Animal Science Papers and Reports vol. 42 (2024) no. 2, 203-216
DOI: 10.2478/aspr-2023-0031
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A mouse model of uterine exposure to long-term hyperglycemia and a high-fat diet*

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(Accepted May 16, 2024)

A growing body of literature has shown that type 1 diabetes (T1D) and high-fat diet (HFD) affect female reproductive function and may be involved in a chronic inflammatory state. Our previous studies indicated that T1D as well as HFD may evoke perturbations in the receptor for advanced glycation end products (RAGE) signaling pathway. The aim of the study was to determine the amount of RAGE protein and its proinflammatory ligands in uterine tissues harvested from T1D and HFD/pre-diabetic mice (n = 5 per group). We sought the impact of T1D and HFD on the activity of the RAGE signaling pathway in uterine tissues during the estrous cycle. The abundance of RAGE and its ligands were estimated using immunohistochemical staining. However, we also performed nerve conduction velocity studies to confirm diabetic neuropathy. The highest amount of RAGE and its ligands were observed in uterine tissues of T1D mice. Moreover, myometrial activity of the RAGE signaling pathway was increased in HFD in comparison to the control group (P≤0.05). We observed a strong relationship between RAGE, Nε-(carboxymethyl)lysine (CML) and tumor

* The study was supported by the National Science Centre, Poland (Grant UMO-2018/30/E/NZ5/00458)
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necrosis factor alpha (TNFα) proteins in mice myometrium. These data suggest that T1D and HFD could modulate the activity of RAGE and thus RAGE signaling pathway in uterine tissues during estrous cycle. Long-term diabetes and HFD may induce malfunctions in the uterine milieu. In the future RAGE protein may serve as a molecular marker in the diagnosis of malfunctions in pre- and diabetic uterus milieu.

KEY WORDS: RAGE / diabetes / uterus / mouse / high-fat diet

Diabetes and obesity are vast topics where knowledge is being often updated. In depth coverage of these topics may be voluminous. Nevertheless, obesity and diabetes are associated with many pathological conditions, such as: neuropathy, cardiovascular disease or cancer [Bierhaous *et al.* 2005, Jaroslawska *et al.* 2021, Juranek *et al.* 2022, 2013, Zglejc-Waszak *et al.* 2023a, 2023b, 2021]. Many studies have focused on the role of the receptor for advanced glycation end products (RAGE) signaling pathway in diabetic and obesity complications [Bierhaous *et al.* 2005, Jaroslawska *et al.* 2021, Juranek *et al.* 2022, 2013, Zglejc-Waszak *et al.* 2023a, 2023b, 2021]. Our previous studies indicated that type 1 diabetes (T1D) and high-fat diet (HFD) may trigger perturbations in RAGE signaling pathway in sciatic nerve and induce diabetic length-dependent neuropathy (DLDN) [Jaroslawska *et al.* 2021, Juranek *et al.* 2022, 2013, Zglejc-Waszak *et al.* 2023a, 2023b, 2021].

Nevertheless, a growing body of literature has shown that diabetes mellitus (DM) and HFD also affect female reproductive function [Chakraborty *et al.* 2016, Garris 1985, Jing *et al.* 2016, Wilson *et al.* 2022]. Moreover, DM and HFD are associated with a chronic low-grade inflammatory state that is involved in alternations in the mouse endometrial transcriptome, hormone levels, as well as mice estrous cycle [Chakraborty *et al.* 2016, Garris 1985, Jing *et al.* 2016, Wilson *et al.* 2022].

Transcriptome analysis of cells harvested from the obese endometrial epithelia revealed that S100 Calcium Binding Protein A8 (S100A8) and S100 Calcium Binding Protein A9 (S100A9) were upregulated in these cells [Wilson *et al.* 2022]. Moreover, that study showed that S100A8 and S100A9 were increased in endometrium harvested from women with recurrent early pregnancy loss as well as in bovine uterine during bacterial infection [Ledgard *et al.* 2015, Nair *et al.* 2013]. These two proteins create a molecular complex known as calprotectin [Ledgard *et al.* 2015, Nair *et al.* 2013, Wilson *et al.* 2022]. Calprotectin is present in monocytes and neutrophils and thus plays a role in immune and immunopathological reactions. The elevated level of calprotectin in serum or body fluids may indicate inflammation [Ledgard *et al.* 2015, Nair *et al.* 2013, Wilson *et al.* 2022]. It is commonly known that long-term inflammation may induce malfunctions in uterine milieu and thus trigger infertility [Chakraborty *et al.* 2016, Fazeli *et al.* 2008, Wilson *et al.* 2022].

