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# ORIGINAL ARTICLE

# Hypoxic mesenchymal stem cell-derived secretome and alkaline water synergistically reduce apoptosis and insulin resistance in type 2 diabetes mellitus rat model

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## ABSTRACT

**BACKGROUND**

Type 2 Diabetes Mellitus (T2DM) is characterized by chronic inflammation and insulin resistance. These factors contribute to pancreatic β-cell apoptosis, reducing insulin production and impairing glucose homeostasis. This study aims to evaluate the protective effects of hypoxic mesenchymal stem cell-derived secretome (HMSCS) and alkaline water on inflammation, apoptosis, and insulin resistance in a T2DM rat model.

**METHODS**

An experimental study was conducted involving 24 male Wistar T2DM model rats (aged 6-8 weeks, 200-250g). They were randomized into four groups: T2DM rats only as negative control (K-), T2DM rats with metformin as positive control (K+), HMSCS treatment (P1), and HMSCS plus alkaline water group (P2). Caspase-3 expression was measured to assess apoptosis levels using RT-PCR, while homeostatic model assessment for insulin resistance (HOMA-IR) was measured using ELISA. One way ANOVA followed by a post hoc LSD test were used to analyse the data.

**RESULTS**

The P1 group (3.03 ± 1.26 a.u) and P2 group (2.93 ± 0.52 a.u.) had significantly lower caspase-3 expression compared to K- group (6.66 ± 2.76 a.u.) ($p<0.05$), but were not significantly different from K+ group (3.83 ± 1.61 a.u.) ($p>0.05$). Additionally, P2 group (6.76 ± 0.96) had a significantly lower HOMA-IR than K- group (18.92 ± 2.63) and K+ group (10.85 ± 1.39) ($p<0.05$), and similarly the P1 (7.71 ± 0.53) group also showed significant difference from K- and K+ groups ($p<0.05$).

**CONCLUSION**

Higher doses of HMSCS and alkaline water are associated with reduced pancreatic β-cell apoptosis and improved insulin sensitivity, highlighting its potential as a novel therapeutic approach for T2DM.

**Keywords:** Caspase-3, HOMA-IR, inflammation, insulin resistance, HMSCS, T2DM, rats

## INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disorder characterized by insulin resistance and progressive pancreatic β-cell apoptosis, ultimately leading to chronic hyperglycemia. The pathophysiology of T2DM involves persistent inflammation, oxidative stress, and dysregulated insulin signaling, all of which contribute to impaired glucose homeostasis.[1] Caspase-3, a crucial effector of apoptosis, plays a significant role in pancreatic β-cell destruction, exacerbating insulin deficiency and disease progression. The homeostatic model assessment of insulin resistance (HOMA-IR) is widely utilized as an index to evaluate insulin resistance, serving as a critical parameter in assessing metabolic dysfunction in T2DM.[2,3]

Recent advances in regenerative medicine have highlighted the therapeutic potential of mesenchymal stem cells (MSCs) and their secretome in metabolic disorders, including T2DM. The secretome derived from hypoxia-conditioned MSCs (HMSCS) exhibits anti-inflammatory, anti-apoptotic, and insulin-sensitizing properties due to its rich composition of bioactive molecules, including cytokines, growth factors, and extracellular vesicles.[4] Previous studies have demonstrated that HMSCS can enhance pancreatic β-cell survival and function, thereby improving glycemic control in diabetic rat models.[5]

In addition, alkaline water has been reported to exert antioxidant and anti-inflammatory effects, which may contribute to enhanced insulin sensitivity and glucose metabolism. By neutralizing reactive oxygen species (ROS) and modulating inflammatory pathways, alkaline water has the potential to mitigate oxidative stress-induced pancreatic β-cell apoptosis, thereby promoting metabolic homeostasis.[6,7]

Previous studies have shown that HMSCS and alkaline water possess beneficial effects in T2DM. For example, Nasution et al.[8] reported that their combination reduced oxidative stress and inflammation by lowering MDA levels and suppressing p65 mRNA expression, while Al-Azzawi et al.[9] demonstrated that MSC secretome protected β-cells from cytokine-induced apoptosis through an IL-10-dependent pathway. These findings consistently indicate protective, anti-inflammatory, and anti-apoptotic effects. However, prior studies did not evaluate their combined effects on both apoptotic markers and insulin resistance in vivo.

