ISSN : 0974 - 7435 Volume 8 Issue 11

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 8(11), 2013 [1561-1566]

Transport stress causes damage in rats' liver and triggers liver autophagy

Peng Yin, Changrong Wan, Shasha He, Xiaolong Xu, Mingjiang Liu, Shixiu Song, Xiaoxi Liu, Xiaoyu Jiang, Jianqin Xu*

CAU-BUA TCVM Teaching and Researching Team, College of Veterinary Medicine, China Agricultural University (CAU), Beijing 100193, (P.R.CHINA)

E-mail: xujianqincau@126.com

ABSTRACT

Transport stress would do great economic damage to animal husbandry. To discover the mechanism of this problem, this study aims to explore the damage and the autophagy level after transport stress in Sprague Dawley (SD) rats' liver. Our previous work has successfully built a stable transport stress model. This study repeated transport stress rats model; detected body weight, rectal temperature and Serum biochemical indexes. The results showed significantly difference between stress group (SG) and control group (CG), suggested the model was successfully built. Histological observation showed that the rat's liver in SG was obviously damaged. Autophagy-related indicator LC3 was evaluated by immunohistochemistry test, and was accumulated within the central vein of liver where autophagy was triggered. RT-PCR analysis of gene expression during transport stress showed that the mRNA level of mTOR, Beclin-1 and LC3 were significantly decreased. These results suggested that transport stress induced systemic reaction in rats, caused liver damage, and triggered autophagy. The mechanism of this autophagy process may be regulated by the low expression of mTOR instead of Beclin-1. This study is the first demonstration of autophagy in liver in transport stress. It lays the theoretical foundation for the future research and new drugs development.

KEYWORDS

Transport stress;
Liver;
Damage;
Autophagy;
LC3.

INTRODUCTION

It is well known that transport stress in summer is caused by stimuli such as hunger, fear, handling, thirst, high temperature etc. Those stress factors can cause stress syndrome, including behavior change, physiology change and tissue damage[1,2]. Transport stress is considered to be a synthetic procedure that not only causes tissue damage, but also affects animal growth, development, immunity and production, and even leads to sudden death. As the increasing demand of livestock products, the economic losses in transport stress are incalculable[3-5]

Transport stress has been reported to cause pigs' multi-organ's acute injury, including myocardial fiber damage, small intestinal villi injury, muscle injury etc.[5,6]. However, there are no previous study reports the effect of liver after transport stress in rats. Liver is one of the ma-

FULL PAPER

jor metabolic organs in the body. It produces acute phase protein and plays an important role in stress[7]. However, overloading in stress may cause acute damage in liver and may trigger autophagy in order to remove damaged organelles and misfolded proteins which accumulating in the hepatocytes[8]. Until now, there is no report focus on the autophagy of liver in transport stress.

The study repeated the model of transport stress as previous report by applying high temperature shaker, simulated summer transport with the stimuli such as heat, handling, shock, shaking and other factors in rats[6,9]. This study aims to explore the potential mechanisms of liver damage after transport stress in rats, and laid a theoretical foundation for futher investigation.

MATERIALS AND METHODS

Animal care and experimental groups

Twelve male Sprague Dawley rats weighing 200±20 g (Beijing Vital River Laboratory, Animal Technology Co., Beijing, P. R. China) were housed [25°C, 60%relative humidity (RH)]for 3days. On the 4th day, the rats were randomly divided into the following two groups: control group (CG) and 3-day stress group (SG). Six rats in each group were housed in plastic cages (400 mm × 300 mm × 180 mm) with a layer of soft woodchips and provided free access to food and water.