Further studies revealed that T1D effects the proteome of mouse oocytes and elevates expression of the calprotectin complex [Jing *et al.* 2016]. However, S100 Calcium Binding Protein A6 (S100A6) was downregulated in oocytes harvested from T1D mice [Jing *et al.* 2016]. Moreover, S100A6 is an extracellular ligand of RAGE and engages with its receptor in inflammatory responses under hyperglycemia milieu

[Jaroslawska *et al.* 2021, Juranek *et al.* 2022, 2013, Zglejc-Waszak *et al.* 2023a, 2023b, 2021]. Thus, we may suppose that T1D and HFD have a negative effect on the female reproductive tract by disrupting inflammation processes [Chakraborty *et al.* 2016, Garris 1985, Jing *et al.* 2016, Wilson *et al.* 2022]. However, to date there are no studies about active advanced glycation end-products (AGEs) molecular pathways in uterine tissues harvested from T1D as well as HFD mice. We hypothesize that hyperglycemic and high-fat milieu may activate RAGE molecular pathways in the mouse uterus.

In this study we sought to characterize the effects of HFD and T1D on the RAGE signaling pathway in uterine tissues harvested from female mice during estrous cycle. We examined the presence of RAGE and its proinflammatory ligands in diabetic as well as in HFD uterine tissues. Finally, we aimed to determine the level of tumor necrosis factor alpha (TNFα) in mouse uterine tissues as proinflammatory cytokine and part of the regulatory processes in the female reproductive tract. The results described herein elevate our knowledge about the impact of T1D and HFD on uterine tissues during estrous cycle.

Material and methods

Animals

The experiment was approved by the Local Ethics Committee of Experiments on Animals in Olsztyn (Poland; decision no. 57/2019). Eight weeks old C57BL/6 females were randomly divided into three experimental groups: 1) control, 2) T1D and 3) HFD (n = 5 per group) as described [Jaroslawska *et al.* 2021].

Animals with a blood glucose level ≥13 mmol/L (260 mg/dL) were considered diabetic. Mice were sacrificed at 24 weeks post the last STZ injection (six months of rendered diabetes, Fig. 1). Sections of the middle part of uterine horn harvested from cyclic mice. Tissues from female mice were fixed immediately in 4% paraformaldehyde in PBS and then transported to the laboratory on ice.

The mouse estrous cycle

The mouse estrous cycle was identified in vaginal smear images before mice were sacrificed. Briefly, a vaginal swab was harvested from each mouse of groups with a cotton tipped swab (Citotest Labware Manufacturing CO., LTD; China) wetted with PBS. Vaginal swabs were transferred to a dry glass slide. The slide was stained with suitable buffers (HemaVet – Sigmed, Poland) according to the manufacturer's protocol.

Hematoxylin and eosin staining

Parts of uterine wall cross-sections containing both endometrium and myometrium were frozen in -20 °C and then cut in cryostat (Leica, Wetzlar, Germany) into 8-µm-thick slices. Next, the slides were immersed in hematoxylin solution (Sigma-Aldrich, USA) for 1 min. Consequently, the slides were washed under tap water and immersed

