Protective effect of Calpain2 on mouse fibroblast L929 from injury induced by mechanical force

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Abstract

Introduction and Hypothesis: To investigate the protective effect of Calpain2 on the mechanical force induced apoptosis in L929 cells, we compared the difference of Calpain2 in pelvic floor tissues between patients with uterine prolapse and those without uterine prolapse.

Methods: The cultured L929 cells were subjected to gene silencing treatment. The cells were grouped into control group, mechanical force group, sh-Capn2 group and sh-Capn2+ force group. Western blot and cell immunofluorescence assay were used to detect protein content: Calpain2, caspase3, Bcl2, Bax, cell apoptosis was detected by Annexin V-FITC. Each set of experiments was repeated three times.

Results: The expression of Calpain2 in pelvic floor tissues of patients with uterine prolapse was lower than that of patients without uterine prolapse. There was a negative correlation between Calpain2 and apoptosis, the decrease of Calpain2 was accompanied by the increase of apoptosis after exposing to mechanical force. There was no difference in Calpain2 expression between the sh-Capn2 group and the sh-Capn2 force group, but the latter had significant apoptosis, caspase3 was higher than the former, and the ratio of Bcl2/Bax was decreased.

Conclusions: The content of Calpain2 in pelvic floor tissue of normal and POP patients is different., and its expression has a protective effect on L929 cells from injury induced by mechanical force.

Key words： POP Mechanical force Fibroblasts Apoptosis

Brief summary: Compare the different content of Calpain2 in the pelvic floor tissue

and explored the relationship between Calpain2 and apoptosis in cells level

Introduction

Pelvic organ prolapse (POP) is a group of gynecological diseases, which are caused by the weak pelvic organs and abnormal function of the pelvic organs due to the weakness of the pelvic floor and the fascia, including the prolapse of the uterus, the bulging of the anterior wall of the vagina, the bulge of the posterior wall of the vagina, etc. Epidemiological studies have revealed that age and estrogen levels, pregnancy and vaginal delivery, constipation, and other chronic diseases, obesity, and pelvic surgery, etc. which cause increased abdominal pressure, are the pathogenic factors of the pelvic floor dysfunction (PFD) [1]. It is believed that [2]the pathogenesis of the PFD is related to the progressive decline of the pelvic floor support tissue, and the female pelvic organ and the pelvic floor tissue are in a complex biomechanical environment that causes a change in the intra-abdominal pressure due to pregnancy, labor, etc. The basal cell of the pelvic floor is an important stress-sensing cell, and the mechanical signal is converted into a biochemical signal, therefore, our research group has shown in previous studies that mechanical force can lead to fibroblast injury, thus simulating the damage of pelvic floor tissue caused by increased abdominal pressure, and as the mechanical force increases within a certain range, fibroblast proliferation is reduced and senescence is increased[3; 4].

Calpains are widely founded in biological tissues and can be classified as either no or tissue-specific protease according to the distribution characteristics. Calpain1 and Calpain2 are widely distributed and studied in two non-tissue-specific isoenzymes,

mainly concentrated in nerve and muscle tissues, and are widely involved in different signal transduction pathways and apoptosis [5]. Some studies have shown that [6] inhibition of calpain activity can enhance cardiomyocyte apoptosis, aggravate myocardial dysfunction and mortality of mice after injection of doxorubicin. It has been proved that Calpain2 can be used as an anti-apoptotic agent. In the induction of heat stress, the overexpression of Calpain2 in cardiomyocytes can prevent the apoptosis of cardiomyocytes and improve the cardiac function of heatstroke mice[7]. In this context, we compared the changes of Calpain2 content in pelvic floor tissue of normal and POP patients, and explored the relationship between Calpain2 and apoptosis in cells level, when the L929 cells are exposed to mechanical forces.

