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Research article

Efficacy of mesenchymal stem cells in treating tracheoesophageal fistula via the TLR4/NF-κb pathway in beagle macrophages

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ABSTRACT

Background: Tracheoesophageal fistula (TEF) remains a rare but significant clinical challenge, mainly due to the absence of established, effective treatment approaches. The current focus of therapeutic strategy is mainly on fistula closure. However, this approach often misses important factors, such as accelerating fistula contraction and fostering healing processes, which significantly increases the risk of disease recurrence.

Methods: In order to investigate if Mesenchymal Stem Cells (MSCs) can enhance fistula repair, developed a TEF model in beagles. Dynamic changes in fistula diameter were monitored by endoscopy. Concurrently, we created a model of LPS-induced macrophage to replicate the inflammatory milieu typical in TEF. In addition, the effect of MSC supernatant on inflammation mitigation was evaluated. Furthermore, we looked at the role of TLR4/NF-κB pathway plays in the healing process.

Results: Our research revealed that the local administration of MSCs significantly accelerated the fistula's healing process. This was demonstrated by a decline in TEF apoptosis and decrease in the production of pro-inflammatory cytokines. Furthermore, *in vivo* experiments demonstrated that the MSC supernatant was effective in suppressing pro-inflammatory cytokine expression and alleviating apoptosis in LPS-induced macrophages. These therapeutic effects were mainly caused by the suppression of TLR4/NF-κB pathway.

Conclusion: According to this study, MSCs can significantly improve TEF recovery. They achieve this via modulating apoptosis and inflammatory responses, mainly by selectively inhibiting the TLR4/NF-κB pathway.

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1. Introduction

Tracheoesophageal fistula (TEF) is a life-threatening and debilitating condition in pulmonary and critical care medicine with a notably high mortality rate [1,2]. Benign TEF often results from adverse tracheal intubation events, such as endotracheal tube, unintentional hyperventilation, or extended intubation efforts [1]. In addition, tracheal intubation following mechanical ventilation can lead to iatrogenic TEF. Despite the application of various therapeutic strategies, including surgery, endoscopic techniques, and radiotherapy, the median survival time for patients remains as short as around 35 days. Without timely intervention, the survival time hardly be extended longer than 3–4 months [1,2].

Healing of fistula requires multifaceted coordination of coagulation, inflammation, and angiogenesis. Inflammation, an early and critical response in this process, determines the stage of adequate wound healing. The extent and chronic nature of inflammation significantly affect the healing velocity, with chronic inflammation often leading to persistent, nonhealing wounds, thus impeding the overall healing process.

Currently, it has been found that mesenchymal stem cells (MSCs) played critical role in modulating immune responses and attenuating inflammation [3]. These cells exhibit pronounced anti-inflammatory capabilities, which are instrumental in reducing organ damage and enhancing wound healing in various injury models [4,5]. The integral role of MSCs in tissue repair is closely linked to their effects on resolution inflammatory responses, including the facilitation of dead cell clearance and the activation of MSCs themselves [6,7]. Prolonged inflammation, however, can adversely affect their performance by damaging their cellular microenvironment or directly impeding their differentiation pathways [8].

There are two subtype of macrophage categorization, which are remarked as M1 and M2. Typically, M1 macrophages are predominant in propagating inflammatory responses, whereas M2 macrophages plays essential role in tissue repairing as well as secretion of anti-inflammatory cytokines [9]. M1 macrophages can exacerbate chronic inflammation, thereby obstructing tissue regeneration [10]. On the contrary, M2 macrophages contribute significantly to restoration by secreting growth factors and cytokines that activate MSCs, which facilitates the rejuvenation of damaged tissues [11,12]. Furthermore, it has shown that MSCs can reprogram macrophages for anti-inflammatory phenotypes, thus enhancing their role in tissue repair and regeneration [13–16].

Here, we established a TEF model in beagle dogs. Human adipose-derived mesenchymal stem cells (aMSCs) were injected to examine the regulatory impacts of aMSCs on fistula healing and inflammation. Additionally, we assess the influence of stem cells on macrophages within an inflammatory environment. We found that aMSCs can mitigate inflammation and enhance fistula healing by modulating macrophage inflammatory phenotypes.

2. Materials and methods

2.1. Cellular and animal models

aMSCs were procured with precision from Saliai Stem Cell Science and Technology Co. LTD. RAW264.7 cell line was grown in RPMI Medium 1640 (1640 DMEM, Gibco, USA), supplemented with 10 % fetal bovine serum (FBS, AusgeneX, Australia), 1 % penicillin/streptomycin (Solarbio, China). Cell cultures were maintained at 37 °C in an environment of 5 % CO^2 and 95 % air to ensure optimal cell growth and stability.

