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Basic research

Changes in epidermal thickness and cutaneous innervation during maturation in long-term diabetes

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Abstract *Aim:* Peripheral nerve fiber depletion in patients with chronic diabetes mellitus (DM) was linked to neuropathic symptoms, development of pain, foot ulcerations and lower extremity amputation. The aim of this study was to analyze cutaneous changes, including paw epidermal thickness and intraepidermal nerve fiber (IENF) density in long-term diabetes, in rats 6 months and 12 months after induction of diabetes.
Materials and methods: Epidermal thickness and IENF density were studied in Sprague—Dawley diabetic rats 6 months and 12 months after diabetes induction with streptozotocin. Epidermal thickness was evaluated using hematoxylin and eosin staining. Peripheral nerve fibers were stained with polyclonal antiserum against protein gene product 9.5 (PGP 9.5). Successful diabetes induction was validated by measuring plasma glucose and body mass regularly throughout the experiment.
Results: This study showed that long-term diabetes, induced in Sprague—Dawley rats with streptozotocin, is characterized with significant epidermal thinning and reduction of intraepidermal nerve fibers, 6 months and 12 months after induction of diabetes.
Conclusion: Long-term studies of streptozotocin models of diabetes could be used for making normative IENF densities that can be later used as age-dependent normative values for studying new treatment modalities.

Introduction

Common complication of both type 1 and type 2 diabetes mellitus (DM) is neuropathy, with predominant involvement of small fiber, beginning at

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the distal extremities and becoming progressively more proximal with longer duration of diabetes. Common symptoms described by patients suffering from diabetic neuropathy are "burning" and "prickly" feet [1]. Diabetic neuropathy is the most common form of peripheral neuropathy in developed countries. The presence of peripheral neuropathy in diabetic patients increases their risks for developing foot ulceration and subsequent necrosis that necessitates lower limb amputation [2].

Glucose is the major fuel for most mammalian cells, and its utilization is regulated by a wide range of factors. However, abnormally and chronically increased levels of glucose, as seen in diabetes, may lead to the development of chronic complications, such as neuropathy. As much as 56% of patients with idiopathic neuropathy, suffering from burning feet, have impaired glucose tolerance, although their neuropathy is milder than the neuropathy associated with DM. In neuropathic patients with impaired glucose tolerance, small nerve fibers are predominantly affected and this may be the earliest detectable sign of neuropathy in glucose dysmetabolism. Additionally, impaired glucose tolerance is associated with a decrease in intraepidermal nerve fiber density [3].

Hyperglycemia and impaired insulin signaling may be directly involved in the pathogenesis of chronic diabetic complications related to skin, by impairing glucose utilization in skin keratinocytes, and negatively affecting skin proliferation and differentiation [4]. For the study of peripheral diabetic neuropathy, the streptozotocin (STZ)-induced diabetic rat model is widely used. It has been reported that the early decrease in mechanical and thermal thresholds, combined with distinctive nerve degeneration in diabetic rats, corresponds with the frequently observed painful symptoms and peripheral nerve fiber depletion in patients with chronic DM [5]. However, most of the studies involving skin of diabetic rats are of short duration, which cannot provide sufficient information about the disease that is by definition chronic. Therefore, the aim of this study was to analyze cutaneous changes, including paw epidermal thickness and peripheral nerve distribution in long-term diabetes, in rats 6 months and 12 months after induction of diabetes.

Material and methods

Ethics

Experimental procedures and protocols were approved by the Ethical Committee of the University of Split, School of Medicine. The animal care was in accordance with institution guidelines.

Animals

Male Sprague—Dawley rats (6 weeks old) weighing 160—200 g were included in the study and raised under controlled conditions (temperature: 22 ± 1 °C; light schedule: 12 h of light and 12 h of dark) at University of Split Animal Facility. Rats were housed in pairs in plastic cages with sawdust and corn bedding in the ratio 3:1. The duration of experiments was 6 and 12 months after induction of diabetes. Rats were divided into four groups: 6-month DM ($N = 5$), 6-month control ($N = 5$), 12-month DM ($N = 5$) and 12-month control group ($N = 5$).

Diabetes induction

The DM1 was induced as described previously [6,7]. After overnight fasting rats were injected intraperitoneally with 55 mg/kg of streptozotocin (STZ) freshly dissolved in citrate buffer (pH = 4.5). Control rats were injected intraperitoneally with pure citrate buffer solution. All rats were fed *ad libitum* with standard laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy).

Body mass and plasma glucose were measured regularly. Single touch glucometer (One Touch VITa, Life Scan, High Wycombe, UK) was used to determine plasma glucose collected from the tail vein of rats. Body mass was measured with a technical laboratory scale.

Tissue processing

At the end of the experiment rats were anesthetized (Isoflurane; Forane, Abbott Laboratories Ltd, Queenborough, UK) and sacrificed. Skin samples from plantar surface of the both hind paws were removed and postfixed in the Zamboni's fixative [2% paraformaldehyde and 15% picric acid in 0.01 M phosphate buffered saline (PBS) at pH 7.4] for 2—5 h at 4 °C and then transferred to 0.01 M PBS. Tissues were cryoprotected in 30% sucrose overnight and skin was embedded in Optimal Cutting Temperature (OCT) freezing medium (Tissue Tek, Tokyo, Japan). The skin was sectioned perpendicular to the skin surface. For each sample of the skin, sequential 8 μm-thick sections were collected and every fifth section was stained with hematoxylin and eosin. Skin sections were examined under a microscope (BX61, Olympus, Tokyo, Japan) and microphotographs were