Materials and Methods

Collection of clinical specimens

Multiple leiomyoma patients who underwent transvaginal hysterectomy and POP III ~IV patients in Renmin Hospital of Wuhan University from September 2018 to June 2019 were selected. Patients who had received estrin treatment within the past three months were excluded. Uterosacral ligaments and cardinal ligaments were obtained from patients following the receipt of informed consent. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China). The tissue was routinely fixed, and the tissue sections were subjected to tunel assay to extract tissue proteins, and the contents of Calpain2 were detected and compared. all methods were performed in accordance with the relevant guidelines and regulations.

Cell culture and group treatment

Mouse fibroblast L929 (purchased from China Center for Type Culture Collection ,Wuhan)was cultured in RPMI 1640(Genom Biotech Ltd. Hangzhou, China), plus 10% fetal bovine serum(FBS, Gemini Bio-Products, California, USA) and 1% antibiotic (100 KU/ml penicillin G and 100 mg/ml streptomycin, Genom Biotech Ltd); the incubator was set to 37 ℃,5% CO2. L929 cells were cultured to 80% for passage, and the cells were divided into the normal group and the gene silencing group, which were the control group, Mechanical force group, sh-Capn2 group and sh-Capn2 force group.

Gene silencing

Collecting cells, inoculating 2 ml of 4 * 10^9/ ml cell suspension into a six-well plate, and replace the medium 1ml with the transfection reagent HitransG (40ul) and the infection multiplicity of 60 after the cells are attached. the normal culture medium is changed after 12 h and the cell state was observed. The infection rate was detected 72 hours later. The constructed mouse Calpain2 gene silencing shRNA sequence (Shanghai GeneChem Co, Ltd. Shanghai, China):gcGGTCAGATACCTTCATTAA .

Cell mechanical force loading

Using four-point bending cell mechanics loading system(Chengdu Miracle Technology Co, Ltd. Chengdu, China), the mechanical parameters of the experimental model were: 5333μstrain (corresponding to the deformation displacement of the culture plate was 4 mm), the frequency was 0.1 Hz, the treatment time was 4h, and the corresponding indexes of the cells on the crawling plate were detected after the force was added to the culture plate. The parameter selection and test method refer to the

previous study of our research group [3; 4].

Cell apoptosis

Apoptosis was detected by Annexin V-FITC (Boster Biological Technology Co, Ltd. Wuhan), and the cells were treated with apoptosis kit to detect apoptosis. The cells were washed once with PBS, and the cells were digested with an appropriate amount of trypsin cell digest (Genom Biotech Ltd.). Incubate at room temperature until gently blowing the adherent cells. Aspirate the digestive juice of the trypsin cells, add the culture solution to stop the digestion, gently puff the cells down, transfer to a 4 ml centrifuge tube, centrifuge at 1000 g for 5 minutes, discard the supernatant. The cells were collected, and the cells were gently resuspended in PBS (Genom Biotech Ltd.) and counted. The 5-100000 suspended cells were centrifuged for 5 minutes at 1000 g for 5 minutes. The cells were discarded and the cells were gently suspended by adding 195μl Annexin V-FITC binding solution. Add 5μl of Annexin V-FITC and mix gently. add 10μl of propidium iodide (PI) staining solution and mix gently. Incubated at room temperature (20 ℃~ 25 ℃) for 10 min, then placed in an ice bath. On the flow cytometer, Annexin V-FITC was green fluorescence, and propidium iodide (PI) was red fluorescence.

Immunofluorescence

The cells in each group were placed on six-well plates after treatment, and pre-cooled 4% paraformaldehyde (Servicebio Technology Co, Ltd. Wuhan) was added. The cells were incubated on ice for 15 minutes and washed with PBS for 5 min /3 times. 0.5ml pre-cooled 0.5% triton-100(Servicebio Technology Co, Ltd.) was added and incubated

on ice for 5 minutes and washed with PBS for 3 times. Under normal temperature, add 2 ml of PBS containing 10% FBS, incubation for 30 minutes, wash once with PBS. Dry the glass slides, add corresponding 100ul diluted primary antibody, overnight at 4 ℃; The next day, the primary antibody was washed 3 times with PBS, incubated with room temperature secondary antibody for 1 hour, and washed with PBST for 3 times; 0.5 ml of DAPI working solution(Servicebio Technology Co, Ltd.) was added, incubated at room temperature for 5 minutes in the dark, washed 3 times with PBST, and mounted under a fluorescence microscope.