To address this gap, the present study evaluated caspase-3 expression and HOMA-IR simultaneously, highlighting a novel therapeutic approach that integrates anti-apoptotic and insulin-sensitizing mechanisms in T2DM models.

## METHODS

### Research design

An in vivo experimental study using a post-test only control group design was conducted at the Animal Research Centre, Semarang, Indonesia, between January and March 2025.

### Experimental animals

A total of 24 male Wistar rats (aged 6-8 weeks, 200-250g) were used. The sample size was determined using Federer's formula (t-1)(n-1) ≥15, where *t* is the number of groups and *n* is the number of animals per group. With four groups, the calculation required at least six rats per group, resulting in a total of 24 rats. The animals were acquired from the Animal Research Centre laboratory, SCCR Indonesia. Environmental conditions were carefully regulated. The indoor temperature was maintained at 26°C, with 12-hour light/dark cycles, and humidity of 50-60%. The animals had free access to food and water. The feed and bedding were regularly replaced to ensure healthy and stable growth. The rats were randomly divided into four groups: T2DM control (STZ-induced diabetes, no treatment); T2DM + metformin (45 mg/kg/day, orally); T2DM + HMSCS (500 µL, intraperitoneally); and T2DM+HMSCS+alkaline water (500 µL HMSCS intraperitoneally+5 mL alkaline water orally). Given dosage was based on previous research.[10]

### Diabetes induction

T2DM was induced using streptozotocin (STZ, 50 mg/kg body weight) dissolved in sodium citrate buffer (0.1 M, pH 4.5) and administered intraperitoneally.[11] The rats were provided with a 30% sucrose solution ad libitum for 48 hours post-injection. Fasting blood glucose (FBG) levels were measured after seven days, and rats with FBG ≥ 126 mg/dL were considered diabetic.[12]

### HMSCS administration

All animal procedures were conducted at the Animal Research Centre, SCCR Indonesia, in

accordance with established ethical guidelines. HMSCS was administered to the treatment groups (P1 and P2) by a veterinarian as single 500 µL intravenous injection. The injections were administered under sterile conditions to ensure precision and safety. Following administration, the rats were maintained for 14 days before being sacrificed for further analysis.

**Pancreatic insulin levels**

Blood samples were collected from the orbital vein using hematocrit tubes on day 7 after STZ induction. Samples were centrifuged at 3000 rpm for 10 minutes to obtain serum for insulin analysis. Pancreatic tissue was collected on day 30 post-treatment. Rats were euthanized using cervical dislocation before tissue extraction. Samples were stored in RNAlater® (Ambion) and frozen at -80°C until further analysis. Insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA), then HOMA-IR was calculated using formula (1): [13]

$$\text{HOMA} - \text{IR} = \frac{\text{Fasting Insulin } \left(\mu\frac{\text{U}}{\text{mL}}\right) \text{ x Fasting Glucose } \left(\frac{\text{mmol}}{\text{L}}\right)}{22.5} \quad (1)$$

**Caspase-3 expression levels using RT-PCR method**

For RT-PCR analysis of caspase-3 expression, 100 mg of adipose tissue was placed in a tube containing 50 µL RNA Iso Plus (Takara) and homogenized using a micropestle, followed by incubation at room temperature for 5 minutes. After adding 20 µL chloroform and incubating for 2-3 minutes, the samples were centrifuged at 15,000 rpm for 15 minutes at 4°C to separate RNA, DNA, and debris. The RNA-containing top layer was transferred to a new tube, and an equal volume of isopropanol was added. The samples were mixed until white strands formed, then centrifuged at 15,000 rpm for 10 minutes at 4°C. The RNA pellet was washed with 70% ethanol in diethyl pyrocarbonate (DEPC)-treated water and centrifuged again at 15,000 rpm for 5 minutes at 4°C. The RNA was resuspended in 30-50 µL DEPC-treated water and incubated at 55°C for 10 minutes. RNA concentration was quantified using Nanodrop, targeting 3000 ng, followed by cDNA synthesis using Oligo(dT) primers and ReverTra Ace enzyme.

