The Effect Of Recombinant Human Sirt1 On Apoptosis And Autophagy Of Human Nucleus Pulposus Cell With Low Nutritional Condition

Shingo Miyazaki1, Kenichiro Kakutani2, Koichiro Maeno1, Toru Takada1, Zhongying Zhang1, Takashi Yurube1, Takuto Kurakawa1, Yoshihiko Terashima1, Masaaki Ito1, Koki Uno2, Tepppei Suzuki2, Masahiro Kurosaka1, Kotaro Nishida1.

1Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, Kobe, Japan, 2Department of Orthopaedic Surgery, National Hospital Organization Kobe Medical Center, Kobe, Japan.


Introduction: The degeneration of intervertebral disc (IVD) is characterized by the IVD cells apoptosis and extra cellular matrix degradation. Nutrition disturbance for IVD cells is one of the most important initiations for IVD degeneration [1]. It has been reported that SIRT1 is a key regulator of several cellular processes, such as the maintenance of energy homeostasis and cellular survival in response to calorie restriction. These functions were considered to be accomplished by the crosstalk between SIRT1 and autophagy [2]. We hypothesized that SIRT1 contributes nucleus pulposus (NP) cells survive in the early disc degeneration by the autophagy-dependent mechanism. Previously the authors reported that low nutritional (LN) condition induced apoptosis thorough a mitochondrial pathway in human NP cells from degenerated IVD cells. Whereas recombinant human SIRT1 (rhSIRT1) prevented apoptosis of human degenerated NP cells by accelerated autophagy in LN condition. In this study, we investigate the effect of rhSIRT1 on autophagy and apoptosis with LN condition in human NP cells of different degeneration grade of IVD.

Methods: Culture of NP cells: Thirty-one human NP cells were obtained from consented patients during surgical procedures for idiopathic scoliosis (n=7), lumbar disc herniation (LDH; n=8), lumbar canal stenosis (LCS; n=16). The degeneration grade of IVD was classified according to Pfirrmann’s grading system [3]. The degeneration grades were rated as grade 2 in 7 discs, grade 3 in 13 discs, and grade 4 in 11 discs. Idiopathic scoliosis patients were significantly younger than the LDH and LCS patients, and all IVDs of idiopathic scoliosis patients were categorized as Pfirrmann grade 2. Whereas among LDH and LCS patients, there was no statistical significance in age and Pfirrmann grade (mean age: grade 2, 16.7 ± 6.9 yo, grade 3, 36.0 ± 17.0 yo, grade 4, 39.9 ± 18.9 yo, P < 0.01).

After 3 days pre-culture, NP cells were cultured in the absence or presence of 10μM rhSIRT1 (Sigma-Aldrich, St Louis, MO, USA) in two different conditions; DMEM with 10% FBS defined as normal nutritional condition (N) and DMEM with 1% FBS defined as low nutritional condition (LN). Furthermore, 3-MA (Santa Cruz, CA, USA), which is a specific inhibitor of the autophagic process, was used to inhibit autophagy. In the present study, four culture types were assigned as following: Group N; 10% FBS without treatment. Group LN; 1% FBS without treatment. Group LN+SIRT1; 1% FBS with 10μM rhSIRT1. Group LN+SIRT1+3MA; 1% FBS with 10μM rhSIRT1 and 5mM 3-MA.

Total number of NP cells
After 3, 7 and 14 days of the treatment, cells were counted using a microscope.
Autophagic activity
Autophagic activity was assessed by Autophagy Staining Kit. Absorbance of Monodansylcadaverine (MDC), which is autophagic marker, was measured using a plate reader. The value of autophagy level was normalized by the cell number in each well, and defined it as the normalized autophagic activity.

Apoptotic incidence
Apoptotic incidence was assessed using Annexin V-FITC Staining Kit after 3, 7 and 14 days of the treatment. Apoptotic cells, including those staining positive for either Annexin V-FITC or propidium iodide and those that were double positive, were counted by flow cytometry and represented as a percentage of the total cell count.

Statistical analysis: Dates were expressed as mean ± standard error. and analyzed using ANOVA with Fisher’s PLSD post hoc test. P values of <0.05 were regarded as statistically significant.