Male beagles from the Guangdong Laboratory Animal Monitoring Institute, they were aged 3–4 years and weighing 8–11 kg. The animals were assigned to three distinct groups: a normal control group (n = 6), a TEF group (n = 6, subjected to a TEF operation followed by PBS injection) and an MSCs group (n = 6, subjected to TEF operation followed by stem cell injection).

2.2. LPS intervention and supernatant transfer experiment

Macrophages were stimulated with 20 ng/ml LPS for 24 h. The MSC supernatant was harvested using high-speed centrifugation, in order to separating cells and debris. This supernatant, enriched of secretions from MSCs, was utilized to culture macrophages for further experimental investigation.

2.3. Western blotting technique

A refined version of the established Western blot protocols were followed [24]. In brief, an extended incubation period of nitrocellulose membranes with primary and secondary antibodies (Affinity, S0001/2, 1:5000 dilution). To accommodate proteins of varying molecular sizes, we horizontally segmented the membranes for targeted antibody incubation. In particular, for proteins with approximate molecular weights, such as NF-κB and p–NF–κB, we implemented a sequential blotting method using a specialized stripping buffer (CWBIO, 01427, China) to ensure precise protein identification. The membranes were subjected to enhanced chemiluminescence (ECL) detection and detailed analysis using advanced image system software (Protein Simple, USA). Primary antibodies used included IL-1β (ab283818, USA), TNF-α (ab183218, USA), NF-κB (CST, #6956), p–NF–κB (ab194726), Bcl-2 (ab194583), Bax (ab32503) and β-actin (Sigma, A5441) for accurate protein characterization.

2.4. Enhanced RT-qPCR analytical approach

For RNA analysis, an advanced RNA extraction process by using RNAiso Plus (TAKARA, Japan) coupled with an optimized purification method were used. This was followed by cDNA synthesis using Revertra Ace with a gDNA remover (TOYOBO, Japan) to enhance transcription accuracy. For quantitative PCR (qPCR), we prepared a precise 20 μL reaction mixture based on the specifications of the GoTaq qPCR Master Mix (A6001, Promega, USA). A comprehensive gene expression analysis was conducted by using the refined 2-ΔΔCt method, employing β-actin as the normalization standard. The sequences of qPCR primers are shown in Table 1.

2.5. Construction of the TEF model

The surgical procedure for the construction of the TEF model was conducted under general anesthesia, induced by intravenous injection of ketamine (10 mg/kg) and thiazine hydrochloride (1 mg/kg) and maintained with isoflurane (1%–5%). Each animal was intubated and provided additional oxygen (30 ml/kg, 15/min) while continuously monitored by electrocardiography. The surgical approach involved a lateral incision parallel to the left sternocleidomastoid muscle, meticulous tracheal resection, esophageal dissection, creation of a fistula using an electrotome, and subsequent meticulous suturing. Postoperative care included administering prophylactic antibiotics (ampicillin sodium, 1.0 g).

2.6. Stem cell transplantation protocol

Each canine in the MSC group received a targeted injection of 1 × 10^6 aMSCs (2 ml) into the tracheal membrane and esophageal submucosa. The TEF group received an equivalent volume of PBS at the corresponding anatomical locations.

2.7. Endoscopic evaluation

Endoscopic examinations were performed using a MINDSION TF52 device, initially on the first postoperative day, to confirm the successful construction of the TEF and subsequently once per week for 35 days. Fistula diameter were dynamic assessment over time.

3. Results

3.1. 1. influence of MSCs on macrophage inflammation and apoptosis

In our innovative supernatant transfer experiment, we elucidated the profound impact of MSCs on macrophage behavior within the milieu of the TEF. This experiment was critical in revealing the bioactive capacity of MSCs to modify inflammatory and apoptotic responses. In Western-blot analysis, we observed increased protein expression of pro-inflammatory cytokines, such as IL-1β and TNF-α in LPS-stimulated macrophage (Fig. 1a and b). The high protein expression of TLR4 and phosphorylated NF-κB can be observed in LPS-stimulated macrophage (Fig. 1c and d).