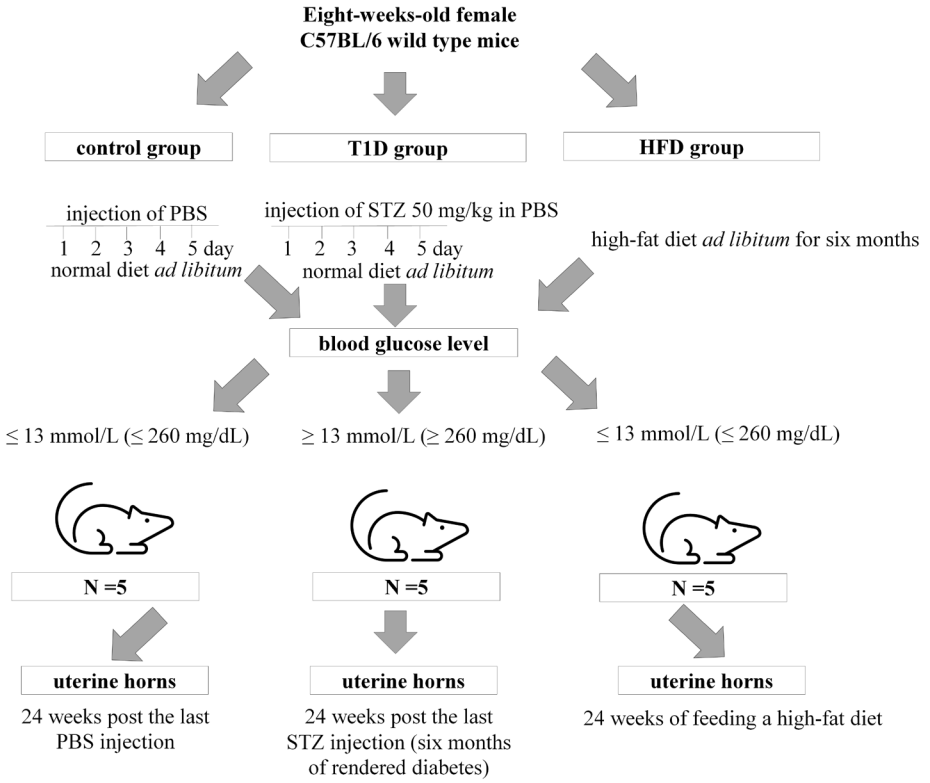


Fig. 1. Study design. In the control group, the vehicle was administered daily for five days. In T1D group, streptozotocin (STZ) was administered daily to female mice for five days. In the HFD group females fed a high-fat diet (HFD) *ad libitum* for six months. Tissues were harvested after 6 months of the experiment.

in eosin solution (Sigma-Aldrich, USA) for 2 min. Finally, the slides were washed in PBS for 5 min and distilled water and then dehydrated.

Nerve conduction velocity (NCV)

In vivo measurement of sciatic nerve electrophysiological activities (n = 5) was performed using Nicolet Viking Quest Apparatus (CareFusion, San Diego, CA, USA) as previously described [Jaroslawska *et al.* 2021, Schulz *et al.* 2014, Zglejc-Waszak *et al.* 2023a]. Needle electrodes were cleaned with ethanol between the animals (n = 5). Briefly, for motor NCV (MNCV) studies (n = 5), the sciatic nerve was stimulated twice (proximal as well as distal stimulation) [Jaroslawska *et al.* 2021, Zglejc-Waszak *et al.* 2023a]. For sural NCV (SNCV, n = 5), the sural nerve was stimulated orthodromically using needle electrodes placed in the fourth toe of the foot, with recording *via* needle electrodes in the gastrocnemius muscle [Jaroslawska *et al.* 2021, Zglejc-Waszak *et al.* 2023a]. MNCV as well as SNCV were calculated by dividing the distance between the

stimulating and recording electrodes by this latency [Jaroslawska *et al.* 2021, Schulz *et al.* 2014, Zglejc-Waszak *et al.* 2023a]. The experiment was approved by the Local Ethics Committee of Experiments on Animals in Olsztyn (Poland; decision no. 57/2019).

Determination of RAGE, High Mobility Group Box 1 (HMGB1), S100 calcium-binding protein B (S100B), Nε-(carboxymethyl)lysine (CML), TNFα proteins amount in mice uterine tissues

Presence of RAGE, HMGB1, S100B, CML and TNFα proteins in uterus sections were determined using a two-day procedure for indirect immunohistochemistry (IHC) staining. The reaction time of IHC staining was the same in all groups according to the manufacturer's protocol and as described [Zglejc-Waszak *et al.* 2023a, 2023b]. Briefly, slides were incubated with primary antibodies (Tab. 1) diluted in 0.1% BSA at 4°C overnight. For negative controls (NCs), tissue slices were incubated only with 2.5% normal horse serum. NCs confirmed antibody specificity (Tab. 1). Consequently, sections were washed in PBS and incubated with secondary anti-rabbit antibodies (Vector Laboratories, USA). The sections were incubated with 3,3' diaminobenzidine tetrahydrochloride solution (DAB, Sigma Aldrich, USA) and immersed in distilled water as soon as the brown coloring was visible. Next, slides were immersed in hematoxylin solution (Sigma-Aldrich, USA) for 1 min and washed under tap water. Finally, slides were mounted with a mounting medium (DPX; Sigma-Aldrich, USA) and examined under light microscope. Images were taken under 40× objective with 0.75 numerical aperture (40×/0.75). The amount of proteins were determined with the ImageJ software which automatically converted the positive signal of IHC staining into the corresponding range of gray values. The total percentage of staining area was calculated per ROI.

