Original Article

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Effects of proprotein convertase subtilisin/kexin type9 inhibitors on salivary glands of obese rats induced by high-fat diet consumption

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Background and Objective(s): Obesity could alter cellular metabolism, leading to insulin resistance and increased vulnerability to oxidative stress, inflammation, and impairment of peripheral organs. Atorvastatin and proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors have been approved as lipid-lowering agents for patients with dyslipidemia and have shown potential in enhancing metabolic, brain, and cognitive functions in obese patients. However, the impact of atorvastatin and PCSK9 inhibitors on salivary glands in an obesity model is still insufficiently explored.
Material and Methods: Twenty-four female Wistar rats (*Rattus norvegicus*) were divided into two groups: those fed a normal diet (ND) and those fed a high-fat diet (HFD), for 16 weeks. During the final four weeks of the study, ND-fed rats received subcutaneous injections of normal saline solution (NDV), while HFD-fed rats were further subdivided into three treatment groups: normal saline solution (HFV), atorvastatin (40 mg/kg/day; HFA), and PCSK9 inhibitor (4 mg/kg/day; HFP). Submandibular salivary glands were removed to evaluate oxidative stress, inflammation, and apoptosis.
Results: Consumption of the HFD led to obesity and damage to the salivary glands, evidenced by increased inflammation and reduced anti-apoptotic expression. Conversely, treatment with atorvastatin and PCSK9 inhibitors improved salivary gland conditions in obese rats, as shown by decreased inflammation and enhanced anti-apoptotic expression.
Conclusion: Atorvastatin and PCSK9 inhibitors prevented salivary gland injury associated with obesity.

Keywords: atorvastatin, high fat diet, obesity, proprotein convertase subtilisin/kexin type 9 inhibitors, salivary glands

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Introduction

The epidemic of obesity is currently a major challenge in healthcare and chronic disease prevention worldwide [1]. Obesity is associated with an increased risk of multiple chronic diseases, including diabetes mellitus, dyslipidemia, coronary artery disease, cognitive dysfunction, and nonalcoholic fatty liver disease [2, 3]. Because of its impact on metabolism and insulin resistance, obesity could also lead to susceptibility to oxidative stress, inflammation, and impaired peripheral organs, such as the brain, heart, and salivary glands [4-6].

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Salivary glands are exocrine glands responsible for secreting saliva into the oral cavity. Saliva plays a crucial role in oral cavity function and maintenance, including lubricating food, hydrating oral tissues, protecting the buccal mucosa and teeth, and promoting speech and taste sensitivity. Moreover, saliva possesses antibacterial properties [7, 8].

Previous studies have shown that consuming a high-fat diet (HFD) for 12-16 weeks could cause salivary gland dysfunction, evidenced by increased oxidative stress, impaired mitochondrial function, inflammatory alterations, and cellular apoptosis [5]. Additionally, obesity altered the morphology and function of salivary glands, resulting in a decrease in glandular mass, an increase in granule size, and a decrease in mitochondrial size [9].

Atorvastatin is a lipid-lowering medication used to treat hyperlipidemia and prevent cardiovascular disease in patients with type II diabetes mellitus [10]. By inhibiting the activity of the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase enzyme responsible for cholesterol biosynthesis in the liver, atorvastatin significantly reduces low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) levels in the bloodstream [11]. Several studies demonstrated that atorvastatin had beneficial effects on cardiac, pancreatic, and renal function in obese models [4, 12, 13]. However, it is essential to note that atorvastatin may have adverse effects, including myopathy, rhabdomyolysis, and abnormalities in liver function tests [14]. On the other hand, proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, a new class of lipid-lowering drugs, have been approved as effective therapeutic agents for dyslipidemia patients, and their use may offer an alternative therapeutic approach to enhance metabolic, brain, and cognitive functions in obese patients [15, 16]. Nevertheless, the impact of atorvastatin and PCSK9 inhibitors on salivary gland conditions in obese rats has not been investigated. Thus, the present study hypothesizes that treatment with atorvastatin and PCSK9 inhibitors can improve salivary gland conditions in obese rats, particularly concerning inflammation and apoptosis.

