Anti-apoptotic effect of resveratrol: regulate of mitochondrial pathway in high tensile stress-induced goat temporomandibular joint disc cells

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Article

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Abstract

**Background:** Attenuating of temporomandibular joint disc (TMJ disc) cells apoptosis may be an effective strategy to reduce abnormal stress-induced disc degeneration. However, a potential target to regulate the apoptosis of TMJ disc cells under abnormal stress is still inconclusive. Resveratrol (RSV) is a polyphenol with multiple effects such as anti-inflammatory, antioxidant, and anti-apoptosis. In this study, an appropriate periodic tensile stress was selected to induce apoptosis in goat TMJ disc cells. To examine the effect and mechanism of RSV on cells via apoptosis under high tensile stress.

**Results:** An experimental model of high tensile stress-induced cells were successfully constructed with 8% stretching stimulus. CCK-8 and Flow cytometry assay showed that 8% tensile stress significantly promoted the apoptosis of TMJ disc cells. However, RSV (7.5 μM) attenuated cell apoptosis under high tensile stress, reflected by the increased ability of cell proliferation and decreased apoptosis rate. Furthermore, the gene expression of pro-apoptotic molecules (Bax and Caspase-3) was down-regulated, whereas that of anti-apoptotic molecule (Bcl-2) was up-regulated. It can be concluded that the mitochondrial pathway exerted an essential role in the regulation of apoptosis by RSV.

**Conclusion:** High-intensity stress effectively promotes the apoptosis of goat TMJ disc cells, while RSV is able to reverse cell apoptosis through the mitochondrial pathway. This work provides a new reference for the clinical treatment of abnormal stress-induced TMJ disc degeneration.

**Background**

The temporomandibular joint (TMJ), one of the most complex joints in the human body, is constantly under loading during jaw movement [1, 2]. In order to maintain the normal mechanical properties of TMJ, the fibrocartilage articular disc between the mandibular condyle and the temporal fossa plays a crucial role in stress-buffering [2]. It is subjected to a multitude of different loading regimens during mandibular movements, including tension, compression, and shear force. When the loading remains at a reasonable level, the disc experiences no injury generally [3]. However, when suffering from excessive force or abnormal force direction such as occlusal trauma, bruxism, clenching, and abnormal condyle morphology, the most frequently seen is disc displacement, causing a clicking sound during jaw movement. When it develops to the disc displacement without reduction, the disc is permanently displaced or dislocated, resulting in limited mouth opening in severe cases. At this time, it has been speculated that the disc is damaged to varying degrees by excessive mechanical stress, in most cases, becomes deformed, or even perforated. In addition, disc displacement is associated with tissue degeneration, and it is considered to be a risk factor for the occurrence and development of osteoarthritis [4]. It has been shown that dynamic mechanical loading is a crucial stimulus to maintain the homeostasis of TMJ cartilage. When the loading is applied at a low intensity, it effectively protects inflammatory tissue by antagonizing IL-1β. However, frequent overloading accelerates cell death and causes cartilage degeneration [5].
Apoptosis is a programmed death mode, distinct from cell necrosis, which involves the process of cell death under the high regulation of specific signals and genes in cells. It plays a vital role in removing harmful, damaged, or unwanted cells without causing an inflammatory response. However, excessive or weak apoptosis is likely to lead to uncontrolled cellular homeostasis [6]. At present, apoptosis has been documented to have a central role in TMJ disorders. After injecting monosodium iodoacetate into the upper cavity of rat TMJ, the intermediate zone of the disc loosened within one day, gradually thinned, and osteoarthritis could be induced within 4 weeks [7]. HE staining showed that disc cells underwent apoptosis within 24 hours, and chondrocyte apoptosis reached a peak after 3 days. It indicated that early apoptosis may be an essential initiator of cartilage degeneration [7]. In addition, under abnormal mechanical loading, cells can sense and convert mechanical signals into biological signals, thereby changing cell biochemical reactions by cell proliferation, apoptosis, and metabolism, then to tissue degeneration [8]. A previous study established a flow fluid shear stress model in vitro and a unilateral anterior crossbite animal model [9]. The results demonstrated that abnormal biomechanical stimulation could activate and regulate MTORC1 signaling, which modulated the autophagy and apoptotic programs in TMJ chondrocytes, leading to cartilage degeneration [9]. As mentioned above, attenuating cells apoptosis may be an effective strategy to reduce abnormal stress-induced tissue degeneration. Therefore, it is necessary to explore a potential target to regulate the disc cells apoptosis under abnormal stress to develop an effective therapy for TMJ disorders.

