Objectives: Sengldeng-4 (SD-4) has a long history of use in Mongolian medical clinics for the treatment of rheumatic disorders. The objective of this study was to investigate the therapeutic effect and mechanism of SD-4 on rabbits with knee osteoarthritis induced by Hulth’s method.

Methods: SD-4 (0.428 and 0.857 g·kg⁻¹) or Zhuangguguanjie pill (0.514 g·kg⁻¹) were orally administered in rabbits for four weeks after the induction of knee osteoarthritis using Hulth’s method. Inflammation factors (TNF-α, IL-1β, and NO) and histopathological lesions in the knee joint were examined by ELISA and HE staining. The expression of type II collagen mRNA in the articular cartilage was detected using real-time qPCR.

Results: SD-4 ameliorated the articular lesion damage caused by Hulth’s method in rabbits and reduced the serum levels of inflammatory cytokines TNF-α, IL-1β, and NO. The qPCR results showed that the mRNA expression of type II collagen (COL2) and miRNA-140 gene in the model group was significantly lower than that in the control group (P<0.01), while SD-4 treatment significantly elevated their expressions in the articular cartilage.

Conclusion: SD-4 can improve articular cartilage morphology and inflammation by regulating miRNA-140 and COL2 gene expressions, and thus ameliorating knee osteoarthritis of rabbits induced by Hulth’s method.

Keywords: Mongolian medicine, Sengldeng-4, Knee osteoarthritis, Inflammation
individuals experiencing osteoarthritis, with the knee joint
being the most often afflicted anatomical location [1]. Typically,
the initial conservative approach for osteoarthritis involves the
administration of antibiotics, which may yield significant adverse
effects and pose potential injury to the human organism. When
osteoarthritis progresses to a state of metaphase or severity, the
surgical procedure of joint replacement may impose additional
risks and economic burdens on the affected individuals [2].
Seng Ideng-4 (SD-4) is one of the well-known traditional
Mongolian medicines used for the treatment of various yellow
water diseases. It consisted of five herbs including Xanthoceras
sorbifolia Bge, Terminalia chebula Retz, Gartdebia jasminoides
Ellis and Terminalia Billerica Roxb at a mixing ratio of 5:3:1:1
with the therapeutic functions of clear heat, drying yellow water,
and swelling [3-5]. SD-4 has been used in clinical treatment
of rheumatic diseases for many years, and it was revealed to
have a relieving effect on patients with knee osteoarthritis
(KO) [6]. It was also reported that SD-4 exhibited beneficial
effects against bacterial infection [7]. Modern pharmacological
studies have shown that active ingredients of SD-4 are primarily
phenolic acids, sitosterols, emodin, benzoquinone, quercetin and
garminoside, which have a wide range of bioactivities, including
antioxidant, antineuritic, antitumor, immunomodulatory,
and anti-inflammatory activities [8]. However, the potential
pharmacological mechanism of SD-4 in the treatment of KO
remains to be explored.

The objective of this study was to assess the extent of
alterations observed in the knee joint during the initial phases
of osteoarthritis. Additionally, the study aimed to examine the
impact of SD-4 administration on the structural integrity of the
joint in a rabbit model of KO, with the intention of verifying its
therapeutic efficacy against KO. The current study utilized the
modified Hulth’s model to create a KO model. Subsequently,
the therapeutic impact and underlying mechanism of traditional
Mongolian medicine SD-4 on KO were investigated using a
molecular pharmacology approach.

Materials and methods

Animals
Forty healthy male Japanese white rabbits (body weights ranged
from 2–3 kg upon receipt; Liaoning Chang Sheng experimental
animal center, China) were used after 1 week of acclimatization
to the laboratory environment. Animal quality certificate
number: SCXX (Liao)-2015-0001. The animal experiment was
conducted in accordance with the Declaration of Helsinki, and
the study protocol was approved by the Ethical Committee of the
Mongolian National University of Medical Sciences (Protocol №
2020/Д-09).

