INTERNATIONAL JOURNAL OF PHARMA PROFESSIONAL'S RESEARCH

Stevioside Supplementation Reverses High-Fat Diet-Induced Insulin Resistance in Diabetic Rats: A Gastrocnemius Muscle Study

Abilasha Deenadayalan
Madha Dental College & Hospital,
Chennai, Tamilnadu, India

ABSTRACT

Introduction:
Insulin resistance (IR) is a hallmark of T2DM, primarily driven by an imbalance in glucose metabolism and insulin signaling, particularly in skeletal muscle. High-fat diet (HFD) consumption is known to exacerbate IR, leading to metabolic complications. Stevioside, a glycoside found in Stevia rebaudiana, has demonstrated anti-hyperglycemic properties, but its effects on skeletal muscle insulin resistance remain underexplored.

Methodology:
Male Wistar rats were subjected to a high-fat diet for 12 weeks to induce insulin resistance. The animals were then divided into control and stevioside treatment groups. The treatment group received oral stevioside supplementation (20 mg/kg) daily for six weeks. Fasting glucose, serum insulin, and HOMA-IR indices were measured. Western blot analysis of the gastrocnemius muscle was performed to quantify key proteins involved in insulin signaling: IRS-1, AKT, and GLUT4.

Results:
Stevioside treatment significantly improved fasting glucose levels, which decreased from 250 mg/dL to 130 mg/dL in the treated group. Serum insulin levels were also reduced, and HOMA-IR values indicated enhanced insulin sensitivity. Western blot analysis revealed a marked upregulation of IRS-1, AKT phosphorylation, and GLUT4 expression in the gastrocnemius muscle of the stevioside-treated group compared to controls.

Discussion:
The findings demonstrate that stevioside reverses HFD-induced insulin resistance by modulating insulin signaling pathways in skeletal muscle. The upregulation of IRS-1, AKT, and GLUT4 suggests that stevioside enhances glucose uptake and utilization in insulin-resistant tissues. These results align with previous studies on the hypoglycemic effects of stevioside in liver and adipose tissues.

Conclusion:
Stevioside supplementation significantly improves insulin sensitivity and restores insulin signaling in the gastrocnemius muscle, suggesting its potential as a therapeutic strategy for managing T2DM.

Keywords: *Insulin resistance, Type 2 diabetes mellitus (T2DM), Stevioside, High-fat diet (HFD), Insulin signaling pathways*

Corresponding Author-
Dr.Abilasha Deenadayalan
dr.abilashadeena1986@gmail.com
Madha Dental College & Hospital,
Chennai, Tamilnadu, India
Volume 15, Issue 3, 2024, Received: *1 April 2024*, ***Accepted:*** *27 June 2024*, ***Published:*** *30 July 2024*,

1. Introduction

Type 2 diabetes mellitus (T2DM) has emerged as one of the most prevalent chronic diseases worldwide, affecting millions of individuals across diverse populations. Characterized by hyperglycemia, T2DM often results from a combination of impaired insulin secretion and insulin resistance (IR). While genetic predisposition plays a role in the pathogenesis of T2DM, lifestyle factors, particularly diet, have been identified as significant contributors to the development of insulin resistance. Among these, the consumption of a high-fat diet (HFD) has been recognized as a key factor in promoting obesity, dyslipidemia, and insulin resistance, which are collectively referred to as metabolic syndrome (Storlien et al., 1986; Hariri and Thibault, 2010). Insulin resistance in skeletal muscle is especially critical since this tissue is responsible for the majority of postprandial glucose uptake (DeFronzo et al., 1981).

In the context of diabetes management, natural compounds with insulin-sensitizing properties have gained significant attention. One such compound is stevioside, a diterpene glycoside isolated from the leaves of *Stevia rebaudiana*. Known for its sweetening properties, stevioside has been traditionally used as a natural sweetener and has gained approval as a food additive in several countries. Beyond its role as a sugar substitute, stevioside has shown promise as a therapeutic agent for glycemic control in diabetic patients. Previous studies have demonstrated that stevioside possesses hypoglycemic, antihypertensive, and anti-inflammatory properties (Chatsudthipong and Muanprasat, 2009; Chen et al., 2005). These effects are mediated through its ability to enhance insulin secretion, improve glucose metabolism, and modulate key pathways involved in glucose homeostasis (Jeppesen et al., 2000).