Resveratrol (RSV) is a natural polyphenolic compound that exists in more than 70 plant species, especially in grapes, berries, and peanuts [10]. Recently, RSV is reported to affect apoptosis, autophagy, and matrix synthesis through different signaling pathways, having been shown to possess anti-inflammatory and antioxidant effects [11]. A large number of medical studies have supported that it is beneficial to improve cardiovascular diseases, diabetes, osteoarthritis, and neurodegenerative diseases [10, 11]. Even more, RSV could represent a potential therapeutic strategy involved in TMJ disorders [12]. Yuce et al. [13] injected RSV in rats of TMJ osteoarthritis induced by Freund’s complete adjuvant. The results verified that chondrocyte apoptosis and MMP-13 expression were lower than those in arthritis groups without RSV injection. Furthermore, the inflammation-induced disc thickening was significantly reduced. In Li et al. study [14], inflammation promoted the degradation of condylar cartilage matrix both in vitro and vivo. While RSV exerted antioxidant effects to reverse the matrix degradation and inhibited the apoptosis of chondrocytes by down-regulating the COX-2/NF-κB pathway. In addition, Zhang et al. [15] demonstrated that RSV could attenuate mechanical overloading-induced nucleus pulposus cell apoptosis by regulating the ERK1/2 signaling pathway. Based on the above statements, we speculate that RSV may be an effective drug to attenuate stress-induced cells apoptosis. Therefore, this study mainly explored the effect and mechanism of RSV on the apoptosis of goat TMJ joint disc cells under high-intensity stress, thus providing a new reference for clinical treatment.

**Results**

**Effects of stretch loading on goat TMJ disc cells**
After loading tensile stress for 3 days, the cytoskeleton morphology was completely different in the experimental groups and the control groups according to the results of immunofluorescence staining (Fig. 1A). Control cells showed a clear filamentous structure with a disorderly arrangement. In the 2% tensile group, the fiber network structure was still intact and clear, and the fiber filaments were increasingly elongated. With the increase of the stretching rate, the actin skeleton gradually dissociated. And the fiber skeleton was highly elongated, even pulled to break in the 8% tensile group. The stretched cells were rearranged in a specific direction similar to a school of fish. As exhibited in Fig. 1B, 2% tension could promote cell proliferation (P<0.01), while 4% and 8% tension inhibited cell proliferation compared with the control group (P<0.01). The results of flow cytometry suggested that the highest apoptotic rate (about 42%) was reached in the 8% stretching group (Fig. 1C).

Taken together, these results suggested that 8% tensile stress could significantly inhibit cell proliferation and promote apoptosis. Therefore, it proved that 8% tensile stress was feasible as high-intensity stress.

**Screening out the optimal concentration of RSV**

The cells proliferation ability against different concentrations (0, 2.5, 5, 7.5, 10, 20 μM) of RSV for 1, 2, and 3 days is shown in Fig. 2A. In the range of 2.5-7.5 μM, RSV promoted cell proliferation and inhibited cell growth significantly when ≥10μM. Therefore, the optimal concentration of RSV under high-intensity stress was selected up to 2.5-7.5μM. While under 8% tensile stress, the proliferation of cells increased linearly after RSV at 0, 2.5, 5, 7.5μM added to the cells for 3 days (Fig. 2B). Therefore, 7.5μM RSV was selected to intervene in cell growth.

**Effects of RSV on TMJ disc cells under high tensile stress**

**Effects of RSV on the proliferation of high tensile stress induced-TMJ disc cells**

The results of CCK-8 showed that 8% tensile force significantly inhibited cell proliferation when compared with the control group (P<0.01). The cell proliferation ability in the 8%+RSV group was enhanced compared with the 8% stretching group (P<0.05), as shown in Fig. 3A.