Western blotting

After treatment of each group of cells, the cell suspension was collected, centrifuged at 1000 rpm for 5 minutes, the supernatant was discarded, and an appropriate amount of RIPA lysate (Saiville Biotechnology Co, Ltd.) was added, the protein concentration was measured by BCA Protein assay kit (Beyotime Institute of Biotechnology ,Wuhan), and the sample volume was calculated. Proteins were separated by 10% SDS-PAGE gel electrophoresis, protein molecules were transferred to PVDF membranes by whole-wet electroporation method, and blocked with 5% skim milk powder (Saiwei Biotechnology Co, Ltd.) for 1 h at room temperature. After the completion of the blocking, the membrane was washed 3 times with TBST (5 min/time), and then the primary antibody (1:1000) was incubated at 4 ℃ overnight. After washing the membrane 3 times (5 min/time) with TBST the next day, the corresponding secondary antibody dilution (1:10000) with goat anti-rabbit fluorescent label was placed in the dark at room temperature for 1 h, and TBST was washed 3

times (5 min/time), the fluorescence signal was collected by Odyssey two-color infrared laser imaging system, and the gray ratio of the target protein band to the GAPDH band of the internal reference was used as the relative expression amount of each protein.

Statistical analysis

All data are expressed as the mean ± SD. Statistical analysis was performed using Graphpad prism 7.0 . One-way ANOVA and t test were used to determine the statistical significance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Calpain2 decreased and apoptosis increased in the ligamentous ligament tissue of patients with POP.

The sacral ligament tissues of patients with clinical POP III ~IV and multiple uterine leiomyoma were selected, and the Calpain2 expression level and apoptosis of the two samples were compared after treatment. Tunel assay showed significant apoptosis in POP group compared with non-pop group (con) (Fig 1a,1b), and Calpain2 expression was significantly lower than that in non-pop group ($P<0.05$) (Fig 1c,1d). The results showed that the expression of Calpain2 in the two tissues was different, and the apoptosis was different. The content of Calpain2 in POP tissues decreased, while the apoptosis increased.

Calpain2 was not expressed after Capn2 gene silencing in L929 cells.

In order to explore the relationship between Calpain2 and apoptosis, Capn2 gene

177 silencing was carried out on L929 cells to prevent them from expressing Calpain2. Fig.
178 1e showed the silencing of Capn2 gene after transfection. Western blot was used to
179 detect the non-expression of Calpain2 in the sh-Capn2 group and the normal
180 expression in the control group($P<0.05$) (Fig 1e,1f,1g). This indicates that the Capn2
181 gene has been silenced and the cells can be used in subsequent experiments.

182 **Calpain2 decreased in L929 cells after mechanical action, aggravating apoptosis.**

183 In order to simulate the mechanical damage of the pelvic floor tissue in the human
184 abdominal pressure, L929 cells were mechanically loaded. The western blot and
185 immunofluorescence assay showed that the expression of Calpain2 protein in the
186 mechanical force group decreased compared with the control group ($P<0.05$),caspase3
187 increased ($P < 0.05$) (Fig. 2a, 2b, 2c,2d), Bcl2/Bax ratio decreased ($P < 0.05$), and
188 apoptosis increased (Fig. 3a,3b). Under the action of mechanical force, the content of
189 Calpain2 in L929 cells decreased, and the apoptosis increased, which was consistent
190 with the findings in tissues.

191 **After Capn2 gene silencing, L929 cells showed a significant increase in apoptosis**
192 **under mechanical force.**

193 In the absence of expression of Calpain2, the cells were mechanically damaged. The
194 Calpain2 protein content in sh-Capn2 group was significantly decreased ($P<0.05$), and
195 the caspase3 was significantly increased ($P<0.05$) (Fig.2a,2b,2c,2d), and the Bcl2/Bax
196 ratio was significantly reduced ($P<0.05$) (Fig.2a,2b), apoptosis increased(Fig.3b, 3c).
197 Given the same mechanical injury, Calpain2 showed more obvious apoptosis without
198 expression of Calpain2 (Fig 3e), indicating that Calpain2 played a certain role in the

process of inducing cell apoptosis by mechanical injury.