In qPCR analysis, we observed significant increased gene expression of pro-inflammatory cytokines, such as IL-1β (M: 5.975 ± 0.568, M + LPS: 1.135 ± 0.651, $p < 0.05$), TNF-α (M: 0.948 ± 0.198, M + LPS: 0.104 ± 0.028, $p < 0.05$), and IL6 (M: 4.211 ± 0.889, M + LPS: 1.551 ± 0.105, $p < 0.05$) in LPS-stimulated macrophages. The increased gene expression of TLR4 (M: 1.200 ± 0.277, M + LPS: 5.712 ± 0.626, $p < 0.05$) and NF-κB (M: 0.278 ± 0.029, M + LPS: 1.623 ± 0.068, $p < 0.05$) also can be observed in LPS-stimulated macrophages (Fig. 1e).

The decreased gene expression of IL-1β (M + LPS + MSC: 3.212 ± 0.32, M + LPS: 5.975 ± 0.568, $p < 0.05$), TNF-α (M + LPS + MSC: 0.074 ± 0.024, M + LPS: 0.948 ± 0.198, $p < 0.05$), IL6 (M + LPS + MSC: 2.201 ± 0.214, M + LPS: 4.211 ± 0.889, $p < 0.05$) and increased gene expression of IL-10 (M + LPS + MSC: 35.342 ± 0.543, M + LPS: 1.073 ± 0.115, $p < 0.05$) also can be observed in LPS-stimulated macrophages with MSCs supernatant. The decreased gene expression of TLR4 (M + LPS + MSC: 3.221 ± 0.302, M + LPS: 5.712 ± 0.626, $p < 0.05$) and NF-κB (M + LPS + MSC: 0.606 ± 0.082, M + LPS: 1.623 ± 0.068, $p < 0.05$) also can be observed in LPS-stimulated macrophages (Fig. 1e).

This was complemented by a notable decrease in apoptotic markers, with a marked reduction in proteins such as Bax (M + LPS + MSC: 1.517 ± 0.191, M + LPS: 21.442 ± 3.183, $p < 0.05$), while antiapoptotic markers such as Bcl-2 (M + LPS + MSC: 2.896 ± 0.372,

Table 1
The Sequences of qPCR Primers.

Genes	Forward primer	Reverse primer
IL-1β	AGTTGACGGACCCCAAA	TCTTGTTGATGTGCTGCTG
IL-6	ATGAGACTGGGGATGTCTGT	TCTTGTTGATGTGCTGCTG
TNF-α	GAAACACAAGATGCTGGGA	TTGCAGAACTCAGGAATGG
IL-10	GGAAGAGAAACCAGGGAGA	CCACAGTTTTCAGGGATGA
TLR 4	CCTGAGCAAACAGCAGAGGA	CCATGTGTTCCATGGGCTCT
NF-κB	TTAAAAGGTGTCGCTGTCC	GCGCATCAAGGTACTCATT
Bax	GTCTCCGGCGAATTGGAT	TTGGATCCAGACAAGCAG
Bcl-2	TCTTATCCCCTGTCCCCCA	ACATCTCAAGCCTTCACGC