Treatment and sampling

Rats in CG were housed in a controlled environment (25°C, 60%RH). Rats in SG were housed under CG's conditions and stimulated on a shaker. The stress condition was set on 60r/min, 35°C stress for 2h, from 9:00 to 11:00 daily, and treated for 3 days separately [6]. Their body weight and rectal temperature were measured before the first treatment (Day 0) and after the last treatment (Day 3). Rats from the CG and SG were exsanguinated and then sacrificed after the last treatment. 1ml blood samples were collected. Sections of the liver were rapidly excised and washed with physiological saline and divided into the following two parts: 1) A 1x0.5x0.5cm^3 section was fixed for 48h in 10% buffered formalin phosphate for paraffin embedding; and 2)Some 0.5x0.5x0.25 cm^3 sections were separated into sample tubes, frozen in liquid nitrogen, and stored at -80°C for DNA microarray and RT-PCR analysis.

Test of biochemical indexes

Blood was immediately collected and centrifuged at 3, 000g for 15 min. Serum was analysis for biochemical indexes by an automated biochemical analyzer (TBA-40FR, Toshiba, Tokyo, Japan). The indexes including total protei (TP), glutamic-pyruvic transaminase (ALT), glutamic-oxal (o)acetic transaminase (AST), albumin (ALB), globulin (GLO)and alkaline phosphatase (ALP)

Morphological observation of liver

Formalin-fixed samples were embedded in paraffin and transversely sectioned (4-μm thickness). After deparaffinizationand dehydration, some paraffin sections were stained with hematoxylin and eosin (HE) (Sigma, St. Louis, MO, USA) for histology observation by BH2 Olympus microscope (DP71, Olympus, Tokyo, Japan)then photographed and analyzed by Olympus Image Analysis System (version 6.0).

Immunohistochemistry test of LC3

The step of immunohistochemistry test is referring to previous study[3]. Reagents were bought from KeyGen Biotech (#KGSP04 histostain-plus kit, KeyGen Biotech, Nanjing, China). To localize LC3 expression, antibody (L8918, Sigma) were used at a dilution of 1:200.

Total RNA isolation and reverse transcription

Total RNA was isolated from the liver using a phenol and guanidine isothiocyanate-based TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity were assessed by a spectrophotometer (SmartSpec plus, Bio-Rad Laboratories, Inc., Hercules, CA, USA) based on an OD260/OD280 ratio.

The total RNA reverse-transcription procedure was performed according to the manufacturer's instructions (Promega, USA); the protocol was as follows: 70°C for 5 min and 42°Cfor 1h. The RT products (cDNA) were stored at -20°C.[4]

Gene mRNA expression analysis by real-time PCR

Expression levels of LC3, mTOR and Beclin-1 were

determined by real-time PCR (RT-PCR) analysis. Quantitative PCR analysis was carried out using the DNA Engine Mx3000P® (ANJIEUN USA) fluorescence detection system against a double-stranded DNA-specific fluorescent dye (Stratagene, USA) according to optimized PCR protocols. β-actin was amplified in parallel with the target genes and used as a normalization control. The protocol and the condition of dissociation curve were as previous reported[4]. Expression levels were determined using the relative threshold cycle (CT) method as described by the manufacturer (Stratagene, USA). Each gene was calculated by evaluating the expression 2-ΔΔCT, which calculated as follows: [CTgene – CTβ-actin] (transport stress) – [CTgene – CTβ-actin] (control). The cDNA of each sample was subjected to RT-PCR using the primer pairs listed in TABLE 1. The PCR reaction (20μL) contained 10μL of SYBR Green PCR mix (Invitrogen, USA), 0.3μL of reference dye, 1μL of each primer (both 10μmol/L), and 1μL of cDNA template.

TABLE 1 : Primers used for real-time PCR.

Gene	Primer sequence 5'~3'
β-actin-F	CCC ATC TAT GAG GGT TAC GC
β-actin-R	TTT AAT GTC ACG CAC GAT TTC
mTOR-F	CTG ATG TCA TTT ATT GGC ACA AA
mTOR-R	CAG GGA CTC AGA ACA CAA ATG C
Beclin-1-F	GCC ACA AGC ATC TCA TCT CAA A
Beclin-1-R	AGC ACG CCA TGT ATA GCA AAG A
LC3-F	GGT CCA GTT GTG CCT TTA TTG A
LC3-R	GTG TGT GGG TTG TGT ACG TCG

Statistical analysis

All results are presented as the mean ± SD. Statistical analysis was performed by independent-sample T-tests using SPASS17.0 software (SPSS, Inc. an IBM Company, Chicago, IL, USA). A *P*-value of < 0.05 was considered significant.