Table 1. Characteristic of antibodies used in immunohistochemical (IHC) staining

Antibody	Type	Species	Dilution	Manufacturer and catalog no.
RAGE	polyclonal	rabbit	1: 50	Abcam, ab37647
CML	polyclonal	rabbit	1: 200	Abcam, ab27684
HMGB1	polyclonal	rabbit	1: 200	Abcam, ab18256
S100B	monoclonal	rabbit	1: 100	Abcam, ab52642
TNFα	polyclonal	rabbit	1: 100	Abcam, ab9739

Abbreviations: RAGE – receptor for advanced glycation end products, CML – carboxymethyl-lysine, HMGB1 – high-mobility group box 1, S100B – S100 calcium-binding protein B, TNFα – tumor necrosis factor alpha.

Statistical analysis

All datasets were tested for presence of outliers by using the Grubbs' test (value: $\alpha = 0.05$). Next, we performed Shapiro-Wilk test. The Shapiro-Wilk test is a test of

normality. The effect of T1D and HFD on the NCV as well as protein amounts in uterus tissues harvested from mice was analyzed with *one-way ANOVA* followed by the Tukey's HSD post-hoc test or nonparametric equivalent, *Kruskal-Wallis* test (the type of test was selected based on the Shapiro-Wilk test). Correlations (parametric Pearson or non-parametric Spearman) were conducted to investigate the linear dependence between RAGE and another protein. Analyses with *P* values ≤0.05 were considered as statistically significant. All data were presented as the mean±SEM and analyzed using GraphPad Prism 9.1.0. (CA, USA).

Results and discussion

Description of blood glucose level and body weight of female mice

Blood glucose was increased by over two folds at six months post-STZ injections in diabetic group compared to the control group and HFD group (P≤0.0001, P≤0.0001, respectively, Fig. 2A). There was no difference in the weight of females between the control group and the T1D group (P≥0.05, Fig. 2B), however there was an increase in body weight in the HFD group (P≤0.001, Fig. 2B).

The impact of diabetes and HFD on NCV

We found that in the T1D group, NCV was reduced both in motor nerve conduction velocity (MNCV; Fig. 2D) as well as sural nerve conduction velocity (SNCV; Fig. 2C). The SNCV was significantly lower in T1D group as compared to HFD (P≤0.01, Fig. 2C). In T1D and HFD groups no significant alteration of SNCV as well as MNCV were observed compared to the control group (P≥0.05, Fig. 2C-D).

Morphological characteristic of uterus harvested from cyclic mice

We observed three cell types in vaginal smear images, such as: leukocytes, cornified as well as nucleated epithelial cells. Vaginal cytology presenting diestrus stage in all groups (Supplementary Fig. 1A). Microscopy images of hematoxylin and eosin stained sections of the uterus indicated lumen, endometrial cells as well as endometrial epithelium, stromal compartment, uterine glands and myometrium cells (Supplementary Fig. 1D). We did not observe any morphological changes in the bright-field microscopy images of the uterus harvested from the three groups of females (Supplementary Fig. 1D).

Localization and presence of RAGE, HMGB1, S100B, CML, TNFα proteins in the endometrium and myometrium

Representative images presenting the localization of RAGE, HMGB1, S100B, CML and TNFα in the endometrium and myometrium are shown on Fig. 3A-F. Notably, studied proteins were presented in epithelium, stromal compartment, uterine glands cells of the endometrium as well as in myometrial cells especially in T1D females (Fig. 3A-F). IHC staining indicated that both endometrial and myometrial

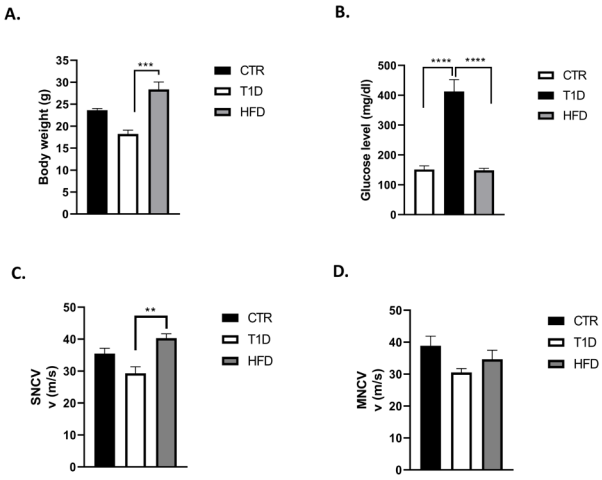
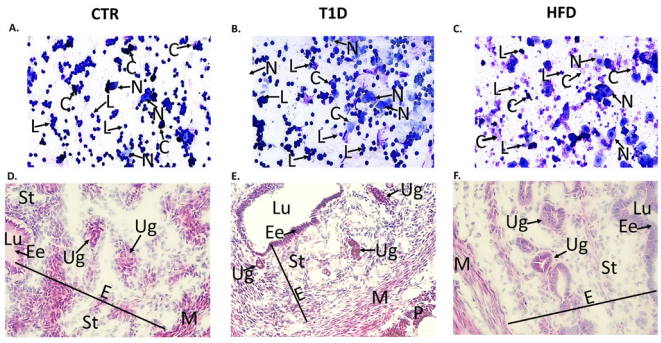


