

Long-Term Effects of Diabetes Mellitus on Skin Melanocytes in Rats

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Abstract. The key component of the skin pigmentation is melanocytes due to ability in melanin production. Pigmentation disorders are more common in the diabetes mellitus (DM), especially hyperpigmentation. It is caused by an increased number or activity of the melanocytes. The present study aimed to investigate the effects of long-term DM, chemically induced by streptozotocin (STZ), on the pathological changes of melanocytes in the rat skin. Seven adult male Sprague-Dawley rats were randomly divided into two groups: non-diabetic control and STZ-induced diabetic groups. In the diabetic rats, an increased amount of melanocytes at the dermoepidermal junction with increased melanin granules in the basal layer of keratinocytes were demonstrated. Moreover, the diabetic melanocytes became hypertrophy and presence of large clear area in their cytoplasm around the nucleus. It was suggested that diabetes caused hyperpigmentation. Its potential mechanism will be investigated in the future research.

1. Introduction

Numerous dermatologic lesions associated with diabetes mellitus (DM). One of which is pigmentation disorder. Abnormal pigmentation reveals either increased or decreased pigment levels, known as hyperpigmentation and hypopigmentation, respectively. Although skin pigmentation disorders seem to be harmless, hypopigmentation is a great risk in developing skin cancer due to high sensitivity to sunlight exposure. Moreover, excessive pigmentation in an area of sun-damaged skin is a warning sign of benign or malignant melanocytic lesions [1]. Approximately 30% of people with diabetes has hyperpigmentation [2], while hypopigmentation has been reported to occur in 10% [3], [4]. Variations in skin color relate to melanin pigments, produced by melanocytes in terms of the amount of melanin within melanosomes, the degree of melanization, and the distribution of the epidermal melanin unit in the keratinocytes [5]. As a part of our pilot study in short-term group, the morphological change of melanocyte in the diabetic condition was not different, when compared with control group. In addition, it has been found that long-term diabetes leads to pyknotic melanocytes in the stratum basale of diabetic rat [6]. Therefore, it is particularly interesting in an altered melanocyte caused by diabetes. This study was focused on long-term effects of diabetes induced by streptozotocin (STZ) on the structural changes of pigment-producing cells or melanocytes in their number and size. The pathogenesis of melanocyte in diabetic rats of this study can be generalized to diabetic patients and may serve as new information justifying the appropriate treatment for pigmentary changes in people with prolonged diabetes, who suffer from physiological, emotional, and social problems of pigmentation disorders.

2. Materials and methods

2.1 Animal model of the DM

Seven healthy male Sprague-Dawley rats, 5-8 weeks, 200-270 g obtained from the National Laboratory Animal Center, Mahidol University, Thailand, were used in this study. All animals were treated according to the Mahidol University Councils for Care and Use of Laboratory Animal. Rats were divided into two groups. The first group served as control ($n = 3$), that was dosed with vehicle only. The second group was diabetic group ($n = 4$), that received an intraperitoneal injection of STZ at a dose of 60 mg/kg body weight dissolved in citrate buffer. Blood glucose levels in serum samples were measured at 72 hours after induction to confirm diabetic state (>300 mg/dl). The experimental period was 24 weeks. At the end of the experimental period, the animals were sacrificed and skins at the soles were removed and prepared for the histological examination.

2.2 Histological study

In the light microscopic study, the specimens were fixed in Bouin's solution. Embedded paraffin tissue blocks were made after dehydration in ethanol, cut in 10 μm thick and stained with hematoxylin and eosin (H&E). The skin sections were examined and photographed under a light microscope (AxioStar plus, Jena, Germany).