Medicine and reagents
SD-4 (20180823) was provided by Affiliated Hospital of Inner
Mongolia University of Nationalities; the positive control medicine
Zhuangguguanjie pill (gzyz44023377) was produced by CR San
Jiu Pharmaceutical Co., Ltd; Tumor necrosis factor-alpha (TNF-α),
interleukin-1 beta (IL-1β) and nitric oxide (NO) ELISA kits were
purchased from Jing Mei Biotechnology (Jiangsu, China); total
RNA extraction kit (dp431), cDNA first strand synthesis kit
(Kr116), fluorescence quantitative detection kit (fp209) and
DNA enzyme (Rt411) were purchased from Tian Gen biochemical
technology (Beijing, China); the primers were designed and
synthesized by Ying Jun company (Shanghai, China). The primer
sequence is as follows:

Type II collagen
Upstream: 5'-TCCTAAGGGTGCCAATGGTGA -3'
Downstream: 5'-AGGACCAACTTTGCCCTTGAGGAC-3'

MiRNA-140
Upstream: 5'-GCCTCAGTGGTTTTACCCTA -3'
Downstream: 5'-GTGCAGGGTCCGAGGT -3'

GAPDH
Upstream: 5'-GCACCGTCAAGGCTGAGAAC -3'
Downstream: 5'-TGGTGAAGACGCCAGTGGA -3'

Induction of knee osteoarthritis disease model in rabbits
A group of forty male Japanese white rabbits, with weights
ranging from 2.5 to 3.0 kg, were subjected to an 8-hour fasting
period, during which they were allowed to consume water.
In this study, animals were subjected to anesthesia with the
administration of 1.53 ml/kg propofol via the marginal ear vein.
Additionally, lidocaine was promptly administered into the right
knee joint. The integumentary tissue covering the articulation of
the left knee was appropriately primed and sterilized. The method
employed in this study was derived from Hulth’s approach as
described in reference [9]. However, modifications were made to
account for the specific anatomical features of the rabbit knee
joint and to address ethical considerations pertaining to the use
of experimental animals. The methodology employed to establish
the model can be described as follows: The subject of the study
underwent a surgical procedure that involved the severing of the medial collateral ligament and anterior cruciate ligament of the knee joint. It is worth noting that this procedure deviates from the existing literature as it did not involve the severing of the posterior cruciate ligament but did involve the removal of the medial meniscus. In order to prevent the occurrence of an infection, a daily injection of penicillin at a dosage of 20,000 units per kilogram was administered for a duration of three days subsequent to the surgical procedure. After a period of four weeks following the induction of knee osteoarthritis (OA) in the subjects, the circumference of the knee was measured on a weekly basis to observe any swelling in the knee joint. Additionally, the weight-bearing value of the bilateral knee joints was measured, and an evaluation of pain in the right knee was conducted, resulting in a corresponding score.

Drug administration
After a period of four weeks following the KO procedure, a total of 32 rabbits were assigned randomly to four distinct groups: the KO surgery group (referred to as the model group), the SD-4 low-dose group, the SD-4 high-dose group, and the Zhuangguguanjie pill group (referred to as the positive control group). An additional eight rabbits comprise the control group for normal conditions. The rabbits in the SD-4 L dose group and SD-4 H dose group were subjected to oral administration of 0.428 g·kg⁻¹ and 0.857 g·kg⁻¹ of SD-4 decoction, respectively. On the other hand, the Zhuangguguanjie pill group got an oral dose of 0.514 g·kg⁻¹ of Zhuangguguanjie pill, which was dissolved in water. Both the blank group and the model group were administered distilled water. The animals received treatment on two occasions per day over a period of four weeks. Throughout the course of the treatment, the overall condition of the rabbits was monitored, and their body weight was assessed on a weekly basis.