Fig. 2. The impact of T1D and HFD on blood glucose level and body weight as well as NCV of female mice. **A.** The blood glucose level. **B.** The body weight of female mice. **C.** The effect of T1D and HFD on sural nerve conduction velocity (SNCV). **D.** Changes in motor nerve conduction velocity (MNCV). The data are expressed as means ± SEM; *P≤0.05, **P≤0.01; ***P≤0.001; ****P≤0.0001; n = 5 mice in each group. Abbreviation: CTR – control, T1D – type 1 diabetes, HFD – high-fat diet, V – velocity.



Supplementary Fig. 1. A – C. Vaginal cytology presents the diestrus stage of the estrous cycle in three groups of mice. The images of vaginal swabs were captured at 40 × objective lens by a light microscope (BX51 Trinocular Transmitted Light Microscope, Olympus, Tokyo). **D – F.** Microscopy images of hematoxylin and eosin stained sections of the uterus indicated lumen, endometrial cells as well as endometrial epithelium, stromal compartment, uterine glands and myometrium cells. We did not observe any morphological changes in the bright-field microscopy images of the uterus harvested from the three groups of females (D–F). Abbreviation: CTR – control, T1D – type 1 diabetes, HFD – high-fat diet.
Images were taken under 40× objective with 0.75 numerical aperture (40×/0.75). Scale bar = 50 μm. Abbreviations: L – leukocytes, C – cornified cells, N – nucleated cells, Lu – lumen, E – endometrium cells, Ee – endometrial epithelium, St – stromal compartment, Ug – uterine glands, M – myometrium cells, P – perimetrium, CTR – control, T1D – type 1 diabetes, HFD – high-fat diet.

amount of RAGE was elevated in diabetic mice compared to control mice (P≤0.01, P≤0.0001, respectively, Fig. 3F). Moreover, HFD induced alteration in myometrial level of RAGE protein compared with control and diabetic group (P≤0.01, P≤0.001, respectively, Fig. 3F). We did not observe any differences between three groups in the amount of HMGB1 and S100B proteins in uterine tissues (P≥0.05, Fig. 3F). But

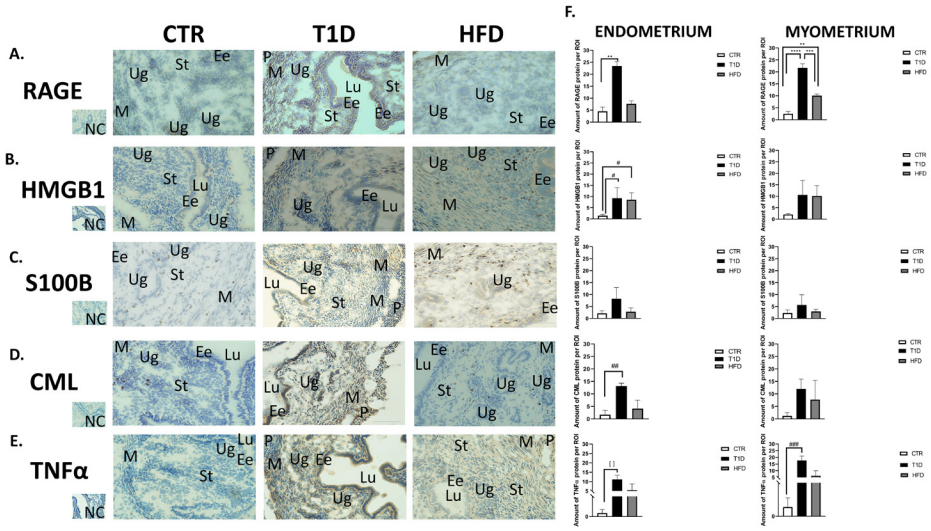


Fig. 3. A-F. The effect of T1D as well as HFD on the abundance of the RAGE, HMGB1, S100B, CML, and TNFα proteins in mice uterus (**A-F**, respectively). Representative images showing localization of proteins in mice uterine tissues. **A-E.** Brown color demonstrated immunoreactive area of stain. Blue color demonstrated hematoxylin staining (**A-E**). Images were taken under 40× objective with 0.75 numerical aperture (40×/0.75). Scale bar = 20 µm. **F.** Data are presented as the mean ± SEM; *P≤0.05; **P≤0.01, ***P≤0.001; ****P≤0.0001, [] – P = 0.095, # – P = 0.085, ## – P = 0.065, ### – P = 0.053. The amount of proteins were determined with the ImageJ software which automatically converted the positive signal of IHC staining into the corresponding range of gray values. The total percentage of staining area was calculated per ROI.