2.3 Quantitative analysis of melanocyte number

Melanocytes in the whole area of the sole including toruli digitales, tori metatarsales, and tori talsales were counted. Although there were a few melanocytes in the skin of the soles, the changes in their number were mostly obvious in the area of the very low melanocyte. Thus, this study focused on the skin of the soles. Fifty slides per experimental group were examined microscopically (high-power field, X600 magnification, equivalent to $250 \times 350 \mu\text{m}^2$) to count number or density of epidermal melanocytes. To avoid counting the same cells twice, melanocytes were counted on one in every ten section. Then, the numbers of melanocytes in each area were calculated for average and standard deviation (SD).

2.4 Statistical analysis

The data were analyzed by unpaired t-test using SPSS 19.0 software. The p -value ≤ 0.001 was set as statistically significance. Data were expressed as the mean \pm SD.

3. Results

All rats in diabetic group developed diabetes rapidly after injection of STZ as evidenced by hyperglycemia, polydipsia, polyphagia, and diuresis. In the histological analysis of skin on the sole in non-diabetic control rats, normal structures of the epidermis and dermis layers of skin were revealed (Fig. 1A). The epidermis was composed of five layers. The stratum basale was single low columnar cells with basal oval nuclei, closest to the dermis. Next to this layer was the stratum spinosum, that consisted of several layers of polygonal cells with central round nuclei. The stratum granulosum was easily identified by basophilic keratohyalin granules inside the cells. A pale pink layer of stratum lucidum was found between the stratum granulosum and the thickness of the stratum corneum. Dermis consisted of densely packed and variably oriented connective tissue. The junction between epidermis and dermis was clearly demarcated. Melanocytes were observed in the layer of stratum basale. They were characterized by clear space around the nucleus. The nucleus was smaller and more deeply basophilic than that of a basal keratinocyte (Figs. 1A, 3A). At 24 weeks after the induction of diabetes, the epidermis of the STZ-induced diabetic rats was thinner than that of the control. The density of melanocytes of all areas in diabetic soles significantly increased, when compared to the control in each area (Fig. 2).

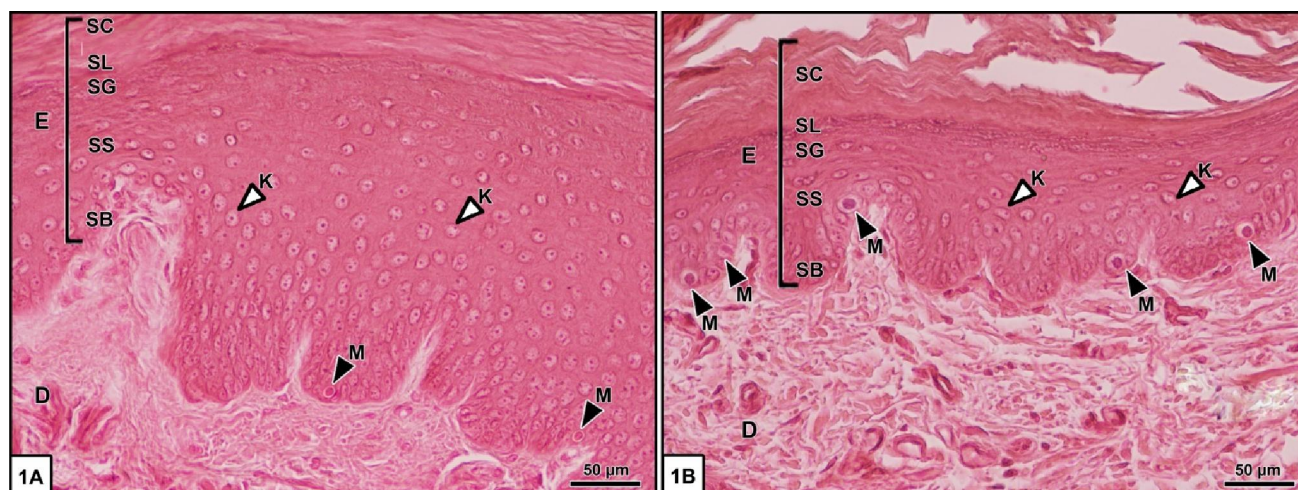


Fig. 1. Histological observation of skin in the control (1A) and diabetic (1B) rats. In the skin of the diabetic rat, a decrease in epidermal (E) thickness and an increase in the number of melanocyte cells (M), which were located in the basal layer of the epidermis (stratum basale: SB), were shown. Stratum corneum (SC); stratum lucidum (SL); stratum granulosum (SG); stratum spinosum (SS); dermis (D); keratinocyte (K).