Materials and Methods

Ethical approval and animal preparation

All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the NIH and approved by the Laboratory Animal Center, Chiang Mai University, and the Animal Care and Use Committee, Chiang Mai University (approval no. 2561/RT-0002 3rd extension).

Twenty-four female Wistar rats (weighing 200-220 g) were obtained from the National Animal Center, Salaya Campus, Mahidol University, Bangkok, Thailand. They were acclimated under temperature-controlled conditions with a light-dark cycle of 12:12 hours for one week. After the acclimation period, the rats were randomly divided into two groups: the normal diet-fed group (ND; n=6) and the high-fat diet-fed group (HFD; n=18).

Experimental protocol

The ND-fed group received standard laboratory feed (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand) containing 19.77% energy from fat for 16 weeks, whereas the HFD-fed group was given a HFD containing 59.28% energy from fat.

At week 13, all groups underwent the collection of metabolic data. Subsequently, ND-fed rats were administered normal saline solution (NSS) as a vehicle (NDV; n=6), while HFD-fed rats were further subdivided into three groups, to receive either normal saline solution (NSS) as a vehicle (HFV; n=6), atorvastatin (HFA; n=6; 40 mg/kg/day of atorvastatin, Sandoz Company, Ljubljana, Slovenia), or PCSK9 inhibitor (HFP; n=6; 4 mg/kg/day of

PCSK9 inhibitor, SBC115076, Selleck chemicals, Texas, USA) through subcutaneous injections for a duration of 4 weeks. After week 16, blood samples were collected from the tail vein for metabolic analysis. Subsequently, the rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and euthanized. The submandibular glands were excised and stored at -80°C for subsequent analysis of oxidative stress, inflammation, and apoptosis.

Assessment of metabolic marker

Plasma glucose, total cholesterol, and triglyceride levels were measured using a colorimetric assay kit (ERBA Mannheim, Mannheim, Germany). Insulin level was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, Inc., St. Charles, MO, USA). HDL and LDL levels were measured using a colorimetric assay kit (Biovision, Inc., Milpitas, CA, USA).

Measurement of salivary gland Malondialdehyde (MDA) concentration

The Thiobarbituric Acid Reactive Substances Assay (TBARS Assay; MAK085 Lipid Peroxidation Assay Kit, Sigma-Aldrich Co. LLC, MO, USA) was used to determine the submandibular gland MDA level, an indicator of oxidative stress. Briefly, each salivary gland serum (20 µl) was combined with 500 µl of 42 mM sulfuric acid and 125 µl of phosphotungstic acid solution (PTA), followed by centrifugation at 13,000 rpm for 5 minutes at room temperature. After that, resuspended the serum pellet on ice with the 102 µl water/Butylated hydroxytoluene (BHT) solution, adjusted the sample volume to 200 µl with water, and then incubated at 37 °C for 2 hours. Subsequently, an MDA-TBA adduct was produced by combining 600 µl of TBA solution with 200 µl of MDA/serum, incubating at 95 °C for 1 hour, and then placing the mixture in an ice bath for 15 minutes. Each reaction mixture was pipetted into a 96-well plate with 200 µl and measured for absorbance at 532 nm. TBARS (Thiobarbituric acid reactive solution) concentration was calculated from the standard curve and reported as MDA concentration.

Salivary gland inflammation

Tumor Necrosis Factor-α (TNF-α) serves as a biomarker of inflammation, therefore the levels of TNF-α in the submandibular glands were quantified using the ELISA method, employing the rat TNF-α ELISA kit designed for cell and tissue lysates (Sigma-Aldrich Co. LLC, MO, USA).

