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RESEARCH ARTICLE

Effect of Streptozotocin- and Alloxan-Induced Hyperglycemia on the First Anagen Cycle in Skin of Mice, *Mus musculus*

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Abstract

The present investigation was conducted to investigate the effects of both diabetogenic agents streptozotocin- and alloxan-induced hyperglycemia on postnatal skin development of hair follicles at 10th day of delivery during the first anagen cycle. Pups from the same mother were classified and injected from the first day of birth into three groups: control, streptozotocin- and alloxan-treated groups. Pubs were sacrificed at 10th day of birth, the STZ-treated pubs showed elevation in the blood sugar than alloxan-treated pubs as compared to control. The skin of STZ-treated pubs showed several complications, losing of hair coat, bald and wrinkled skin, thickened epidermis and decrease in the content of carbohydrate in the outer root sheath of hair follicles. In addition, the immunohistochemical studies showed the decrease in GDNF, GFRα1, EGF, CTGF and BCL-2 in STZ-treated group as compared to both the control and alloxan-treated pubs. These findings showed that STZ is more effective than alloxan in the induction of skin abnormalities in hyperglycemic model animals. The observed findings were attributed to the inhibition of the growth factors that are essential during hair follicle development in STZ- induced hyperglycemia compared to both the control and alloxan-treated pubs.

Introduction

The skin is the heaviest organ of the body. Some of the many functions of skin include protection against physical, chemical, and biologic assaults; providing a waterproof barrier; absorbing ultraviolet radiation; vitamin D synthesis; excretion and thermoregulation. There are three main phases of the hair growth cycle; anagen, catagen and telogene. Anagen is the active and the longest phase in which RNA and DNA synthesis is initiated during cell proliferation and differentiation. Also several expression of polypeptide growth factors as cytokeratines for hyperproliferative of keratinocytes were included during this phase (Powell *et al.,* 1991). Anagen is followed by catagen, a period of controlled regression of the hair follicle. Ultimately, the hair follicle enters a resting state telogene (Militzer, 2001).

Members of transforming growth factors (TGF-β) were found to play a role in hair follicle differentiation during anagen phase (Zwick *et al.,* 2002; Jamora *et al.,* 2003; Albers *et al.,* 2006). Glial cell line-derived neurotrophic factor (GDNF) is a member of the GDNF family of neurotrophic factors which is distally related to TGFβ superfamily (Widenfalk *et al.,* 1997; Luukko *et al.,* 1998). GDNF and its receptor GFRα1 are essential for the development of organs which are formed on the basis of complex epithelial-mesenchymal interaction such as kidney, hair follicle and human skin (Suvanto *et al.,* 1996; Nosrat *et al.,* 1997; Adley *et al.,* 2006). In addition, epidermal growth factor (EGF) has a role in embryonic tissue maturation (Gospodarowicz, 1981) including the development of skin (Dolling *et al.,* 1983; Green *et al.,* 1984; Herbst, 2004). Moreover, connective tissue growth factor (CTGF/CCN2) exhibits diverse biological activities *in vitro*, such as cell proliferation, adhesion, migration

and extracellular matrix (ECM) production (Brigstock, 1999; Gupta *et al.,* 2000; Chen *et al.,* 2001). CTGF is required for the coordination of chondrogenesis (Ivkovic *et al.,* 2003). Also, During normal healing, CTGF expression is activated at the wound site and then switched off once the repair process is complete (Igarashi *et al.,* 1993). BCL-2 family proteins are inhibitors and inducers of cell death (Hardwick and Soane, 2013). At the same time, BCL-2 were found to promote cell survival as opposed to promoting cell proliferation (Vaux *et al.,* 1988; Tsujimoto, 1989). BID and BAD are members of BCL-2 family, BAD is known to promote cell death by antagonizing anti-death BCL-2 proteins, and a number of studies showed that BAD has a normal physiological role in healthy cells (Seo *et al.,* 2004). BID has a "day-job" in healthy cells, in addition to its apoptotic function. At the same time, BID has a nonapoptotic role in regulation of the DNA damage response (Zinkel *et al.,* 2005).

Streptozotocin and alloxan induce insulin deficiency. Streptozotocin was originally identified in the late 1950s as an antimicrobial and an antibiotic (Vavra *et al.,* 1959) and used as a cancer chemotherapeutic agent (Brentjens and Saltz, 2001). Alloxan was discovered since 1828 and is one of the oldest named organic compounds. The alloxan model of diabetes was first described in rabbits with concomitant effects on pancrease (Dunn *et al.,* 1943; Bailey *et al.,* 1950). Reactive oxygen species in the case of alloxan and DNA alkylation in the case of streptozotocin mediate the toxic action of these glucose analogues (Bolzan and Bianchi, 2002; Lenzen, 2008). Hence, the aim of the present study is to evaluation the effect of both diabetogenic agents STZ and alloxan for induction of hyperglycemia during skin development of mice at the first anagen phase of hair follicle at histological, histochemical, ultrastructure and immunohistochemical investigations.

