Original Article

pISSN, eISSN 0125-5614
M Dent J 2023; 43 (Suppl) : S27-S36

Mitochondrial dysfunction in the salivary glands of type 1 diabetes mellitus rats: effects of insulin and atorvastatin treatment

Benyanoot Kingkaew[1], Kawisara Wannasawet[1], Patchanee Chuveera[1], La-Ongdao Thongnak[2], Anusorn Lungaphin[2], Wasana Pratchayasakul[2,3,4], Siriporn Chattipakorn[3,4,5], Jitjiroj Ittichaicharoen[5]

[1] *Division of General Dentistry, Department of Family and Community Dentistry, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand*
[2] *Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
[3] *Neurophysiology Unit, Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand*
[4] *Center of Excellence in Cardiac Electrophysiology Research, Chiang Mai University, Chiang Mai, Thailand*
[5] *Department of Oral Biology and Oral Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand*

Background and Objective: Type 1 Diabetes mellitus (T1DM) involves pancreatic beta-cell dysfunction and inadequate insulin production, leading to disrupted blood glucose levels and subsequent organ dysfunction. The aim of this study was to examine the influence of insulin and statins on oxidative stress and mitochondrial dysfunction in the salivary glands of streptozotocin-induced diabetic rats.

Material and Methods: Twenty male rats were divided into control (n=4) and experimental groups (n=16) of streptozotocin-induced diabetic rats. Diabetic rats were further divided into four groups (n=4/group) receiving vehicle, low-dose atorvastatin, low-dose insulin, or combined drugs for 4 weeks. Metabolic parameters were assessed via blood samples. Mitochondrial function in submandibular salivary glands was evaluated for reactive oxidative stress (ROS), mitochondrial membrane potential, and swelling analysis.

Results: Streptozotocin-induced T1DM rats displayed hyperglycemia, indicating heightened mitochondrial dysfunction in salivary glands. Mitochondrial ROS production and membrane depolarization were significantly elevated compared to normal rats ($p<0.05$). Atorvastatin, insulin, and combination therapy similarly mitigated mitochondrial dysfunction in induced T1DM rats ($p<0.05$). All three treatment groups significantly reduced plasma glucose, while combined therapy was the most effective.

Conclusion: Combined drug therapy demonstrated the highest efficacy in improving metabolic parameters. Atorvastatin, insulin, and combined therapy were equally effective in mitigating mitochondrial dysfunction in the salivary glands of induced T1DM rats. These findings suggest the potential of combination therapy for T1DM management. Further investigations are needed to understand their impact on salivary gland function and implications for oral health and overall well-being in individuals with T1DM.

Keywords: atorvastatin, insulin, mitochondria function, salivary gland, type 1 diabetes mellitus

How to cite: Kingkaew B, Wannasawet K, Chuveera P, Thongnak L, Lungaphin A, Pratchayasakul W, Chattipakorn SC, **Ittichaicharoen J**. Mitochondrial dysfunction in the salivary glands of type 1 diabetes mellitus rats: effects of insulin and atorvastatin treatment. M Dent J 2023;43(Suppl):S27-S36.

Corresponding author: Jitjiroj Ittichaicharoen
Department of Oral Biology and Oral Diagnostic Sciences, Faculty of Dentistry, Chiang Mai University
239 Huay Kaew Rd., Muang District, Chiang Mai 50200, Thailand.
Tel.: +66 5394 4451, Fax: +66 5322 2844
E-mail: jitjiroj.itti@cmu.ac.th
Received: 17 August 2023 **Revised:** 15 September 2023 **Accepted:** 18 September 2023

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder that results in the destruction of insulin-producing pancreatic beta cells, leading to chronically elevated blood glucose levels and chronic inflammation in critical organs such as the heart and brain [1, 2]. Untreated hyperglycemia can cause long-term complications, including retinopathy, nephropathy, and cardiovascular diseases. Oxidative stress has been found to underlie the inflammation observed in these complications, and mitochondrial dysfunction has been linked to oxidative stress, characterized by mitochondrial ROS production, mitochondrial membrane depolarization, and mitochondrial swelling [3-6].

Salivary glands play a vital role in maintaining oral homeostasis. Studies have shown that patients with T1DM often experience reduced salivary flow rates and xerostomia [7-9]. Additionally, an in vivo experiment demonstrated that salivary gland dysfunction in T1DM rats was linked to elevated oxidative stress, leading to decreased saliva production [10]. However, the extent of salivary mitochondrial dysfunction in T1DM remains largely unexplored.