Salivary gland apoptotic expression

Western blot analysis was used to evaluate the expression of apoptotic proteins. Fifty to 80 mg of total protein isolated from salivary gland proteins extracted with loading buffer (5% betamercaptoethanol, 0.05% bromophenol blue, 75 mMTris-HCl (pH 6.8), 2% SDS and 10% glycerol), and loaded onto 12.5% SDS-acrylamide gels. Then, the proteins were separated by electrophoresis and transferred on to nitrocellulose membranes as previously described [17]. Immunoblotting was conducted using the following antibodies: rabbit polyclonal anti-Bax (N-terminus) (1:1000 dilution, Millipore corporation, CA, USA), mouse monoclonal anti-Bcl-2 Clone AW604 (1:1000 dilution, EMD Millipore corporation, CA, USA), rabbit polyclonal anti-Caspase-3, active (cleaved) form (1:500 dilution, EMD Millipore corporation, CA, USA), rabbit polyclonal anti-Caspase-3 (1:1000 dilution, EMD Millipore corporation, CA, USA), and mouse monoclonal anti-actin (1:1000 dilution, Santa Cruz biotechnology, CA, USA). Bound antibodies were detected using horseradish peroxidase conjugated with either anti-rabbit IgG or anti-mouse IgG (1:2000 dilution, EMD Millipore corporation, CA, USA). Then, the membranes were visualized by ClarityTM 192 Western ECL Blotting Substrate (Bio-Rad Laboratories Ltd., CA, USA). The densitometric analysis was performed using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories, CA, USA).

Statistical analysis

IBM SPSS Statistics 24 software (IBM SPSS Science, Chicago, Illinois, USA) was used for statistical analysis. Data were presented as mean ± SE. One-way ANOVA followed by Fisher's least significant difference post hoc was performed to examine the difference between the groups. The statistical significance was considered at $p < 0.05$.

Results

Atorvastatin and PCSK9 inhibitor improved metabolic parameters in obese rats, induced by HFD consumption

In this study, HFD-fed rats exhibited the development of obesity over a 16-week dietary intervention period. This was evidenced by a substantial increase in parameters such as body weight, plasma glucose, plasma insulin, total cholesterol, LDL, and triglycerides when compared to NDV-fed rats, as detailed in Table 1. However, upon administration of medication, either atorvastatin or a PCSK9 inhibitor, a notable reduction was observed in body weight, plasma insulin, total cholesterol, and LDL levels in comparison to HFD-fed rats. Notably, the PCSK9 inhibitor exhibited a superior efficacy compared to atorvastatin in lowering body weight, total cholesterol, and LDL levels.

No variation in salivary oxidative stress was observed among the groups.

HFD-fed rats treated with the vehicle did not significantly enhance the concentration of salivary gland MDA compared to ND-fed rats treated with the vehicle, nor did it differ from HFD-fed rats treated with atorvastatin or PCSK9 inhibitor (Figure 1). These findings suggest that both atorvastatin and PCSK9 inhibitor led to similar salivary gland oxidative stress production.

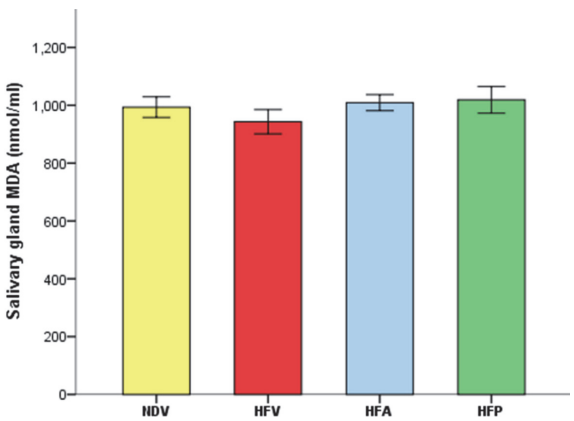

Figure 1 The effect of atorvastatin and PCSK9 inhibitor on salivary gland MDA of obese rats

Table 1 The effect of atorvastatin and PCSK9 inhibitor on metabolic parameters of HFD-fed rats

Parameters	NDV	HFV	HFA	HFP
Body weight (g)	250.58 ± 7.07	322.46 ± 8.48 *	292.27 ± 6.33 *,†	271.18 ± 4.41 †,‡
Plasma glucose (mg/dl)	138.64 ± 25.02	292.86 ± 7.99 *	250.48 ± 21.52 *	247.45 ± 14.93 *
Plasma insulin (mg/dl)	1.44 ± 0.64	7.98 ± 1.27 *	3.85 ± 1.27 †	3.24 ± 0.56 †
Total cholesterol (mg/dl)	115.31 ± 12.80	313.04 ± 30.67 *	234.35 ± 22.14 *,†	182.75 ± 18.75 *,†,‡
HDL (mg/dl)	29.16 ± 2.75	29.38 ± 2.87	29.34 ± 3.42	26.59 ± 2.19
LDL (mg/dl)	82.49 ± 14.46	268.54 ± 33.42 *	191.91 ± 21.59 *,†	98.53 ± 4.36 †,‡
Triglyceride (mg/dl)	36.20 ± 2.51	76.79 ± 10.00 *	72.73 ± 0.55 *	73.68 ± 4.74 *