Materials and Methods

Experimental animals

Mature males and virgin females of mice, *Mus musculus*, weighing approximately 30-35g were obtained from animal house, Faculty of medicine, Assiut University for experimentation. Two females were mated with one male and zero day of pregnancy was determined by appearance of vaginal plug. Pregnant females were kept for delivery. Pups of the same mother were divide into three groups, the control, streptozotocin-and alloxan-treated pubs at least three individuals in each group. Injection of pups was done for three consecutive days from day zero of delivery with 100 mg/kg of STZ (Ganda *et al.,* 1976; Katsumata *et al.,* 1992; Szkudelski, 2001) and 150 mg/kg of alloxan (Katsumata *et al.,* 1993; Szkudelski, *et al.,* 1998). Blood glucose level was tested using blood glucose meter (Be Smart instrument, USA) at 10^{th} day of delivery. Specimens of skin from mid-dorsal back of previous groups were fixed in Carnoy's fluid, dehydrated, cleared and processed for sectioning. Sections were stained with Haematoxylin and Eosin stain for general histological picture, periodic acid Schiff's reaction (PAS) for polysaccharide detection, Masson trichrome stain for collagen (Drury and Wallington, 1967), aldehyde–fuchsin staining to visualize the morphology of the various skin layers (Geerligs *et al.,* 2011), acridine orange/ethidium bromide staining to visualize the viability of cells (Ribble *et al.,* 2005) by using fluorescent microscope (Axio Scope A1, Zeiss, Germany). To study the ultrastructure of the growing hair follicles during its first anagen phase, specimens of skin from previous groups were fixed in 2.5% gluteraldehyde then post-fixed in 1% osmium tetraoxide at 4 ˚C for two hours, dehydrated in ascending grades of ethyl alcohol and embedded in epoxy-resin. Ultrathin sections were cut using ultratome (Reichert, Supernova, Germany). Ultrathin sections were mounted in grids, stained with uranyl acetate and lead citrate. Grids were examined under a Joel Transmission Electron Microscopy (JEM 1010, Japan).

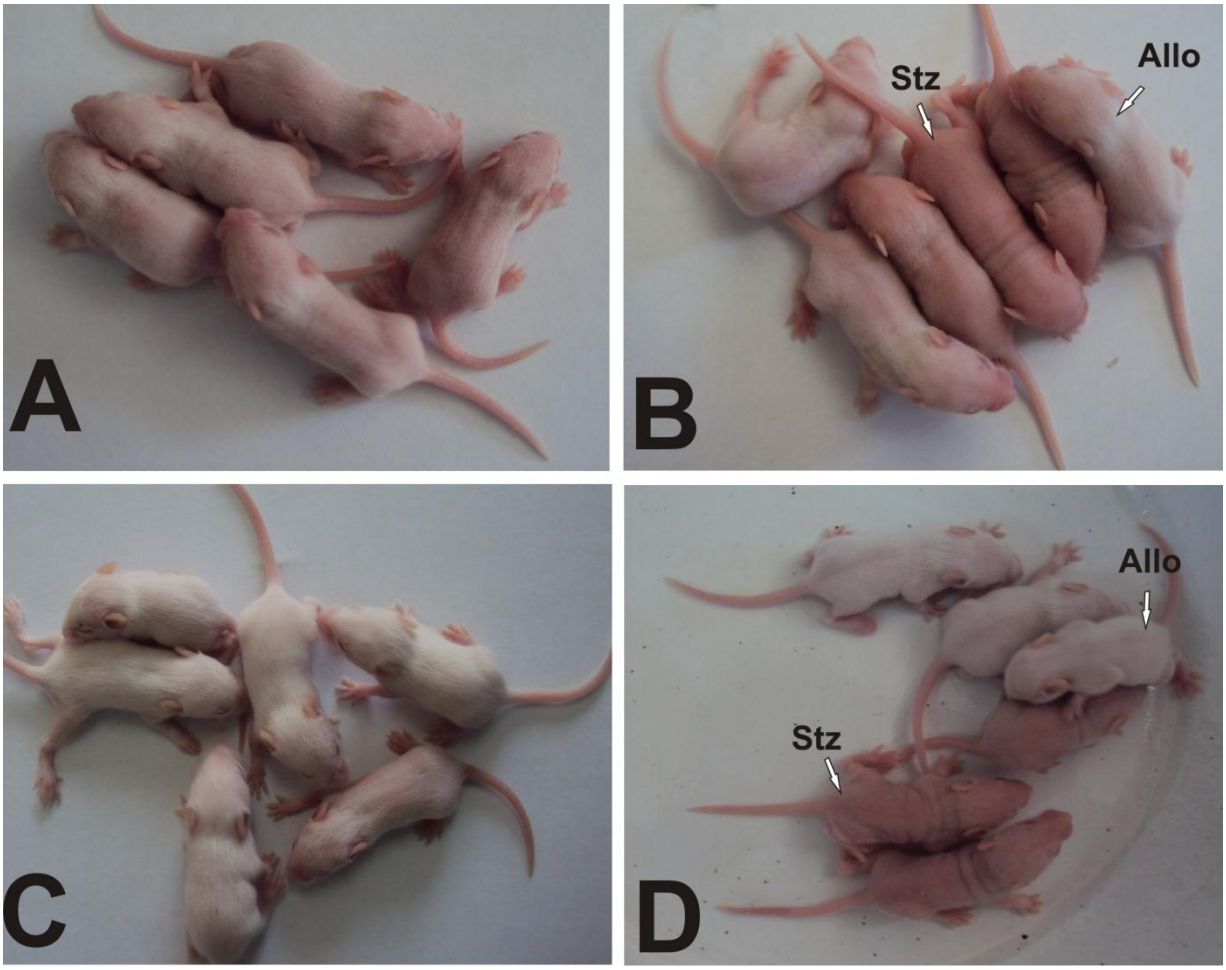
For immunohistochemical investigation, sections were mounted on Superfrost/Plus glass slides. The slides were deparaffinized in xylene, rehydrated and retrieved for re-antigenicity using 10 mM citrate buffer at pH6 in 100 ˚C for an hour (Buchlowalow and Bocker, 2010). Sections were incubated with specific primary antibody against GDNF and GFRα1(Rabbit anti-GDNF and GFRα1, Sigma Aldrich, USA), CTGF (Rabbit anti-Cyr61, spring, Bioscience, USA), EGF (Rabbit anti-EGFL7, spring, Bioscience, USA), for three hours at room temperature. Sections were then washed using phosphate buffer and incubated with secondary antibody (Biotinylated Goat Anti-polyvalent HRP DAB detection system, Spring Bioscience, USA). For The immunohistochemistry of BCL-2 family, sections were incubated with specific primary antibody (Bid and Bad Goat polyclonal antibody, Santa Cruz, Biotechnology, INC., Germany) for three hours at room temperature, then washed using phosphate buffer and incubated with secondary antibody (Econo Tek HRP (DAB) anti-polyvalent, Logan, Utah, USA). DAB and chromogen were mixed to visualize the color of reaction. Sections were dehydrated, cleared, mounted. Selected sections at the level of histological, immunohistochemical and ultrastructure were photographed and processed as required.

