

Original Research

# Conditioned Media from Deer Antler Stem Cells Effectively Alleviate Type 1 Diabetes Mellitus Possibly via Inhibiting the NF- $\kappa$ B Signaling Pathway

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## Abstract

**Background:** Type 1 diabetes mellitus (T1D) represents a severe threat to human health. Persistent hyperglycemia and dyslipidemia can lead to damaged liver function, while effective interventions for these complications are currently lacking. Deer antler stem cells (AnSCs), a novel type of adult stem cells, significantly reduced liver injury, which was speculated to be achieved through the paracrine pathway. **Methods:** In this study, AnSC-conditioned medium (AnSC-CM) was used to treat C57BL/6 mice with T1D symptoms induced by streptozotocin (STZ). The therapeutic effects of AnSC-CM on T1D were evaluated, and the underlying mechanism was investigated. **Results:** It was shown that AnSC-CM alleviated the T1D symptom: decreased body weight, increased blood glucose levels and islet lesions, and reduced insulin secretion. Moreover, AnSC-CM treatment improved liver function and mitigated liver injury in T1D mice. Impressively, the therapeutic effects of AnSC-CM on T1D were better than those of bone marrow mesenchymal stem cell-CM (BMSC-CM). The mechanistic study revealed that AnSC-CM significantly downregulated the NF- $\kappa$ B signaling pathway in both pancreatic and liver tissues. **Conclusions:** Therapeutic effects of AnSC-CM on STZ-induced T1D and liver injury may be achieved through targeting the NF- $\kappa$ B signaling pathway.

**Keywords:** deer antler stem cells; conditioned medium; type 1 diabetes mellitus; liver injury; NF- $\kappa$ B signaling pathway

## 1. Introduction

Type 1 diabetes mellitus (T1D) is a severe threat to human health. The most direct result is absolute insulin deficiency, which leads to elevated blood glucose levels and the development of life-threatening complications from diabetes, such as liver injury [1,2]. The liver is a vital organ that metabolizes glucose and lipids; persistent hyperglycemia and dyslipidemia can attenuate metabolic pathways affecting the liver. People with diabetes have a substantially increased risk of liver damage and liver fibrosis, which can progress to cirrhosis and even liver cancer if left untreated [2,3]. However, effective early interventions for T1D and related induced liver injuries are currently lacking. NF- $\kappa$ B is one of the primary signaling pathways to regulate the inflammatory response, which promotes the release of inflammatory mediators in the body, activates immune-inflammatory responses, and is involved in insulin resistance in diabetic patients [4]. Thus, NF- $\kappa$ B may be a potential targeted signaling pathway for treating T1D.

Of all the currently available treatments for T1D and its induced liver injury, mesenchymal stem cell (MSC) transplantation has repeatedly been proven as an effective

approach in translational and clinical practices due to the regenerative and immunomodulatory capabilities of MSCs [5,6]. Thus far, the accumulated evidence has demonstrated that the effects of transplanted MSCs are mainly punctuated through the paracrine pathway [5]. Therefore, conditioned medium from MSCs (MSC-CM) is reported to have similar therapeutic effects as MSCs since it would contain the paracrine factors released from cultured MSCs [7–9]. Moreover, MSC-CM provides several advantages over using MSCs, such as a lower risk of tumorigenesis, a simpler handling process, and negligible immunogenicity [1].

Deer antlers are the only mammalian organs that regenerate each year, a process known to be mediated by antler stem cells (AnSCs) [10–12]. Compared to the other types of stem cells, AnSCs have the advantages of easier acquisition and *ex vivo* expansion [13]. In one of our previous studies, AnSCs demonstrated significant efficacy in treating liver injuries [14]. Thus far, the role of AnSC-conditioned medium (AnSC-CM) in treating liver injury, especially in treating diabetic liver injury associated with T1D, has not been studied.

This study focused on the effect of AnSC-CM on T1D and diabetic liver injuries. Studies have shown that AnSC-



CM not only relieves T1D symptoms but also T1D-induced liver injury and is more effective than bone marrow MSC-conditioned medium (BMSC-CM). The mechanistic study suggests that the therapeutic effect of AnSC-CM may be achieved by targeting the NF- $\kappa$ B signaling pathway. Overall, our study provides a new strategy for applying alternative stem cell CMs to treat T1D and diabetic liver injuries effectively in the clinical setting.

