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Brief Communication

The streptozotocin-high fat diet induced diabetic mouse model exhibits severe skin damage and alterations in local lipid mediators

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ABSTRACT

Background: Type 2 diabetes (T2D) can go undiagnosed for years, leading to a stage where produces complications such as delayed skin wound healing. Animal models have been developed in the last decades to study the pathological progression in this disease. Streptozotocin (STZ), that has a selective pharmacological toxicity toward pancreatic β cells, in addition to high fat diet has been widely used to induce diabetes however no evidence has shown its effects on the skin integrity.
Methods: Eighteen C57BL/6J male mice, were divided in 3 groups; the first was fed with chow diet and the second was kept on a high fat diet and the third injected with STZ intraperitoneal for 5 days consecutively before starting the diet protocol with high fat. Mice were maintained 5 weeks in total.
Results: We show that animals treated with STZ-high fat diet exhibit skin injuries without significant alterations on basal insulin and triglycerides, compared to the control. The skin from these animals presents higher levels of oxidative stress, lower levels of adhesion proteins and alterations in lipid mediators, effects that are not produced by the high fat diet itself.
Conclusion: Our results suggest that this *in vivo* model represents a relevant approach for studying skin damage induced by diabetes.

Diabetes affects over 340 million people worldwide. It is characterized by sustained hyperglycemia and can go undiagnosed for years [1]. Its chronicity leads to delayed wound healing due to skin complications increasing the risk of amputation; the foot ulcer is a leading cause of hospital admissions in the developed world and is a major morbidity associated with diabetes, often leading to pain, suffering, and a poor quality of life [2].

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The oxidative stress and lipids content has been considered as the mechanism underlying the abrogated skin integrity [3]. However, there is no report showing the local alterations in the skin of diabetic animal models.

Several non-genetic mice models have been established. The most widely used are based on high fat (HF) diet with or without a chemical ablation of the pancreatic β cells using streptozotocin (STZ) [4]. Nevertheless, no characterization of the skin alterations on the models have been reported [5].

We used two diabetic-induced mice models to study the skin condition in addition to the oxidative stress and local levels of lipid mediators that might contribute to the skin disruption.

Materials and methods

Eighteen C57BL/6J male mice of 15–21 weeks of age (average weight 23 ± 0.5 g), were divided in 3 groups. The first group was fed with a low fat chow D12450B as a control, the second was kept on a HF diet D12492. The content in fat percentage of the diets are 10% kcal% fat and 60% kcal% fat, respectively. The third was injected with STZ (40 mg/kg) (Cayman Chemicals, catalog no. 18883664) intraperitoneal (IP) for 5 days consecutively before starting the diet protocol with HF. The compound was dissolved in 0.05 M citrate buffer, pH 4. In parallel the control and HF groups were injected with the vehicle during the pretreatment. Mice were maintained 5 weeks in total, since STZ or vehicle treatment.

Parameters were controlled weekly after 6 h of fasting. The study protocol was approved by the Ethics Committee for Animal Experimentation at Juntendo University, Japan.

Triglycerides and blood glucose levels were measured using CardioChek® PA (catalog no. 0197) and the compatible PTS Panel® test strips. Insulin was measured using the Ultrasensitive Mouse ELISA kit (Mercodia, article no. 10-1249-01).

Ex vivo trans-epithelial electrical resistance (TEER) measurement was performed in skin samples with an 8 mm diameter and 1 mm thickness obtained from the back of the animal using disposable biopsy punches (Kai Medical, catalog no. BP-80F). Samples were placed facing up onto a 12 mm polycarbonate filter (0.4 μm pore) (Millicell Merck Millipore, catalog no. PIH01250) suspended inside a cell culture well containing 1X PBS (500 μL). The TEER was measured immediately using the Millicell® ERS-2 Voltohmmeter (Millipore, catalog no. MERS00002).

Oxidative stress was measured using TBARS assay Kit (Cayman Chemicals, catalog no. 10009055).

Histological analysis was performed in skin samples fixed with 10% formalin, paraffin-embedded, and stained with hematoxylin eoisin (HE) and masson's trichrome (MT). Images were taken using Keyence BZ-9000 microscope.

Western blot used anti-ZO-1 pAb (Thermo Fisher Scientific-Invitrogen catalog no. 61–7300), anti-E-Cadherin mAb (Cell Signaling Technology catalog no. 3195), anti-Occludin pAb (Thermo Fisher Scientific-Invitrogen catalog no. 71–1500), anti-Claudin-4 pAb (Thermo Fisher Scientific-Invitrogen catalog no. 36–4800) or anti-β-actin mAb (Sigma–Aldrich catalog no. A2228) as primary antibodies all at 1/500 dilution. Chemiluminescence was detected by the Chemidoc-IT Imaging System (UVP, LLC) and immunoreactive bands were analyzed with ImageJ software (National Institutes of Health).

