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ORIGINAL ARTICLE

Sex-based differences in streptozotocin-induced type 2 diabetes rat models

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Abstract

Background: Diabetic foot ulcers (DFUs) are a severe complication of diabetes mellitus, leading to chronic infections, amputations, and increased mortality despite existing treatments. More effective therapeutics are urgently needed, and animal models provide a critical platform for investigating the molecular mechanisms underlying DFUs. Streptozotocin (STZ)—administered at a low dose to induce type 2 diabetes (T2D) and at a high dose to induce type 1 diabetes—is commonly used in mice and rats in DFU research. **Aim:** The objective of this study is to highlight the importance of including male and female rats in DFU research. **Methods:** Both male and female Sprague–Dawley rats, 6–8 weeks old, were fed a high-fat diet for 9 weeks. STZ (25 mg/kg intraperitoneally) was administered weekly from the 6th week to induce T2D. **Results:** Female rats required a higher dose of STZ compared to male rats. The induction of T2D correlated positively with weight gain, which was greater in males than in females. **Conclusion:** The findings suggest that in addition to gender and weight, other factors may influence the induction of T2D in rats. Most studies in the literature do not use both sexes in DFU research. The distinct responses to STZ and weight gain observed emphasize the need to include both sexes and employ a more detailed approach in preclinical studies to enhance the understanding of DFU wound healing and translate the findings into potential treatments. **Relevance for patients:** The multifactorial effect on diabetes development, which differs in males and females, suggests the need to consider etiological, physiological, and demographic factors, such as body weight, gender, age, and body mass index, in the prevention and treatment of diabetes. This will also help in planning the individualized treatment for DFUs.

Keywords: Diabetic foot ulcer; Animal model; Type 2 diabetes; Streptozotocin; Gender

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1. Introduction

Diabetic foot ulcers (DFUs) represent one of the most severe complications of diabetes mellitus, often resulting in chronic infections, amputations, and significantly

increased mortality rates.[1] DFUs are the leading cause of hospitalization among all diabetes complications,[2] placing a substantial burden on healthcare systems due to their complex treatments and heightened risk of life-threatening outcomes. DFUs affect approximately 19 million people worldwide, including around 1.6 million in the United States, significantly burdening healthcare costs, with annual estimated treatment expenses ranging from USD 9 to USD 13 billion.[3] Despite current treatment, which includes wound debridement, antibiotics, growth factors, hyperbaric oxygen therapy, negative pressure wound therapy, topical oxygen, and skin grafts, the amputation rate of the lower extremity ranges from 3.34% to 42.83%.[4] Furthermore, the recurrence rate in DFU after treatment is 40% within 1 year, 60% in 3 years, and 68% within 6 years.[5] This suggests the need for improved treatment strategies. Moreover, chronic wounds, such as DFUs, present a major challenge for clinicians, complicating the understanding of their pathophysiology, hindering effective interventions, and highlighting the urgent need for targeted research and innovation in this field. Animal models are useful for studying the underlying molecular mechanisms and for investigating the effects of drugs and small compounds, which are tested for targeted therapy, in improving DFU healing.[6-8] Investigating underlying molecular mechanisms, therapeutic targets, and potential drugs in animal models, particularly in rodents and pigs, raises interest in translating these insights to enhance human patient care. While these models have offered valuable insights into the mechanisms and therapeutic approaches for wound healing, it is important to note that animal healing processes differ significantly from those in humans, especially given the chronic nature of human wounds that are often absent in animal models.[9]

Animal models used in preclinical studies of DFU healing, including mice, rats, pigs, guinea pigs, dogs, rabbits, zebrafish, or sheep, have their advantages and limitations.[8,10] The widely used Zucker diabetic Sprague–Dawley (ZDSD) rat model, along with the ob/ob and db/db mouse models, carries genetic defects in the leptin signaling pathway, leading to obesity and insulin resistance (IR).[8] However, these mutations do not fully represent the typical pathophysiology of human type 2 diabetes (T2D), making these models less ideal for capturing the complexity of T2D development in humans. To mimic diabetic ulcers in humans, inducing diabetes in mice or rats is a common practice in DFU research. Streptozotocin (STZ) is commonly used to induce diabetes in rodent models,[11] and research suggests that a high dose of STZ (50 mg/kg body weight) induces type 1 diabetes (T1D) while a low dose (25 mg/kg body weight) induces T2D.[8] Female rodents tend to be more resistant to low-dose STZ-induced T2D compared to males.[7] Males tend to develop severe metabolic disturbances, including IR, hyperglycemia, and obesity, while females often experience a delayed onset of hyperglycemia and milder IR. This difference is particularly evident in younger male rats, where hyperglycemia occurs earlier and more frequently than in females, likely due to the protective effects of estrogen.[12] This hormone has been shown to enhance insulin sensitivity, reduce inflammation, and offer protection against metabolic syndrome,[13] which explains why female ZDSD rats tend to display milder diabetes symptoms compared to males. Along with the role of estrogen in mice, the role of estrogen in insulin sensitivity in male and female rats has also been discussed,[14-16] suggesting its role in rats. Male rats also tend to gain more weight and accumulate more visceral fat,[17] a major contributor to IR development. Since obesity is a key driver of T2D in ZDSD rats, males typically display more extreme obesity-induced metabolic impairments than females. This suggests that induction of diabetes in rodents may be affected by age, hormones, metabolism, and weight.