Results:

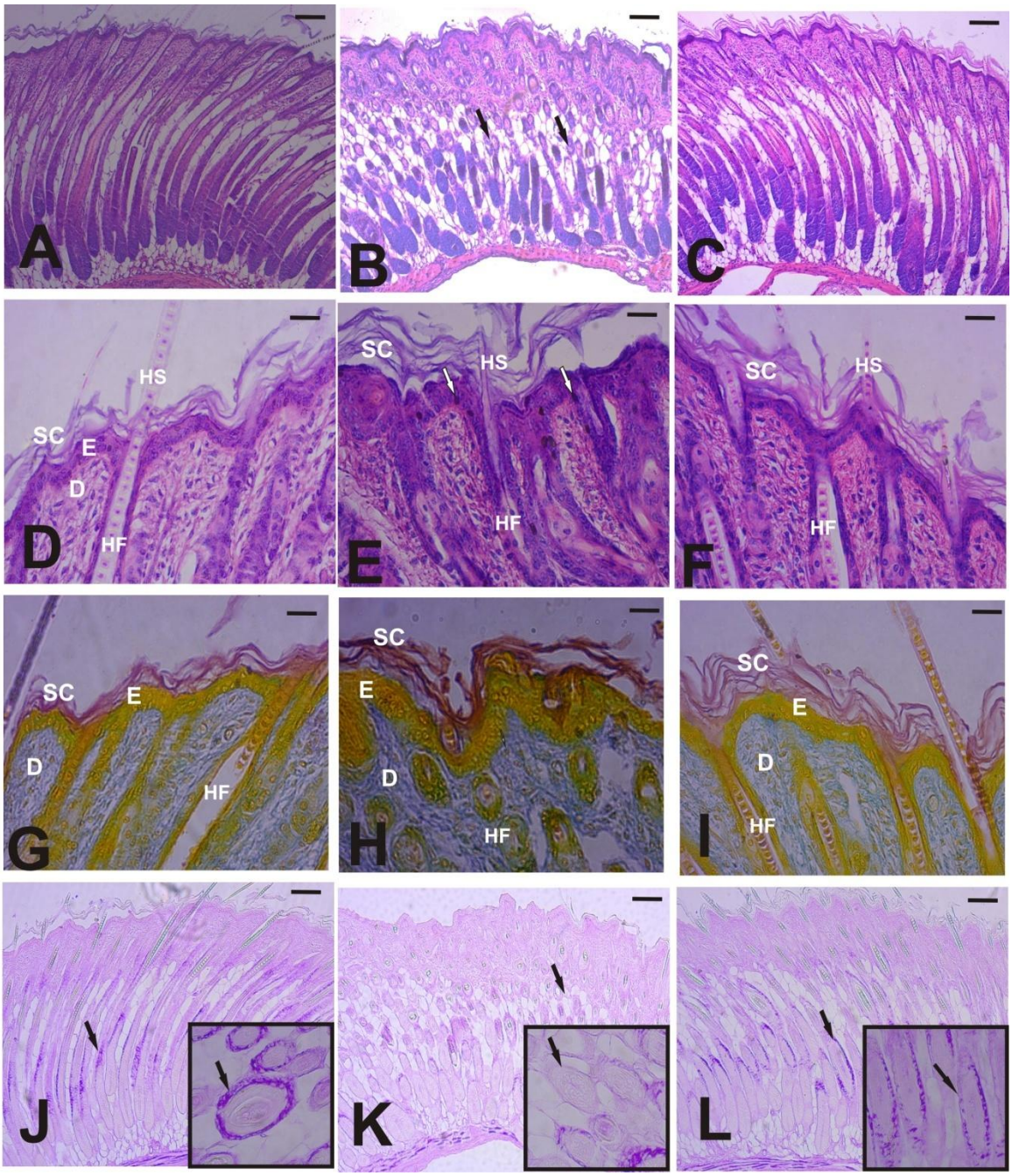
Morphological, Histological, Immunohistochemistry and TEM observations:-