RESULTS

Model evaluation

Every rat was healthy before the stress treatment in CG and SG, and no significant difference in their body weight and rectal temperature. However, they show abnormal behaviors during transport stress, such as grooming, trying to escape, incontinence, nervous, fatigue, and so on. Because of the heat treatment and lacking sweat glands, the body weight of the rats declined 13.3% in SG (*P*<0.01;Figure 1A), and the rectal temperature of rats in SG was increased 1.43°C on average (*P*<0.01; Figure 1B).

Changes of biochemical indexes

The content of ALT is increased in SG (P<0.05); AST is significantly increased (P<0.01); ALB, GLO, ALB/GLO, ALP is significantly decreased compared with CG (P<0.01); and no significant difference in TP and AST/ALT. (Figure 1C)

Histological observation in liver during transport Stress

Paraffin sections of transport liver were stained with HE for histology observation. The result of histology observation under optical microscope showed that in CG, the structure and hepatocytes of liver is integral, hepatic cords and hepatic sinusoids were normal (Figure 2 A, D). However, in SG, hepatocytes and the tissue were severe damaged, appear swelling and granular degeneration, (Figure 2 B, E, C, F).

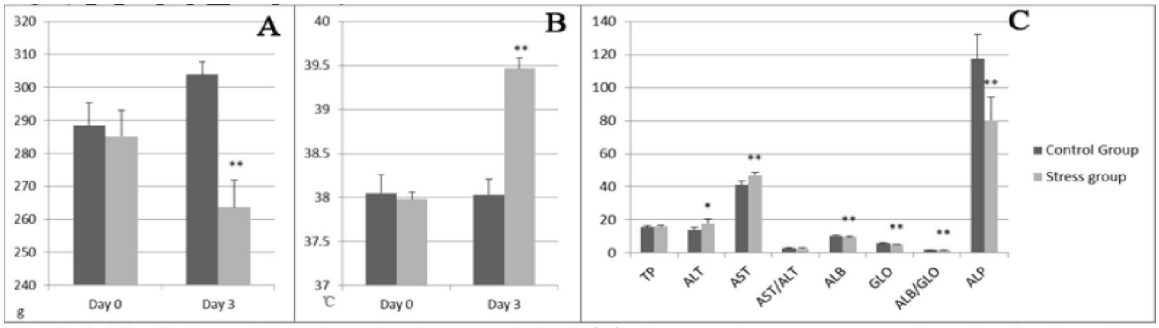

Figure 1 : The body weight of rats in day 0 and day3 (A), the rectal temperature of rats in day 0 and day3 (B), and The Changes of biochemical indexes (C). Values represent the mean ± SD, n=6 rats for each group. *P<0.05, **P<0.01.