Sample collection and preparation
Blood serum collection: Following the cessation of drug administration, the rabbits in each experimental group had an 8-hour fasting period (with the exception of water consumption). Subsequently, a 5 ml blood sample was obtained from the marginal ear vein of each rabbit. Following the incubation of the collected blood at ambient temperature for a duration of 60 minutes, the entire blood sample was subjected to centrifugation at a speed of 3500 revolutions per minute for a period of 10 minutes. The supernatant samples were held at a temperature of -30°C until they were subjected to analysis. Following the cessation of drug treatment, the right knee joint capsule of the rabbits was subjected to injections of saline with a volume of 1.0 ml. Subsequently, the joint fluid was extracted. Following centrifugation at a speed of 3500 revolutions per minute for a duration of 10 minutes. The volume of the supernatant was approximately 200 µl and it was held at a temperature of -30°C until it was subjected to analysis. Histologic assessment was conducted on the knee joint cartilage of the rabbits to examine its collection. The samples were immersed in a 10% neutral buffered formalin solution for fixation and subsequently treated with a 5% nitric acid solution for decalcification. Upon the completion of the decalcification process, the specimen underwent a gradual dehydration procedure using a series of ethanol solutions with varying concentrations. Subsequently, the specimen was rendered transparent by treating it with xylene, and finally, it was embedded in paraffin. Furthermore, the tissue of joint cartilage was subjected to a temporary placement in liquid nitrogen to facilitate quick freezing, followed by storage at a temperature of -80°C until it was ready for analysis.

Morphological and histopathological examination
After sacrifice, knee joints were dissected, and the gross appearance of the femoral condyle was assessed and photographed. For histological analysis, five µm thick sections were stained with hematoxylin-eosin staining. The morphological features of the articular cartilage of the knee were identified using digital images of the stained section. Images are acquired using an optical microscope (200x magnification objective is appropriate).

Enzyme-linked immunosorbent (ELISA) assay
The contents of IL-1β and TNF-α in serum and the contents of NO in synovial fluid were measured by ELISA kits according to the ELISA kit (JingMei Biotechnology, Jiangsu, China) manufacturer’s instructions. Absorbance at a wavelength of 450 nm was detected using a UV-microplate reader.

Real-time qPCR
Total RNA was extracted by the Trizol method and reversely transcribed according to the instructions on the reverse transcription kits. The total mRNA quantitative analysis and the agarose gel electrophoresis qualitative analysis were conducted by the ultramicro spectrum scanner. The cDNA first strand synthesis kit was used to reverse transcribed into cDNA, and the real-time fluorescence quantitative PCR kit was used. SYBR method was used to amplify and collect fluorescence
signals. GAPDH was used as an internal reference to calculate the target gene 2-ΔΔCt; the relative expression level of mRNA of each target gene was compared and analyzed. Each sample was repeated in 3 replicate wells to obtain the average value. ΔΔCt = (Ct value of the target gene in the experimental group - Ct value of the internal reference) - (Ct value of the target gene in the control group - Ct value of the internal reference). The gene regulation was described by the comparison between groups.

Statistical analysis
Experimental data are expressed as mean ± standard deviation (X±S). Statistical analysis was performed using SPSS20.0 software package. Kruskal-Wallis non parametric one-way analysis of variance was used to compare multiple groups of data with Dunn’s post-hoc test. A p<0.05 was considered statistically significant.

Results
Morphological and pathological observation
Following the cessation of drug treatment, the knee articular cartilage in the control group had a smooth and glossy appearance, characterized by a light blue-white hue. In the experimental model group, the observed cartilage surface exhibited a yellowish-white coloration, rough texture, lack of luster, and the presence of cartilage flaws. In the context of the treatment, both the SD-4 group and the Zhuangguguanjie pill group exhibited cartilage lesions associated with osteoarthritis; however, the severity of these lesions was comparatively less severe when compared to the model group (Figure 1).

Histological examination
The histological analysis using HE is staining revealed that the articular structure in the model group exhibited incomplete development, varying degrees of surface damage, ulceration, shallow fissure formation, reduced cell density, mild staining loss, and incomplete tidal mark. When comparing the model group to the SD-4 groups, small observations were made under a microscope regarding the morphological structure and surface damage. Additionally, it was seen that the cells were grouped in clusters. The cellular morphology of the SD-4 groups exhibited superior characteristics compared to the model group (Figure 2A-E).
Biochemical parameters

In comparison to the control group, the model group exhibited a significant increase in TNF-α levels, whereas treatment with SD-4 resulted in a significant decrease (P < 0.05) in TNF-α levels. Furthermore, the levels of IL-1β and NO exhibited a substantial increase in the model group but had a significant reduction (P < 0.05) in both the SD-4 groups and the Zhuangguguanjie pill group.