The STZ-diabetic pubs at 7^{th} and 10^{th} day of birth showed complete loss of hair coat (Pl.1 B,D), and their bald skin was wrinkled as compared to alloxan-treated (Pl. 1 B, D) and control pubs (Pl. 1A,C). The level of blood glucose was highly significant in STZ-treated pubs (283±29.9 mg/dl), significant in alloxan-treated pubs (133±6.48 mg/dl) as compared to control (96±2.3 mg/dl). The normal structure of skin was detected in control pubs at 10^{th} day of delivery (Pl.2 A,D). On the other hand, STZ-treated pubs (Pl.2 B,E) showed alopecia, hairless skin accompanied with follicle degeneration, thickening stratum corneum, thinning and fragile hair shaft, wrinkling epidermis with increase melanocytes. In contrast, alloxan-treated pubs showed similarity with control (Pl. 2C,F). Also, the same symptoms were noted in aldehyde fuchsin-stained sections of STZ-treated pubs (Pl. 2 H) as compared to control pubs (PL.2 G) and alloxan-treated pubs (Pl.2 I). In addition, PAS-staining showed positive reaction in the outer root sheath of the developing hair follicles in the control (Pl.2 J) and alloxan-treated pubs (Pl.2 L). STZ-treated pubs showed decreasing in carbohydrate contents of the outer root sheath of the follicles (Pl.2 K). Masson trichrome staining exhibited degeneration of hair follicles in STZ-treated pubs and degeneration of collagen at the level of the hair bulb (Pl.3 B,E) as compared to the control (Pl.3 A,D) and alloxan-treated pubs (Pl.3 C, F). Acridine orange/ethidium bromide staining revealed the healthy hair follicles and viability of their cells in the control (Pl.3 G) and alloxan-treated pubs (PL.3 I). On the other hand, the STZ-treated pubs showed the degeneration of hair follicles and decreased viability of their cells (Pl.3 H). The ultrastructural abnormalities of the follicles were investigated using transmission electron microscope. In control, the hair follicle consists of five distinctive layers: outer root sheath; inner root sheath; cuticle; cortex and medulla (Pl.4 A,B). In STZ-treated pubs, the outer root sheath was noticeably thinner and distinctive vacuolation through cuticle, cortex and medulla (PL.4C) as compared to alloxan-treated (Pl.4 D) and control pubs (Pl. 4 B).

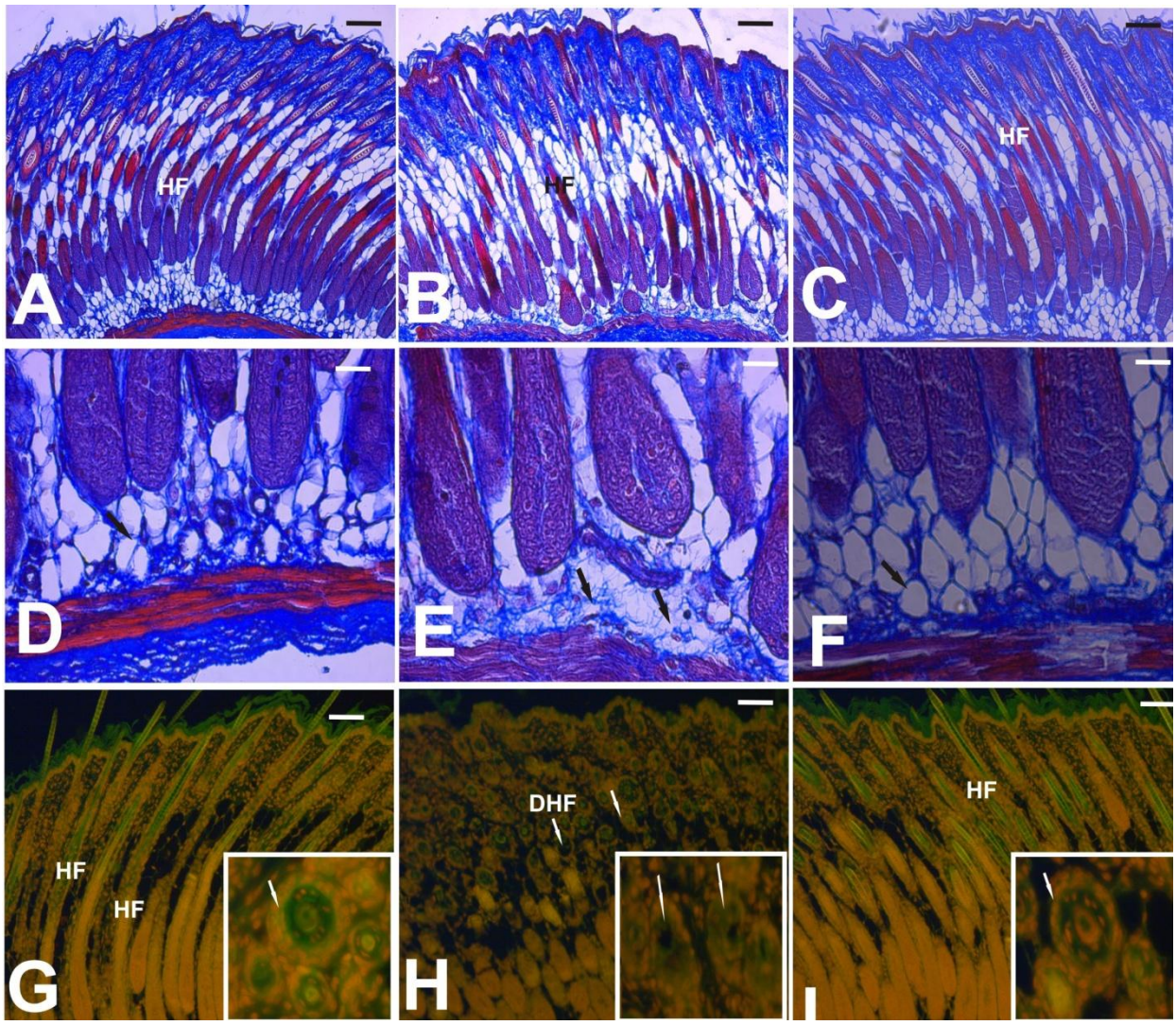
Immunoreactivity of glial cell line-derived neurotrophic factor (GDNF), its receptor (GFRα1) and EGF were expressed in the epidermis and dermis of control skin pubs at 10^{th} day of birth (Pl. 5 A,D,G). On the other hand the STZ-treated pubs showed the reduction in the expression GDNF, GFRα1 and EGF (Pl. 5 B,E,H), the expression in alloxan treated-pubs (Pl. 5 C,F, I) appeared similar to that of control. CTGF, BID and BAD expression were widely distributed in the epidermis and a long-side of the growing follicles invading the dermis of the control pups skin at 10^{th} day of birth (Pl.6 A,D,G). STZ-treated pubs showed reduction in CTGF expression in the epidermal portion of the skin and the expression was restricted only on the dermal fibroblast cells (Pl.6 B). Also, STZ-treated pubs showed reduction in the immunoreactivity of both BAD and BID expression in the epidermal portion and the expression was restricted only on melanocytes that scattered in the basal and suprabasal layer of epidermis (Pl.6 E,H). on the other hand, alloxan-treated pubs exhibited the same expression as control (Pl.6C,F,I).