**Effects of RSV on apoptosis of high tensile stress induced-TMJ disc cells**

Hoechst 33259 staining was shown in Fig. 3B, and the cells were bright blue as apoptotic cells. Fewer apoptotic cells were observed in the control and the 2% tension group, while the 8% tension group showed much more apoptosis cells. After adding RSV, the apoptosis was significantly reduced in the 8%+RSV group. The results of flow cytometry were shown in Fig. 3C, it was basically consistent with the staining result.

**RT-PCR assay of apoptosis-related genes Bcl-2, Bax, and Caspase-3 expression**

The expression levels of apoptosis-related genes Bcl-2, Bax, and Caspase-3 detected by RT-PCR were shown in Fig. 4. Compared with the control group, the gene expression of the anti-apoptosis molecule
(Bcl-2) was up-regulated and the pro-apoptosis molecules (Bax and caspase-3) were down-regulated in the 8% tension group. The expression of Bcl-2 in the 8%+RSV group was increased ($P>0.05$), and the expressions of Bax and Caspase-3 were significantly decreased ($P<0.05$).

**Discussion**

A healthy TMJ typically experiences a variety of functional stresses and continuously adapts to changing functional demands to maintain structural and functional stability and integrity [16]. However, the mechanical environment of the TMJ disc is completely complex because of stress-strain interactions on nutrient gradients and metabolism where cells and tissues are sensitive to mechanical loading. More specifically, the balance between nutrient transport and consumption establishes a concentration gradient throughout the articular disc. This gradient is modulated by mechanical loading, affecting cell viability, metabolism, matrix synthesis, and responses to inflammatory factors [17]. In recent years, studies on the biomechanics of living cells have found that Young's modulus of cells has undergone dramatic changes in the process of apoptosis [18]. This suggested that there was a certain relationship between cell mechanics and apoptosis. Currently, it is believed that abnormal mechanical loading is involved in activating multiple inflammatory pathways and downstream signaling pathways, such as IL-1β, TNF-α, NF-κB, Wnt, microRNA, and oxidative stress, as well as regulating the key degrading enzymes in articular cartilage, which induces cellular apoptosis, and matrix degradation [19].

In our study, 8% uniaxial cyclic tensile strain was applied to establish a high-intensity stress model. Initially, this study proved the response of apoptosis of disc cells to 8% tension. The dissociation of the actin skeleton was seen from our results of immunofluorescence staining. Previous studies have found that there are not only changes in the mechanical properties of cells, but also the remodeling and conformational changes of the cellular actin microfilament skeleton in the process of apoptosis [20]. Obviously, 8% tensile stress played a significant role in promoting cell apoptosis. Furthermore, this conclusion was also based on a previous study [21]. It was demonstrated that in the strain range of 2%-4%, the typical corrugated structure of collagen fibers in the joint disc was observed and interacted with proteoglycans and interstitial fluid to withstand stress and strain without causing tissue damage. While with increasing strain, collagen fibers were straightened, the tensile stiffness of the disc tissue increased rapidly, and the tissue was gradually deformed or even destroyed [21].

Apoptosis in the TMJ disc occurs mainly through the endogenous (mitochondrial-mediated) and the exogenous (death receptor-mediated) pathways [22]. The endogenous pathway is triggered by intracellular damage, cytokine shrinkage, DNA damage, oxidative, endoplasmic reticulum stress, and overload of cytoplasmic calcium. This pathway is influenced in part by members of the mitochondrial outer membrane-bound Bcl family, including Bcl-2 and Bax, which act as anti- and pro-apoptotic regulatory proteins, respectively [23]. Huang et al. [24] were the first to describe these molecules in normal rabbit TMJ. And the balance of high Bcl-2 and low Bax expression ensured their critical roles in maintaining fibrochondrocyte survival and metabolism. However, the imbalance of Bcl-2 results in the loss of mitochondrial wall integrity. Then Cytochrome c is released into the cytoplasm, and it activates...
Caspase-9, which in turn activates the executor Caspase-3, thereby inducing apoptosis. On the other hand, the extrinsic pathway is triggered by injury or pathogen-associated molecules and the tumor necrosis factor receptor family. Although the two pathways activate different cascades, both ultimately activate Caspase to result in cell apoptosis [25]. TMJ disc degeneration appears to be associated with decreased cell numbers through apoptosis-related processes, which in turn lead to impaired extracellular matrix production, organization, and repair [16]. Another finding has also suggested that apoptosis is associated with disc degeneration, especially through the mitochondrial pathway [26]. In a model of hydrostatic pressure-induced apoptosis of rat condylar chondrocytes, it was found that the expressions of Bax and cleaved-Caspase-3 were increased, while the expression of Bcl-2 was decreased with increasing pressure [27]. In our study, TMJ disc cells remained a low expression of Bcl-2 and high expression of Bax and Caspase-3 under high-intensity stress. It mediated apoptosis through the mitochondrial pathway.