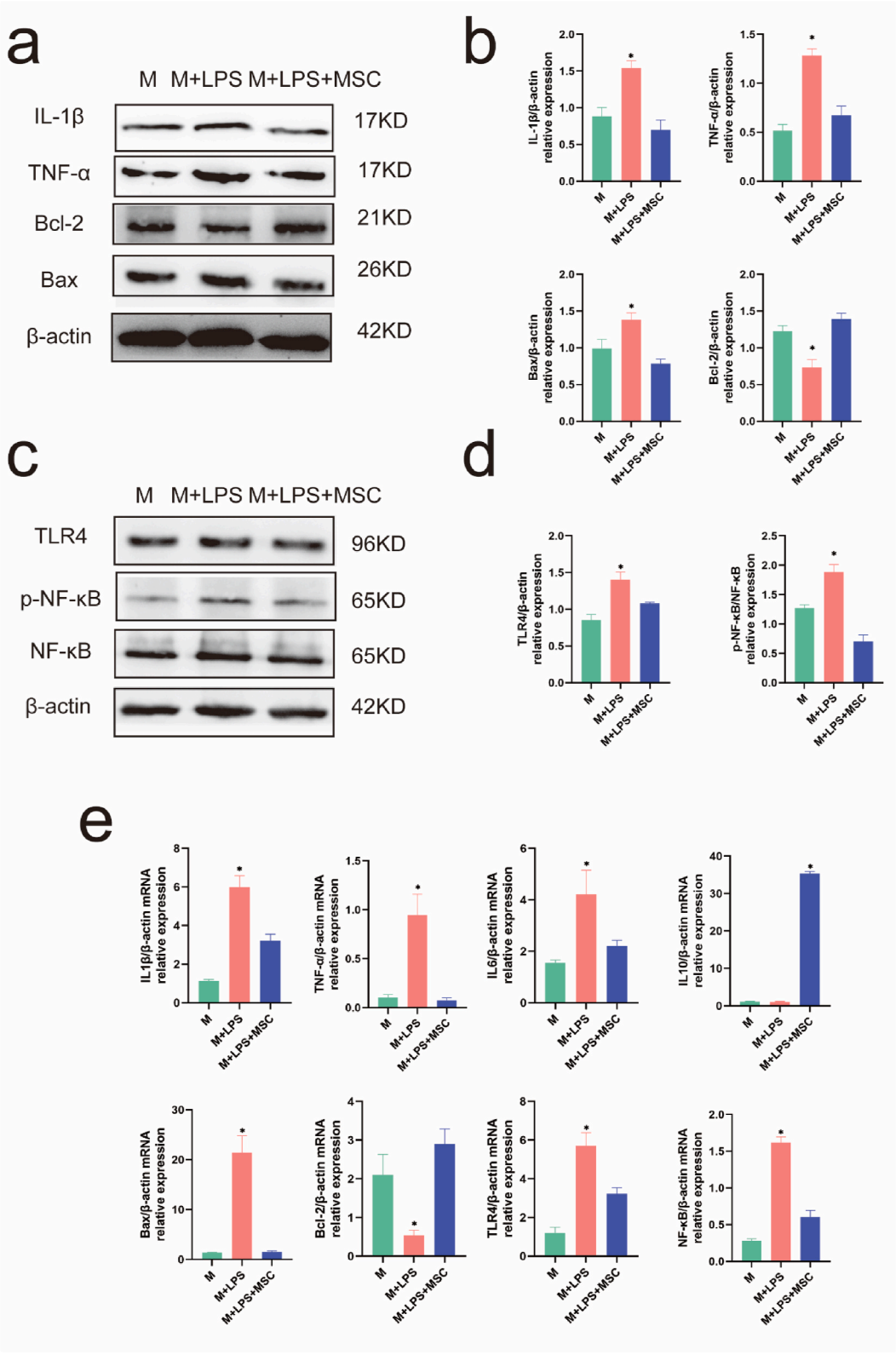

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Fig. 1. Effects of MSCs on Inflammation and Apoptosis in Macrophages. 1a, 1b: Quantification of IL-1β, TNF-α, Bax, and Bcl-2 expressions by western blotting, presented in bar graphs. Significance levels are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. 1c, 1d: Quantification of TLR4/NF-κB signaling pathway by Western blot, displayed in bar graphs. The significance levels were set as above. 1e: qPCR quantification of genes related to inflammation, apoptosis, and the TLR4/NF-κB pathway, as shown in bar graphs. The significance levels are as above. These results indicate a decrease in pro-inflammatory protein expression and suppression of apoptosis in the fistula after injection of MSC, potentially linked to NF-κB pathway inhibition. The qPCR results supported these findings. Groups: M (macrophages), M + LPS (LPS-induced macrophages), and M + LPS + MSC (LPS-induced macrophages treated with MSC supernatant).

M + LPS: 0.536 ± 0.128, $p < 0.05$) showed an increase (Fig. 1e). These findings, based on qPCR analysis, underscore the anti-inflammatory and cytoprotective properties of MSCs.

3.2. 2. efficacy of MSCs in promoting fistula healing

The initial construction of the TEF models in beagles, showing a uniform fistula diameter of 8 mm, laid the foundation for our comprehensive study (Fig. 2a-d, Fig. 2f, and Fig. 3a). Endoscopic evaluation over time provided to dynamically evaluate the fistula healing (Fig. 2e). We observed a discernible increase in body temperature, consistent coughing, and increased sputum production in these animals. These symptoms, indicative of respiratory distress and infection, were valuable in assessing the physiological impact of TEF. Masson's trichrome staining were performed to demonstrated structural changes in the tracheal and esophageal walls, corroborating the endoscopic findings and providing a histopathological perspective of the progression of TEF (Fig. 3b).