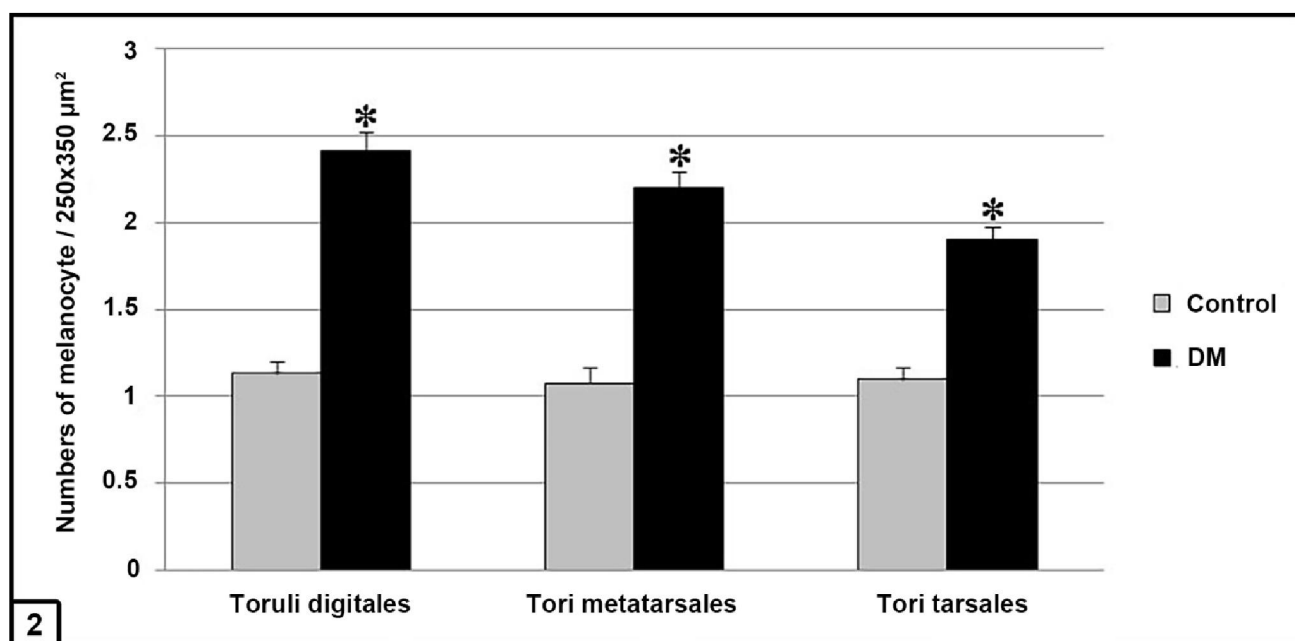


Fig. 2. Comparison in melanocyte numbers/250 x 350 µm² in three areas on the sole; toruli digitales, tori metatarsals, and tori tarsales. In the STZ-induced diabetic rats, the increased numbers of melanocytes in all areas on the soles were compared to the control rats. * p-value ≤ 0.001 vs. the control group

In the high magnification images, hypertrophy of melanocyte was identified by a wide nucleus and large cytoplasmic area. The darkened region was presented inside the nucleus of melanocyte during diabetic condition. Their cytoplasmic area became pale eosinophil and turbid with red-fine granules, unlike translucent cytoplasm in control melanocytes (Fig. 3B). This result was implied that diabetes enhanced melanogenesis in the melanocyte. Furthermore, an increased melanin in the basal cell layer of epidermis was observed as evidenced by dark-red diffuse cytoplasmic staining, compared to these in the control (Figs. 1B, 3B)