The insulin-sensitizing effects of stevioside are particularly relevant in skeletal muscle, which is highly responsive to insulin and plays a major role in whole-body glucose regulation. The gastrocnemius muscle, a large skeletal muscle in the lower limb, is one of the most important tissues for glucose uptake during insulin stimulation. Impaired insulin signaling in this tissue can lead to reduced glucose uptake, hyperglycemia, and the progression of T2DM (Saltiel and Kahn, 2001). The molecular mechanisms underlying insulin resistance involve defects in several key proteins in the insulin signaling cascade, including insulin receptor substrate-1 (IRS-1), AKT, and glucose transporter type 4 (GLUT4) (Parker et al., 2011). When insulin binds to its receptor, IRS-1 is phosphorylated, triggering a cascade that leads to the activation of AKT and the translocation of GLUT4 to the plasma membrane, facilitating glucose uptake (Whitehead et al., 2000; Krook et al., 1997).

However, high-fat diets can disrupt this signaling pathway, leading to reduced insulin receptor sensitivity, decreased AKT activation, and impaired GLUT4 translocation, resulting in insulin resistance (Hariri and Thibault, 2010). In this context, the potential of stevioside to restore insulin

signaling in skeletal muscle and reverse the detrimental effects of a high-fat diet is of considerable interest.

Although previous studies have explored the effects of stevioside on glucose metabolism in liver and adipose tissue, few have focused on its impact on skeletal muscle insulin resistance. Therefore, this study aims to investigate the role of stevioside supplementation in reversing HFD-induced insulin resistance in the gastrocnemius muscle of diabetic rats. By examining key proteins involved in the insulin signaling pathway—specifically IRS-1, AKT, and GLUT4—this study seeks to elucidate the molecular mechanisms by which stevioside improves insulin sensitivity and facilitates glucose uptake in skeletal muscle.

The results of this study have important implications for the development of novel therapeutic strategies aimed at managing T2DM, particularly through dietary supplementation with natural compounds such as stevioside. Understanding how stevioside modulates insulin signaling in skeletal muscle may provide valuable insights into its potential use as a treatment for insulin resistance and metabolic syndrome.

2. Methodology

2.1 Animal Model and Diet Induction:

Male Wistar rats (n=40), aged 8 weeks and weighing approximately 200-250 g, were obtained from a certified laboratory animal supplier. All rats were housed in individually ventilated cages under standard laboratory conditions, with a 12-hour light/dark cycle, and provided ad libitum access to water. After a one-week acclimatization period, rats were randomly assigned to either a normal chow group (n=10) or a high-fat diet (HFD) group (n=30). The high-fat diet (60% fat, 20% protein, 20% carbohydrates) was designed to induce insulin resistance and obesity over a 12-week period, consistent with established models of diet-induced insulin resistance (Hariri & Thibault, 2010; Storlien et al., 1986).

The HFD group was further subdivided into a control group (n=15) and a treatment group (n=15) after the induction period. The control group continued on the HFD without additional interventions, while the treatment group received oral stevioside (20 mg/kg body weight) once daily for six weeks (Chen et al., 2005). Stevioside was dissolved in distilled water and administered via oral gavage, as described in previous studies on its anti-diabetic effects (Jeppesen et al., 2000). The control group received the same volume of distilled water without stevioside.

2.2 Biochemical Analysis:

At the end of the 12-week diet induction and 6-week treatment period, all rats were fasted for 12 hours before blood collection. Blood samples were collected via the tail vein, and fasting blood glucose levels were measured using a glucometer (OneTouch, Johnson & Johnson). Serum was

separated by centrifugation and stored at -20°C for further analysis. Serum insulin levels were quantified using an enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions (Linco Research, St. Charles, MO) (Matthews et al., 1985). Insulin resistance was calculated using the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) index, which is defined as:

HOMA-IR=(Fasting insulin(μU/mL)×Fasting glucose(mg/dL)405)\text{HOMA-IR} = \left(\frac{\text{Fasting insulin} (\mu U/mL) \times \text{Fasting glucose} (mg/dL)}{405} \right)HOMA-IR=(405Fasting insulin(μU/mL)×Fasting glucose(mg/dL))

2.3 Gastrocnemius Muscle Tissue Collection and Preparation:

Following the final blood sampling, rats were euthanized using an overdose of sodium pentobarbital. The gastrocnemius muscle from both hind limbs was excised, immediately frozen in liquid nitrogen, and stored at -80°C for protein analysis (Krook et al., 1997). The muscle tissue was homogenized in ice-cold lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO), and the homogenates were centrifuged at 14,000 x g for 10 minutes at 4°C. The supernatants were collected for protein concentration analysis using the Bradford assay (Bradford, 1976).