NDV = ND-fed rats treated with vehicle; HFV = HFD-fed rats treated with vehicle;
HFA = HFD-fed rats treated with atorvastatin 40 mg/kg/day; HFP = HFD-fed rats treated with PCSK9 inhibitor 4mg/kg/day;
HDL = high density lipoprotein; LDL = low density lipoprotein
*p<0.05 compared with NDV; †p<0.05 compared with HFV; ‡p<0.05 compare with HFA

The effects of atorvastatin and PCSK9 inhibitor on inflammation marker in obese rats, induced by HFD consumption

This study investigated TNF-α concentration as an indicator of salivary gland inflammation. HFD-fed rats exhibited the significant increase in the TNF-α concentration, compared to NDV-fed rats. However, after the HFD-fed rats were treated with Atorvastatin for 4 weeks, the TNF-α concentration was significantly reduced compared to HFD-fed rats. On the other hand, the TNF-α concentrations of HFD-fed rats treated with a PCSK9 inhibitor tended to decrease, but they were not significantly different from those of HFD-fed rats (Figure 2).

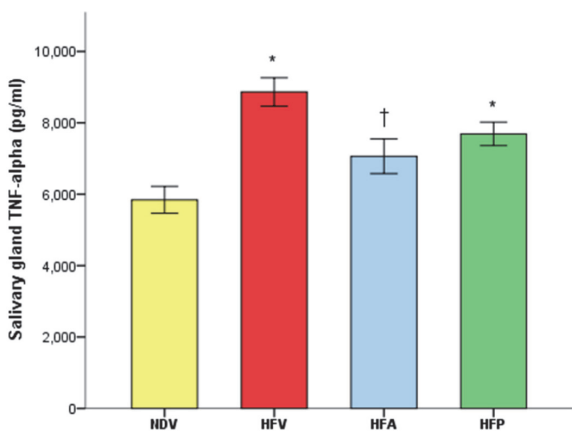

Figure 2 The effect of atorvastatin and PCSK9 inhibitor on salivary gland TNF-α of obese rats *p<0.05 compared with NDV; †p<0.05 compared with HFV

These findings indicated that atorvastatin and PCSK9 inhibitor could reduce inflammation in the salivary glands of HFD-fed rats.

The effects of atorvastatin and PCSK9 inhibitor on apoptosis marker in obese rats, induced by HFD consumption

The expression of pro-apoptotic markers, including caspase 3 and Bax, as well as an anti-apoptosis marker, Bcl-2, in the submandibular glands, was also evaluated. The results indicated that the expression of cleaved caspase 3/caspase 3 and Bax was not significantly different among the groups (Figure 3A and 3B). In contrast, the expression of Bcl-2 in the salivary gland of HFD-fed rats was significantly decreased compared to the NDV group. However, the HFD-fed rats treated with either atorvastatin or PCSK9 inhibitor showed a significant increase in Bcl-2 expression compared to the HFV group (Figure 3C). These findings demonstrate that atorvastatin and the PCSK9 inhibitor promoted anti-apoptotic activity against HFD-induced salivary gland injury.