Insulin replacement therapy is crucial for treating T1DM by normalizing blood insulin levels. However, one of its main side effects is weight gain, as the result of hyperinsulinemia and fat accumulation in peripheral tissue. Consequently, statins, known as hydroxy-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are recommended as a primary preventive therapy in DM patients to reduce fatal cardiovascular events. Studies have indicated that insulin and statins have favorable effects on metabolic parameters and brain apoptosis in T1DM rats [2]. Furthermore, a study has suggested that statins may offer clinical benefits in reducing radiation treatment-induced side effects on salivary gland function [11]. However, the specific impact of insulin and statins on salivary gland function in the T1DM model requires further investigation. Therefore, it was hypothesized that the administration of insulin and statins may influence salivary gland function in the T1DM model.

Materials and Methods

Animal models and experimental protocols

The Faculty of Medicine, Chiang Mai University authorized all experimental procedures and ensured that they were carried out under international and national standards for the care and use of animals. This approval was based on the findings of the Institutional Animal Care and Use Committee with Ethical No.AF01-009. Twenty male Wistar rats weighing 170-200 grams (aged approximately 6 weeks old) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All rats were accommodated separately in a controlled environment with a 12:12 light-dark cycle for 7 days. Body weight and food consumption were monitored weekly. Then the rats were divided into a control group (n=4) and an experimental group (n=16). The control group was injected intraperitoneally with citrate buffer (10 mM, pH 4.5), while the experimental group received a single injection of streptozotocin (65 mg/kg) intraperitoneally to develop diabetes. Following the diabetes condition progression for 1 week, the experimental group was equally divided into four subgroups and given either vehicle (DM), atorvastatin (10 mg/kg/day; p.o.: DS), insulin (s.c.: DI), or the combination of atorvastatin and insulin (10 mg/kg/day; p.o., s.c.; accordingly: DIS) through 4 weeks. The first 40 U/kg dose of insulin was administered in the case of insulin injection after the individual was diagnosed with diabetes (fasting plasma glucose level > 250 mg/dl).

Afterward, daily fasting plasma glucose was investigated before an insulin injection. According to maintain the normal plasma glucose level (70-120 mg/dl) after treatment with insulin, if the fasting plasma glucose level had been 200-250 mg/dl, the dose of insulin injection was 30U/kg. If the fasting plasma glucose level had been 150-200 mg/dl, the dose of insulin injection was 20 U/kg, and if the fasting plasma glucose level had been 100-150 mg/dl, the dose of insulin injection was 10 U/kg. The intention of the study's low-dose insulin intervention was to avoid hypoglycemia, a critically adverse effect of pharmacological therapy for people with diabetes [12-14]. All rats fasted for 5 hours as the experiment was finalized. To give the rats deep anesthesia, 2-3% isoflurane was administered. To investigate metabolic parameters, blood samples were taken (insulin, glucose, total cholesterol levels). The right submandibular glands of each rat were then removed and separated into two pieces before the rat was sacrificed. One was used for the experiment and another one was for a precautionary approach to mitigate potential experimental failures, thereby retaining the supplementary. The experiment's protocol is displayed in (Figure 1).