Endoscopic observations played a pivotal role in differentiating the healing process between experimental groups. In the group treated with MSCs, there was a remarkable acceleration in the healing process (Fig. 4a–l), with complete fistula closure observed at 3 weeks postoperatively (Fig. 4d). This contrasted sharply with the TEF group, where the fistula remained considerably patent. The

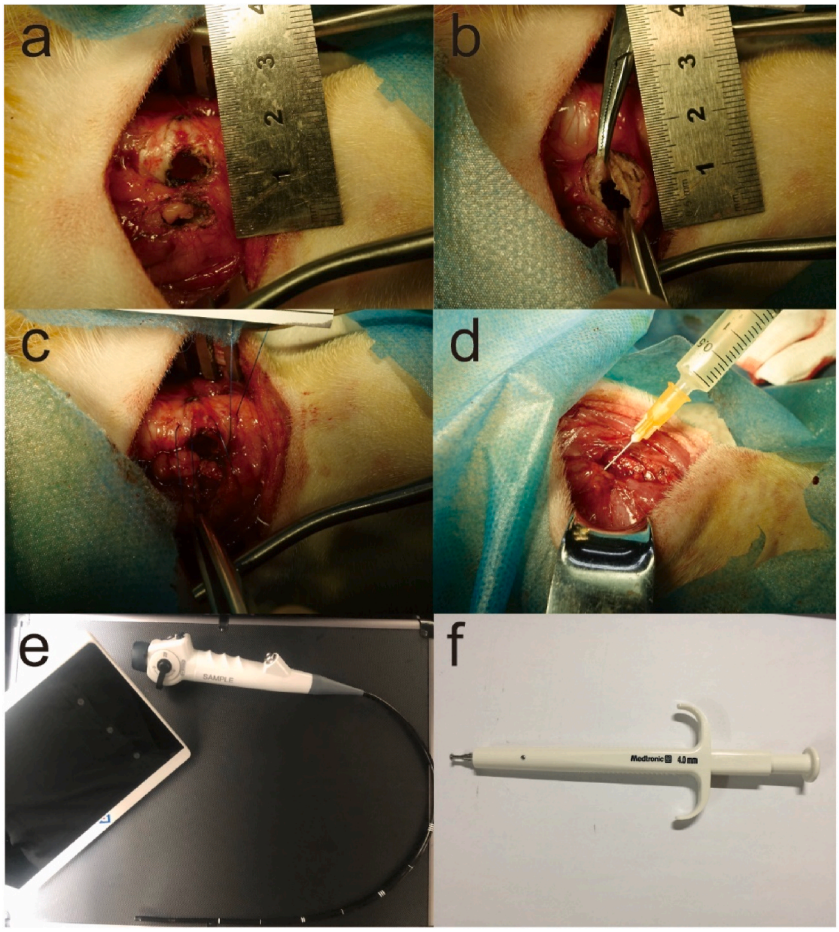

Fig. 2. TEF Construction Procedure. 2a: Measurement of the tracheal fistula. 2b: Measurement of esophageal fistula. 2c: Suturing of tracheal and esophageal fistulas to form a TEF. 2d: Local injection of MSCs at the fistula site. 2e: Endoscopic observation of the healing of the fistula over time. 2f: Tools used for fistula construction. Abbreviations: TEF (tracheoesophageal fistula), MSCs (mesenchymal stem cells).

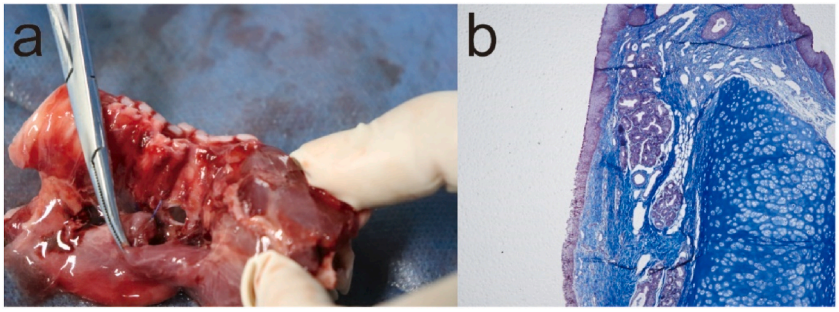

Fig. 3. TEF Formation in Gross Specimens and Masson Stain. 3a: Gross specimen showing TEF formation. Sutures connect the tracheal and esophageal fistulas, forming a fistula with a fibrous structure. 3b: Junction of the tracheal and esophageal epithelium, illustrating the coexistence of the ciliated columnar and squamous epithelium due to TEF. Abbreviations: TEF, tracheoesophageal fistula.

analysis of the gross specimen, particularly the examination of the tissue sections, further confirmed these observations (Fig. 5a–d). The MSC-treated group showed substantial epithelial regeneration and decreased fibrosis around the fistula site, while the TEF group exhibited persistent inflammation and limited epithelial repair. These findings highlighted the regenerative potential of MSCs in the treatment of TEF, suggesting their role in enhancing tissue repair and reducing pathological inflammation.