## 2. Materials and Methods

### 2.1 Preparation of Conditioned Media

AnSC-CM was prepared using AnSCs (2–5th passage), described in our previous study [15]. Briefly, AnSCs were cultured in Dulbecco's Modified Eagle Medium (DMEM, 01-052-1ACS, BI, Ness Ziona, Israel) supplemented with 10% fetal bovine serum (FBS, 04-001-1ACS, BI, Brisbane, Australia) until they reached 90% confluency, then, the medium was replaced with an FBS-free medium and the cells cultured for another 48 h. The supernatants were collected and concentrated via ultrafiltration using a spin column with molecular weight cut at 3 kDa (Millipore Corp, Billerica, MA, USA). The AnSC-CM concentration was measured using the BCA assay (P0012, Beo Tianmei, Shanghai, China).

Mouse BMSC-CM was prepared following the same procedure as for AnSC-CM.

All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO<sub>2</sub>.

### 2.2 Animals and Treatment

Female C57BL/6 mice, 6–8 weeks old, were chosen for this study, and the procedure for handling animals was approved by the Animal Ethics Committee of Changchun Sci-Tech University (No. CKARI202110). In total, 24 mice were allocated to the treatment groups and injected intraperitoneally with streptozotocin (STZ, A610130, Shangon Biotech, Shanghai, China) (35 mg/kg) for five days (1/day) to induce T1D [16]; 8 mice were in the control group and injected intraperitoneally with PBS for five days (1/day). The STZ-treated mice were divided into three groups: STZ (negative control), BMSC-CM (positive control), and AnSC-CM conducted preventive treatment with PBS, 100  $\mu$ g/50  $\mu$ L of BMSC-CM, and 100  $\mu$ g/50  $\mu$ L of AnSC-CM, respectively, in the second week. The control (CTRL) group mice were injected with PBS. All injections were performed through the mouse tail vein for 4 weeks (2/week), and the mice were euthanized on day 5 after the last CM injection. Body weight and blood glucose changes in each mouse were measured once a week. Four weeks after treatment, an oral glucose tolerance test (OGTT) was performed [17].

### 2.3 Biochemical Analysis

The blood samples were collected and centrifuged to separate the serum (1200 g, 10 min). Then, the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), and superoxide dismutase (SOD) were measured using the respective kits (COO9-1-1, CO10-1-1, A001, and A003-1, Nanjing Jiancheng, Nanjing, China).

### 2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

A total of 0.5 mL of physiological saline was added to 50 mg of mouse liver tissue for homogenization, then centrifuged at 3000 rpm and 4 °C for 10 min to collect the supernatant. Next, the levels of interleukin 1 $\beta$  (IL-1 $\beta$ ), interleukin 6 (IL-6), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were measured by ELISA assay, using the specific procedures illustrated in the instruction manual of each kit (SEKM-0002, SEKM-0007, and SEKM-0034, Solarbio, Beijing, China).

### 2.5 Histological and Immunohistochemical Analyses

Pancreas and liver tissues were fixed for 24 hours (10% formalin was chosen as the fixing solution) and dehydrated by a gradient of ethanol solutions. Then, the tissues were embedded in paraffin, cut into 5  $\mu$ m sections, and stained with hematoxylin and eosin (H&E) before immunohistochemical (IHC) analysis was performed.