Abbreviations: Lu – lumen, Ee – endometrial epithelium, St – stromal compartment, Ug – uterine glands, M – myometrium cells, P – perimetrium, CTR – control, T1D – type 1 diabetes, HFD – high-fat diet, NC – negative control.

during the diestrus stage of the estrous cycle, we observed an endometrial trend (no static differences) to increased HMGB1 immunoreactivity in the group of females with HFD compared to the control group (P = 0.085, Fig. 3F). Moreover, diabetes induced a trend (no static differences) to increase levels of CML as well as TNFα proteins in endometrium when compared to the control group (P = 0.065, P = 0.095, respectively; Fig. 3F). However, we did not observe any differences between three groups in the myometrial abundance of CML protein (P ≥0.05, Fig. 3F). The myometrial abundance of TNFα protein was elevated in the T1D group of females compared to the control group (P = 0.053, Fig. 3F). We did not observe any effect of HFD on abundance of TNFα protein in uterine tissues compared to other groups (P≥0.05; Fig. 3F).

Linear model of RAGE and its ligands as well as proteins associated with inflammation

We sought to determine if amount of RAGE protein in mice uterine tissues is correlated with HMGB1, S100B, CML or TNFα proteins (Fig. 4A-H). In endometrium, we only observed a tendency to correlate RAGE with CML and TNFα proteins (r =

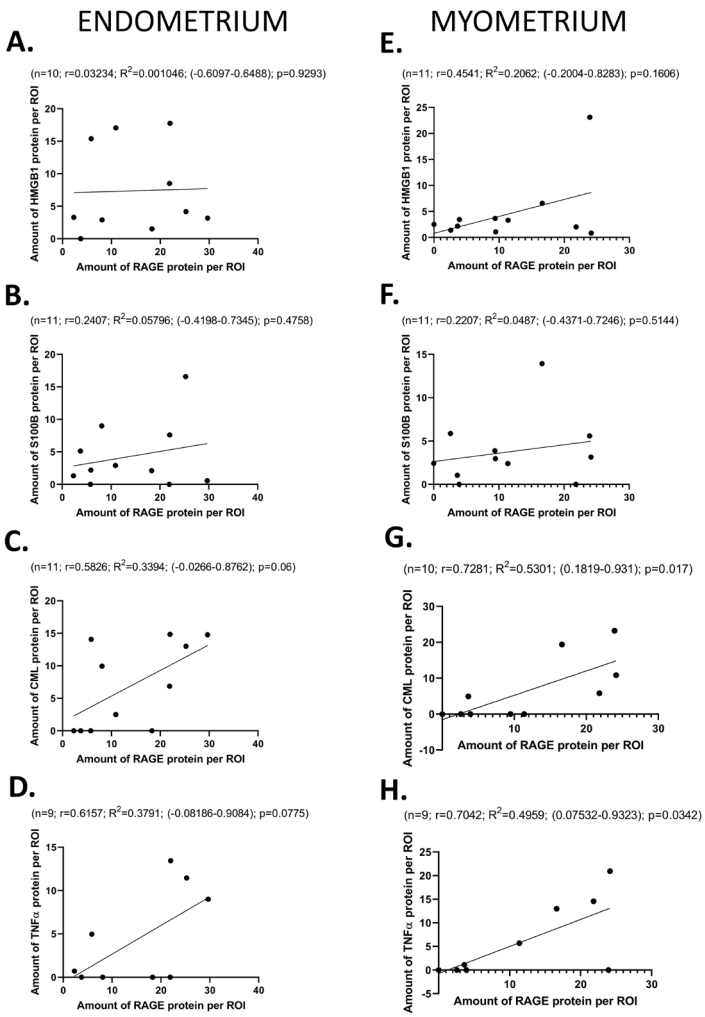


Fig. 4. RAGE with its ligands and proinflammatory cytokine (TNFα). The results demonstrated the correlation in endometrium (A-D) as well as in myometrium (E-H) harvested from cyclic mice. In our study, the correlation analysis is statistical relationship between obtained data from IHC staining.