In recent years, numerous vitro and animal models have investigated the role of RSV in TMJ disorders. RSV (22.5 mg/kg and 45 mg/kg) supplementation in mice for 12 weeks prevented osteoarthritis progression by reducing type II collagen degradation and inhibiting chondrocyte apoptosis [28]. RSV treatment in rats with traumatic osteoarthritis inhibited apoptosis by upregulating miR-18a expression and increasing the proportion of chondrocytes in the S phase of the cell cycle [29]. In addition, the ability of RSV to protect articular chondrocytes was associated with the inhibition of metalloproteinase expression by inhibiting IL-1β-induced IKB activation and regulating the NF-κB inflammatory pathway [30]. In the present study, our results revealed that RSV partly inhibited the apoptosis of TMJ articular disc cells under high-intensity stress, mainly regulating apoptosis through the mitochondrial pathway. However, existing human clinical trial results are controversial regarding the protective effect of RSV on diseases and their sequelae. The reasons for these controversies are diverse, and possible reasons that have been proposed include differences in patient characteristics, RSV doses, and duration of action. Most importantly, the optimal RSV dose that maximizes benefits without causing toxicity issues remains an area of extensive research. Varies in RSV concentration have different effects on cell growth, proliferation, or apoptosis. This is consistent with our findings showing that in the range of 2.5–7.5µM, RSV promoted TMJ disc cell proliferation and inhibited cell growth significantly when \( \geq 10\mu M \).

Notably, RSV is also a potent autophagy regulator, acting on cells by regulating autophagy-related signaling pathways. There are reports that the effects are related to the activation of SIRT1 [31]. On the other hand, the interaction between gut microbiota and TMJ disorders deserves attention, and the RSV test is a meaningful start. In a mouse model of TMJ arthritis induced by Freund's complete adjuvant, not only persistent joint pain was observed, but also a decrease in intestinal short-chain fatty acids, including acetate, propionate, butyrate, and changes in related microbiota were detected. Interestingly, RSV treatment suppressed inflammation and reversed the reduction of intestinal short-chain fatty acids and microbiota, dose-dependently. The results suggested that gut microbiome disturbance was critical for the development of TMJ inflammation [32]. We summarized the anti-apoptotic effect of resveratrol on temporomandibular joint disc cells induced by abnormal stress, as shown in Fig. 5.
Our study also has several limitations. First, it is difficult to simulate the stress state of TMJ disc cells as that of the human body. Second, TMJ disc cells include fibroblast-like cells and chondrocyte-like cells. We did not discuss the difference between the two cells under stress and drug intervention. Third, no specific signaling pathways in the process of apoptosis were explored. We will continue to study the effects of RSV on disc cell biology in future studies.

Conclusions

In conclusion, this study successfully established a model of TMJ disc cell via apoptosis induced by high-intensity stress. Moreover, RSV effectively reverses cell apoptosis through the mitochondrial pathway, which provides a reliable experimental basis for future clinical treatment.

Methods

Main reagents and instruments

Human fibronectin (Solarbio, China), CCK-8 (Solarbio, China), Hoechst 33259 (Solarbio, China), FITC-phalloidin (UE, USA), DAPI solution (Solarbio, China), Annexin V-FITC Apoptosis Detection Kit (Yeasen, China), mRNA Extraction Kit (Vazyme, USA), cDNA Synthesis Kit (Vazyme, USA), RT-qPCR Kit (Vazyme, USA), Inverted fluorescence microscope (CX31, Olympus, Japan), B-Bridge cell stretcher (ST-190-XY, USA), Multi-dimensional panoramic flow cytometer (FlowSight, USA), Real-time PCR instrument (ABI, USA).