For the determination of lipid content, it was performed an extraction from skin with methanol containing deuterium-labeled internal standards. Each sample was diluted with water to yield a final methanol concentration of 20%, and then loaded on Oasis HLB cartridges (Waters). The column was subsequently washed with petroleum ether and water containing 0.1% formic acid. The samples were eluted with 200 μl of methanol containing 0.1% formic acid. Eicosanoids in each sample were quantified by LC-MS/MS using a Shimadzu liquid chromatography system and tandem-connected a TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with an electrospray ionization system (Thermo Fisher Scientific). Each sample was injected into the trap column, an Opti-Guard Mini C18 and concentrated sample was analyzed with an analytical column, a Capcell Pak C18 MGS3 (Shiseido, Tokyo, Japan). Separation of lipids was achieved by a step gradient with 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The LC column eluent was introduced directly into a TSQ Quantum Ultra. All compounds were analyzed in a negative ion polarity mode.

Results are presented as mean ± SEM. Statistical analyses were performed using ANOVA. Post hoc tests were also performed. Statistical significance was set at $p < 0.05$. All statistics were calculated using Prism GraphPad (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Mice fed with HF diet showed a higher weight gain ($p < 0.001$) from the second week onwards. The animals pretreated with STZ (Type 2 diabetes (T2D) mice) exhibited an early weight loss followed to a normalization towards values similar to control [Fig. 1A]. The HF diet induced higher glucose basal levels, effect that was enhanced by the STZ from the third week [Fig. 1B]. Moreover, the STZ treatment did not altered the triglycerides and insulin basal levels ($p = 0.1$), parameters affected by the HF diet [Fig. 1C and D; respectively].

The skin from diabetic mice exhibited lower TEER ($p < 0.05$) than control, effect that is potentiated by STZ ($^{**}p < 0.01$) [Fig. 1E, grey bar and black bar; respectively]. However, the levels of TBARS were significantly higher only in the skin from the HF + STZ animals ($p < 0.05$) [Fig. 1F, black bar]. The macroscopic view showed severe spontaneous skin lesions on animals pretreated with STZ and histological sections suggested an atrophic epidermis without signs of inflammatory cell infiltration or vasculitis with a reduction of hair follicles [Fig. 1G]. Consistent with the observations, the skin from mice under HF and STZ showed lower adhesion protein levels of ZO-1, E-Cadherin, Occludin and Claudin-4 [Fig. 1H].

In addition, the skin from STZ-HF mice showed lower levels of lipid mediators docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), 12-hydroxyheptadecatrenoic acid