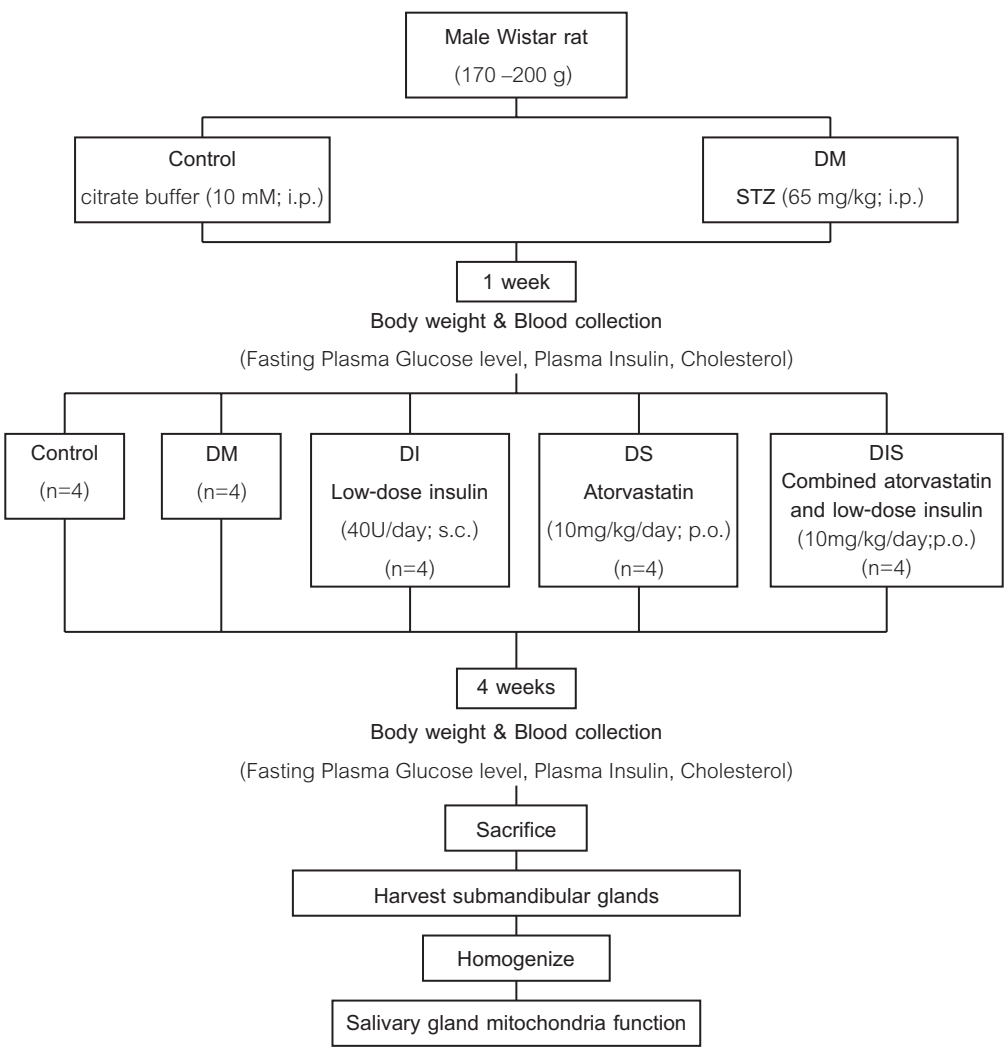

Figure 1 The experimental protocol of the study.
STZ = Streptozotocin; C = Control rats; DM = Diabetic rats; DI = Insulin-treated diabetic rats; DS = Atorvastatin-treated diabetic rats; DIS = Combined drug-treated diabetic.

Blood sampling

Blood was collected from the tail vein at the beginning of the study (week 0), week 12, and at the end of the treatment period. The collected blood was transferred into tubes and centrifuged at 6,000 rpm for 10 minutes at 4°C to separate the plasma. The plasma was then stored at -80°C until analysis. Plasma insulin levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit developed by Willa Hsueh (2005). Plasma glucose and total cholesterol levels were measured using colorimetric assay kits from ERBA Mannheim (Mannheim, Germany).

Determination of Malondialdehyde (MDA) Level

Submandibular gland MDA levels were measured as an assessment of oxidative stress. MDA is produced during lipid peroxidation, and its level was determined using a colorimetric (532 nm) assay that measures the product formed by the reaction of MDA with thiobarbituric acid (TBA). The lipid peroxidation assay was conducted using the MAK085 – Lipid Peroxidation (MDA) Assay Kit. The procedure involved the combination of 20 µl of serum from the salivary gland was mixed with 500 µl of a 42 mM sulfuric acid solution and 125 µl of a phosphotungstic acid solution (PTA). Subsequently, centrifugation was performed at 13,000 rpm for 5 minutes at room temperature. Following this, the serum pellet was resuspended on ice using a 102 µl water/Butylated hydroxytoluene (BHT) solution. The sample volume was then adjusted to 200 µl with water, and incubation was carried out at 37 °C for 2 hours. Following incubation, the formation of an MDA-TBA adduct was achieved by combining 600 µl of a TBA solution with 200 µl of the MDA/serum mixture, followed by incubation at 95 °C for 1 hour. Subsequently, the mixture was placed in an ice bath for 15 minutes. Each reaction mixture was then pipetted into a 96-well plate in a 200 µl volume, and its absorbance was measured at 532 nm. The concentration of TBARS (Thiobarbituric acid reactive substances) was determined using a standard curve and expressed as MDA concentration (nmol/µl).

Preparation of Salivary Gland Mitochondria

After the rats were sacrificed, their submandibular glands were rapidly harvested and homogenized in iced-cold TES buffer (300 mmol/L sucrose, 5 mmol/L TRIS, and 0.2 mmol/L EGTA, pH 7.2 at 4°C). The homogenized gland samples were centrifuged at 800 g for 5 minutes, and the supernatants were collected and centrifuged again at 8,800 g for 5 minutes. The mitochondrial pellets were then resuspended in the ice-cold buffer and centrifuged again at 8,800 g for 5 minutes.

Determination of Reactive Oxygen Species (ROS)

ROS levels were determined using the dye dichlorohydrofluorescein diacetate (DCFDA). The isolated salivary mitochondria were incubated with 2 µM DCFDA for 25 minutes at 25°C and then examined using a fluorescent microplate reader at 485 nm (bandwidth 5 nm) and 530 nm for emission (bandwidth 10 nm).