4. Discussion

The TEF is predominantly the result of emergency or prolonged endotracheal intubation [17–20]. Historically, surgery has been the primary intervention for TEF; however, traditional thoracotomy operations are not universally applicable, especially in cases where the vascular supply of the trachea are compromised [20–22]. This emphasizes the necessity of innovative treatment strategies.

Regenerative medicine provides a new strategy for the treatment of fistula. In previously study, administration of autologous stem cell transplantation has been considered as an effective method for fistula closure [23]. In the study of bronchopleural fistula (BPF), Aho et al. has been described a patient with bronchopleural fistula (BPF) who treated with autologous mesenchymal stem cell. The patient remained asymptomatic during the clinal follow-up of 1.5 years [24]. The Díaz-Agero Álvarez et al. described the patient suffering from BPF who administered with adipose tissue-derived stromal cells leading to 80 % closure of the fistula [25]. Meanwhile, Zeng et al. also described a case report of successful closure of a BPF who treated by MSC [26]. While, the clinical cases involving humans for the treatment of TEF with regenerative medicine approaches are still relatively few. It has been described that a patient with congenital TEF was alleviated by autologous adipose-derived mesenchymal stem cell [27].

MSCs have emerged as a critical player in regenerative medicine due to their ability to secrete proangiogenic cytokines and reprogram recruited cells for effective wound healing [28]. MSCs are recognized for their dual role in stimulating angiogenesis and vasculogenesis while simultaneously suppressing inflammation and apoptosis [29]. Their ability to modulate the local immune response by altering the inflammatory phenotype of macrophages is particularly relevant in the context of TEF. In this study, we explored the attributes of MSCs in TEF beagle model. Both the effects of MSCs on the healing of fistula tissue and their influence on inflammatory and apoptotic processes were examined.

MSCs played essential role in repairing damaged tissues [30]. Numerous studies have documented that MSC transplantation accelerates tissue regeneration and shortens the healing process [31]. However, the specific evidence of MSC treatment in cases of TEF is sparse. In analogous studies focused on bronchopleural fistula, a specific form of tracheal fistula, successful alleviation through autologous adipose-derived MSC transplantation has been reported [24]. Similar findings have been reported in other cases of bronchopleural fistula [32,33], bone marrow-derived MSCs effectively closed these fistulas [25].

The intricate healing process involves a dynamic change in macrophage subsets during the inflammatory phase, distinguishing it from the subsets observed in non-inflammatory conditions [34]. A crucial element in the resolution of inflammation is the transformation of macrophages from a pro-inflammatory to an anti-inflammatory phenotype, which not only quells inflammation but also fosters tissue healing [35]. Furthermore, macrophages play a vital role in triggering the activation of stem cells in various tissues, contributing to their regeneration [36–38]. There is a synergistic interaction between macrophages and stem cells, reminiscent of their interaction with hematopoietic stem cells in the bone marrow or osteoblasts on bone surfaces [39]. It has previously been established that bone marrow-derived MSCs can steer macrophages toward M2 polarization, an action that is primarily attributable to the suppression of the NF-κB signaling pathway [40].

Our investigation reveals that MSC supernatant treatment markedly decreases the expression of pro-inflammatory markers such as p–NF–κB, TLR4, IL-1β, and TNF-α, attenuating the pathological inflammatory response in TEF. The inherent plasticity of macrophages allows them to adapt to various phenotypes and be shaped by the immune microenvironment [41–43]. The balance between M1 and M2 macrophages were critical in different phases of fistula healing. In particular, macrophage NF-κB pathway favors the polarization toward the M1 phenotype. A significant reduction in the inflammatory response of TEF was observed after 35 days of MSC treatment, primarily through the decrease in the abundance of macrophages M1 and their pro-inflammatory activity.