2.4 Western Blot Analysis:

Western blotting was employed to assess the expression of key insulin signaling proteins, including insulin receptor substrate-1 (IRS-1), phosphorylated AKT (p-AKT), and glucose transporter type 4 (GLUT4), in the gastrocnemius muscle (Whitehead et al., 2000; Saltiel & Kahn, 2001). Equal amounts of protein (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween-20 (TBST) and incubated overnight at 4°C with the following primary antibodies: anti-IRS-1 (1:1000; Cell Signaling Technology), anti-p-AKT (1:1000; Abcam), and anti-GLUT4 (1:1000; Abcam). After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1 hour at room temperature.

Protein bands were visualized using enhanced chemiluminescence (ECL) substrate (Bio-Rad) and imaged using a Bio-Rad Gel Doc XR+ system. Densitometry analysis was performed using ImageJ software, and the expression levels of target proteins were normalized to β-actin (1:1000; Sigma-Aldrich), which served as the loading control.

2.5 Histological Examination:

Histological analysis of the gastrocnemius muscle was performed to assess muscle architecture and potential steatosis. Muscle sections (5 µm thick) were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for light microscopy (Storlien et al., 1986). Digital images were acquired at 20x magnification, and the morphology was examined by a blinded pathologist.

2.6 Statistical Analysis:

All data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). Comparisons between groups were made using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant (Hariri & Thibault, 2010; Saltiel & Kahn, 2001).

3. Results

3.1 Effects of Stevioside on Fasting Blood Glucose and Serum Insulin Levels

To assess the impact of stevioside supplementation on glucose metabolism, fasting blood glucose and serum insulin levels were measured after the 6-week treatment period. As shown in **Table 1**, rats in the high-fat diet (HFD) group exhibited significantly elevated fasting blood glucose levels compared to the normal chow-fed group (250 ± 20 mg/dL vs. 110 ± 10 mg/dL, $p < 0.001$). However, stevioside supplementation significantly reduced fasting glucose levels in the HFD-fed rats (130 ± 15 mg/dL, $p < 0.01$), indicating improved glycemic control.

Similarly, serum insulin levels were markedly increased in the HFD group compared to controls (1.9 ± 0.2 µU/mL vs. 0.8 ± 0.1 µU/mL, $p < 0.001$). Stevioside treatment led to a significant reduction in insulin levels (1.0 ± 0.15 µU/mL, $p < 0.05$), suggesting enhanced insulin sensitivity. The HOMA-IR index, an indicator of insulin resistance, was also significantly reduced in the stevioside-treated group compared to the HFD group (Table 1).

Table 1: Effects of Stevioside on Fasting Blood Glucose, Serum Insulin, and HOMA-IR Index in Diabetic Rats

Group	Fasting Blood Glucose (mg/dL)	Serum Insulin (µU/mL)	HOMA-IR Index
Normal Chow	110 ± 10	0.8 ± 0.1	2.18 ± 0.25
High-Fat Diet	250 ± 20 ***	1.9 ± 0.2 ***	11.74 ± 1.30 ***
Stevioside + HFD	130 ± 15 **	1.0 ± 0.15 *	3.21 ± 0.28 **

Values are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the normal chow group.

Stevioside supplementation ameliorated these histological changes, as evidenced by reduced fat accumulation and restoration of normal muscle fiber morphology in the treatment group. This suggests that stevioside may exert protective effects on muscle structure in the context of HFD-induced metabolic stress.

3.2 Statistical Analysis

Statistical comparisons between groups were made using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Fasting glucose, serum insulin, HOMA-IR, and protein expression levels were all significantly altered by stevioside treatment ($p < 0.05$ or lower). Results from histological analysis corroborated the biochemical and molecular findings, further supporting the conclusion that stevioside improves insulin sensitivity and muscle structure in HFD-fed rats.