T2D in rodents is induced with low-dose STZ, inducing β-cell dysfunction after the rodents are fed a high-fat diet (HFD) for at least 8 weeks, while T1D can be induced without HFD.[18,19] After STZ injection, severe T1D develops in nearly 50% of mice around 3 weeks after injection,[20] while the rate of diabetes induction in rats is 76% and 89% for T1D and T2D, respectively.[21] A study by Zhang *et al.*[22] reported that repeated injections of low-dose STZ are a better way to induce diabetes for developing a stable animal model of T2D. Consumption of HFD is associated with weight gain and adiposity, and it may depend on the rate of consumption and compositional changes.[23] Most studies in DFU research using rat models have either not mentioned the sex of the rats being used, used only female rats, induced T1D, or induced T2D in male rats after a single STZ injection (dose varying between 30–35 mg/kg). In the case of more than one STZ injection, the studies mentioned multiple injections but not the exact numbers or timing. Furthermore, the association or correlation between weight gain after HFD, the number of STZ injections, and the development of T2D in male versus female rats has not been mentioned.[24-31]

In this observational study, we used Sprague–Dawley (SD) rats to induce T2D for DFU healing research. We observed that after 8 weeks of HFD, male and female rats did not develop diabetes at the same rate, and female rats required more STZ injections over a longer period to develop T2D. Furthermore, the weight gain in the rats also differed between males and females. The findings of this study underscore the importance of including both sexes

in DFU healing research and provide valuable insight into sex-specific responses to STZ during diabetes induction.

2. Materials and methods

2.1. Animal model

SD rats (n = 42; 21 males and 21 females), aged 6–8 weeks and weighing approximately 175 g, were obtained from Charles River Laboratories (United States of America). The animals were housed in the Animal Resource Facility at the Western University of Health Sciences, Pomona, California, under standardized conditions, including a constant temperature of 22°C and a 12-h light/dark cycle, following the facility's standard operating procedures. The research involving these rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Western University of Health Sciences with protocol approval # R24IACUC013. The rats were randomly divided into two groups: a control non-diabetic group (14 rats) and a diabetic group (28 rats). The control group was fed a normal diet (20% protein, 70% carbohydrate, 10% fat; Research Diet Inc., United States of America) and water *ad libitum*. The diabetic group rats received an HFD (35% carbohydrate, 20% protein, 45% fat; 5.7 kcal/g total; Research Diet Inc., United States of America), and water was available *ad libitum*. The rats were weighed at three time points: at the start of the experiment, after 6 weeks of feeding (before diabetes induction), and at the end of the experiment (week nine, following the development of diabetes). The blood glucose levels in the rat tail vein were also measured initially, before inducing diabetes, 2 weeks after the first STZ injection, and at the end of the experiment. All of the diabetic rats were utilized for further study.

We performed power analysis considering the primary outcome of animal weight and the secondary outcome of blood glucose levels and chose the sample size that would suffice for all experiments. Based on the power analysis using G*power software (Version 3.0.10, University of Kiel, Germany) with a value of 0.05, the sample size required to have at least 95% power to detect a significant change is 10 in each group. Based on our previous experience with mortality, we used a minimum of 14 rats in each group.