After the Capn2 gene was silenced, L929 cells did not express Calpain2, but the apoptosis was different.

Compared with the sh-Capn2 group and the sh-Capn2 force group, neither group expressed Calpain2 ($P>0.05$), and there should be no difference in their apoptosis, but in fact, the apoptosis situation of the two groups was not the same(Fig 3e).Caspase3 was higher than the former ($P<0.05$), and Bcl2/Bax ratio decreased ($P<0.05$) (Fig. 2b, 2c), the latter has obvious apoptosis (Fig.3c,3d).This indicates that Calpain2 is not the only reason for inducing apoptosis after mechanical force loading, but also other factors.

Discussion

Female pelvic floor disorders (PFD) refers to a series of conditions in which pelvic floor support tissue degeneration, damage leads to weak pelvic floor support tissue, and pelvic organ displacement occurs, mainly in stress urinary incontinence (SUI) and POP are common, mostly in middle-aged and older women. 71% of POP patients were accompanied by SUI, and 59% of SUI patients were complicated with POP[8]. POP is a common disease in the clinic, which not only reduces the quality of life of patients, but also affects the emotional mood of patients, so it is necessary to study the disease.

Calpain is a calcium-dependent family of neutral cysteine proteases involved in regulating a variety of physiological and pathological processes [9], including structural reconstruction of cytoskeleton and cell membrane adhesion, apoptosis, and

different signal transduction pathways. etc. Calpain1 and Calpain2 are two proteins that are commonly expressed and require micromole and millimole concentrations of calcium for activation respectively. Genetic studies have found that mice lacking Calpain1 grow normally and mouse embryos are dying without Calpain2 gene [10; 11]. This shows the importance of Calpain2 to the body. Previous studies have shown that Calpain1 and Calpain2 react differently to apoptosis and viability under different stimuli, they can be synergistic or antagonistic under different conditions[12; 13]. Under pathological conditions, Calpain1 can activate and promote myocardial apoptosis [14; 15; 16; 17], while Calpain2 up-regulated can inhibit apoptosis, protect myocardial cells through membrane repair, activate AKT signaling pathway and inhibit caspase3[6], thereby regulating the balance of pro-apoptotic and anti-apoptotic proteins[18] . The protective effect of Calpain2 on the heart was observed in heat stress induction, and caspase3 was inhibited by attenuating p38 phosphorylation[7]. A similar study[19] demonstrated that doxorubicin damages the heart and leading to myocardial dysfunction, but this damage was protected by Calpain2. Inhibition of apoptosis is mediated through the induction of MKP-1 in cardiomyocytes, and activation of Akt signaling promotes MKP-1 expression[20].

Apoptosis is a kind of active cell death regulated by gene, and caspase3 is the key enzyme and executive molecule of cell apoptosis. It is generally believed that apoptosis signals can lead to cascade activation of irreversible limited hydrolyzed substrates of Caspase family members through different pathways, and finally lead to activation of caspase3 and cell apoptosis. In this experiment, we observed that the

expression level of Calpain2 in the sacral ligament tissues of POP patients was significantly reduced compared with normal subjects, and the apoptosis was obvious. At the cellular level, Calpain2 gene silencing treatment of L929 cells inhibited the expression of Calpain2 in L929 cells, and then the cells were mechanically loaded to simulate the mechanical damage of the pelvic floor tissue when the abdominal pressure increased. The expression of Calpain2 was observed in the control group. After the force loading, the expression of caspase3 was significantly lower than that of the sh-Capn2 group, that is, the apoptosis was inhibited. On the contrary, the apoptosis of sh-Capn2 group is obvious and the content of caspase3 is higher than that of the control group, suggesting the importance of Calpian2 and its protective effect on L929 cells after mechanical injury. It was also found that in the absence of expression of Calpian2, apoptosis was still increased after mechanical force loading, indicating that Calpian2 is not the only factor inducing apoptosis induced by mechanical damage.