0.5826, $R^2 = 0.3394$, (-0.0266 – 0.8762), P = 0. 06, Fig. 4C, r = 0.6157, $R^2 = 0.3791$, (-0.08186 – 0.9084), *P* = 0. 0775), Fig. 4D, respectively). However, in myometrium we found that RAGE was significantly and positively correlated with CML as well as TNFα proteins (r = 0.7281, $R^2 = 0.5301$, (0.1819 – 0.931), P = 0. 017, Fig. 4G, r = 0.7042, $R^2 = 0.4959$, (0.07532 – 0.9323), *P* = 0. 0342, Fig. 4H, respectively). No other statistically significant correlations were observed for these comparisons in uterine tissues (Fig. 4A-H).

In the present study we revealed malfunctions in RAGE signaling pathways that take place in uterine tissues under hyperglycemic milieu and HFD. We showed the localization and abundance of RAGE, HMGB1, S100B, CML as well as TNFα in mice uterus during estrous cycle. Furthermore, our studies provided data concerning correlation between RAGE and its ligands in uterine tissues. Nevertheless, based on our experience, we believe that correlation alone may not be enough to advance our understanding of the RAGE signaling pathway in uterine tissues. The result of the correlation should be supported by additional analysis.

STZ-treated female mice developed T1D, while HFD mice showed pre-diabetes (not fully developed T2D) and obesity. Our studies demonstrated the impact of pre- and diabetes on the RAGE signaling pathway in mouse uterus during DLDN.

RAGE belongs to the immunoglobulin family and has the ability to bind many ligands, especially: HMGB1, S100B as well as CML. Our previous studies revealed that RAGE and its mentioned ligands were elevated during DLDN [Zglejc-Waszak *et al.* 2023a, Juranek *et al.* 2013, 2010]. We suppose that RAGE and its proinflammatory ligands are engaged in malfunctions in the uterine milieu during progression of DLDN.

NCV results revealed that females during studies suffered from DLDN. According to the international consensus, only one symptom is enough to diagnose DLDN [Yu 2021]. NCV studies revealed that SNCV was declined in T1D female mice. We observed an altered amount of RAGE protein in uterine tissues harvested from T1D females when compared to the control group. Given only the statistically insignificant trend (no static differences) of HMGB1, S100B, CML and TNFα proteins, we observed an elevated amount of chronic mediators of inflammation in uterine tissues in hyperglycemic milieu during estrous cycle.

Holistic view of the local alternations in the uterine horn during early pregnancy indicated that the embryo may attenuate the uterine local immune system, including downregulation of molecules involved in the inflammation process [Almiñana *et al.* 2012]. However, during pregnancy loss we may observe an elevated level of proteins that trigger inflammation in both female and fetal tissues [Buhimschi *et al.* 2009]. Therefore, the immune system homeostasis in female reproductive tract is necessary to achieve reproductive success [Fazeli *et al.* 2008, Almiñana *et al.* 2012]. T1D and accumulation of AGEs trigger local inflammation and thus disrupt local tissue immune homeostasis [Juranek *et al.* 2022]. Moreover, Buhimschi *et al.* [2009] revealed that RAGE and HMGB1 may be crucial biomarkers of preterm birth. Our results showed an elevated level of RAGE and HMGB1 proteins in endometrium as well as in myometrium harvested from diabetic female mice during estrous cycle.

Herein we address for the first time in mice uterine tissues some of the complex cross-talk between proteins involved in RAGE signaling pathway. RAGE is considered the first receptor of AGEs under high glucose milieu and also acts as regulator of cytokine network in inflammation process [Juranek *et al.* 2022]. We documented strong and positive correlation between RAGE and CML in the myometrium harvested

during the estrous cycle. Thus, further studies are necessary to clarify the cross-talk between these proteins and need to be supported by additional analysis. However, the molecular mechanism of RAGE contributing to local inflammation in uterine tissues is still unknown. RAGE is mainly expressed in healthy lung type 1 pneumocytes as well as embryonic cells. Nevertheless, it protein elevates quickly during progression of inflammation [Juranek *et al.* 2022, Sparvero *et al.* 2009, Zglejc-Waszak *et al.* 2023a, 2023b]. Thus, based on our results, we may suppose that elevated level of RAGE protein in uterine tissues may trigger chronic inflammation and thus disturbs homeostasis in uterine milieu during estrous cycle.