2.2. Induction of T2D

After 6 weeks of HFD, both male and female rats in the diabetic group were injected intraperitoneally (IP) with a low dose of STZ (25 mg/kg, dissolved in 0.1 M sodium citrate buffer, pH 4.4; Sigma-Aldrich, United States of America) to induce T2D. Our previous experience using IACUC protocol R22IACUC020 showed that SD rats did not develop T2D after a single STZ injection; a second injection was required 2 weeks after the first injection (unpublished own data). Based on this experience, we administered a second STZ injection in the current study to induce T2D in SD rats (n = 28, 14 males, and 14 females). The second dose was administered 1 week after the first STZ injection. Rats in the control group were injected with vehicle citrate buffer (0.25 mL/kg) on the same day. Hyperglycemia developed consistently in diabetic rats around 2-week post-induction. The blood glucose levels were monitored in both groups using tail vein blood samples and measured with an AlphaTrak glucometer (Zoetis Inc., United States of America). Rats with a blood glucose level exceeding 250 mg/dL 2 weeks after the first injection in males and after the third injection in females were considered diabetic.

2.3. Statistical analysis

Data were presented as mean ± standard deviation. The statistical significance was analyzed using a Student's t-test to compare two groups. A $p<0.05$ was considered statistically significant.

3. Results

3.1. HFD-induced greater weight gain in males than in females but did not induce diabetes

The initial weight for male and female rats was 195.06 ± 13.48 g and 152.21 ± 25.21 g, respectively, while the random blood glucose levels were 118.9 ± 2.29 mg/dL and 116.48 ± 5.15 mg/dL (Figure 1A). After 6 weeks of HFD, male SD rats gained more weight compared to female rats. The average weight was 303.93 ± 27.68 g in females and 492.28 ± 35.12 g in males, while the average blood glucose was 144.57 ± 16.33 mg/dL in females and 137.93 ± 19.66 mg/dL in males. The weight gain in female and male rats was statistically significant ($p<0.0001$ for both weight and blood glucose in female rats and $p<0.0001$ for weight and $p<0.01$ for blood glucose in males, respectively) (Figure 1B). Though the change in weight and blood glucose was significant, neither male nor female rats developed diabetes (Figure 1).

3.2. Males developed diabetes after two STZ injections but not females

All males developed diabetes after two injections of STZ (25 mg/kg), while the majority of the females did not develop diabetes (3 out of 14 females developed diabetes) (Figure 2A). The 11 females were injected with a third injection 1 week after the second STZ injection. The female rats developed diabetes after the third injection (Figure 2B). It was also evident from the results that male rats weighed more than females (Figure 2A and B), and

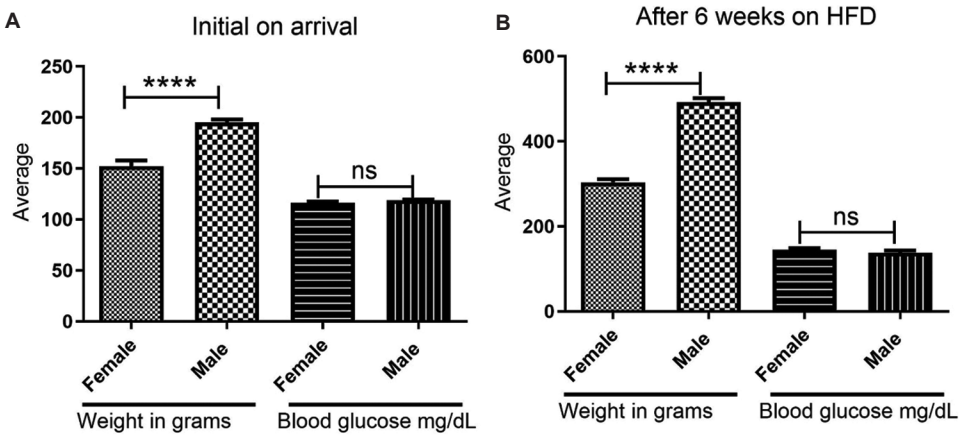

Figure 1. Weight and blood glucose at the start of the experiment and after 6 weeks of a high-fat diet (HFD). The data are presented as mean ± standard deviation, with $p<0.05$ indicating statistical significance. (A) Weight and blood glucose at the start of the experiment. (B) Weight and blood glucose after 6 weeks of HFD.
Notes: ****$p<0.0001$.
Abbreviation: ns: non-significant.