Results: Total number of NP cells
In Pfirrmann grade 2 disc, the number of the NP cells of Group N showed the time dependent increase. Whereas it of Group LN showed slightly increase and was significantly lower than Group N at every time point. Although Group LN+SIRT1 significantly increased the cell number compared to Group LN, however the number of Group LN+SIRT1 was also significantly lower than Group N. Whereas it of Group LN+SIRT1+3MA were significantly decreased than Group LN+SIRT1 at every time points.

In Pfirrmann grade 3 or 4 discs, the number of the NP cells of Group N showed the time dependent increase. The number of the NP cells of Group LN were significantly decreased compared with it of Group N at every time point and did not show any significance increase between day 3 and 14. Whereas it of Group LN+SIRT1 showed the time dependent increase with significant difference compared to Group LN and was almost comparable with it in Group N. It of Group LN+SIRT1+3MA was significantly decreased than in Group LN+SIRT1 and Group LN at 7 and 14 days. (Fig.1)

Autophagic activity
In Pfirrmann grade 2 disc, the normalized autophagic activity of NP cells of Group N was not seen the significant difference at every time point. It of Group LN and Group LN+SIRT1 was significantly up-regulated than in Group N at 24 hours after treatment. However, there was no significant difference in the normalized autophagic activity of NP cells between Group LN and Group LN+SIRT1 at 0-72 hours after treatment. It in Group LN+SIRT1+3MA was significantly decreased than Group LN+SIRT1.

In Pfirrmann grade 3 or 4 discs, the normalized autophagic activity of NP cells of Group N was not seen the significant difference at every time point. It of Group LN was significantly up-regulated than in Group N at 72 hours after treatment. Moreover, it of Group LN+SIRT1 was significantly stimulated compared to Group N and Group LN. Whereas, it of Group LN+SIRT1+3MA was significantly down-regulated than in Group LN+SIRT1. (Fig.2)

Apoptotic incidence
In Pfirrmann grade 2 disc, the apoptotic incidence of Group LN was significantly increased than Group N at day 14. Whereas it of Group LN+SIRT1 was significantly decreased than Group LN, it of Group LN+SIRT1+3MA was significantly increased than in Group LN+SIRT1.

In Pfirrmann grade 3 or 4 discs , both Group LN and Group LN+SIRT1+3MA showed significant apoptotic alterations compared with Group N at the every time point. Whereas the apoptotic incidence of Group LN+SIRT1 was significantly lower than both of Group LN and Group LN+SIRT1+3MA. (Fig.3)
**Discussion:** Previously we revealed that, in human NP cells from Pfirrmann grade 3 and 4 discs, LN condition induced apoptosis thorough a mitochondrial pathway, moreover rhSIRT1 prevented apoptosis of human NP cells by accelerated autophagy with LN condition. The present study revealed that the effects of rhSIRT1 on autophagy and apoptosis in human NP cells were stronger in Pfirrmann grade 3 and 4 discs than in Pfirrmann grade 2 disc. The reactivity of rhSIRT1 to human NP cells was considered to be depended on degeneration grade of IVD. The role of SIRT1 may be altered according to the degeneration grade of IVD. SIRT1 may play the key role in the pathomechanism of IVD degeneration characterized by apoptosis due to low nutrition supply. rhSIRT1 may become the potential candidate as a novel treatment of IVD degeneration.

**Significance:** The study of SIRT1 function in NP cells may lead to new approaches to study the pathomechanism of IVD degeneration.

![Figure 1](image.png)

*Figure 1* The effect of rhSIRT1 on total number of NP cells

Total number of NP cells after 3, 7, and 14 days of treatment

A: Pfirrmann grade 2, B: Pfirrmann grade 3 and 4
Figure 2 The effect of rhSIRT1 on autophagic activity:
Normalized autophagic activity, which is absorbance of Monodansylecadaverine (MDC), measured by Autophagy Staining Kit (0-96 hours) A: Pfirrmann grade 2; B: Pfirrmann grade 3 and 4.
Figure 3 The effect of rhSIRT1 on apoptosis
Apoptotic incidence was assessed by Flow cytometry after 3, 7, and 14 days of treatment
A: Pfirrmann grade 2, B: Pfirrmann grade 3 and 4

ORS 2015 Annual Meeting
Poster No: 1604