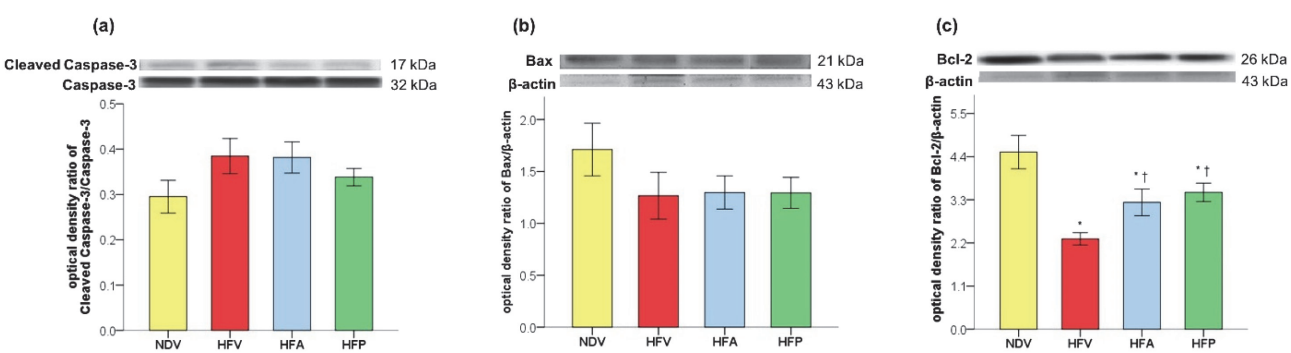

Figure 3 The effect of atorvastatin and PCSK9 inhibitor on salivary gland apoptotic markers of obese rats (a) Cleaved Caspase-3/Caspase-3 (b) Bax (c) Bcl-2 *p<0.05 compared with NDV; †p<0.05 compared with HFV

Discussion

This study demonstrates that 1) Chronic HFD consumption led to salivary gland damage indicated by an increase in inflammation level and a decrease in anti-apoptosis level. 2) Atorvastatin and PCSK9 inhibitors both alleviated metabolic disruption and reduced inflammation in the salivary gland of obese rats.

Excessive consumption of a high-fat diet leads to obesity, dyslipidemia, and peripheral insulin resistance [6]. This study also revealed that rats fed with HFD exhibited substantial increases in body weight, plasma glucose, plasma insulin, total cholesterol, LDL, and triglyceride levels, when compared to rats fed with ND. The current investigation exhibited a notable rise in plasma glucose levels among HFD-fed rats, in contrast to an alternate study that did not find such an increase [6, 17-19]. However, subsequent treatment with either atorvastatin or a PCSK9 inhibitor led to improvements in the metabolic parameters, with a significant reduction in plasma insulin levels being particularly noteworthy. A study indicated that atorvastatin not only improved dyslipidemia but also targeted insulin resistance by inhibiting sterol regulatory element binding protein-1 (SREBP-1) expression in the liver, subsequently leading to the inhibition of gluconeogenesis [20].

The current study illustrates that consuming of a high-fat diet resulted in inflammation of the salivary glands. This observation is corroborated by a separate study that revealed damage to the salivary glands at the cellular level in obese insulin-resistant rats. This damage was evidenced by elevated oxidative stress, inflammation, and apoptosis [21]. However, the level of oxidative stress observed in this study did not reach a point that would induce cell death. The expression of pro-apoptotic markers, Bax and cleaved caspase-3, did not exhibit significant differences. Interestingly, the expression of the anti-apoptotic marker, Bcl-2, showed a noteworthy decrease in HFD-fed rats. In contrast, treatment with either atorvastatin or a PCSK9 inhibitor not only enhanced peripheral insulin sensitivity but also averted salivary gland injury in the context of obesity. This pathway is recognized as the intrinsic pathway of apoptosis or mitochondrial-mediated apoptosis. Furthermore, oxidative stress triggers apoptosis via the extrinsic pathway of apoptosis or Fas ligand-mediated apoptosis [22, 23]. Hence, our findings suggest that both atorvastatin and PCSK9 inhibitor diminish apoptosis in obese rats through modulation of both intrinsic and extrinsic pathways.

Conclusion

The salivary glands of obese rats, induced by the consumption of the HFD, exhibited cellular-level damage, as evidenced by elevated expression of inflammatory and apoptosis-related proteins. Treatment with either atorvastatin or a PCSK9 inhibitor not only improved metabolic impairments but also reversed the injury to salivary glands in the context of obesity. Intriguingly, PCSK9 inhibitor showed a greater efficacy in restoring metabolic parameters compared to atorvastatin. However, atorvastatin therapy demonstrated both anti-inflammatory and anti-apoptotic effects, whereas PCSK9 inhibitor therapy primarily exerted an anti-apoptotic effect in mitigating salivary gland injury induced by the HFD.

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