A hallmark of adult stem cells, including MSCs, is their ability to self-renew after injury and their capacity to inhibit apoptosis [44,45]. Recent studies have highlighted that MSCs effectively reduce cell death, improve angiogenesis, and minimize the size in central

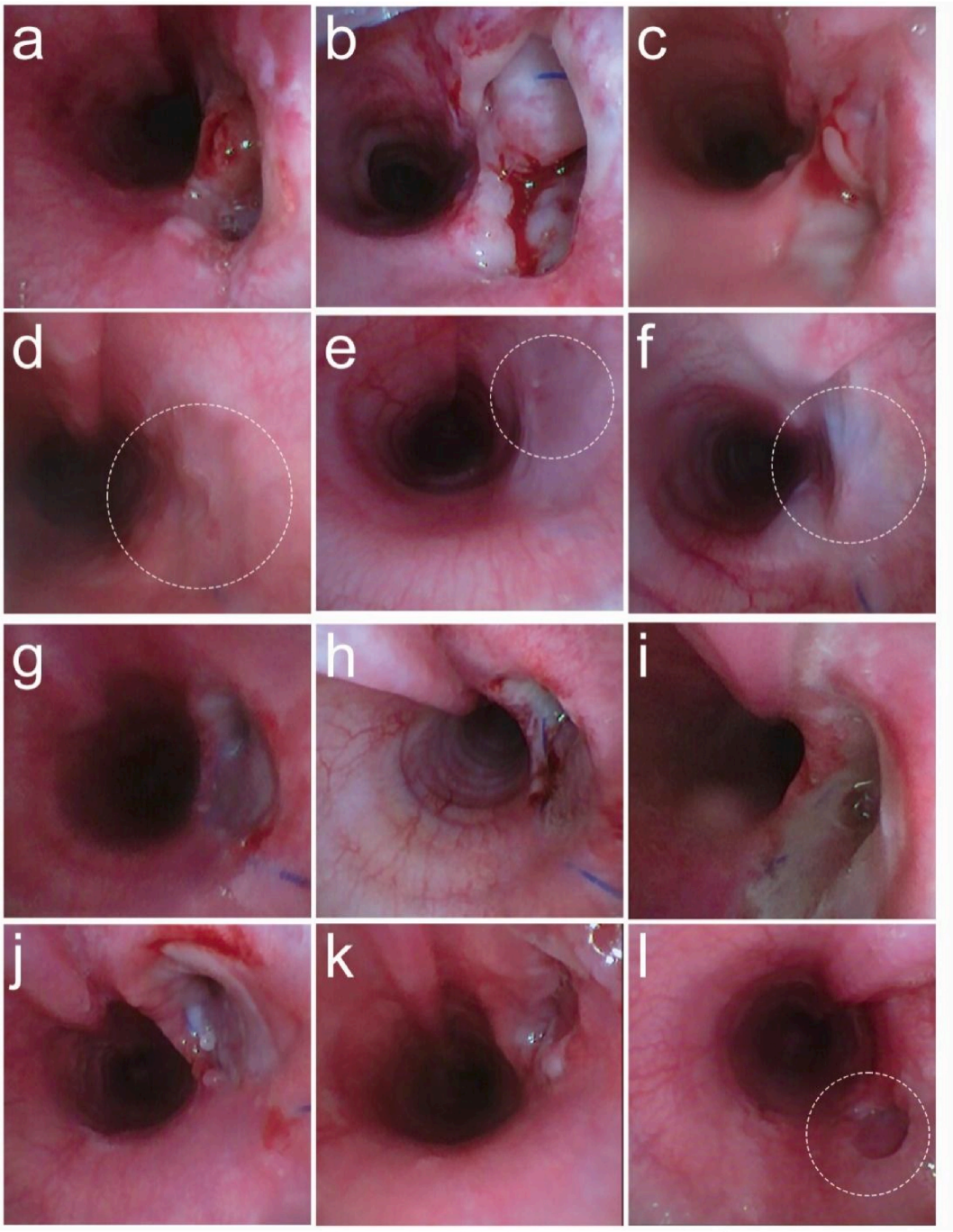

Fig. 4. Dynamic Endoscopic Observation of Fistula. 4a–f: TEF post-construction and at 1/2/3/4/5 weeks in the MSC group. Notable healing was observed at 3 weeks (4d), the structure of the tracheal fistula returned to normal (4e) and complete recovery at 5 weeks (4e, 4f). 4 g–l: TEF post-construction and at 1/2/3/4/5 weeks in the TEF group. Persistent inflammation was observed with no fistula closure at 5 weeks (4 l). Abbreviations: TEF, tracheoesophageal fistula.

nervous system injuries. For example, bone marrow-derived MSCs have been shown to mitigate oxidative stress and reduce cell death in rat models of spinal cord injury [46]. In the context of liver injury, MSCs have shown efficacy in improving conditions in mouse models of acute liver failure, characterized by decreased mortality rates, reduced apoptotic activity, and increased liver regeneration [47]. Moreover, stem cell exosomes have been identified as conveyors of anti-apoptotic signals to damaged heart tissues [48,49].