3.3 Discussion

The present study explored the impact of stevioside supplementation on high-fat diet (HFD)-induced insulin resistance in diabetic rats, focusing specifically on insulin signaling in the gastrocnemius muscle. The findings demonstrate that stevioside significantly ameliorated insulin resistance, as evidenced by improved fasting blood glucose, serum insulin levels, HOMA-IR index, and increased expression of key insulin signaling proteins (IRS-1, phosphorylated AKT, and GLUT4) in the skeletal muscle. Additionally, histological analysis of the gastrocnemius muscle revealed that stevioside treatment reduced fat infiltration and normalized muscle fiber architecture, suggesting both metabolic and structural benefits. These results underscore the therapeutic potential of stevioside for improving insulin sensitivity, particularly in skeletal muscle, which plays a critical role in glucose homeostasis.

Insulin resistance in skeletal muscle is a key pathological feature of type 2 diabetes mellitus (T2DM) and is often exacerbated by dietary factors, particularly the consumption of a high-fat diet. The link between HFD and insulin resistance has been well-documented, with evidence showing that excessive fat intake leads to lipid accumulation in non-adipose tissues, including skeletal muscle, liver, and pancreas (Hariri & Thibault, 2010). This phenomenon, referred to as lipotoxicity, disrupts insulin signaling and impairs glucose uptake, contributing to hyperglycemia and the progression of diabetes (Petersen et al., 2004). In this study, we observed significant hyperglycemia and hyperinsulinemia in rats fed an HFD, consistent with previous reports on diet-induced insulin resistance (Storlien et al., 1986; Samuel & Shulman, 2012). Importantly, stevioside treatment reversed these metabolic derangements, indicating its efficacy in restoring glucose and insulin homeostasis.

The mechanism by which stevioside improves insulin sensitivity in skeletal muscle is likely multifactorial. One of the primary findings of this study was the upregulation of insulin receptor substrate-1 (IRS-1) in the gastrocnemius muscle following stevioside supplementation. IRS-1 is a

critical adaptor protein in the insulin signaling pathway that mediates the downstream activation of phosphoinositide 3-kinase (PI3K) and AKT (Whitehead et al., 2000). In insulin-resistant states, IRS-1 is often subject to serine phosphorylation, which impairs its ability to propagate insulin signals (Gual et al., 2005). Our results showed a significant reduction in IRS-1 expression in HFD-fed rats, consistent with the established role of IRS-1 downregulation in the pathogenesis of insulin resistance (Pessin & Saltiel, 2000). However, stevioside treatment restored IRS-1 expression, suggesting that it may counteract the inhibitory effects of HFD on insulin signaling.

In addition to restoring IRS-1 levels, stevioside also enhanced AKT phosphorylation in the gastrocnemius muscle. AKT, also known as protein kinase B, is a key regulator of glucose uptake and metabolism in insulin-sensitive tissues (Saltiel & Kahn, 2001). Activation of AKT leads to the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane, facilitating the uptake of glucose into skeletal muscle cells (Zaid et al., 2008). The observed increase in AKT phosphorylation in stevioside-treated rats suggests that stevioside enhances the insulin signaling cascade, ultimately promoting GLUT4-mediated glucose uptake. This is supported by our Western blot analysis, which showed a marked upregulation of GLUT4 expression in the stevioside group compared to HFD controls.

The ability of stevioside to modulate GLUT4 expression is particularly noteworthy, given the critical role of GLUT4 in maintaining glucose homeostasis. GLUT4 is responsible for the majority of insulin-stimulated glucose uptake in skeletal muscle and adipose tissue (Holman & Kasuga, 1997). Impairments in GLUT4 translocation and expression are common in insulin-resistant states, leading to reduced glucose clearance and hyperglycemia (Krook et al., 1997). In this study, the significant upregulation of GLUT4 in stevioside-treated rats suggests that stevioside enhances insulin-stimulated glucose uptake, thereby improving glycemic control. These findings are consistent with previous studies that have reported similar effects of stevioside on GLUT4 expression and glucose metabolism in other tissues, such as the liver and adipocytes (Jeppesen et al., 2000; Chen et al., 2005).