FULL PAPER

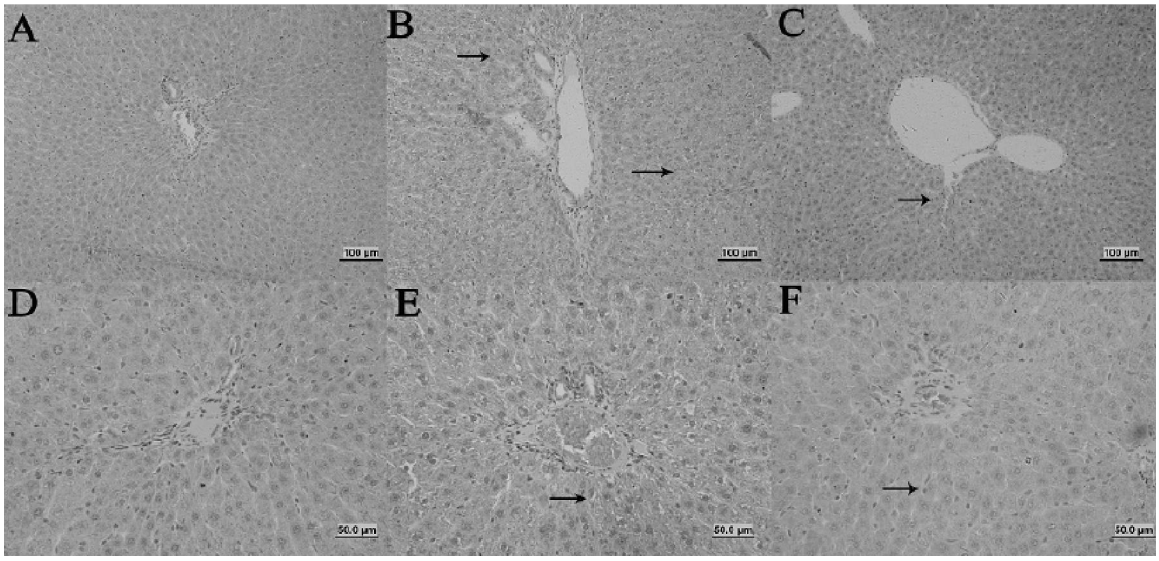

Figure 2 : Histological changes in liver during transport stress. Photomicrographs of HE stained sections of the liver from CG (A, D) and SG. (B, C, E, F)0(A, B, C:200×;D, E, F:400×)

The content of LC3 detected by immunohistochemistry test

Immunohistochemistry results showed that in SG, positive material obviously adhered near liver central vein, while there are still small amount of positive materials near portal areas (Figure 3 B, E, C, F).

Autophagy related genes detected by Real-Time PCR

mTOR, Beclin-1, and LC3 mRNA expression levels were down-regulated in liver after vibration-induced treatment ($P<0.05$; Figure 4).

DISCUSSION

Assessment of transport stress model

During transport stress, the body immediately mobilizes energy, enhances metabolism, and increases heat production to protect itself from handling, high temperature and other stimulation. Rats were grooming, trying to escape, incontinence, nervous, fatigue, and show some other abnormal behavior in the stress, which lead to diarrhea, dehydration and nutrients decomposition. Eventually, rats' body weight significantly decreased after

