) & fibroblast growth factor (FGF) thus inducing angiogenesis, which plays a crucial role in wound healing (Mohammad et al., 2008). Angiogenesis in granulation tissues improves circulation to the wound site thus, providing oxygen and nutrients essential for the healing process (Mahmood et al., 2009).

**Remodeling phase**

Estrogen can affect collagen content, tensile strength, and macroscopic appearance of scar tissue. It is established that the use of estrogens in intact skin increases its collagen content (Brincat et al., 1983).

**Healing of wounds in senile people**

There is a general decrease in the number and size of dermal fibroblasts with age. Aged fibroblasts are also shown to exhibit a diminished response to growth factors and diminished replicative capacity (West, 1994).