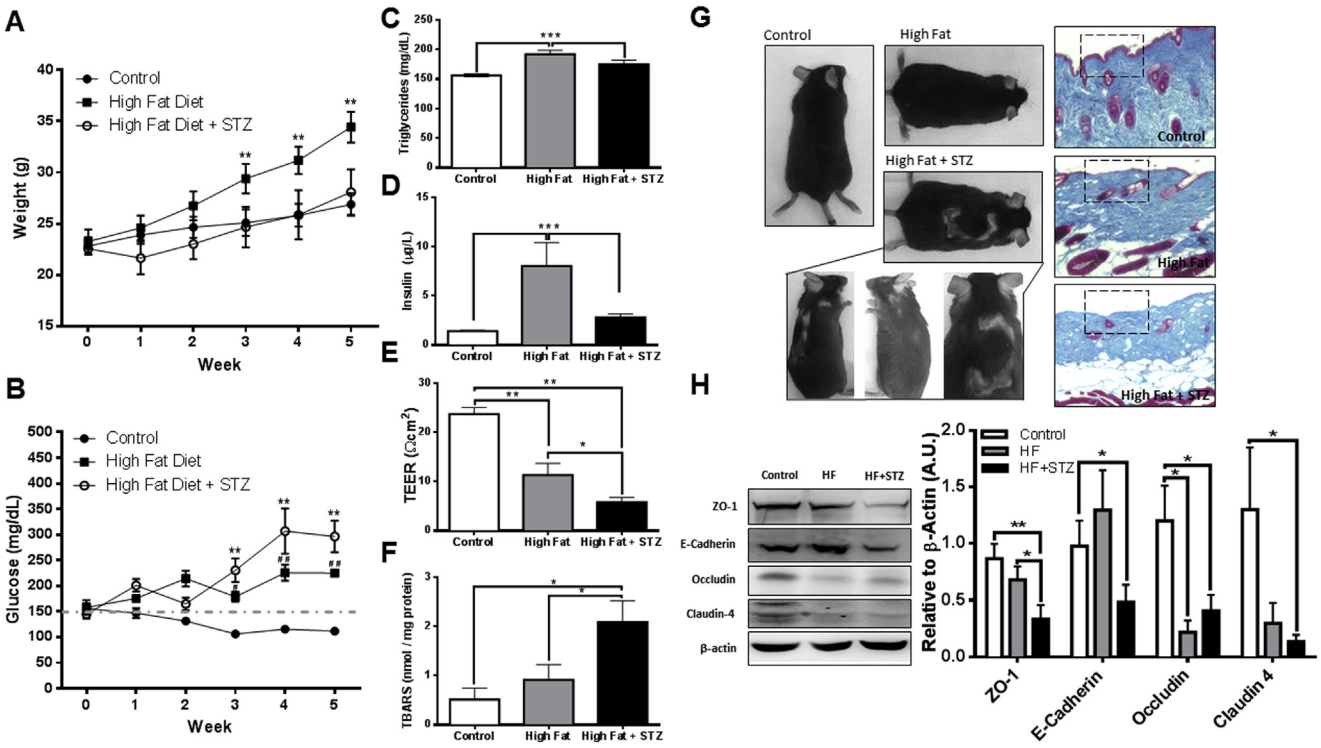

Fig. 1 Diabetes induced by STZ-HF diet generates several skin alterations on mice. (A) Weekly weight measurements in non-sedated animals for 5 weeks, (B) weekly basal glucose levels in non-sedated animals after 6 h starving for 5 weeks, (C) basal blood triglycerides and (D) blood insulin levels in non-sedated animals after 6 h starving at 5th week of treatment. (E) *Ex vivo* TEER and (F) TBARS in skin from animals after 5 weeks of treatment. (G) Representative images of the animals after the protocol and microphotographs of skin MT stains at 20x magnification. (H) Representative western blots and densitometric quantification of adhesion proteins relative to total β-actin. Data expressed as mean ± SEM, n = 6 per group $^{*}p < 0.05$ ** or $\#\#p < 0.01$, $^{***}p < 0.001$, One or 2-Way ANOVA, Tukey's post hoc test.

(HHT), leukotriene C4 (LTC_4), protectin D1 (PD1), thromboxane B2 (TXB_2), 6-keto-$PGF_1\alpha$, 5-oxoeicosatetraenoic acid (KETE), 12-KETE and 8,9 epoxyeicosatrienoic acids (EET) and higher amount of PGD_2, PGE_2, 9-hydroxyoctadecadienoic acid (HODE), 13-HODE and 7-hydroxydocosahexaenoic acid (HDoHE), compared to HF and control skin [Table 1].

Discussion

Skin changes result from an impaired skin barrier that is caused by either diabetes-induced skin metabolism abnormalities or diabetic complications, such as foot ulcer [6].

Previous results using the HF diet model have described mild skin alterations associated with reduction on TEER, epidermis thickness and increased inflammatory markers [7]. Using a STZ type 1 diabetic rat model it was described in skin an increase on inflammatory cells infiltration and oxidative stress [8]. Moreover, increased TBARS have been reported in the skin of T2D patients [9] and in STZ diabetic rats [10,11].

Furthermore, it was recently described in a type 1 diabetic mice model a disruption on the skin barrier accompanied by disorganization on the tight junctions characterized by a change on ZO-1 protein localization [12]. In agreement with the previously mentioned observations, we show a dramatic reduction in the levels of ZO-1, E-Cadherin, Occludin and Claudin-4 in the STZ-HF T2D model, a condition that may be associated with the reduction on TEER. It is important to highlight that our model would represent T2D in a sever and uncontrolled glycaemia state, as the main trigger of the conditions presented in this study. The severity of the phenotype could be attributed to the synergic effect to the glucose level, that has the high fat diet after the β cells ablation.

Interestingly, the reduction on skin DHA and EPA has been associated with alteration in the epidermal barrier [13], the decrease in PD1, PGF_1, KETE and EET with inflammation [3,14,15], TXB_2 indicating lesions [15] and 12-HHT through BLT2 receptor, has been described as a pro regenerative lipid mediator [16]. On the other hand, the local increase of PGD_2, PGE_2, and HODEs has been associated with hair loss, inflammation and oxidative stress [17–19].