For PCR analysis, the reaction mixture contained 3 µL cDNA, 12.5 µL Taq master mix, 0.6 µL primers (forward and reverse), and 8.3 µL nuclease-free water. The primers for caspase-3 were: Forward: 5'-GGA GCA ATA AGT GCA GCA GCA-3' and Reverse: 5'-GGT CGT AGT TCA GGT CAT CAG-3'. PCR products were analyzed using qRT-PCR Illumina, and caspase-3 expression was normalized to housekeeping gene levels and analyzed using EcoStudy software (Illumina).

**Statistical Analysis**

Data were analyzed using one-way ANOVA followed by a post hoc LSD test. Results were expressed as mean ± standard deviation (SD), with significance set at $p<0.05$.

**Ethical approval**

All procedures involving animals adhered to the ethical standards and guidelines established by the Institutional Research Bioethics Commission of Universitas Islam Sultan Agung No.10/ I/2025/KomisiBioetik.

## RESULTS

**Validation of type 2 diabetes mellitus model in Wistar rats**

The induction of T2DM in Wistar rats using streptozotocin (STZ) resulted in a significant increase in fasting blood glucose (FBG) levels, confirming the establishment of the diabetic model. Table 1 shows that the FBG levels in the diabetic control group reached 450.29 ± 41.7 mg/dL, significantly higher than in the healthy control group (105 ± 28.4 mg/dL, $p<0,05$). In addition, HOMA-IR values were markedly elevated in the diabetic control group (20.21 ± 3.1) compared with the healthy control group (0.77 ± 0.12, $p<0.001$).

Table 1. The T2DM rat validation results

| Variables | Healthy rats (n=6) | STZ induced rat (n=6) | p value |
|---|---|---|---|
| Fasting blood glucose (mg/dL) | 105.0 ± 28.4 | 450.29 ± 41.7 | 0.0001 |
| Homa-IR | 0.77 ± 0.12 | 20.21 ± 3.1 | 0.0001 |

Note: T2DMT: type 2 diabetes mellitus; data presented as mean ± SD

**Effect of HMSCS and alkaline water on caspase-3 expression**

The analysis of caspase-3 gene expression data showed that the negative control group (K-) had the highest value (6.66 ± 2.76 a.u.), followed by the positive control group (K+) (3.83±1.61 a.u.), treatment group 1 (P1) (3.03 ± 1.26 a.u.), and treatment group 2 (P2), which had the lowest mean value (2.93 ± 0.52 a.u.). The Shapiro-Wilk normality test indicated that the data were normally distributed ($p>0.05$), while the Levene's homogeneity test confirmed that the data had homogeneous variance ($p>0.05$). Furthermore, the one-way ANOVA results revealed a statistically significant difference in caspase-3 gene expression among the groups ($p<0.05$), indicating that the treatments exerted a significant effect. Post hoc analysis using the LSD test showed that both P1 and P2 differed significantly from K- ($p<0.05$) but not from K+ ($p>0.05$). No significant difference was observed between P1 and P2 ($p>0.05$).

The expression levels of caspase-3, an apoptotic marker, were significantly increased in the T2DM control group, as shown in Figure 1. However, administration of HMSCS resulted in a notable decrease in caspase-3 expression. The combination of HMSCS and alkaline water demonstrated the most significant reduction, suggesting a synergistic protective effect against pancreatic β-cell apoptosis.

**Effect of HMSCS and alkaline water on HOMA-IR index**

The results of the HOMA-IR analysis indicate that the K- group had the highest mean value (18.92±2.63), followed by K+ (10.85±1.39), P1 (7.71±0.53), with P2 having the lowest value (6.76±0.96). The Shapiro-Wilk normality test showed that the data were normally distributed ($p>0.05$), whereas Levene's homogeneity test indicated that the data were not homogeneous ($p<0.05$).

Based on the significant ANOVA test results and the normally distributed but non-homogeneous data, the Tamhane post hoc analysis was used after a significant one-way Analysis of variance (ANOVA) to identify which specific group pairs are significantly different from each other. As shown in Figure 2, P1 was significantly different from K-, K+, and P2 ($p<0.05$). Likewise, P2 was significantly different from K- and K+ ($p<0.05$) but not significantly different from P1 ($p>0.05$).