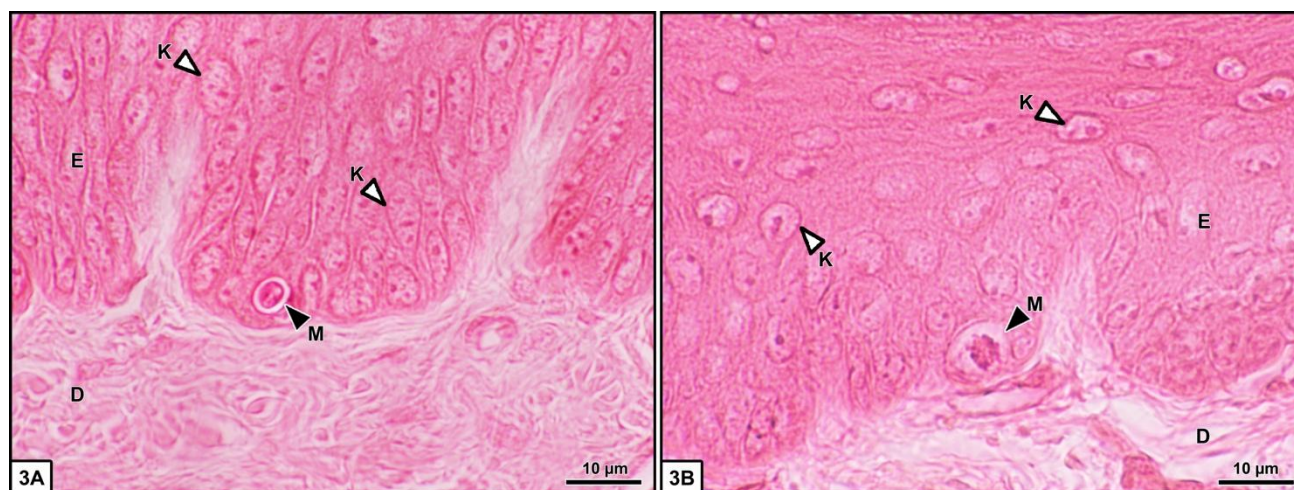


Fig. 3. High magnification of the skin at stratum basale of the control (3A) and diabetic (3B) rats. Enlarged melanocyte (M) with acidophilic granules in a large volume of cytoplasm was found in the diabetic state. Epidermis (E); dermis (D); keratinocyte (K).

4. Discussion

In the hyperglycemic condition, melanocytes in stratum basale increased in number, which associates with elevated reactive oxygen species (ROS) via polyol and advanced glycation end product pathways [7]. The ROS directly affects Langerhans cells in the epidermis, which release pro-inflammatory cytokines such as interleukin (IL)-6 and IL-11 [8]. Next, these pro-inflammatory cytokines bind to their receptors on the cell membrane of melanocyte and then recruit the Src family of protein tyrosine kinase, leading to nuclear transcription of activated Yes-associated protein (YAP)/transcriptional co-activator with a PDZ-binding motif (TAZ). Then, the complex can bind to and activate TEA domain family member (TEAD) transcription factors and form the complex of YAP/TAZ-TEAD, which enhances the cyclin D1 (CCND1) and forkhead box protein M1 (FOXO1) genes. Firstly, the CCND1 gene is the gene encoding cyclin D1, which activates cyclin-dependent kinase 4 to in turn phosphorylate the retinoblastoma protein, resulting in activated E2F transcription factor. The activation of E2F causes G_0 to G_1 phase of cell cycle. Secondly, the FOXO1 gene increases to progress G_1 to S phases and subsequently transit from G_2 phase to mitosis [9]. Ultimately, the proliferation of melanocytes leads to numerous numbers of cells in diabetes.