Table 1. Contents of IL-1β, TNF-α and NO

<table>
<thead>
<tr>
<th>Groups</th>
<th>TNF-α (pg/mL)</th>
<th>IL-1β (pg/mL)</th>
<th>NO (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank group</td>
<td>108.76±28.26</td>
<td>26.41±14.09</td>
<td>97.90±9.22</td>
</tr>
<tr>
<td>Model group</td>
<td>394.17±54.67##</td>
<td>93.59±12.65##</td>
<td>105.46±7.06#</td>
</tr>
<tr>
<td>Chinese medicine group</td>
<td>389.78±81.86##</td>
<td>69.95±18.34##*</td>
<td>91.44±12.56*</td>
</tr>
<tr>
<td>Sengdeng-4 low group</td>
<td>328.86±38.36##*</td>
<td>69.58±8.90##*</td>
<td>93.42±21.41*</td>
</tr>
<tr>
<td>Sengdeng-4 high group</td>
<td>330.57±42.78##**</td>
<td>74.78±18.79##*</td>
<td>89.47±14.77*</td>
</tr>
</tbody>
</table>

Note: # compared with blank group p < 0.05. ## Compared with the blank group, P < 0.01. * Compared with the model group, P < 0.05, ** compared with the model group, P < 0.01.

Expression of Type II collagen (COL2) and miRNA-140 genes

In comparison to the control group, the model group exhibited a substantial decrease in mRNA expression of COL2 and miRNA-140 (P < 0.05). However, the administration of SD-4 and Zhuangguguanjie pill treatments resulted in a significant increase in the expression of these two genes (Table 2).

Table 2. mRNA expression level of COL2 and miRNA-140 in articular cartilage

<table>
<thead>
<tr>
<th>Groups</th>
<th>COL2</th>
<th>miRNA-140</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Group</td>
<td>4.70±2.56</td>
<td>1.51±0.32</td>
</tr>
<tr>
<td>Model Group</td>
<td>0.06±0.06##</td>
<td>0.70±0.43##</td>
</tr>
<tr>
<td>Control Group</td>
<td>4.76±4.95*</td>
<td>2.46±1.84*</td>
</tr>
<tr>
<td>Sengdeng-4 low dose</td>
<td>5.4±3.56**</td>
<td>1.36±0.52*</td>
</tr>
<tr>
<td>Sengdeng-4 high dose</td>
<td>4.76±3.9##*</td>
<td>1.97±1.12*</td>
</tr>
</tbody>
</table>

Note: ## compared with the blank group P < 0.01. * Compared with the model group P < 0.05, ** compared with the model group P < 0.01.