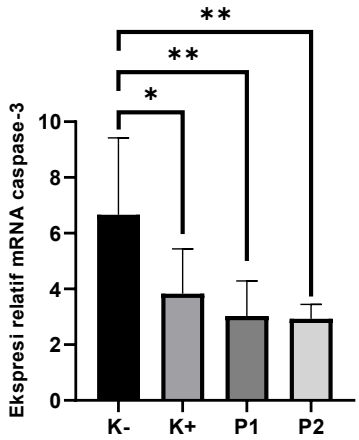


**Figure 1.** The significant results from the ANOVA test prompted further analysis using the post hoc LSD test to examine intergroup relationships. P1 and P2 groups differed significantly from K- ($p<0.05$) but did not differ from K+ ($p>0.05$). Additionally, no significant difference was found between P1 and P2 ($p>0.05$). These findings suggest that the treatment interventions effectively reduced caspase-3 expression compared to the untreated diabetic group but had similar effects to the positive control group. Note= K-: T2DM rats as negative control group; K+: Metformin treatment as positive control group; P1: HMSCS treatment group; P2: HMSCS combined with alkaline water group. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.
"Ekspresi relatif mRNA caspase-3" = "caspase-3 mRNA relative expression"

Based on the intergroup analysis, there is a trend of decreasing HOMA-IR with the given treatments, as shown in the Figure 2. This indicates that the applied interventions contributed to improving insulin sensitivity. The significant differences observed between groups suggest that the treatments may play a role in modulating insulin resistance, potentially providing therapeutic benefits in metabolic disorders such as T2DM.

## DISCUSSION

The results of this study provide strong evidence supporting the therapeutic potential of HMSCS and alkaline water in mitigating pancreatic β-cell apoptosis and improving insulin sensitivity in a T2DM model. The results showed that P1 (3.03 ± 1.26 a.u.) and P2 (2.93 ± 0.52 a.u.) had significantly lower caspase-3 expression compared to K- (6.66 ± 2.76 a.u.), but were not significantly different from K+ (3.83 ± 1.61 a.u.) In parallel, the HOMA-IR index demonstrated that P2 (6.76 ± 0.96) was significantly lower than both

K- (18.92 ± 2.63) and K+ (10.85 ± 1.39). Likewise, P1 (7.71 ± 0.53) also showed significant reductions compared to K- and K+ groups. Collectively, these findings indicate that HMSCS and alkaline water, either alone or in combination, exert pronounced anti-apoptotic and insulin-sensitizing effects.

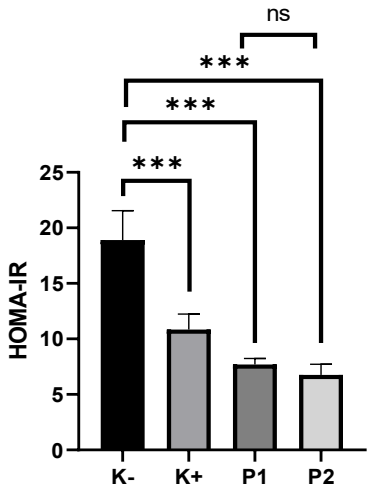


**Figure 2.** Presents the HOMA-IR values across different groups. The diabetic control (K-) group exhibited the highest HOMA-IR values, confirming insulin resistance. Treatment with HMSCS (P1) significantly ($p<0.05$) reduced HOMA-IR, while the combination therapy (P2) significantly ($p<0.05$) enhanced insulin sensitivity, as indicated by a greater reduction in HOMA-IR values. Additionally, no significant difference was found between P1 and P2 ($p>0.05$). These findings suggest that HMSCS and alkaline water improve glucose metabolism by modulating insulin resistance mechanisms. Note= K-: T2DM rats as negative control group; K+: Metformin treatment as positive control group; P1: HMSCS treatment group; P2: HMSCS combined with alkaline water group. Ns: not significant; ***: $p<0.001$

These findings are consistent with those of a previous study that shows hypoxic MSCs inhibiting caspase-3 as apoptosis marker in lung injury.[14] Prior research also showed that alkaline water can act as an antioxidant, which contributes to decreased cleaved caspase-3 expression and improved insulin signaling.[15] The protective mechanism is likely attributed to bioactive components in the HMSC secretome, such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF), which support β-cell regeneration and reduce oxidative stress-induced nuclear factor-kappa B (NF-κB) activation.[16,17] Additionally, alkaline water acts as an antioxidant, lowering ROS levels and inhibiting NF-κB activation, thereby reducing tumor necrosis factor alpha (TNF-α) expression and preventing apoptosis.[18,19] Caspase-3 is a key enzyme in the apoptotic pathway, playing a crucial role in programmed cell death, particularly in diabetic pancreatic β-cells.[20,21]