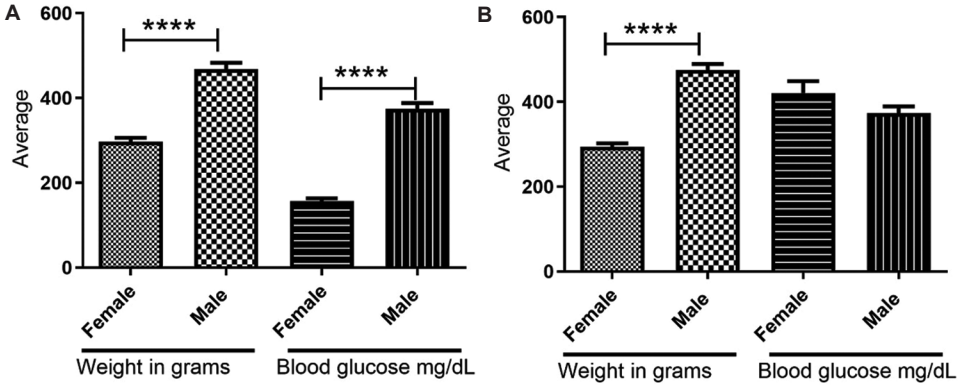

Figure 2. Streptozotocin (STZ)-induced type 2 diabetes in male and female rats. The data are presented as mean ± standard deviation, with $p<0.05$ indicating statistical significance. (A) Weight and blood glucose after two injections of STZ. (B) Weight and blood glucose before inducing ulcers (two STZ injections in males and three STZ injections in females).
Note: ****$p<0.0001$.

after the third injection, blood glucose levels were higher in females compared to males, although not statistically significant. The average blood glucose levels in males were 374.34 ± 48.6 mg/dL, and the average weight was 468.04 ± 51.85 g. The average blood glucose levels in females were 348 ± 51.17 mg/dL in those developing diabetes (n = 3) and 156.63 ± 21.92 mg/dL in those not developing diabetes (n = 11). The average weight of female rats was 296 ± 28.96 g.

3.3. Blood glucose increased after STZ injections and was positively correlated with weight gain

In both male and female rats, increases in random blood glucose levels were positively associated with weight gain and HFD. In male rats, strong positive correlations were observed between initial weight and blood glucose levels (Figure 3A), after 6 weeks of HFD (Figure 3B), and at the time of wound induction (Figure 3C) (r^2 = 0.79, r^2 = 0.85, and r^2 = 0.95, respectively). In female rats, the correlation was weaker at the start of the experiment (r^2 = 0.40; Figure 3A) but strengthened after 6 weeks of HFD (r^2 = 0.81; Figure 3B) and at the time of wound induction (r^2 = 0.96; Figure 3C). These results suggest that weight gain is positively correlated with an increase in blood glucose. Since weight gain with HFD is associated with an increase in blood glucose, it is important to investigate why females require one extra dose of STZ.

4. Discussion

The results of this observational study suggest that STZ is effective in inducing T2D in rats, but female rats require an additional dose of STZ compared to males. In addition, the

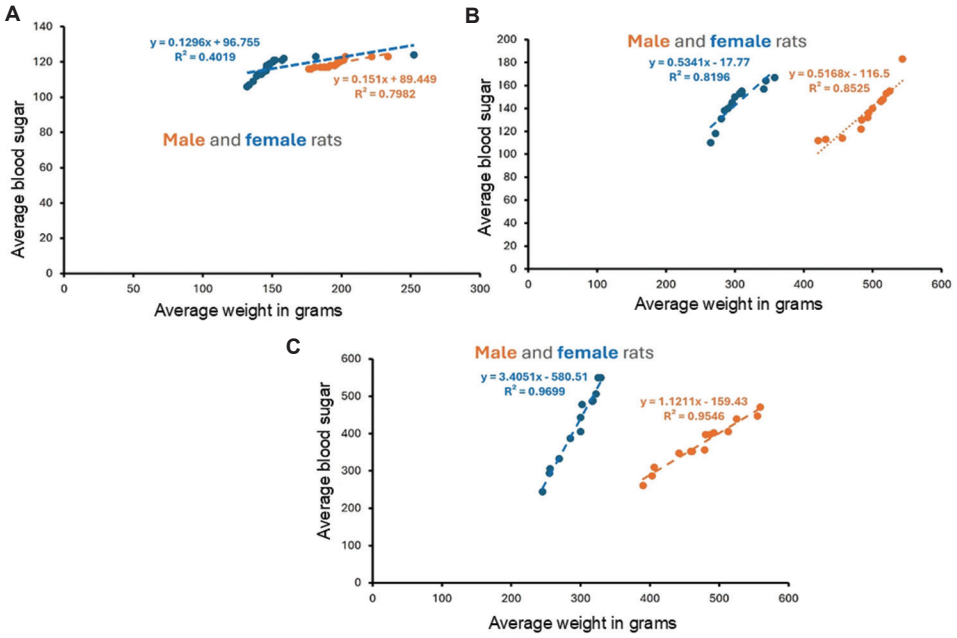

Figure 3. Correlation analysis between weight and random blood glucose levels in the female and male rats fed a high-fat diet. (A) At the start of the experiment (on arrival). (B) After 6 weeks of feeding with a high-fat diet. (C) Before inducing ulcers.