Isolation and culture of goat TMJ disc cells

A fresh 3-month-old goat head was cleaned and soaked in 75% alcohol for 30 minutes. Under sterile conditions, the TMJ disc was taken out as a whole, and the attached muscle and ligament tissue were removed. After being fully rinsed with PBS, it was cut into pieces to about 1mm³ and added to 0.2% type I collagenase to digest. Then, the cells were placed in a constant temperature water bath at 37 °C and 90 r/min for about 18 hours. After centrifugation, the cells were collected and cultured. When approximately 90% adherent cells were found in the flask, they were digested with 0.25% trypsin and passaged. In this study, the third passage cells after passaging were applied.

Screening of high-intensity stress values

Groups of experiment

Using the cell loading test system (B-Bridge cell stretcher), goat TMJ disc cells were inoculated into human fibronectin-treated silicone membrane chambers, and the uniaxial periodic tensile strain was applied (the frequency of 0.5Hz for 1 hour once per day) for 3 days (Fig.6). According to the stretching strain ratio, it was divided into the control group (0% tension) and 2%, 4%, and 8% tension groups. It was preliminarily verified that 8% tensile stress could promote apoptosis as high-intensity stress, which could be applied in the next experiment.
Immunofluorescent staining assay

The cells were seeded in the silicone membrane chambers at a density of $10^4$ cells/ml. After the cells were completely attached to the wall, different groups were given corresponding treatments. After 3 days, 10μl FITC-phalloidin stock solution diluted in 800μl PBS was added to the cells, then incubated for 20 minutes at 37°C protected from light. Finally, 800μl DAPI solution (10ug/ml) was added and incubated for 10 minutes again. The changes of the cytoskeleton were observed under a laser confocal microscope. FITC-phalloidin was stained for the actin skeleton of cells, and DAPI solution was stained for cell nuclei.

CCK-8 assay

The cells were seeded in 96-well plates, 10μL of CCK-8 reagent was added to each well and the plate was incubated at 37°C for 4 hours. Then the optical density (OD) value at 450 nm wavelength was measured on a microplate reader. This result reflected the proliferation of cells.

Assay by flow cytometry

The cells were washed twice with PBS, digested with trypsin and centrifuged, and resuspended in PBS for 2 times. Then the supernatant was discarded, and 100μl of 1×BD buffer was added for resuspending cells. Finally, 5μl Annexin V and 10μl PI were added, respectively. The samples were stained at room temperature for 15 minutes and the apoptosis rate was detected by flow cytometry within 1 hour.

Screening for RSV concentration

Screening of RSV Concentration Ranges that promote cell proliferation

A previous study concluded that low concentrations of RSV inhibit apoptosis; on the contrary, RSV promotes apoptosis at high concentrations [33]. Therefore, the concentration range of RSV to promote the proliferation of TMJ disc cells was firstly screened. At first, the cells were seeded in a 96-well plate at a density of 6000 per well, and different concentrations of RSV (0, 2.5, 5, 7.5, 10, 20μM) were added. Then, 10μL of CCK-8 was added to each well after 1, 2, and 3 days, respectively. Finally, the plate was incubated at 37°C for 4h. The OD value was measured on a microplate reader.

Screening the optimal concentration of RSV under high-intensity stress

The cells were seeded in silicone membrane chambers at a density of $10^4$ cells/ml and loaded with 8% tensile stress. Then, different concentrations of RSV (the concentration range selected in the first step to promote cell proliferation) were added. After 3d, the OD values were measured.

Effects of RSV on apoptosis of goat TMJ disc cells under 8% tensile stress

Groups of experiment
The following experiment was divided into 6 groups: control, RSV, 2% tension, 2% tension +RSV, 8% tension, and 8% tension +RSV. After the cells were grown for 24h, periodic tensile stress was applied, parameters: 0.5Hz, 1h/d, 3d. 8% tension force was intervened as high-intensity stress.

**CCK-8 assay**

The same step as above.

**Hoechst 33259 staining assay**

The culture medium was removed, and 0.5µg/ml Hoechst 33259 solution was added to fully cover the samples, then incubated at 37°C for 15-20 minutes. The samples were rinsed three times with PBS and observed directly under a fluorescence microscope (excitation wavelength 350nm, emission wavelength 460nm).