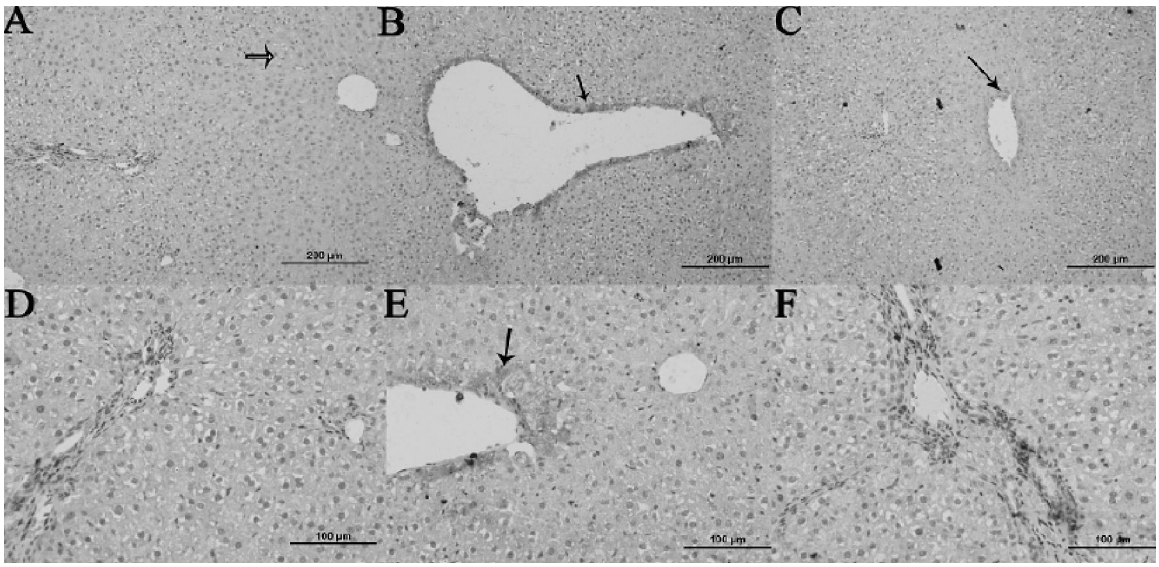

Figure 3 : The content of LC3 in liver during transport stress. Photomicrographs of immunohisto-0chemistry test of the liver from CG (A, D) and SG. (B, C, E, F)0(A, B, C:200×, D, E, F:400×)

transport stress. Additionally, their rectal temperature significantly increased since they were stimulated by high temperature and lacked sweat glands. In this experiment, after transport stress, rats' behavior changed, their body weight decreased and rectal temperature increased ($p<.0.01$). These results were consistent with previous studies, which suggesting that transport stress model in rats was successfully constructed[6,9].

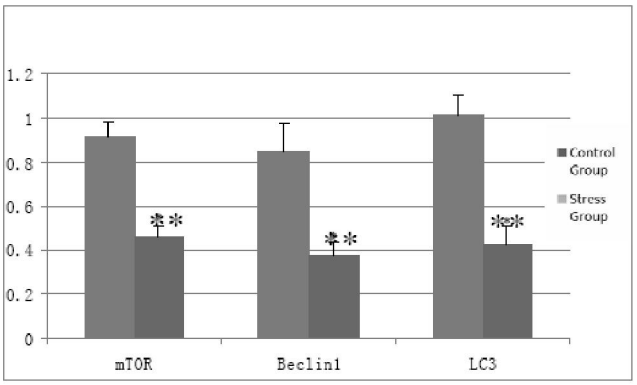

Figure 4 : mRNA expression of mTOR, Beclin-1, and LC3 in liver

Transport stress induced liver damage in rats

After transport stress, liver need to undertake more to meet the need of energy because metabolism of the body was enhanced. With the increasing load of the liver, ALT level in SG was increased ($P<0.05$), AST was significantly increased ($P<0.01$), ALB, GLO, ALB / GLO and ALP levels were significantly lower than CG ($P<0.01$). This result suggests that transport stress causes hepatocytes damage. In addition, ALB decreased in serum may also reveal the reserve function of liver was damaged. It has been reported that early phase of heat stress can lead to liver cells swelling, vacuoles appeared in the cytoplasm, and full of red blood cells in hepatic sinusoid[10]. The result of histology observation under optical microscope showed that in CG, hepatocytes were intact, hepatic cords were clear, hepatic sinusoids were normal (Figure 2 A, D). However, in SG, liver cells were swelling, damaged, appearing granular degeneration near liver central vein; hepatic cords were blurring; hepatic sinusoids were decreased or even disappeared (Figure 2 B, E, C, and F). These results suggest that transport stress causes a serious of pathological injury in liver. Especially near liver central vein.