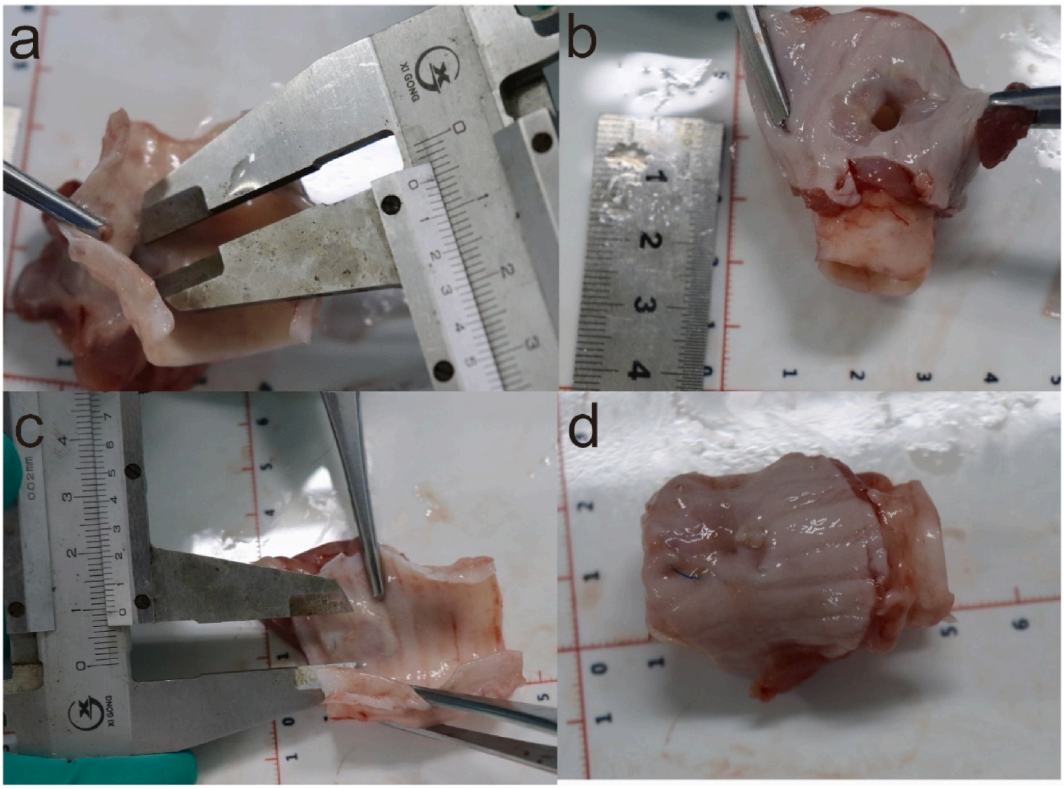

Fig. 5. Observation of TEF After 5 weeks. 5a, 5b: Tracheal and esophageal fistulae in the TEF group after 5 weeks. 5c, 5d: Tracheal and esophageal fistulas in the MSC group after 5 weeks. The gross tissue specimens indicated that the fistula did not heal significantly in 5 weeks in the TEF group, with the diameter remaining less than 8 mm (the initial diameter of the TEF). Abbreviations: TEF, tracheoesophageal fistula.

5. Limitations and future directions

Despite these promising findings, certain limitations persist in our understanding of the specific mechanisms of MSCs in TEF. Technological constraints limit us to trace MSC differentiation in TEF injuries. Therefore, the exact role and differentiation pathways of MSCs in TEF could not be elucidated.

6. Conclusion

In conclusion, our study demonstrated that MSCs can significantly promote TEF healing by mitigating inflammation and apoptosis, mainly by inhibiting the excessive NF-κB pathway in macrophages. These results suggesting the therapeutic potential of MSCs in the management and warrant further investigation to fully harness their regenerative capabilities.

Declarations

Ethics declarations

The study protocols were approved by the IACUC of the Guangdong Laboratory Animal Monitoring Institute (No. IACUC2020132).

Consent for publication

All authors have consented to submit and publishing this manuscript.

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Data availability statement

All relevant data are within the manuscript.

CRediT authorship contribution statement

Jinghua Cui: Software, Methodology, Formal analysis, Data curation. **Yuchao Wang:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Shuixiu Li:** Formal analysis, Data curation. **Yanqing Le:** Methodology. **Yi Deng:** Investigation. **Jingjing Chen:** Investigation. **Qian Peng:** Methodology. **Rongde Xu:** Writing – review & editing, Visualization, Validation, Supervision. **Jing Li:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32903.

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