Determination of salivary gland mitochondrial membrane potential change

The dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to investigate the change in the membrane potential of salivary gland mitochondria. The isolated salivary mitochondria were stained with JC-1 (5 µM) for 30 minutes at 37°C. The salivary mitochondrial membrane potential was calculated as fluorescence intensity using a fluorescent microplate reader. The emission of the JC-1 monomer fluorescent, which appears green in color, was detected at a wavelength of 590 nm after being excited at a wavelength of 485 nm. The emission of the JC-1 aggregated form fluorescent, which appears red in color, was observed at a wavelength of 530 nm, induced at

485 nm. The ratio of red to green fluorescence was used to calculate the change in mitochondrial membrane potential.

Determination of salivary mitochondrial swelling

The isolated salivary mitochondria (0.4 mg/ml) were incubated in 1.5 ml of respiration buffer (100 mM KCl, 50 mM sucrose, 10 mM HEPES, 5 mM KH2PO4, pH 7.4 at 37°C) with the addition of 10 mM pyruvate/malate for 1 minute. The rise in suspension absorbance at 540 nm was analyzed for 30 minutes using a spectrophotometer to investigate salivary mitochondrial swelling.

Statistical analysis

Data was presented as mean ± standard error of the mean (SEM). Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Fisher's post-hoc test for significant differences. A *p*-value less than 0.05 was considered statistically significant.

Results

Metabolic parameters

STZ-induced T1DM rats exhibited typical characteristics of DM, including a decrease in plasma insulin levels, elevated fasting plasma glucose, and a significant decrease in body weight. However, all treatment groups showed a significant reduction in fasting plasma glucose levels and an increase in insulin levels. Moreover, the DIS group showed the most improvement in metabolic parameters among the treatment groups. The cholesterol level was significantly reduced in the group that received statin, DS, and DIS. Furthermore, the DIS group demonstrated a better reduction in cholesterol level than the DS group. These findings suggest that the combination treatment not only cured the diabetic condition but also improved the cholesterol level (Table 1).

Table 1 Outcomes of insulin, atorvastatin, and combination treatment on metabolic parameters. C = Control rats; DM = Diabetic rats; DI = Insulin-treated diabetic rats; DS = Atorvastatin-treated diabetic rats; DIS = Combined drug-treated diabetic.

Parameters	C	DM	DI	DS	DIS
Body weight (g)	428 ± 3.74	198 ± 7.51*	282 ± 9.16*†	258 ± 17.51*†	345 ± 14.66$^{*\dagger\ddagger\#}$
Fasting Glucose (mg/dl)	148.10 ± 14.53	505.14 ± 12.03*	399.38 ± 22.96*†	399.81 ± 28.18*†	231.83 ± 23.97$^{*\dagger\ddagger\#}$
Insulin (U/ml)	1.92 ± 0.31	0.37 ± 0.05*	0.62 ± 0.07*	0.47 ± 0.12*	1.22 ± 0.15$^{*\dagger\ddagger\#}$
Cholesterol (mg/dl)	58.38 ± 7.10	106.27 ± 6.41*	103.19 ± 3.06*	95.37 ± 2.77*	76.74 ± 3.37$^{*\dagger\ddagger\#}$

$^{*}p<0.05$ compared with C group; $^{\dagger}p<0.05$ compared with DM group; $^{\ddagger}p<0.05$ compared with DI group; $^{\#}p<0.05$ compared with DS group

Salivary gland mitochondrial function and oxidative stress

Mitochondrial dysfunction was evaluated by mitochondrial ROS production, mitochondrial membrane potential change (depolarization), and mitochondrial swelling. Our study demonstrated that the submandibular glands of DM rats had a significant increase in mitochondrial ROS production and mitochondrial membrane potential change, but the salivary mitochondrial swelling was not different from the control group (Figure 2a). These findings suggest that T1DM rats had salivary gland mitochondrial dysfunction. On the other hand, the other treated groups showed a significant improvement in salivary mitochondrial function, as indicated by attenuated mitochondrial ROS production (Figure 2a), preserved mitochondrial membrane potential (Figure 2b), and prevented mitochondrial swelling (Figure 2c). These findings suggest that insulin, atorvastatin, and the combination treatment could recover salivary gland mitochondrial function.