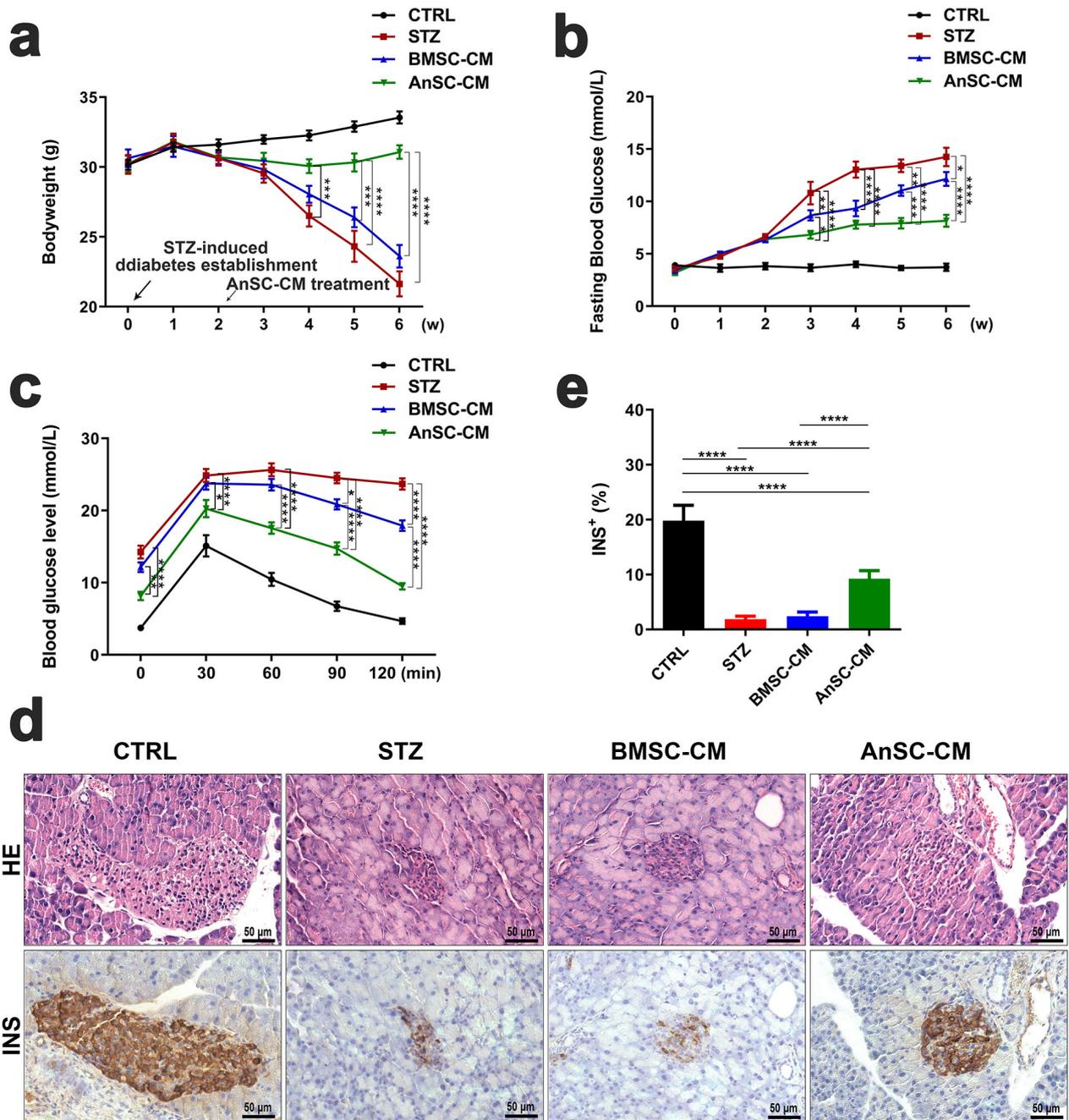
IHC staining was performed using the kit (KIT-9710, Maixin, Fuzhou, China), as described in the instructions. The primary antibodies, anti-PCNA (ab15497, Abcam, Cambridge, UK) and anti-INS (tech66198-1-Ig, Proteintech, Wuhan, China), were used in this experiment. Images were captured under a microscope (M8 PreciPoint, Freising, Germany) and evaluated using Image-Pro Plus 6.0 software (MEDIA CYBERNETICS, Rockville, MD, USA).

### 2.6 Western Blot Analysis

Western blot analysis was performed using the methods reported in a previous study [18]. Briefly, the protein was extracted from the pancreas and liver tissues of mice using radioimmunoprecipitation assay (RIPA) lysis buffer. Proteins were separated on SDS-polyacrylamide gels and then electrically transferred onto a nitrocellulose membrane. Next, membranes were incubated with the indicated primary antibody: p65 (CST8242), p-p65 (CST3033), IKB $\alpha$  (CST4814), p-IKB $\alpha$  (CST2859), and GAPDH (ab8245) at 4 °C overnight, after which incubation with a secondary antibody (ab15007) was conducted at 25 °C for 2 h. Finally, the ECL system (5800, Tanon, Shanghai, China) was used to visualize the bands, and Image J software (LOCI, University of Wisconsin, Madison, WI, USA) was used for quantification.

### 2.7 Statistical Analysis

Data are presented as mean  $\pm$  SD ( $n \geq 3$ ) and analyzed using one-way ANOVA ( $t$ -test); \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , or \*\*\*\* $p < 0.0001$ .