All in all, whether drugs, heat stress and other injury to the heart muscle, or mechanical injury to fibroblasts, Calpian2 plays an important role in the process of cell apoptosis and is worthy of our continued research, and may bring new theoretical support for clinical treatment of pelvic floor diseases.

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Compliance with ethical standards

Conflict of interest: None.

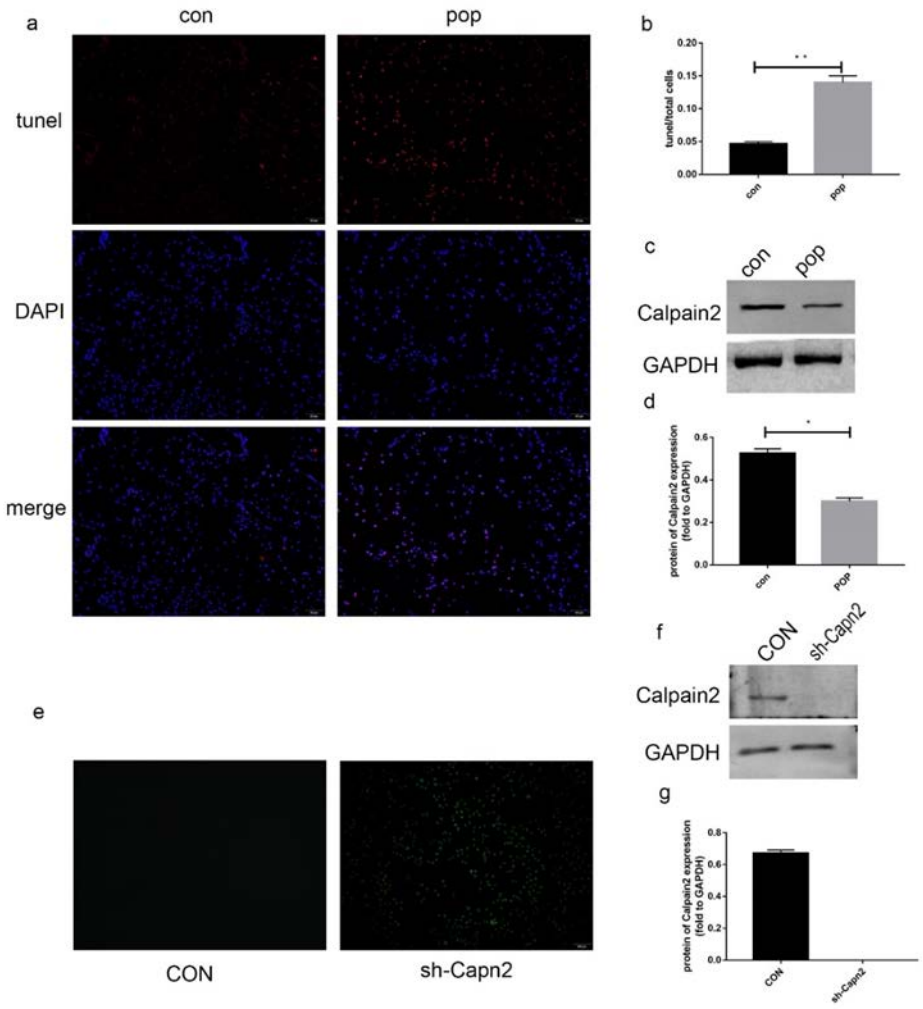
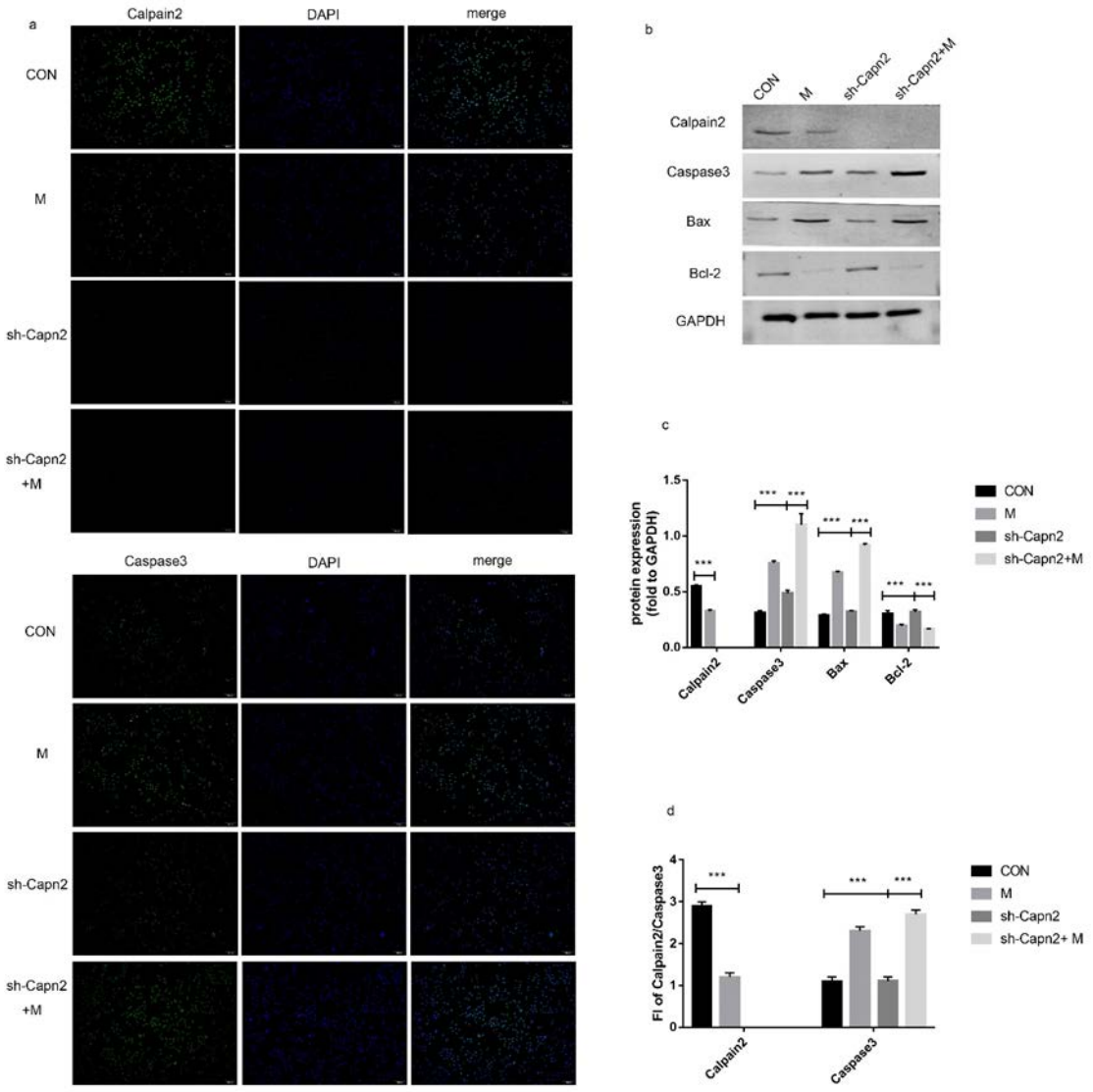