Our data showed elevated level of TNFα protein in myometrium harvested from T1D female. Earlier studies indicated that in non-gravid female TNFα increased uterine production of estrone [Franczak *et al.* 2013a]. We may suggest that T1D may induce perturbations in uterine estrone release by elevated level of TNFα. However, increased amounts of this cytokine may also cause malfunctions in implantation, immune system of female reproductive tract and trigger endometriosis [Hazout 1995, Giacomucci *et al.* 1994, Zhang and Wild 1993]. We observed a positive relationship between RAGE and TNFα in myometrium harvested from mice during estrous cycle. Therefore, based on our results, we may hypothesize that the myometrium is more sensitive to a hyperglycemic milieu.

Furthermore, Franczak *et al.* [2013b, 2014] revealed that the transcriptomic profile of myometrium differs from that of the endometrium. Nevertheless studies indicated that the activity of Cyclooxygenase 2 as well as 3β-Hydroxysteroid dehydrogenase is different between the endometrium and myometrium during early pregnancy and estrous cycle in pigs [Wojciechowicz *et al.* 2013, Franczak *et al.* 2010]. The results indicate that the response of the myometrium may be different than that of the endometrium during estrous cycle and this may be a physiological phenomenon. However, further studies are necessary to explain this phenomenon.

Moreover, RAGE is involved in activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-ƙB) and consequently enhance activity of TNFα and thus progression of inflammation and tissue damage [Zglejc-Waszak *et al.* 2023b, 2021]. We may speculate that in T1D uterine tissues the RAGE signaling pathway is activated and an inflammatory process occurs by increasing the release of proinflammatory cytokine, *i.e.* TNFα. However further studies are necessary to clarify the role of TNFα protein in T1D uterine tissues during estrous cycle.

Schmidt and co-workers [2021] postulated a model of progression of inflammation consisting of two steps. Firstly, they observed elevated amount of RAGE and its proinflammatory ligands in studied tissue/cell. Next, Schmidt *et al.* [2021] showed various forms of stress resulting from malfunctions in tissue/cell homeostasis. In the second step, they observed, among other things, "inflammatory stress" promoting malfunctions and lesions in tissue/cell [Schmidt *et al.* 2021]. Our present results confirm Schmidt *et al.* [2021] hypothesis that inflammatory perturbations in uterine tissues may be mediated by RAGE and its ligand.

Long-term HFD and thus obesity may cause malfunctions in the uterine milieu [Chakraborty *et al.* 2016]. Our results indicated that long-term HFD may induce elevated levels of RAGE in myometrium compared to the control group of female mice. However, we did not observe any differences in RAGE proinflammatory ligands, such as: HMGB1, S100B as well as CML in uterine tissues harvested from HFD mice. Wilson *et al.* [2022] revealed that HFD-induced obesity may alter gene expression patterns associated with innate immunity and leukocyte chemotaxis. Moreover, our previous studies showed that long-term HFD alters blood lipids in mice [Jaroslawska *et al.* 2021]. Our current study indicated that HFD has a profound effect on body weight as previously described by Jaroslawska *et al.* [2021]. Growing number of studies indicated the harmful impact of maternal diabetes and HFD on offspring health [Wilson *et al.* 2022].

Our study revealed that T1D and long-term HFD could modulate the local immune system and activate the RAGE signaling pathway in uterine tissues during the estrous cycle. The presence of RAGE ligands, such as: HMGB1, S100B, CML proteins in epithelium, stromal compartment, uterine glands cells of the endometrium as well as in myometrial cells harvested from HFD mice was confirmed. Activation of RAGE signaling pathways promotes the formation of proinflammatory cytokine, *i.e.* TNFα and maintain inflammation in the uterine milieu. Malfunctions in the uterine immune system may be the reason for infertility or subfertility. In the future RAGE pathway may be a molecular goal in diagnosis of female infertility.

Moreover, previous studies showed that TNFα and thus RAGE signaling pathway, may be involved in the regulation of endometrial Prostaglandin F2α (PGF2α) mechanism [Franczak *et al.* 2012, Zglejc-Waszak *et al.* 2021]. It is well documented that PGF2α is involved in the maintenance of the corpus luteum in early pregnancy or facilitates its regression during the estrous cycle [Franczak *et al.* 2012]. Therefore, RAGE and its proinflammatory ligands may be involved in the maintenance of early pregnancy. However, further studies are necessary to explain this phenomenon.

Our results showed that T1D and HFD alter the amount RAGE as well as act on RAGE signaling pathway in mice in uterine tissues during DLDN. Our studies confirmed Schmidt theory about "two hits" during progression of inflammation. We confirmed our hypothesis that T1D and HFD modulate the local immune system in uterine tissues during estrous cycle.

Data availability statement

All data used and/or analyzed during this study are included in this published article. Further inquiries can be directed to the corresponding author.

Conflicts of interest

The authors have no conflicts of interest to declare.

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