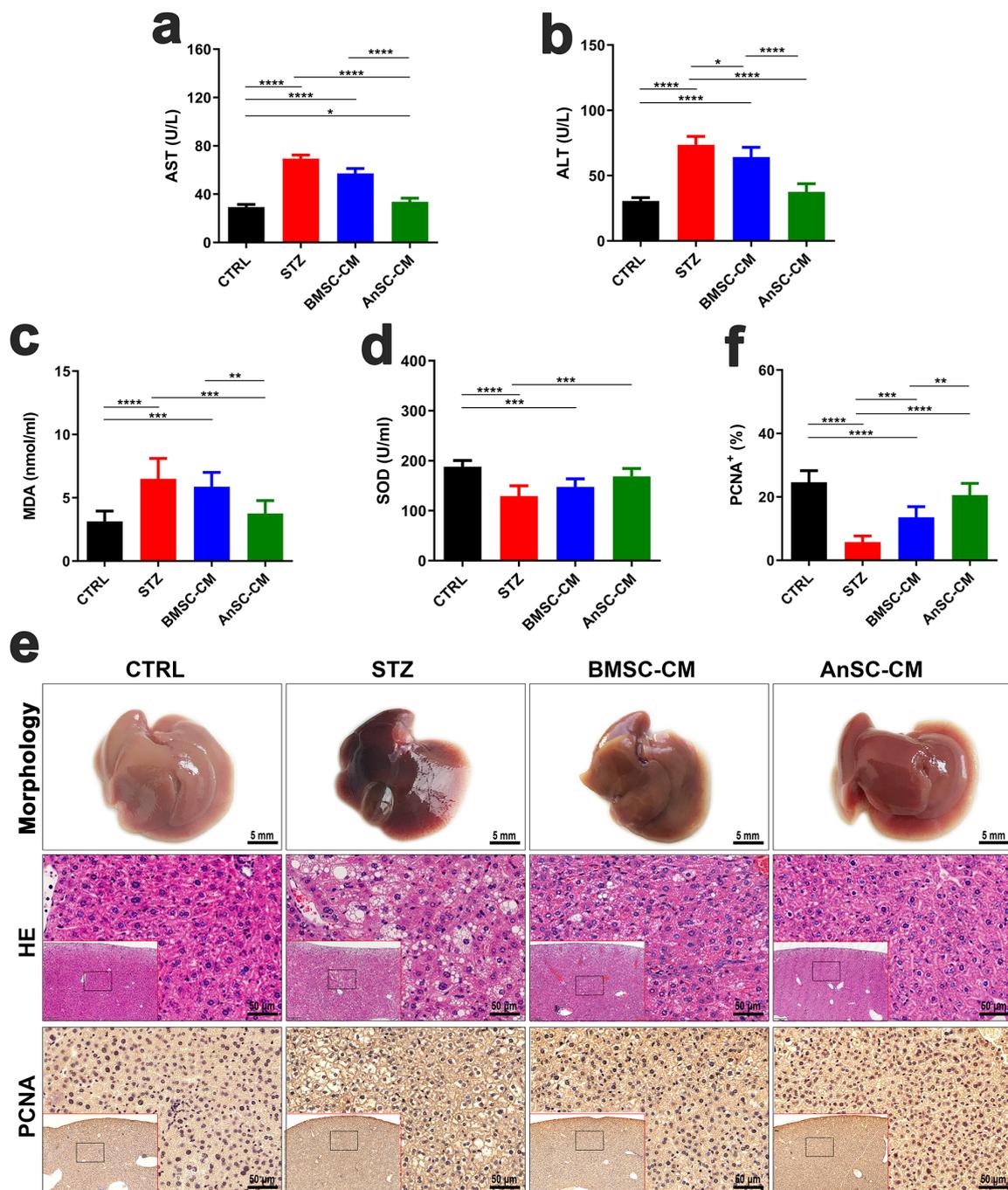
**Fig. 1. Effects of AnSC-CM on mice with T1D.** (a,b) Body weight and fasting blood glucose levels of mice were measured weekly (w). (c) Glucose tolerance levels in each group. (d) H&E staining and immunohistochemical evaluation of INS in the pancreatic tissue; scale bar = 50  $\mu$ m. (e) INS-positive staining in the pancreatic tissue. H&E, hematoxylin and eosin; T1D, type 1 diabetes mellitus; AnSC-CM, antler stem cell conditioned medium; BMSC-CM, bone marrow mesenchymal stem cell conditioned medium; CTRL, control; INS, insulin; STZ, streptozotocin. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ ; mean  $\pm$  standard deviation (SD);  $n = 8$ .