According to increased numbers of melanocytes, they can lead to increased melanogenesis under the hyperglycemic condition as a clear area of cytoplasm in the melanocytes. The elevated ROS, induced by hyperglycemia, in keratinocyte activates both nuclear factor kappa B (NF- κ B) /activator protein 1 and p53 via sirtuin 1 downstream, which enhance transcriptional regulation of proopiomelanocortin (POMC) in keratinocyte. The upregulation of POMC gene stimulates and releases alpha-melanocyte stimulating hormone (α -MSH). Next, the elevated α -MSH binds to melanocortin 1 receptor in the extracellular membranes of melanocytes and then activates adenylate cyclase to convert ATP to cyclic adenosine monophosphate (cAMP), which stimulates protein kinase A (PKA). Subsequently, the PKA phosphorylates the cAMP-responsive binding element and then activates the microphthalmia-associated transcription factor (MITF). Next, the MITF induces the increased expressions of pigment enzymes including tyrosinase and tyrosinase-related protein 1 (TRP1). The tyrosinase, as the main pigment enzyme, converts L-tyrosine to L-DOPA and then to DOPA quinone, which is changed to dihydroxyindole by TRP1, leading to eumelanin synthesis. Next, melanosomes containing mature eumelanin transport along dendrites of melanocytes to neighboring keratinocytes [10], [11]. As a result, there were small red granules scattered in cytoplasm of melanocyte, which represented an increase in melanin production during diabetes.

Due to elevated transportation of melanosomes to adjacent keratinocytes, increased dendritic formation of melanocyte occurs during hyperglycemia. In the keratinocytes, hyperglycemia induces increased inflammatory cytokines including IL-1 and IL-6. These cytokines activate NF- κ B to express cyclooxygenase-2, causing to production of prostaglandin H₂ (PGH₂). After that, the PGH₂ is converted to PGE₂ by PGE synthase. Next, the PGE₂ releases from keratinocyte to intercellular space and then binds to prostaglandin E₃ receptor on the cell membrane of melanocyte [12], [13]. Next, activation of protein kinase C zeta occurs to subsequently stimulate cell division control protein 42 homolog, which activates the neural Wiskott-Aldrich syndrome protein (N-WASP). The WCA domain of N-WASP protein enhances actin-related protein 2/3 (Arp2/3), as the key protein complex for regulation of actin cytoskeleton. Then, activated Arp2/3 binds to linear actin filaments and in turn stimulates actin polymerization [14], [15]. As a result, the elongation of dendritic melanocyte increases during hyperglycemia. Next, the melanosomes in dendritic melanocyte release into extracellular space via increased exocytosis. In the increased exocytosis, the ROS, as mentioned above, induces lipid peroxidation, that causes increased permeability of cell membrane in cytoplasmic organelles; including rough endoplasmic reticulum, Golgi complex, and mitochondria, resulting in release of increased Ca²⁺ from intracellular stores in these cytoplasmic organelles to the cytoplasm of melanocyte [16]. After that, the high intracellular Ca²⁺ mediates the interactions between vesicle-associated membrane protein 2 on the melanosomal membrane and synaptosomal-associated protein 23 on the cell membrane of melanocyte, that form as an exocytotic core complex. As a result, this complex leads to membrane fusion and creating a pore for the release of melanosomes, which are phagocytosed by keratinocyte [17], [18]. In the phagocytosis of keratinocyte, the protease-activated receptor 2 (PAR-2) is activated by serine proteases, which cleave the extracellular amino-terminal domain of PAR-2. Finally, the activated PAR-2 enhances the phagocytosis in keratinocyte, leading to increased uptake of melanosomes into the cell [18], [19]. Finally, a large amount of melanin stored within keratinocyte, that caused the hyperpigmentation in the skin of diabetic group.

5. Conclusion

In summary, the histopathological changes in epidermal melanocytes of the long-term diabetic rats induced by STZ were demonstrated in this study, that raise the awareness of hyperpigmentation in diabetic patients.

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