Chronic inflammation and insulin resistance in type 2 diabetes mellitus (T2DM) are driven by NF-κB activation and increased levels of pro-inflammatory cytokines such as TNF-α and IL-6.[22] These factors contribute to pancreatic β-cell apoptosis, leading to decreased insulin production and impaired glucose homeostasis.[23] In the present study, the administration of HMSCS and alkaline water demonstrated protective effects against inflammation and apoptosis, as assessed by caspase-3 expression. The significant decrease in caspase-3 expression in the HMSCS and alkaline water-treated group suggests a robust anti-apoptotic effect. Previous studies have demonstrated that the HMSCS contains anti-inflammatory cytokines and growth factors such as TGF-β, VEGF, and IGF-1, which play a crucial role in inhibiting apoptotic pathways.[24,25] Additionally, the antioxidative properties of alkaline water may contribute to reducing oxidative stress-mediated apoptosis, further preserving pancreatic β-cell integrity.[7]

The reduction in HOMA-IR in P2 indicates that the combination of HMSCS and alkaline water exerts a synergistic effect in improving insulin sensitivity. The hypoxic MSC secretome contains TGF-β and interleukin 10 (IL-10), which inhibit NF-κB activation and reduce TNF-α and IL-6 levels, known disruptors of insulin signaling.[23,26] Moreover, alkaline water alleviates oxidative stress and suppresses lipopolysaccharide (LPS)-induced inflammation, contributing to enhanced insulin sensitivity.

The improvement in HOMA-IR values in the combination treatment group suggests enhanced insulin sensitivity. The ability of the HMSCS to modulate insulin signaling and promote pancreatic β-cell regeneration has been reported in prior studies.[27] Moreover, alkaline water's role in reducing systemic oxidative stress and inflammation may contribute to an improved insulin response.[28] The combined treatment appears to exert a synergistic effect, outperforming HMSCS or alkaline water alone.

The results of this study provide strong evidence supporting the therapeutic potential of HMSCS and alkaline water in mitigating

pancreatic β-cell apoptosis and improving insulin sensitivity in a T2DM model. The observed reduction in caspase-3 expression and improvement in the HOMA-IR index highlight the combined efficacy of these interventions in preserving β-cell function and modulating insulin resistance pathways.

This study has several limitations. The findings are based on an animal model of T2DM, which may not fully replicate the complexity of the human disease, thereby limiting direct clinical translation. Additionally, only caspase-3 expression and HOMA-IR index were assessed, whereas additional markers of apoptosis, inflammation, and insulin signaling could provide a more comprehensive understanding of the therapeutic effects. Nevertheless, despite these limitations, the findings have significant implications for diabetes management. Current treatment strategies primarily focus on glycemic control without addressing β-cell preservation and regeneration. The combined use of HMSCS and alkaline water presents a novel therapeutic approach that targets both cellular apoptosis and insulin resistance. However, further research is needed to elucidate the precise molecular mechanisms underlying these effects and to validate these findings in clinical trials. Taken together, these results pave the way for future investigations into integrative therapeutic approaches targeting multiple pathways in diabetes pathophysiology.

## CONCLUSION

This study has concluded that HMSCS and alkaline water can mitigate pancreatic β-cell apoptosis and improve insulin resistance in a T2DM rat model. The combination therapy demonstrated a significant reduction in caspase-3 expression and an improved HOMA-IR index, suggesting a protective role in maintaining pancreatic function and enhancing insulin sensitivity. The findings indicate that integrating HMSCS and alkaline water may serve as a novel therapeutic approach for T2DM management.

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**Conflict of Interest**

All the authors declare that there are no conflicts of interest.

**Author Contributions**

MSM conceptualized and planned the study, ES and MSM collected and analyzed the data, MSM and AP calculated the experimental data, and MSM wrote the text and prepared the figures. All authors participated in critical editing of the manuscript. All authors have read and approved the final manuscript.

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**Data Availability Statement**

The supporting data of the findings in this study are available from the corresponding author upon request.

**Declaration the Use of AI in Scientific Writing**

The authors declare that AI tools were only used for grammar and language checking in the preparation of this manuscript. No AI tools were used for data analysis, interpretation, or drawing of scientific conclusions.

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