increase in blood glucose after STZ injection is positively correlated with weight gain after HFD administration in both males and females. Another important finding was that the increase in weight was greater in male rats compared to female rats after 6 weeks of HFD (weight gain of 200% in females and 250% in males) and remained stable until the end of the study (200% in females and 248% in males). The weight gain in both male and female rats may be due to the continuous HFD feeding. The increase in weight gain and a positive correlation in both male and female rats suggest that factors other than weight may affect the induction of diabetes after STZ injection, and these factors may be underlying causes of the need for increased STZ in female rats.

The first reason may be the differential STZ metabolism in male and female rats because sex plays a critical role in diabetes development. A recent study suggests that HFD and low-dose STZ induce hyperinsulinemia and IR in male but not female C57BL/6J mice,[32] where 30 mg/kg STZ was given once daily for 3 consecutive days. Another study reported different responses in IR and glucose metabolism in C57BL/6J female and male mice treated with low-dose STZ (40 mg/kg for 5 days).[33] Sex differences have also been monitored for fasting blood glucose and glucose tolerance between male and female outbred mice after 40 mg/kg STZ injection for 8 days.[34] Furman[20] concluded that C57BL/6, CD-1, or Balb/cJ mice develop T1D with 40 mg/kg STZ, intraperitoneal, injected for 5 days. A high dose of STZ (200 mg/kg) induces T1D within 48 h but has multiple toxic effects. Low-dose STZ is associated with milder toxicity. SD or Wistar male rats develop T1D after 65 mg/kg STZ injections. These studies primarily employed mouse models, focusing on T1D development in mice. These effects may be due to the sensitivity to STZ. Iwase *et al.*[35] reported sex differences in susceptibility to neonatal STZ treatment in inducing diabetes and hypertension in spontaneously hypertensive rats.

Second, the resistance of the female rats to the effects of low-dose STZ may be the reason for the increased number of injections we used in this study. Studies suggest that female rodents (mice and rats) are resistant to low doses of STZ, and this may be overcome by increasing the dose of STZ.[19,20] This notion is supported by the fact that most of the studies cited above used a higher dose of STZ for T1D and multiple injections of low-dose STZ to induce T2D in rodents. In this study, we administered only one additional dose of STZ (25 mg/kg) to induce T2D in rats fed with HFD. STZ sensitivity and resistance may also vary by strain: Wistar and SD rats are more sensitive, whereas Wistar–Kyoto rats exhibit lower sensitivity.[19] Among mice, DBA/2 is the most sensitive, followed by C57BL6, and Balb/cJ are resistant to STZ.[20] This suggests that it is not only the sex of the animal but also the strain that should be carefully considered when selecting the animals for DFU research.

Next, induction of T2D in males with a lower total dose of STZ may also be due to the increased toxicity of STZ to the pancreatic islets of males compared to females.[19,20,33]

This is supported by the fact that lower levels of beta-cell apoptosis occur in female mice compared to male mice after STZ treatment, with the fact that alpha-to-beta-cell transdifferentiation rates are higher in healthy female mice than in male mice, while control male mice have larger pancreatic islets than females.[36] The need for an increased dose or multiple injections of low-dose STZ in female rodents to induce T2D may also be due to the greater effect of STZ on glucose metabolism in male diabetic mice than in female diabetic mice.[34] Furthermore, the response in IR and glucose metabolism differs in male and female mice fed with HFD and treated with low-dose STZ.[32]