Fig 1: the content of Calpain2 in tissue samples was different, and the apoptosis was different. Silencing of Capn2 gene in L929 cells. a: Tunel staining tissue specimen; b: The ratio of Tunel-positive cells to the total number of cells; c, d: Western blot detection of Calpain2 expression; e: fluorescence detection gene silencing Calpain2: f, g: Western blot detection of Calpain2 expression after gene silencing. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; every experiment was repeated for 3 times. (CON: normal L929 cells; sh-Capn2: Calpain 2 silencing in L929 cells.)



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332 Fig 2: Comparison of the expression levels of Calpain2 and caspase3 in different treatment groups.

333 a: Calpain 2, caspase3 immunofluorescence staining of cells after different treatments; b, c:

334 Western blot detection of protein expression: Calpain2, caspase3, Bcl-2, Bax; d: Fluorescence

335 intensity of Calpain 2 and caspase3. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents p

336 < 0.001; every experiment was repeated for 3 times. (CON: normal L929 cells; M: normal L929

337 cells treated with Mechanical force; sh-Capn2: Calpain 2 silencing in L929 cells; sh-Capn2+M:

338 sh-Capn2 cells treated with Mechanical force.)

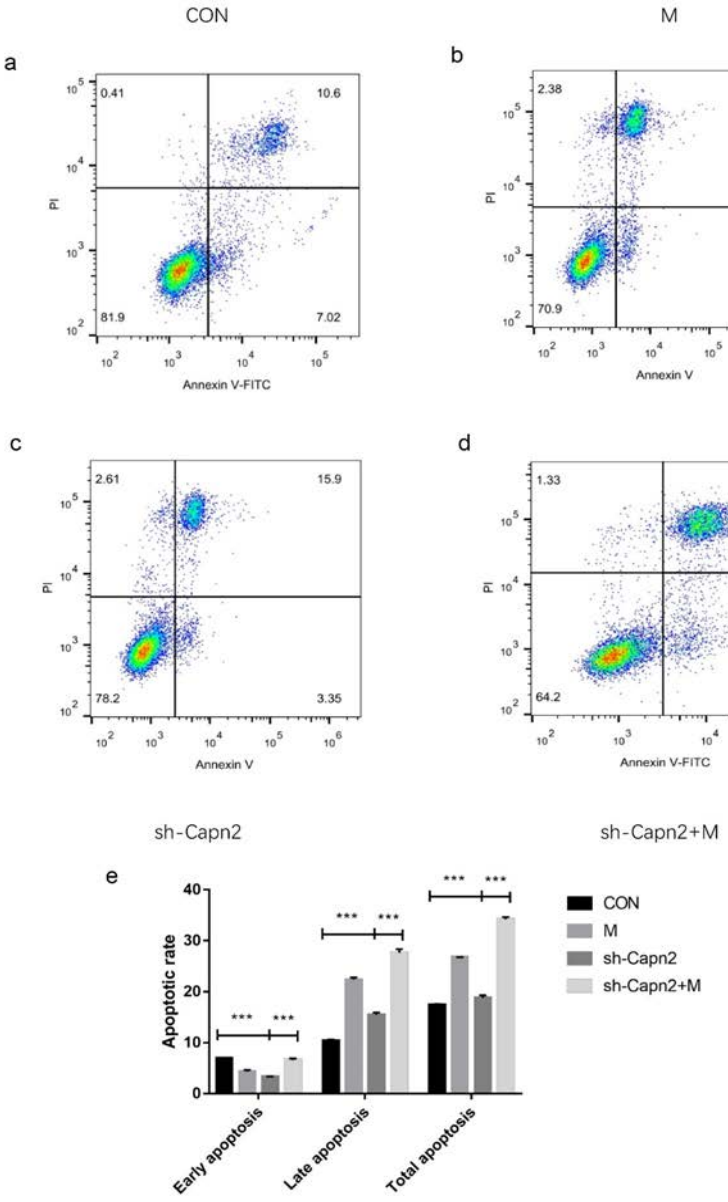

Fig 3: Mechanical force induces cell apoptosis. Calpain2 silencing results in increased apoptotic rates. Cell apoptosis by flow cytometry analysis after Annexin V-FITC assay dual staining. The apoptotic rate is determined as the percentage of Annexin-V-positive cells, with early apoptotic cells being PI negative and late apoptotic cells being PI positive. a, b, c, d: the apoptosis of four groups of cells. e: Quantified apoptotic rates of each group. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; every experiment was repeated for 3 times. (CON: normal L929 cells; M: normal L929 cells treated with Mechanical force; sh-Capn2: Calpain 2 silencing in L929 cells; sh-Capn2+M: sh-Capn2 cells treated with Mechanical force.)