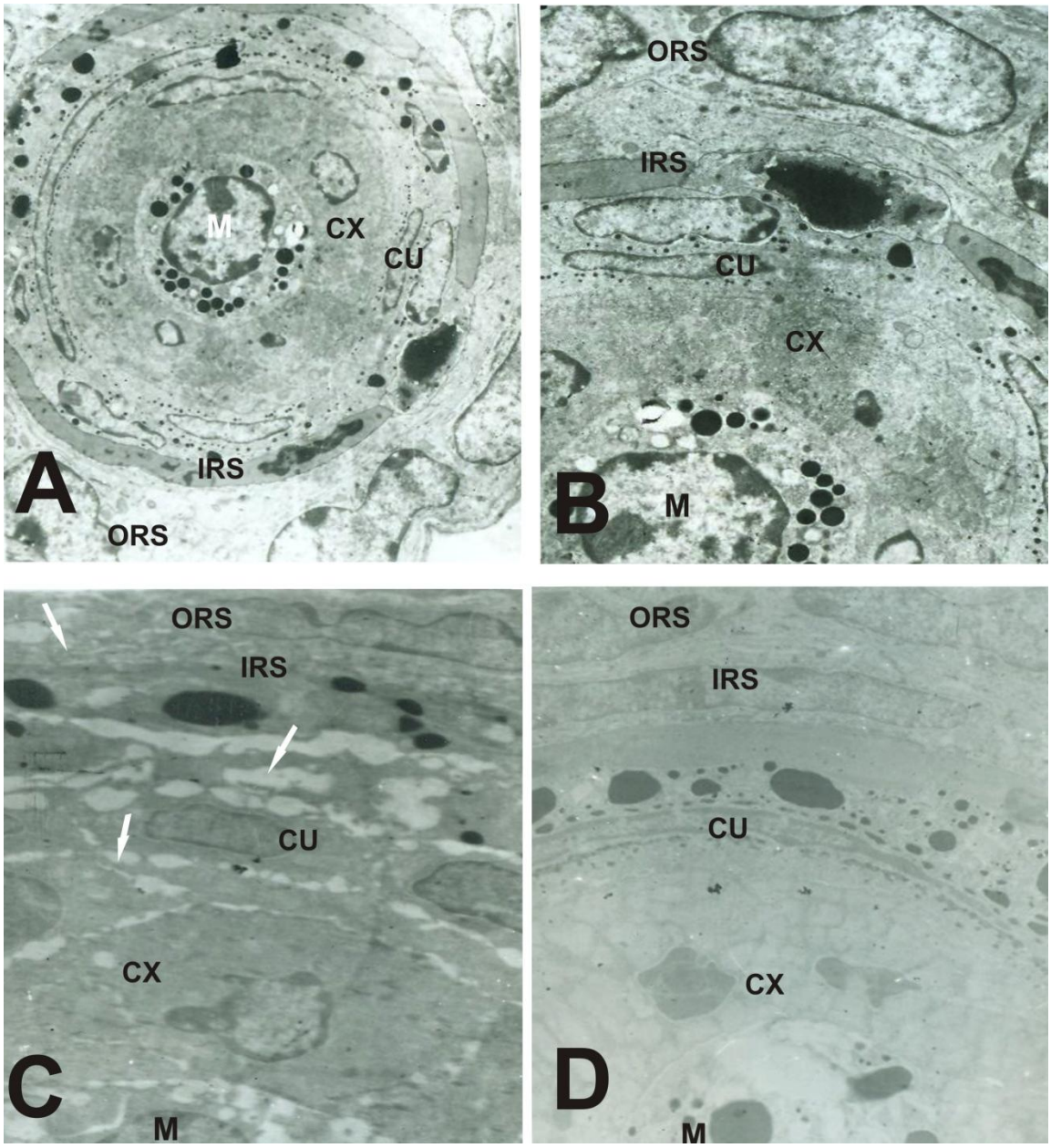
Pl. 1 A - D: Photographs of control pubs at 7th (A) and 10th(C) day of delivery. STZ-treated pubs (STZ) showed losing of hair coat as compared with unaffected alloxan-treated pubs (Allo) at 7th (B) and 10th (D) day of delivery.