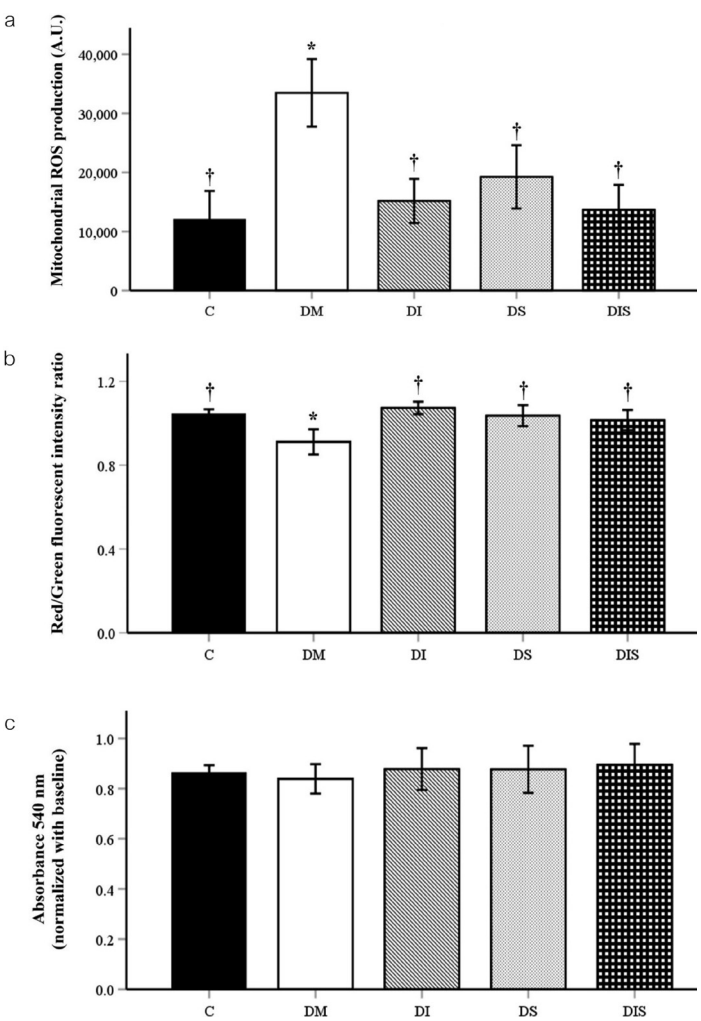

Figure 2 The outcomes of insulin, atorvastatin, and combination treatment on salivary gland mitochondrial ROS production (a), mitochondrial membrane potential changes (b), mitochondrial swelling (c).
C = Control rats; DM = Diabetic rats; DI = Insulin-treated diabetic rats; DS = Atorvastatin-treated diabetic rats; DIS = Combined drug-treated diabetic
$^{*}p<0.05$ compared with C group; $^{\dagger}p < 0.05$ compared with DM group.

Salivary oxidative stress production

Salivary gland oxidative stress was measured by elevated salivary gland MDA levels. When compared to the C group, DM did not demonstrate significantly higher salivary gland MDA levels, and they exhibited no significant difference from DI, DS, and DIS either. These results suggest that similar salivary gland oxidative stress production was addressed by atorvastatin, insulin, and combination therapy (Figure 3).

Discussion

The principal results of this study are listed below;

1) T1DM was associated with accelerated hyperglycemia, impaired salivary gland mitochondrial function, and elevated salivary gland oxidative stress.

2) In T1DM rats, respectively atorvastatin and insulin treatment lowered plasma glucose levels similarly.

3) There were significant differences in weight between individual treatments.

4) The combination of atorvastatin and insulin was shown to be the most effective in reducing hyperglycemia and dyslipidemia when compared to the individual treatments as a single therapy.

5) Salivary gland oxidative stress and mitochondrial dysfunction were all considerably lowered by atorvastatin, insulin, and combination therapy.

Insulin is the main medication for T1DM. The key mechanism of insulin treatment in diabetic conditions appears through the stimulation of glucose and lipid consumption in different parts of the body. Besides that, greater plasma levels of triglycerides, cholesterol, and low-density lipoprotein (LDL) cholesterol are among T1DM's primary characteristics [15, 16]. Atorvastatin is among the most commonly prescribed medications, and also the most commonly prescribed statins globally. In addition to decreasing cholesterol biosynthesis, atorvastatin also has glycemic control effects [2, 17]. In this study, aside from the explained circumstances, it is also determined that both single insulin treatment and atorvastatin treatment led to reduced hyperglycemia. Besides that, neither the plasma insulin nor cholesterol levels of the T1DM rats are impacted by these treatments. Our data's speculative explanation could be that the study's doses of atorvastatin or insulin therapy were insufficient to reduce the hypoinsulinemia and dyslipidemia associated with T1DM. Interestingly, this study revealed that, compared to separate monotherapy, the combination treatment was more effective at reducing hyperglycemia and dyslipidemia. These findings indicate that atorvastatin and low-dose insulin affect glycemic control in T1DM rats differently. So, it might be concluded that the combined treatment group's effect on reducing blood glucose levels appears to be associated with elevated insulin levels.