**Assay by flow cytometry**

The same step as above.

**RT-PCR assay**

RNA was extracted from TMJ disc cells in each group according to the steps of the RNA extraction kit instructions. The concentration of the sample was determined by an ultra-micro spectrophotometer. If the ratio of A260/280 was 1.8-2.0, this showed the purity of the RNA could be guaranteed. The corresponding cDNA was synthesized and amplified according to the reverse transcription instructions.

Bcl-2 forward (5'-3'): 5'-AGTGGGAACCTTTGCGATTTT-3'
reverse (5'-3'): 5'-AAAGCAGGGCACAACACAC-3'

Bax forward (5'-3'): 5'-GAGAGGTCTTTTCCGAGTGG-3'
reverse (5'-3'): 5'-AAGTAGAAAAGGGCACAACC-3'

Caspase-3 forward (5'-3'): 5'-TGCAAAACACACGAGCAGTGA-3'
reverse (5'-3'): 5'-CACCTTTGTCAAGCTTCTTCT-3'

Finally, the results were analyzed using the $2^{-\Delta\Delta CT}$ statistical method.

**Statistical analysis**
Graphpad Prism 8.0 statistical software was used for analysis, and the experimental data were expressed in the form of mean ± standard deviation (Mean ± SD). Paired t-test was used between two groups of data, and a one-way analysis of variance (ANOVA) was used for multiple groups of data. P<0.05 was considered statistically significant.

**Abbreviations**

TMJ disc: Temporomandibular joint disc; RSV: Resveratrol; PBS: Phosphate buffer saline; OD: Optical density; RT-PCR: Reverse transcription polymerase chain reaction; SD: Standard deviation; ANOVA: One-way analysis of variance.

**Declarations**

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**Authors’ contributions**

HR performed all experiments with the help of FM. GB and HK designed and guided the experiments. The manuscript was written by HR and revised by SZ, JH, QL, FM. All the authors reviewed the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

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

**Ethics approval and consent to participate**

In this study, TMJ-disc was extracted using 3-6 month old healthy fresh sheep heads (purchased from the slaughterhouse in Qilihe District, Lanzhou City), approved by the Experimental Animal Ethics Committee of the School of Stomatology, Northwest Minzu University (No. XBMZ YX-2021004), and cultured, prepared and primed by the Key Laboratory of Stomatology. All methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines.

**Consent for publication**
Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


Figures

![Figure 1](image_url)
Effects of stretch loading on goat TMJ disc cells

A. a-d was the microscopic view of immunofluorescence staining B. CCK-8 detected the proliferation ability of TMJ disc cells under stretch loading C. Flow cytometry was used to detect the apoptosis rate under stretch loading. **P<0.01 vs control group, n=3.

![Graph A](image1)

![Graph B](image2)

**Figure 2**

The effect of RSV on the proliferation of goat TMJ disc cells

A. The proliferation ability of TMJ disc cells was detected by CCK-8 at different concentrations (0, 2.5, 5, 7.5, 10, 20μM) of RSV B. The proliferation of TMJ disc cells was detected by CCK-8 at different concentrations (0, 2.5, 5, 7.5μM) of RSV under 8% strain. **P<0.01 vs control group, # P<0.05 vs intergroup, n=4.
**Figure 3**

Effect of RSV on TMJ disc cells under high tensile stress

A. CCK-8 detected cell proliferation B. Microscopic observation of Hoechst 33259 to detect the apoptosis of goat TMJ disc cells C. Cell apoptosis rate was detected by flow cytometry. **P<0.01 vs control group, # P<0.05 vs intergroup, n=3.**
Figure 4

Expression levels of apoptosis-related genes Bcl-2, Bax, and Caspase-3 were detected by RT-PCR

**P<0.01, *P<0.05 vs control group, # P<0.05 vs intergroup, n=3.
Figure 5

Anti-apoptosis effect of resveratrol on abnormal stress-induced TMJ disc cells
Figure 6

A schematic diagram of the cell loading test system

A. The composition of the stretching system of the B-Bridge was shown. B. The silicone rubber membrane did not deform when the cells were not loaded. C. The membrane deformed along the stretch direction, and the TMJ disc cells were stretched.