### 3. Results

#### 3.1 Effects of AnSC-CM on T1D Mouse Symptoms

The changes in body weight and fasting blood glucose level of mice treated with AnC-CM were measured. The results showed that STZ treatment significantly decreased

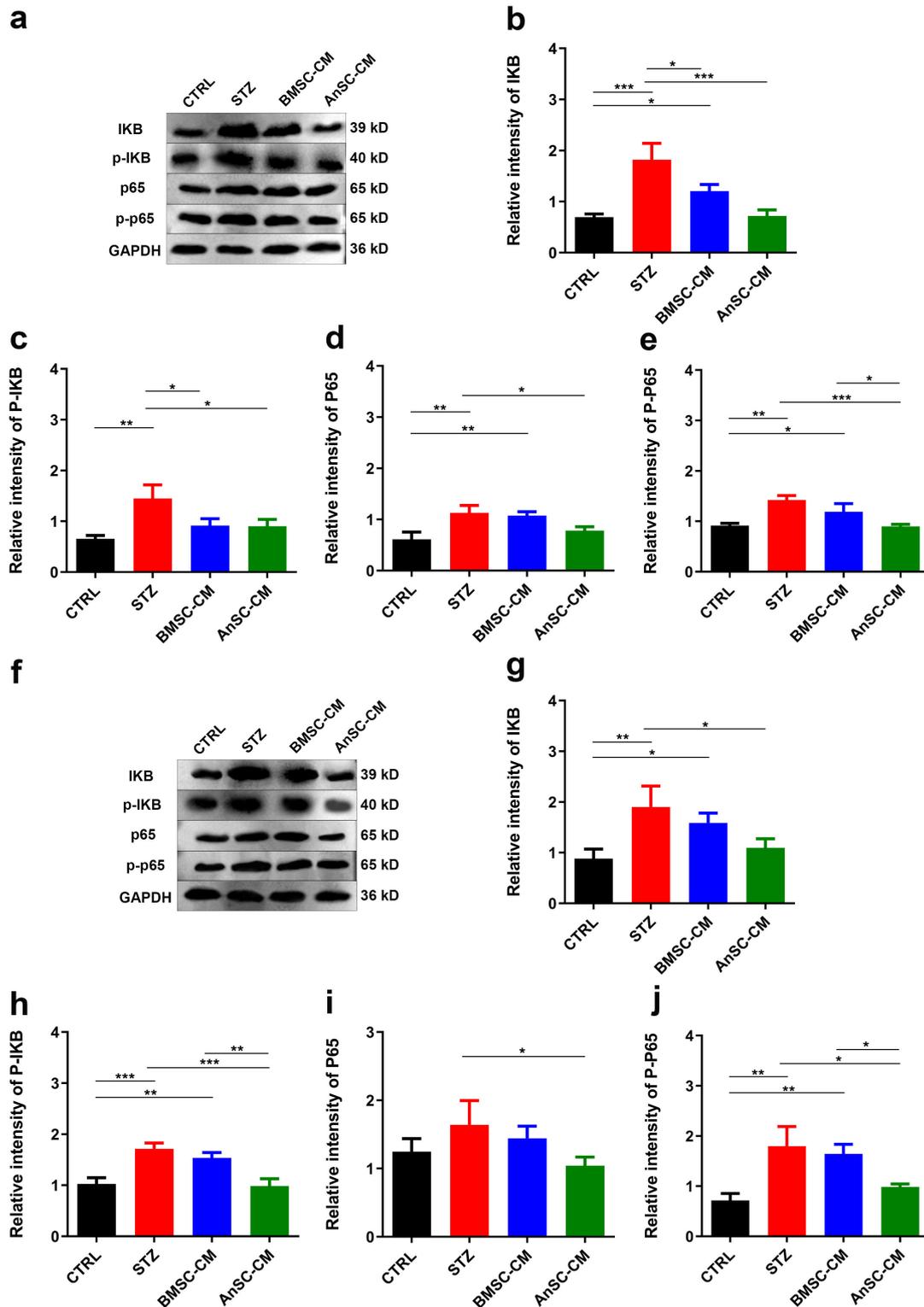
body weight and increased fasting blood glucose levels in mice. Additionally, these changes were significantly attenuated four weeks after the treatment by administering AnSC-CM ( $p < 0.001$ ; Fig. 1a,b). The OGTT results also showed that AnSC-CM significantly counteracted this increase in blood glucose level ( $p < 0.001$ ; Fig. 1c). Im-