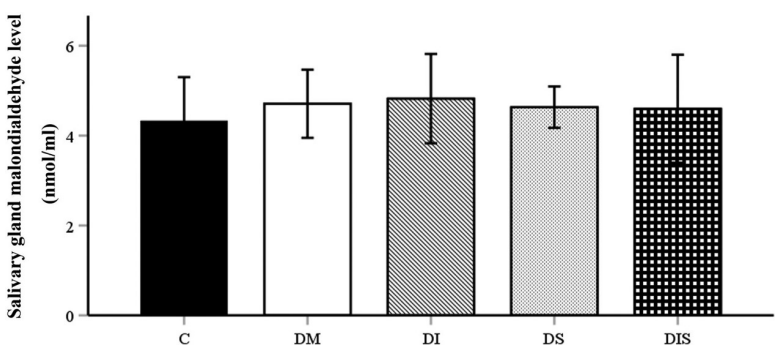

Figure 3 The outcomes of insulin, atorvastatin, and combination treatment on salivary gland oxidative stress production indicated by malondialdehyde level. C = Control rats; DM = Diabetic rats; DI = Insulin-treated diabetic rats; DS = Atorvastatin-treated diabetic rats; DIS = Combined drug-treated diabetic.

The significant weight differences between each experimental group; the DM group had the least weight, which can be the result of the excess sugar failure to serve as a source of energy for the body, prompting a compensatory mechanism wherein the body resorts to metabolizing fat and muscle tissues for energy production, thereby leading to observable weight loss [18]. The DI group had gained weight compared to DM due to insulin-regulating blood glucose levels by facilitating the uptake of glucose into cells, preventing excessive glucose accumulation in the bloodstream. By achieving better blood glucose control with insulin, the body can reduce the reliance on burning fat and muscle for energy. This can help prevent further weight loss and promote weight stabilization [19]. DS and DIS also had weight influences which could be explained that by managing blood glucose levels effectively with insulin and reducing the risk of cardiovascular events with statins, individuals may experience improved overall health and well-being. Better health can promote increased physical activity and appetite, potentially leading to weight maintenance or modest weight gain in individuals who were previously experiencing weight loss due to uncontrolled diabetes [20].

Also, atorvastatin, insulin, and the combination treatment seem to evenly improve salivary gland mitochondrial dysfunction and salivary gland oxidative stress. DM is a metabolic disease correlated with enhanced production of free radicals and reduced antioxidant potential, attending to macro and microvascular complications. The oxidative stress stimulation effect in a DM complication as an explicit mechanism is not completely acknowledged. The cardiovascular complications which are caused by prothrombotic reactions can also be developed by oxidative stress in DM patients. Tissue-oxidative-damaging effects can be found in chronic hyperglycemia damage [21, 22]. Increased intracellular glucose generates too much ROS, which exceeds the capacity of the cell's antioxidants to neutralize them. Oxidative stress, which is a chain reaction of mitochondrial ROS leakage and induced membrane degeneration, can increase lipid peroxidation. These processes are assumed to play an important part in cellular apoptosis or necrosis [23-25].

However, this research demonstrated that insulin, atorvastatin, and the combined drug groups all improved salivary gland mitochondrial dysfunction (by lowering ROS) and mitochondrial membrane potential change but did not differ considerably in mitochondria swelling and MDA levels. The ROS production in this study was probably insufficient to result in the lipid peroxidation detected by MDA levels, which is the hypothetical explanation for the disparate oxidative stress assessment by ROS and MDA [26-28].

It is, therefore, possible that the duration of undergoing STZ-induced diabetes treatment in the diabetic groups (1 week) was inadequate to demonstrate the fibrosis of the salivary glands. Since these medications may initially affect the oxidative stress, as well as the subsequent development of salivary gland fibrosis, the brief time after STZ inducement and/or the short duration of treatment with these therapies may be responsible for the study's findings. Prolonged administration of insulin, atorvastatin, or combined medications may significantly enhance salivary gland mitochondrial function to a level comparable to that observed in the C group. So, further investigation is necessary to obtain more solid evidence that this hypothetical description is legitimate.

Conclusion

In conclusion, the study suggests that both insulin and atorvastatin treatments can reduce hyperglycemia in rats with T1DM, but their effects on plasma insulin and cholesterol levels were limited. The combination of these two therapies was found to be more effective in reducing hyperglycemia and dyslipidemia than individual treatments. Furthermore,

all three treatment groups (insulin, atorvastatin, and combination therapy) improved salivary gland mitochondrial dysfunction, and oxidative stress. The study also suggests that oxidative stress is a crucial factor in the development of diabetic complications, including macro and microvascular complications, and that improved glycemic control through combination therapy may reduce oxidative stress levels. Overall, the findings of this study provide valuable insights into the mechanisms underlying T1DM and its potential treatment strategies.