Discussion

Mongolian medicine exhibits inherent qualities such as being derived from natural sources, possessing few adverse effects, ensuring safety and reliability, and exhibiting low resistance [10, 11]. The treatment of diseases has demonstrated significant advantages and has garnered increasing attention from individuals due to its potential for widespread use [12]. Consequently, it is deserving of further investigation and promotion within therapeutic settings. The present study employed the modified Hulth’s model to develop a KO model, and utilized the molecular pharmacology method to elucidate the therapeutic impact and mechanism of action of traditional Mongolian medicine SD-4 on KO in rabbits. In this study, the ELISA method was employed to identify the presence of IL-1β, a pro-inflammatory cytokine that is closely associated with osteoarthritis [13]. The indexes observed in the model group exhibit a statistically significant increase compared to those in the control group, aligning with the findings reported in the aforementioned literature research. Simultaneously, it is widely acknowledged that IL-1β has the ability to stimulate the expression of NO [14]. The experimental findings indicate that
the concentration of NO in the joint fluid of the model group is significantly higher compared to that of the control group. The level of TNF-α in the model group is observed to be higher compared to the control group, which aligns with previous findings reported in the literature. During the progression of osteoarthritis, IL-1β plays a significant role in the regulation of the proliferation and differentiation of articular chondrocytes and synovial cells [15]. Several studies have indicated a correlation between IL-1β and osteoarthritis; nevertheless, the precise mechanism by which IL-1β influences KO has yet to be fully understood [16-18]. The production of a significant quantity of IL-1β has been observed in the articular cartilage, synovium, and joint fluid of individuals with osteoarthritis, indicating a strong association between IL-1β and the progression of KO [19]. Simultaneously, IL-1β has the capacity to stimulate the production of NO and afterwards trigger chondrocyte death. In other words, the presence of IL-1β results in an elevation in NO levels in both joint fluid and serum. This increase in NO production hinders the synthesis of collagen and proteoglycan, triggers cellular death, and ultimately contributes to the deterioration of cartilage and inflammation of the synovial tissue [20,21]. Furthermore, the presence of IL-1β might induce the breakdown of the cartilage matrix, resulting in the eventual destruction of articular cartilage [22]. Overall, it was observed that the levels of IL-1β, TNF-α, and NO exhibited a considerable increase in the KO model group in comparison to the control group. However, following the administration of SD-4, these levels demonstrated a subsequent decrease. Hence, it is plausible that the effect of SD-4 in a KO animal model could be attributed to its capacity to mitigate inflammatory responses. The initial occurrence of articular cartilage injury is attributed to the degradation of COL2 within the extracellular matrix. COL2 forms the primary structural constituent of cartilage [23]. The primary procedure involves the cleavage of the peptide bond between the collagen peptide chains, forming the characteristic interwoven fiber structure observed in articular cartilage [24]. In contrast, miRNA-140 has the potential to enhance the inflammatory response of chondrocytes. Several studies have demonstrated that the expression of miRNA-140 can be considerably suppressed by IL-1β and other cytokines that are closely related [25,26]. After birth, mice that lacked miRNA-140 exhibited deformities in bone growth and experienced apoptosis in chondrocytes. miRNA-140 holds significant prominence in the context of chondrocyte growth and development [27,28]. Furthermore, it is important to note that COL2 serves as the primary structural constituent of cartilage when considering its structural composition [29,30]. The present study employed the qPCR technique to ascertain alterations in gene expression levels of COL2 and miRNA-140 within the articular cartilage. The findings indicated a considerable downregulation of COL2 and miRNA-140 gene expression in the model group. However, significant increases in the expression of COL2 and miRNA-140 mRNA were observed in SD-4 groups as compared to the model group, suggesting that the SD-4 may possess the ability to safeguard the integrity of articular cartilage structure and potentially serve as a therapeutic intervention for KO [31]. All these evidence strongly suggest that SD-4 possesses the potential to exert an anti-inflammatory effect through the activation of the miRNA-140 and COL2 signaling pathway during the anti-KO process. The present study has some limitations. Although this research has confirmed the anti-KO capability of SD-4, it is important to note that the investigation of the key bioactive components of SD-4 was not conducted in this study. Therefore, additional chemical investigation is required in order to ascertain the primary active chemicals present in SD-4 and validate their anti-inflammatory and anti-KO properties. Furthermore, our study identified two key targets, namely miRNA-140 and COL2 genes, which were hypothesized to be the underlying pharmacological mechanism via which SD-4 improves knee osteoarthritis in rabbits. Nevertheless, the present investigation solely validated the aforementioned genes at the mRNA expression level. Further experiments are required to validate potential drug targets in the protein expression level and to elucidate the underlying mechanisms combining both in vivo and in vitro models.

Conclusion

SD-4 can improve articular cartilage morphology and inflammation by regulating miRNA-140 and COL2 gene expression and to relieve KO in rabbits. The Mongolian medicine prescription SD-4 is a promising anti-KO agent with potential use for treating rheumatic diseases in clinical practice.

Conflict of Interest

The authors declare no conflict of interest.
Acknowledgements

The authors greatly appreciate the editors and anonymous peer reviewers for their critical reading and insightful comments, which are helpful to improve our manuscript substantially.

References