**Fig. 2.** Effects of AnSC-CM on injured livers induced by STZ in T1D mice. (a,b,c,d) Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA), and SOD (superoxide dismutase). (e) Morphology (scale bar = 5 mm), H&E staining (scale bar = 50  $\mu$ m), and immunohistochemical localization of proliferating cell nuclear antigen (PCNA) (scale bar = 50  $\mu$ m) in the liver tissues. (f) Quantification of PCNA-positive staining. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ ; mean  $\pm$  SD;  $n = 8$ .

portantly, AnSC-CM was significantly more effective than BMSC-CM in improving body weight and blood glucose levels in T1D mice ( $p < 0.001$ ; Fig. 1a–c). Histological results showed that STZ treatment caused a significant re-

duction in islet size, incomplete pancreatic structure, inhomogeneous morphology, poorly defined borders, and a significant decrease in islet cell counts or infiltration of the inflammatory cells in the model group (Fig. 1d,e). AnSC-



**Fig. 3.** Effects of AnSC-CM on protein expressions of p65, p-p65, IKB, and p-IKB in liver and pancreatic tissues of T1D mice. (a) Western blot protein bands of p65, p-p65, IKB, and p-IKB in liver tissues. (b–e) Quantification of relative intensities of protein bands in (a). (f) Western blot protein bands of p65, p-p65, IKB, and p-IKB in pancreatic tissues. (g–j) Quantification of relative intensities of protein bands in (f). \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ ; mean  $\pm$  SD;  $n = 3$ .

CM treatment attenuated these pancreatic histopathologic changes. Furthermore, the level of insulin (INS) in the mice was significantly higher than in the model mice when treated with AnSC-CM ( $p < 0.001$ ; Fig. 1d,e), evidenced by immunohistochemical staining.

### 3.2 Effects of AnSC-CM on Parameters of Injured Livers in T1D Mice

Serum parameters (AST, ALT, and MDA), which are associated with liver injury, were measured. The results showed that AST, ALT, and MDA levels in the AnSC-CM group were significantly lower than in the STZ group (Fig. 2a–c;  $p < 0.001$  or  $p < 0.0001$ ); in contrast, SOD levels in the AnSC-CM group were significantly higher than in the STZ group (Fig. 2d;  $p < 0.001$ ). The macroscopic assessment of the injured liver in the STZ group showed obvious atrophy, congestion, necrosis, brittle texture, and a rough appearance (Fig. 2e). Histological results showed that the livers in the STZ group had structural disorganization, irregular arrangement, necrosis, and vacuolated degeneration. AnSC-CM treatment significantly reduced these symptoms caused by STZ (Fig. 2e). In addition, the level of proliferating cell nuclear antigen (PCNA) expression in the liver tissues in the AnSC-CM group was significantly higher than in the STZ group (Fig. 2e,f;  $p < 0.0001$ ). Further, we found that AnSC-CM significantly reduced the levels of proinflammatory cytokines, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , in liver tissues ( $p < 0.0001$ , **Supplementary Fig. 1**). Overall, the effects of AnSC-CM on all parameters, as mentioned above, were better than using BMSC-CM. Therefore, AnSC-CM represents a better choice for attenuating diabetic liver injury in these model mice.

### 3.3 Regulation of NF- $\kappa$ B Signaling Pathway by AnSC-CM in Pancreatic and Liver Tissues

We further measured the expression levels of genes related to the NF- $\kappa$ B signaling pathway in the liver and pancreas. The results showed that the expression levels of p65, p-p65, IKB, and p-IKB in the liver tissues of the AnSC-CM group were significantly lower than in the STZ group ( $p < 0.05$  or  $p < 0.001$ ; Fig. 3a–e). Simultaneously, the expression levels of p65, p-p65, IKB, and p-IKB were also reduced in the pancreatic tissues of mice in the AnSC-CM group compared to the STZ group ( $p < 0.05$  or  $p < 0.001$ ; Fig. 3f–j). Thus, AnSC-CM therapy effectively improved diabetic symptoms, possibly by inhibiting the NF- $\kappa$ B signaling pathway.