We identify as weakness of our study the lack of *in vivo* wound healing studies, however the data shown in this study describes a highly used animal model that would serve as evidence for future research in the dermatoendocrinology field.

Table 1 Lipidomics analysis of the skin.

(pg/mg tissue)	Control	High Fat	High Fat + STZ
AA	10,380 ± 210	8730 ± 150	9650 ± 140
DHA	22,980 ± 660	19,370 ± 360	10,130 ± 180*
EPA	1410 ± 30	1380 ± 20	660 ± 10*
12-HHT	15.22 ± 6.1	6.73 ± 2.3	4.81 ± 1.2*
LTB_4	0.07 ± 0.01	0.05 ± 0.02	0.06 ± 0.02
LTC_4	2.11 ± 0.9	0.38 ± 0.1**	0.41 ± 0.1*
PD1	19.09 ± 3.2	13.29 ± 3.1	3.67 ± 1.1**
TXB_2	2.85 ± 1.2	1.03 ± 0.2*	0.88 ± 0.1*
PGD_2	0.05 ± 0.01	0.03 ± 0.01	0.24 ± 0.06**
PGE_2	51.68 ± 11.5	82.62 ± 26.2	104.0 ± 13.2*
6-keto-$PGF_1\alpha$	5.51 ± 1	3.42 ± 1	2.65 ± 0.4*
$PGF_2\alpha$	2.42 ± 0.5	3.87 ± 2.3	4.59 ± 1.3
5-HEPE	0.13 ± 0.07	0.11 ± 0.07	0.12 ± 0.1
8-HEPE	0.47 ± 0.1	0.53 ± 0.2	0.3 ± 0.1
12-HEPE	0.92 ± 0.2	1 ± 0.3	1.43 ± 0.3
15-HEPE	0.76 ± 0.3	0.6 ± 0.1	0.76 ± 0.2
18-HEPE	0.16 ± 0.02	0.19 ± 0.06	0.4 ± 0.2
9-HODE	340 ± 100	370 ± 100	1040 ± 200**
13-HODE	60 ± 0.5	130 ± 1	410 ± 0.7**
5-HEPE	1.4 ± 0.3	1.3 ± 0.2	1.5 ± 0.5
8-HEPE	10 ± 0.3	10 ± 0.4	4.2 ± 1
11-HEPE	1 ± 0.4	2 ± 0.9	2 ± 0.6
12-HEPE	60 ± 10	40 ± 20	80 ± 20
15-HEPE	20 ± 7	20 ± 8	20 ± 9
7-HDoHE	1.81 ± 0.12	3.74 ± 0.85*	3.34 ± 0.5*
14-HDoHE	0.02 ± 0.01	0.01 ± 0.002	0.02 ± 0.005
17-HDoHE	0.05 ± 0.02	0.04 ± 0.01	0.03 ± 0.01
5-KETE	1.57 ± 0.42	0.48 ± 0.09**	0.25 ± 0.15**
12-KETE	0.54 ± 0.25	0.23 ± 0.12	0.21 ± 0.06*
5, 6 EET	0.09 ± 0.04	0.04 ± 0.02	0.06 ± 0.03
8, 9 EET	0.33 ± 0.08	0.11 ± 0.03	0.03 ± 0.01*

Lipid mediator levels in skin after 5 weeks of controlled diet with or without STZ (average ± SEM), n = 6 per group, *$p < 0.05$, **$p < 0.01$ non parametric one-way ANOVA with Tukey's post hoc test. Abbreviations: AA: Arachidonic acid; LTB4; Leukotriene B4; HEPE: Hydroxyeicosatetraenoic acid;

Conclusion

Animals treated with STZ-high fat diet exhibit skin injuries without significant metabolic alterations, compared to the control. The skin from these animals presents higher levels of oxidative stress, lower levels of adhesion proteins and alterations in lipid mediators, effects that are not produced by the high fat diet itself.

Based on the findings presented in this study and our previous report [7] we suggest that the severe hyperglycemia and no the insulin, in the STZ-high fat diet model, leads to an outburst of pro-inflammatory cytokines and oxidative stress on the keratinocyte, that activates LOX/COX dependent pathways causing changes on local lipid mediators associated with loose on the epidermal integrity that might cause the spontaneous skin injuries observed [20].

The relevance of understanding and characterizing the diverse diabetic animal models represent an important contribution to emulate the pathological condition. The studies on the field are relevant to counteract this important public health issue; and for this reason, the use of proper animal models and more work on this topic is required.

Conflicts of interest

There are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bj.2018.08.005.

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