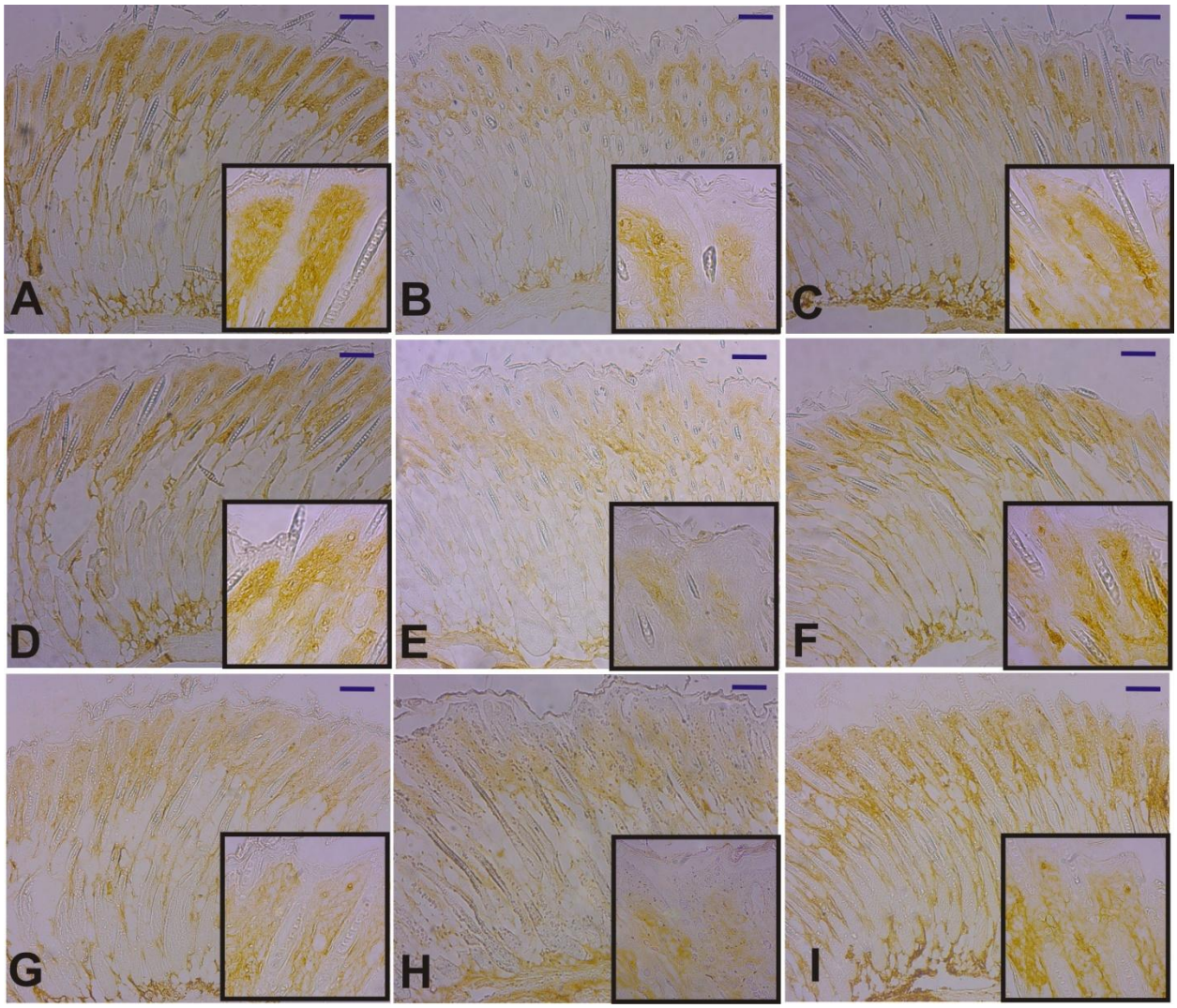
Pl. 2 A- L: Photomicrographs of H&E stained sections through the trunk region of skin in control pubs (A,D) at 10th day of delivery showing skin layers, epidermis (E), dermis(D) and hair follicle (HF) with growing hair shaft (HS) and normal stratum cornium (SC). STZ-treated pubs (B,E) showed a less-abundant hair follicles (HF), thickened stratum cornium (SC) of epidermis; increased melanocytes (arrows,E) and rudimentary hair shafts (HS) (arrows,B) as compared to both the control and alloxan-treated pubs (C,F). Aldehyde fuchsin-stained sections showing thickened stratum cornium (SC), degenerated hair follicles (HF) in STZ-treated pubs (H) as compared to both the control (G) and alloxan-treated pubs (I). PAS stained sections showing the decreased carbohydrate contents in the outer root sheath (arrows) in STZ-treated pubs (K) as compared to both the control (arrows,J) and alloxan-treated pubs (arrows,L). The inserts represents high magnified of the previous treatments. Scale bar in A-C, J-L 50 µm, D-F, G-I 10 µm.