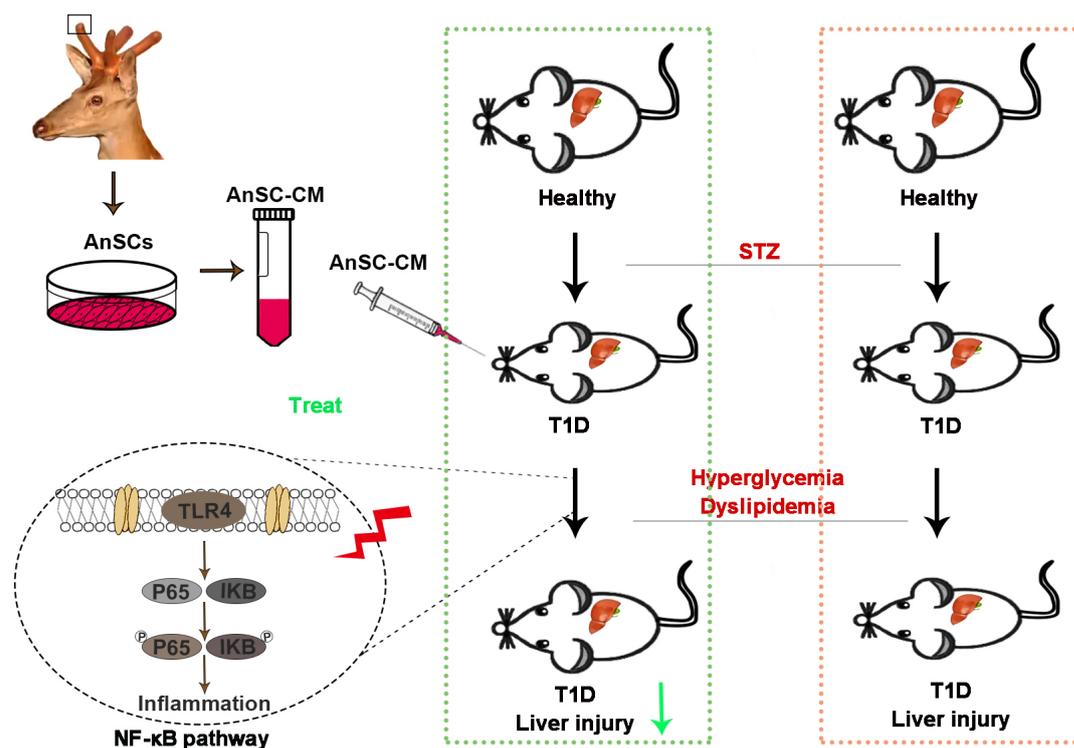
## 4. Discussion

Mesenchymal stem cell therapy has been recognized as a promising strategy over the past several decades for treating a wide range of conditions, especially T1D (mainly caused by autoimmune destruction), owing to the regenerative and immunomodulatory potential of MSCs [5]. Studies have shown that the leading cause of tissue repair *in vivo* is

the paracrine effect by MSCs [5]. Thus, it is evident that CM that contains cultured-MSC paracrine factors can induce tissue repair as effectively as MSCs [15]. In one of our previous studies, we found that AnSCs effectively attenuated liver injury, improved liver function, and reduced inflammation in rats [14]. Thus, we speculate that AnSC or AnSC-CM treatment would effectively alleviate the symptoms of diabetes mellitus and induced liver injury. This study showed for the first time that AnSC-CM effectively improved symptoms related to STZ-induced T1D, which was found to be, at least partially, by inhibiting the NF- $\kappa$ B signaling pathway. More impressively, the therapeutic effect of AnSC-CM was vastly improved in treating T1D mice compared to BMSC-CM. Therefore, this study points to another type of MSC-CM that could be used to treat T1D more effectively.