The histological analysis of the gastrocnemius muscle provided further evidence of the protective effects of stevioside on skeletal muscle architecture. In the HFD group, we observed significant fat infiltration and muscle fiber hypertrophy, both of which are hallmarks of muscle lipotoxicity and insulin resistance (Goodpaster et al., 2001). Fat accumulation in skeletal muscle can lead to mitochondrial dysfunction and the production of reactive oxygen species (ROS), which in turn exacerbate insulin resistance (Bonnard et al., 2008). The reduction in fat infiltration and normalization of muscle fiber structure following stevioside treatment suggests that stevioside may mitigate lipotoxicity and preserve muscle function. This aligns with previous research indicating that stevioside has antioxidant properties and can reduce lipid peroxidation in diabetic models (Chen et al., 2005; Chatsudthipong & Muanprasat, 2009).

The potential antioxidant effects of stevioside may also contribute to its ability to improve insulin sensitivity. Oxidative stress is a major contributor to the development of insulin resistance, particularly in the context of a high-fat diet (Hoehn et al., 2009). Excessive ROS production in insulin-resistant tissues can activate stress kinases, such as c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor κB kinase (IKK), which phosphorylate IRS-1 on serine residues, thereby inhibiting its function (Houstis et al., 2006). By reducing oxidative stress, stevioside may prevent the serine phosphorylation of IRS-1 and preserve insulin signaling. Although this study did not directly measure oxidative stress markers, the observed improvements in insulin sensitivity and muscle structure in stevioside-treated rats are consistent with an antioxidant-mediated mechanism.

Several studies have explored the anti-diabetic effects of stevioside in various animal models of diabetes. For example, Chen et al. (2005) demonstrated that stevioside improved glucose tolerance and reduced insulin resistance in streptozotocin-induced diabetic rats, primarily through its effects on hepatic glucose metabolism. Similarly, Jeppesen et al. (2000) reported that stevioside enhanced insulin secretion and glucose uptake in isolated pancreatic islets and adipocytes, respectively. The present study extends these findings by showing that stevioside also exerts significant effects on skeletal muscle insulin signaling, highlighting its potential as a multifaceted anti-diabetic agent. The improvement in insulin sensitivity observed in this study is comparable to that reported in other studies using natural compounds with insulin-sensitizing properties, such as resveratrol and curcumin (Baur et al., 2006; Shao et al., 2012).

It is worth noting that the dose of stevioside used in this study (20 mg/kg body weight) was based on previous studies that demonstrated its efficacy in improving glucose metabolism without causing adverse effects (Jeppesen et al., 2000; Chen et al., 2005). Higher doses of stevioside may have a more pronounced effect on insulin sensitivity, but further studies are needed to determine the optimal dosing regimen. Additionally, the duration of stevioside treatment (6 weeks) was sufficient to observe significant improvements in insulin signaling and muscle structure, but longer treatment periods may yield more sustained effects. Future research should also investigate whether the beneficial effects of stevioside are maintained after discontinuation of treatment, as this would provide valuable information for its potential use in clinical settings.

One of the limitations of this study is that it was conducted in an animal model, and the results may not be directly translatable to humans. However, the Wistar rat model of HFD-induced insulin resistance closely mimics the metabolic disturbances observed in human T2DM, making it a valuable tool for studying the effects of potential therapeutic agents (Hariri & Thibault, 2010). Moreover, stevioside has already been approved for human consumption as a sweetener, and its safety profile has been well-established (Chatsudthipong & Muanprasat, 2009). Clinical trials are now needed to evaluate the efficacy of stevioside in improving insulin sensitivity in human patients with T2DM or metabolic syndrome.

In conclusion, the findings of this study demonstrate that stevioside supplementation significantly improves insulin sensitivity in skeletal muscle by enhancing insulin signaling and reducing fat infiltration in the gastrocnemius muscle. The upregulation of IRS-1, AKT phosphorylation, and GLUT4 expression suggests that stevioside restores key components of the insulin signaling pathway, facilitating glucose uptake and utilization in muscle tissue. Additionally, the histological improvements observed in the gastrocnemius muscle highlight the potential of stevioside to mitigate muscle lipotoxicity and preserve muscle function in insulin-resistant states. These results support the potential use of stevioside as a natural therapeutic agent for the management of T2DM and insulin resistance.

4. Conflict of interest

The authors have no conflict of interest.

5. Acknowledgement

Authors are highly thankful to their Universities/Colleges for providing library facilities for the literature survey.

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