Another important aspect may be the effect of sex hormones on the metabolism of STZ and glucose. Female hormones may regulate STZ and glucose metabolism regardless of the genetic background.[33,34] This can be due to estrogen. Estrogen's role in glucose metabolism after STZ treatment is supported by the findings of restoration of nitrergic relaxation and estrogen receptor levels in STZ-induced diabetic female mice after 17β-estradiol supplementation.[33] The findings that 17β-estradiol supplementation suppresses gastric inflammatory and apoptotic stress responses in STZ-induced diabetic female mice[37] further support the role of estrogen. Another study further supported its role by reporting higher fasting blood glucose and hemoglobin A1c levels in ovariectomized female mice.[34] Furthermore, estrogen regulates various metabolic processes in tissues, including adipose, liver, muscle, and brain tissues, and dysregulation of estrogen signaling results in metabolic disorders, such as diabetes.[38] This again suggests that estrogen may play a role in the induction of diabetes. In addition, androgens may also sensitize the male mice to glucocorticoid-induced IR and fat accumulation.[39] It should also be noted that STZ-induced T2D rats, as compared to normoglycemic rats, showed comparatively higher levels of variation among biochemical, toxicological, and hematological parameters, suggesting alterations in normal glucose metabolism/homeostasis.[40] Although Rehman *et al.*[40] conducted the study using only male rats, the effects in female rats should be investigated. In the current observational study, induction of diabetes was positively correlated with weight gain in both male and female rats. The positive association in male and female rats may be supported by the secretion of androgens in males[41] and, to a lesser extent, in females.[42] We observed that both male and female rats gained weight on an HFD. However, there was no further weight gain after STZ injection. This aligned with previous observations that rodents experienced weight loss after STZ injections due to the lack of insulin, resulting in impaired glucose uptake and utilization in adipose tissue.[43] The need for a low dose of STZ in males to induce diabetes is supported by the notion that a higher level of body fat can exacerbate the effects of STZ-induced diabetes due to altered insulin sensitivity and changes in adipose tissue function, because adiposity significantly affects the STZ metabolism.[44,45] In our study, male rats gained more weight than female rats.

Altogether, the findings of this study suggest that female rats need a higher amount of STZ to induce T2D. Most studies related to DFU research include either male or female rodents. However, based on our results, both male and female rodents should be used while inducing T2D and working with diabetic ulcers. This is important because the healing pattern may differ due to the differential weight gain (adiposity) in male and female rats before the induction of T2D. One of the important findings we observed was that the rats (both male and female; one female and three males in the control group) were associated with mortality when anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine injected IP before the induction of the wound. We switched the anesthesia to isoflurane only and induced wound on the dorsal surface, and this resulted in no mortality. The mortality in rats injected with ketamine + xylazine may be due to the inhibition of the CYP-mediated ketamine metabolism by xylazine, resulting in a higher ketamine concentration in the body, as reported in humans, dogs, and horses.[46] However, this association and its relationship with adiposity in rodents warrant investigations. Of note, these rats died after inducing the full-thickness dermal wound and not during induction and maintenance of diabetes; thus, the mortality has not affected the statistical analysis of this study.

5. Conclusion

The results of this study suggest that the development of STZ-induced T2D in SD rats on an HFD is positively correlated with weight gain. The female rats need a higher amount of STZ to induce diabetes. These results underscore the importance of including both male and female rats in DFU research. This becomes increasingly important in translational research because the weight, amount of STZ, level of hyperglycemia, and the gender of the animal may influence the effect of the testing article (drug or small molecules).

There are also limitations in this study. Differences in hepatic metabolism, hormonal regulation, and islet cell structure are proposed as potential mechanisms underlying sex differences in STZ sensitivity. However, these remain hypothetical and lack experimental validation. A study evaluating the differences in hepatic metabolism, hormonal regulation, and islet cell structure can be conducted to support this hypothesis with observational

results. In addition, estrogen levels in female rats fluctuate significantly across the estrous cycle, which is known to influence insulin sensitivity and glucose metabolism.[47] Thus, future studies should monitor or control the estrous cycle to investigate the effects of estrogen on assessing STZ sensitivity. Future studies should also incorporate functional analyses focusing on sex hormones and the pharmacokinetics of STZ, as well as experimental designs that include monitoring of the estrous cycle. An additional issue is the difference in the dosage of STZ, which must be taken into consideration. For this study, we used the STZ from the same lot.

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Conflict of interest

Vikrant Rai is an Editorial Board Member of this journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Author contributions

Conceptualization: Vikrant Rai
Formal analysis: Vikrant Rai
Investigation: Ugljesa Malicevic, Jacob Smith
Methodology: Ugljesa Malicevic, Jacob Smith, Vikrant Rai
Writing–original draft: Ugljesa Malicevic, Jacob Smith, Vikrant Rai
Writing–review & editing: All authors

Ethics approval and consent to participate

The study was reviewed and approved by the IACUC at Western University of Health Sciences with protocol approval # R24IACUC013.

Consent for publication

Not applicable.

Availability of data

All data related to this manuscript have been included in the manuscript. Further details may be obtained on request from the corresponding author.

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