In addition to the elevated blood glucose in diabetes mellitus mice, absolute or relative insulin deficiency also leads to imbalances in lipid metabolism and hepatic lipid accumulation [1,2]. The early manifestation of T1D is the fat accumulation in the liver parenchyma. Subsequently, a large quantity of the accumulated lipids progresses to the formation of lipotoxicity. The latter causes dysfunctions in the endoplasmic reticulum, mitochondria, and other cellular organelles and promotes inflammatory reactions, which lead to hepatocyte damage and even cell death [8,14]. It has been reported that paracrine secretions of BMSC and human urine-derived stem cells can improve STZ-induced diabetes and its complications [19,20]. Studies have shown that AnSC-CM reduced fasting blood glucose levels and promoted gains in body weight in T1D mice. Additionally, AnSC-CM was more effective than BMSC-CM in treating these symptoms. AnSC-CM enhanced insulin synthesis and secretion in T1D mice alongside alleviating liver dysfunction and improving lipid metabolism, which could be well explained by a significant decrease in the serum levels of alanine aminotransferase (ALT), alanine aminotransferase (AST), and MDA, and an increase in the serum levels of SOD. Compared to other MSC-CM-based therapies, AnSC-CM-based treatment is likely more effective in preventing and treating T1D. We speculated that AnSC-CM performs better than BMSC-CM, which may be due to the dual properties of both embryonic stem cells and mesenchymal stem cells from AnSCs [21], meaning there may be more active components that are anti-inflammatory in their CM components.

Inflammation is strongly associated with the development of diabetes [22,23]. NF- $\kappa$ B is one of the primary signaling pathways that regulate the inflammatory response, promoting the release of inflammatory mediators in the body, activating immune-inflammatory responses, and is involved in the insulin resistance of diabetic patients [4]. p65, one of the members of the NF- $\kappa$ B family, is located in the cytoplasm under normal conditions and binds to inhibitory proteins IKBs to form a trimeric complex in an in-



**Fig. 4. Schematic diagram of the mechanism through which AnSC-CM alleviates STZ-induced T1D liver injury symptoms.** AnSC-CM alleviates STZ-induced T1D liver injury symptoms by potentially downregulating the NF- $\kappa$ B signaling pathway and oxidative stress. AnSCs, deer antler stem cells; AnSC-CM, AnSC conditioned medium; T1D, type 1 diabetes mellitus; TLR4, toll-like receptor 4.

activated state [24,25]. Evidence from clinical and experimental studies suggests that upon being stimulated, IKBs phosphorylate and dissociate from the trimeric complex, which causes NF- $\kappa$ B to be released from the complex and translocated to the nucleus to stimulate transcription of target genes, thus, causing activation of NF- $\kappa$ B and the induction of the inflammatory response [24,25]. This study showed that the expressions of p65, p-p65, IKB, and p-IKB were decreased in the liver and pancreas tissues after treatment with AnSC-CM, suggesting that AnSC-CM may inhibit the NF- $\kappa$ B signaling pathway and attenuate diabetic liver injury. Groeger *et al.* [26] also found that induced pluripotent stem cells reflect specific effects on insulin signaling, and glucose metabolism was mediated by NF- $\kappa$ B. This enhanced the evidence that suggests mediating the NF- $\kappa$ B signaling pathway is a potential strategy for treating diabetes. Moreover, it indicates that the mechanism involved in stem cell secretions in treating diabetes may have commonness through inhibiting the NF- $\kappa$ B signaling pathway.

## 5. Conclusions

In summary, the results of the present study highlight that AnSC-CM has advantages over BMSC-CM in ameliorating STZ-induced T1D and diabetic liver injury in our mouse model, which is possibly achieved through the

downregulation of the NF- $\kappa$ B signaling pathway and oxidative stress (Fig. 4). We believe that our study would open up new avenues for future selection of alternative types of MSC-CM for more effective treatments of T1D.

## Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; AnSCs, deer antler stem cells; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HE, hematoxylin and eosin; IHC, immunohistochemistry; IL-1 $\beta$ , interleukin 1 $\beta$ ; IL-6, interleukin 6; MDA, malondialdehyde; RIPA, radioimmunoprecipitation; SOD, superoxide dismutase; STZ, streptozotocin; T1D, Type 1 diabetes mellitus; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ .

## Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Author Contributions

GZ and CL designed the study. DW, XL, JL and JR performed the research. ZP, JY, TJ, QG and ZW analyzed the data. DW, GZ, and CL wrote the paper. All authors contributed to editorial changes in the manuscript. All au-

thors give final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

## Ethics Approval and Consent to Participate

The animal experiments were approved by the Animal Ethics Committee of Changchun Sci-Tech University (Approval No.: CKARI202110).

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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2903096>.

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