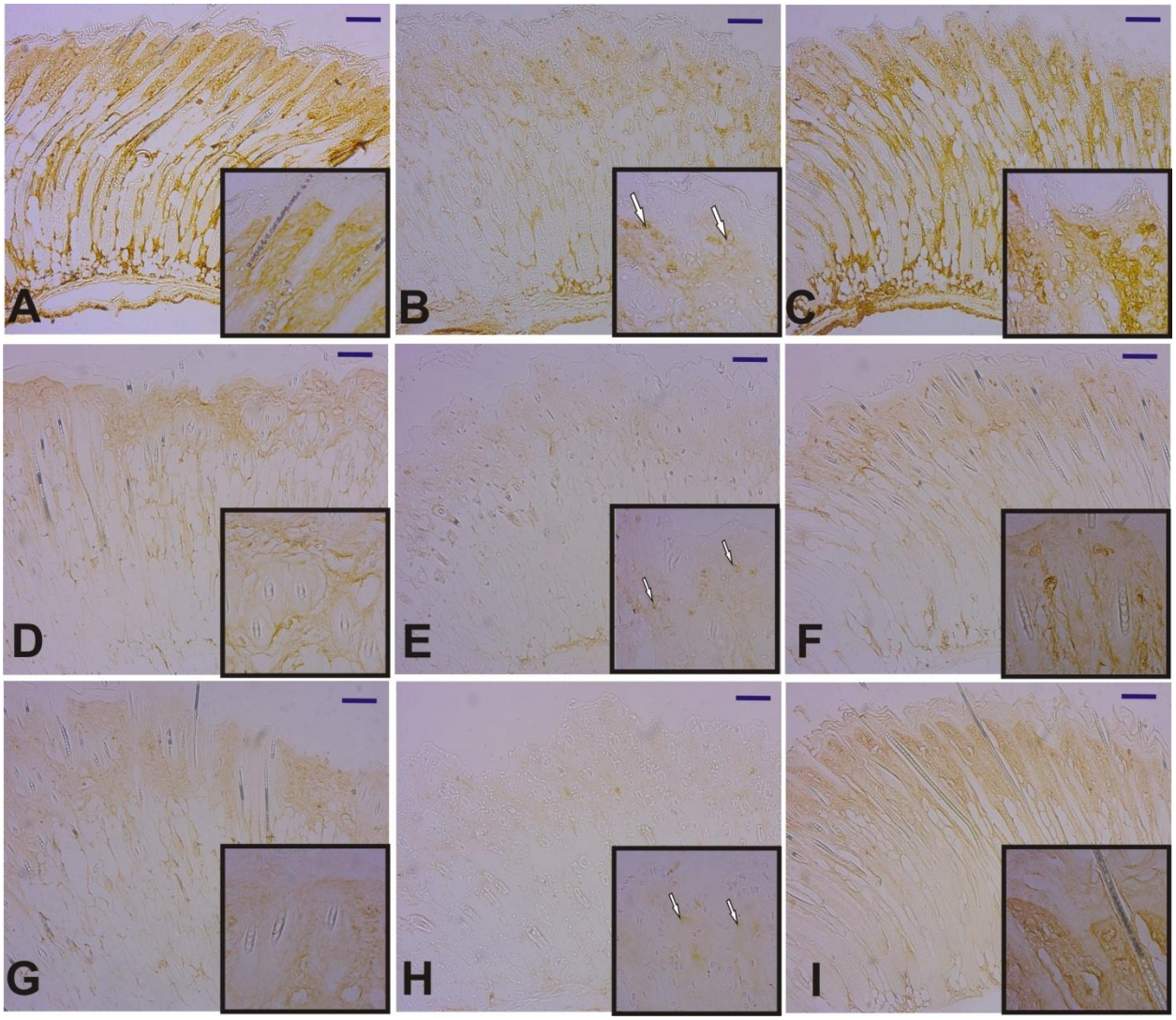
Pl.3 A-I: Photomicrographs of Masson trichrome Stained sections through the trunk region of the skin showing a less abundant hair follicles (HF) in STZ-treated pubs (B,E) at 10th day of delivery as compared to both the control (A,D) and alloxan-treated pubs (C,F) and high magnified field showing degeneration of collagen in the dermis (arrows) of STZ-treated pubs (E). Ethidium bromide/Acridine orange stained sections showing degenerated hair follicles (DHF), reduction in their cell viability in STZ-treated pubs (H) as compared to both the control (G) and alloxan-treated pubs (I) of a well viable cells of hair shafts (HF). Scale bar A-C, G-I 50 µm; D-F 10 µm.



Pl.4 A-D: Transmission electron micrograph of transverse section of hair follicle at 10th day of delivery of control pubs (A) showing the distinct layers; the outer root sheath (ORS); the inner root sheath (IR); cuticle (CU); cortex (Cx) and medulla (M), X 1000. High magnified fields of TEM of transerverse sections through the growing follicles showing vacuolation (arrows) and disorganized follicle layers in STZ-treated pubs (C) as compared to both the control (B) and alloxan-treated pubs (D), X 5000.



Pl.5 A-I: Photomicrographs of GDNF (A-C), GFRα1 (D-F) and EGF (G-I) immunostained sections through the trunk region of control skin (A,D,G), STZ treated pubs (B,E,H) and alloxan treated pubs (G,H,I). STZ-treated pubs showed decreasing in the immunoreactivity of GDNF (B), GFRα1 (E) and EGF (H) in the epidermis, dermis and along side the sheath of the growing follicles as compared to the control (A, D, G) and alloxan-treated pubs (C, F, I) respectively. The inserts represent high magnified fields of the epidermis Scale bar 50 µm.



Pl. 6 A-C: Photomicrographs of CTGF (A-C), BID (D-F) and BAD (G-I) immunostained sections through the trunk region of skin in control pubs at 10th day of delivery showing the expression of CTGF (A), BID (D) and BAD (G) in the epidermis and along side the sheath of the growing follicles. STZ-treated pubs showed reduction in the expression of CTGF (B) and the staining was restricted only to fibroblast cells (arrows). Also, STZ-treated pubs showed reduction in BID (E) and Bad (H) and the staining restricted only to melanocytes cells (arrows in E,H). Alloxan-treated pubs didn't show abnormalities related to the expression of CTGF (C), BID (F) and BAD (I). The inserts represents enlarged part of the epidermis in different treatments. Scale bar 50 µm.