Acknowledgement

I am immensely grateful to my advisors, Assistant Professor Dr. Jitjiroj Ittichaicharoen and Assistant Professor Patchanee Chuveera, for their invaluable patience, constructive feedback, and unwavering support throughout this study. I also extend my gratitude to Professor Dr. Siriporn Chattipakorn, whose expertise has been instrumental in shaping the research questions and methodology. Your insightful feedback compelled me to refine my logical approach and elevate the quality of my work.

I would like to extend my heartfelt appreciation to the dedicated staff members: Professor Dr. Nipon Chattipakorn, Dr. Wasana Pratchayasakula, Dr. Sasiwan Kerdphoo, and Dr. Nanthip Prathumsup at the Cardiac Electrophysiology Research and Training Center, Faculty of Medicine, Chiang Mai University. Their commendable involvement and enduring support have played a pivotal role in this research. I am truly grateful for the abundant opportunities to advance my research and for their expert guidance in the laboratory. I also wish to express my sincere thanks to the Division of General Dentistry, Department of Family and Community Dentistry, Chiang Mai University Faculty of Dentistry, for granting me the platform to pursue progressive studies.

Lastly, I would be remiss not to acknowledge the unwavering support of my family, especially my parents, my friends, and my partner, Jordi van der Schaft. Their unwavering belief in me has been a driving force of motivation and encouragement throughout this journey. Additionally, I extend my appreciation to my furry companions for providing solace and unwavering emotional support.

Sincerely,

Benyanoot Kingkaew

Funding Sources

The Distinguished Research Professor Grant from the National Research Council of Thailand (SCC), the NSTDA Research Chair grant from the National Science and Technology Development Agency Thailand (NC), and the Chiang Mai University Center of Excellence Award (NC)

References

1. Ma Z, Li L, Livingston MJ, Zhang D, Mi Q, Zhang M, *et al.* p53/microRNA-214/ULK1 axis impairs renal tubular autophagy in diabetic kidney disease. ***J Clin Invest.*** 2020Sep;130(9):5011-5026.doi:10.1172/JCI135536.
2. Pratchayasakul W, Thongnak LO, Chattipakorn K, Lungaphin A, Pongchaidecha A, Satjaritanun P, *et al.* Atorvastatin and insulin equally mitigate brain pathology in diabetic rats. ***Toxicol Appl Pharmacol.*** 2018 Mar;342:79-85. doi: 10.1016/j.taap.2018.01.021.
3. Nakaya H, Takeda Y, Tohse N, Kanno M. Mechanism of the membrane depolarization induced by oxidative stress in guinea-pig ventricular cells. ***J Mol Cell Cardiol.*** 1992 May;24(5):523-534. doi: 10.1016/0022-2828(92)91841-r.
4. Peng TI, Jou MJ. Mitochondrial swelling and generation of reactive oxygen species induced by photoirradiation are heterogeneously distributed. ***Ann N Y Acad Sci.*** 2004 Apr;1011:112-122. doi: 10.1007/978-3-662-41088-2_12.
5. Murphy MP. Mitochondrial dysfunction indirectly elevates ROS production by the endoplasmic reticulum. ***Cell Metab.*** 2013 Aug;18(2):145-146. doi: 10.1016/j.cmet.2013.07.006.

6. Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. ***Curr Biol.*** 2014 May;24(10): R453-462. doi: 10.1016/j.cub.2014.03.034.
7. Edblad E, Lundin SA, Sjödin B, Aman J. Caries and salivary status in young adults with type 1 diabetes. ***Swed Dent J.*** 2001;25(2):53-60.
8. Moore PA, Guggenheimer J, Etzel KR, Weyant RJ, Orchard T. Type 1 diabetes mellitus, xerostomia, and salivary flow rates. ***Oral Surg Oral Med Oral Pathol Oral Radiol Endod.*** 2001 Sep;92(3):281-291. doi: 10.1067/moe.2001.117815.
9. Alves C, Menezes R, Brandão M. Salivary flow and dental caries in Brazilian youth with type 1 diabetes mellitus. ***Indian J Dent Res.*** 2012 Nov-Dec;23(6): 758-762. doi: 10.4103/0970-9290.111254.
10. Knaś M, Maciejczyk M, Daniszewska I, Klimiuk A, Matczuk J, Kołodziej U, *et al.* Oxidative damage to the salivary glands of rats with Streptozotocin-induced diabetes-temporal study: oxidative stress and diabetic salivary glands. ***J Diabetes Res.*** 2016;2016:4583742. doi: 10.1155/2016/4583742.
11. Xu L, Yang X, Chen J, Ge X, Qin Q, Zhu H, *et al.* Simvastatin attenuates radiation-induced salivary gland dysfunction in mice. ***Drug Des Devel Ther.*** 2016 Jul;10:2271-2278. doi: 10.2147/DDDT.S105809.
12. Herzog RI, Sherwin RS, Rothman DL. Insulin-induced hypoglycemia and its effect on the brain: unraveling metabolism by in vivo nuclear magnetic resonance. ***Diabetes.*** 2011 Jul;60(7):1856-1858. doi: 10.2337/db11-0498.
13. McCall AL. Insulin therapy and hypoglycemia. ***Endocrinol Metab Clin North Am.*** 2012 Mar;41(1):57-87. doi: 10.1016/j.ecl.2012.03.001.
14. Heller SR, Peyrot M, Oates SK, Taylor AD. Hypoglycemia in patient with type 2 diabetes treated with insulin: it can happen. ***BMJ Open Diabetes Res Care.*** 2020 Jun;8(1):e001194. doi: 10.1136/bmjdrc-2020-001194.
15. Alotaibi A, Sultan BA, Buzeid R, Almutairi M, Alghamdi E, Aldhaeefi M, *et al.* An overview of insulin therapy in pharmacotherapy of diabetes mellitus type I. ***Int J Community Med Public Health.*** *2018 Mar;5(3):834-838.* doi: 10.18203/2394-6040.ijcmph20180418.
16. Hatting M, Tavares CDJ, Sharabi K, Rines AK, Puigserver P. Insulin regulation of gluconeogenesis. ***Ann N Y Acad Sci.*** 2018 Jan;1411(1):21-35. doi: 10.1111/nyas.13435.
17. Parida S, Swain TR, Routray SN, Maiti R. Effect of atorvastatin on glycaemic parameters in normoglycaemic and prediabetic subjects: A prospective, panel study. ***J Clin Diagn Res.*** 2017 Feb;11(2):FC04-FC09. doi: 10.7860/JCDR/2017/23741.9427.
18. Magkos F, Fraterrigo G, Yoshino J, Luecking C, Kirbach K, Kelly SC, *et al.* Effects of moderate and subsequent progressive weight loss on metabolic function and adipose tissue biology in humans with obesity. ***Cell Metab.*** 2016 Apr;23(4):591-601. doi: 10.1016/j.cmet.2016.02.005.
19. Russell-Jones D, Khan R. Insulin-associated weight gain in diabetes--causes, effects and coping strategies. ***Diabetes Obes Metab.*** 2007 Nov;9(6):799-812. doi: 10.1111/j.1463-1326.2006.00686.x.
20. Anyanwagu U, Mamza J, Donnelly R, Idris I. Effects of background statin therapy on glycemic response and cardiovascular events following initiation of insulin therapy in type 2 diabetes: a large UK cohort study. ***Cardiovasc Diabetol.*** 2017 Aug;16(1):107. doi: 10.1186/s12933-017-0587-6.
21. Kawahito S, Kitahata H, Oshita S. Problems associated with glucose toxicity: role of hyperglycemia-induced oxidative stress. ***World J Gastroenterol.*** 2009 Sep;15(33):4137-4142. doi: 10.3748/wjg.15.4137.
22. Yan LJ. Pathogenesis of chronic hyperglycemia: from reductive stress to oxidative stress. ***J Diabetes Res.*** 2014;2014:137919. doi: 10.1155/2014/137919.
23. Fleury C, Mignotte B, Vayssière JL. Mitochondrial reactive oxygen species in cell death signaling. ***Biochimie.*** 2002 Feb-Mar;84(2-3):131-141. doi: 10.1016/s0300-9084(02)01369-x.
24. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. ***Apoptosis.*** 2007 May; 12(5):913-922. doi: 10.1007/s10495-007-0756-2.
25. Kamogashira T, Fujimoto C, Yamasoba T. Reactive oxygen species, apoptosis, and mitochondrial dysfunction in hearing loss. ***BioMed Res Int.*** 2015;2015:617207. doi: 10.1155/2015/617207.
26. Hancock JT, Desikan R, Neill SJ. Role of reactive oxygen species in cell signalling pathways. ***Biochem Soc Trans.*** 2001 May;29(Pt 2):345-350. doi: 10.1042/0300-5127:0290345.
27. Bokoch GM, Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. ***Blood.*** 2002 Oct;100(8):2692-2696. doi: 10.1182/blood-2002-04-1149.
28. Cherian DA, Peter T, Narayanan A, Madhavan SS, Achammada S, Vynat GP. Malondialdehyde as a marker of oxidative stress in periodontitis patients. ***J Pharm Bioallied Sci.*** 2019 May;11(Suppl 2):S297-S300. doi: 10.4103/JPBS.JPBS_17_19.