Discussion:

The present investigation revealed that the induction of hyperglycemia led to skin defects including hyperkeratininzation, wrinkling, thickening of the epidermis, follicle degeneration, decrease of carbohydrates in the outer root sheath, collagen degeneration and inhibition of growth factors under investigations, In this context, the diabetogenic agent STZ was found to display a retardation of the first anagen phase during follicle development and induce histological abnormalities in skin of hyperglycemic pubs. Concerning these observations, hyperglycemia and impaired insulin signaling might be directly involved in the developmental of chronic complications of diabetes by impairing functions of skin keratinocytes as well as skin proliferation and differentiation (Spravchikov *et al.,* 2001). Also, research findings stated that, during the pathogenesis of diabetes, hyperglycemia results in high cellular glucose level, those cells unable to reduce glucose intake (Dominicza, 2003; Brownlee,2005). Hence, increasing of the reactive oxygen species and damaging of DNA (Nathan *et al.,* 2003; Hartog *et al*., 2005; Topol and Califf, 2006). In addition, hyperglycemia was found to alter the structural properties of skin collagen that led to stiffness and reduced elasticity, thus, hyperglycemia and its complex reactions perpetuate the damage of skin collagen, elastin and intercellular matrix and increase the death rate of the fibrocytes (Forbers *et al.,* 2005, Oumeish, 2008). The present study demonstrate more effects of STZ than alloxan that may be attributed to STZ was found to be more stable in aqueous solution after injection than alloxan (Lenzen and Munday, 1991; Lenzen, 2008). In addition, the range of STZ dose is not as narrow as in the case of alloxan, and it is used to induce both insulin-dependent and non-insulin dependent diabetes mellitus that result in hyperglycemia (Ganda *et al.,* 1976; katsumata *et al.,* 1992, Szkudelski, 2001). Also, Saini *et al.,* 1996 showed that STZ at low doses induces apoptosis and at high doses causes necrosis in a murine pancreatic β–cell line. Thus, impaired structural alterations of skin in the present investigation were hyperglycemic-dependent that may result from increased peroxidative stress with resultant destruction of fibrocytes in STZ- than in alloxan-treated pubs skin.

It is well known that, hair cycle relies on an elegant and coordinated balance of stimulatory and inhibitory signals which are spatially and termporally regulated in the hair follicle niche through orchestrated actions of various growth factors (Blanpain and Fuchs 2009; Yamamoto *et al.,* 2011). The present study provide an evidence that GDNF, GFRα1, EGF, CTGF and BCL2 family were expressed in the skin development at 10^{th} day of birth in synchrony with the postnatal follicle cycle and participate in its quality control. The expression of these factors was inhibited in hyperglycemic STZ- than in alloxan-treated pubs. GDNF is broadly distributed in adolescent mouse skin, and expressed in both epithelial and mesenchymal cells whereas the expression of GFR α1 receptor appears in the epithelial skin compartment (Botchkareva *et al.,* 2000a,b). GDNF gene is transcribed in embryonic skin (Trupp *et al.,* 1995; Hellmich *et al.,* 1996), and its receptor (GFR α1) expression was recorded during all stages of hair follicle cycling in normal adolescent C57BL/6 mice (Enomoto *et al.,* 1998; Rossi *et al.,* 1999). The present investigation revealed that STZ-treated pubs showed reduction in the expression of EGF with a concomitant pathologic changes of skin layers. In mouse EGF is known to influence hair development and hair growth cycle (Steidler and Reade, 1980; Moore *et al.,* 1982). EGF is important physiologically in maintaining normal structures of epidermal cells (Tsutsumi *et al.,* 1987) and in the outer root sheath of the growing hair follicles (du Cros *et al.,* 1992). Epidermal growth factor function as a biologic switch that is turned on and off in hair follicles at the beginning and end of the anagen phase of the hair cycle guarding the entry to and exit from the anagen phase (Mak and Chan, 2003). The STZ-treated pubs showed also reduction in the expression of CTGF that was restricted only on fibroblast cells. The crucial role of CTGF in prenatal skin development and in adult skin was reported (Carulli *et al.,* 2005; Jones *et al.,* 2007). CTGF was found to be an effective surrogate marker of fibrosis (Leask *et al*., 2009), CTGF is expressed by embryonic fibroblast cultured from E13.5 mice (Shi-Wen *et al.,* 2006), Interestingly, high levels of CTGF expression have been detected within fibrotic lesion in scleroderma patients (Abraham et al., 2000; Quan *et al.,* 2009). Sonnylal *et al.,* (2010) demonstrated that, selective expression of CTGF in fibroblast alone causes tissue fibrosis. Thus, a dual role of CTGF in both the development and disease manifestation is played. In addition, BCL-2 is important during hair development and it expressed in normal control skin at 10^{th} days of birth that expression is confirmed in the developing hair follicle that in the early stage or in the place where the cells continued to proliferate (Maskey *et al* ., 2009). Developing human skin express Bcl-2 in the epithelial component of the hair germ, localizes to the follicular papilla during fetal life and epidermal portion of hair follicle (LeBrun *et al.,* 1993; Polakowska *et al.,* 1994; Sellheyer *et al.,* 2001). STZ-treated mice showed the reduction in BCL-2 family and the immunoreactivity was restricted only to the melanocytes. These findings were supported by Maskey *et al*. 2009, they reported that the reduction of Bcl-2 expression increase cell death to perform the tissue morphogenesis during development or the organ functions. At the same time, the strong immunoreactivity of Bcl-2 demonstrates

melanocytes that were observed in the basal and suprabasal layer of epidermis as well as in the hair matrix cells similar to the findings of the present investigation. Bcl-2 is also expressed by melanocytes in various studies (Klein-Parker *et al*., 1994; Plettenberg *et al.*, 1995). In conclusion, the present investigation revealed the adverse effects of hyperglycemia on skin during anagen phase of follicle development through the disturbance in the expression of various growth factors under investigation. At the same time, the present investigation showed the strength of STZ in induction of hyperglycemia than alloxan and its complications during skin development at the anagen phase of hair follicle development.

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