Transport stress triggered autophagy in rats' liver

Autophagy is a conservative degradation pathway in eukaryotic cells. It degrades and recycles long-lived organelles and incorrectly folded proteins by merging with lysosomes, which can slow down the various types of cellular stress and plays an important role in body growth and development[17]. Autophagy is an alternative, non-apoptotic route of programmed cell death,[12,18]as well as a mechanism of cell survival and defense[10]. LC3 is the maker autophagy protein in mammalian cells. LC3-I is a soluble form which would change into LC3-II, a fat-soluble form, in autophagy process by the effect of phosphatidylethanolamine (PE), ATG7 and ATG3. LC3-II localizes on the membrane of complete spherical autophagosomes as well as on the isolation membranes[13,14]. Mammalian target of rapamycin (mTOR), a downstream effector of Akt/PKB plays a critical role in cell proliferation, growth, and survival, also influences autophagy[19]. The inhibition of mTOR results in autophagy induction. It can promote the adhesion of ribosome and endoplasmic reticulum (ER), thereby inhibits ER membrane shedding and interferes autophagic membrane formation. In addition, mTOR kinase can directly phosphorylate ATG1, which lead to ATG13 cannot combine with ATGl, which affects the formation of autophagosome[15]. Beclin-1 governs the autophagic process by regulating PtdIns3KC3-dependent generation of phosphatidylinositol 3-phosphate (PtdIns (3)P), guides other ATG proteins locating in autophagosome, and participate in the regulation of autophagy formation[16].

Autophagy provides recycled materials for cells by degrading cells' own metabolites, which plays a key role in the mechanism of liver injury and repair. By detecting autophagy marker protein LC3 can determine the location of liver autophagy. Immunohistochemistry result shows that several positive material attached to liver central vein and rare near the portal area in CG. (Figure 3 A, D). In SG on the contrary, more positive material significantly adhered near liver central vein, while there are still a small amount of positive material in portal area (Figure 3 B, E, C, and F). These results suggest that autophagy mainly concentrates near liver center vein, and few near portal areas. The enhancing of LC3 near central vein perhaps associated with the increasing hepatic metabolism and serious hepatocellular injury, combined with histological result.

The Real-Time PCR results show that, the mRNA

FULL PAPER

level in SG of mTOR, Beclin-1 and LC3 were significantly lower than in CG ($P < 0.05$) (Figure 4). LC3 is the characteristic autophagic marker located in autophagosome membrane. The contents of LC3-II or the ratio of LC3-II/LC3-I are positively correlated with the amount of autophagosome vacuoles. In this case, the decline of the total amount of LC3 did not comprehensively reflect the decline of autophagy. Furthermore, LC3's expression may be different in different location in liver, and this may be the reason of why autophagy was activated in immunohistochemistry test but the total amount of LC3 is decreased. Additionally, high expression of Beclin-1 can induce cell autophagy while mTOR is negatively regulated autophagy. In this experiment Beclin-1 and mTOR were both declined, suggesting that mTOR instead of Beclin-1, participated in autophagy regulation of rats' liver in transport stress. The AKT/TSC/mTOR pathway related with mTOR may play a key role in regulating autophagy in transport stress in rats' liver. The specific mechanism still needs further research.

REFERENCE

[1] L.Zhu, E.Bao, R.Zhao el a1; Expression of heat shock protein 60 in the tissues of transported piglets[J], Cell Stress and Chaperones, **14(1)**, 61—69 **(2009)**.

[2] T.Grandin; Assessment of Stress During Handling and Transport[J],. Journal of Animal Science, **75**, 249-257 **(1997)**.

[3] Jin Yu, Fenghua Liu, Peng Yin el a1; Involvement of oxidative stress and mitogen-activated protein kinase signaling pathways in heat stress-induced injury in the rat small intestine, [J] Stress, **(2012)**.

[4] Jin Yu, Peng Yin, Fenghua Liu el a1; Effect of heat stress on the porcine small intestine: A morphological and gene expression study [J], Comparative Biochemistry and Physiology, Part A, **156**, 119–128 **(2010)**.

[5] D.Mota-Rojas, M.Becerril-Herrera, Effects of long distance transportation and CO2 stunning on critical blood values in pigs [J], Meat Science., **90**, 893-898 **(2012)**.

[6] Banchao Shu, Wuren Ma, Peng Yin; Effects of transportation stress on Rats'Jejunum Morphology and Hsps mRNA expression[J], Chinese Journal of Veterinary Medicine, **48**, **(2012)**.

[7] Carolyn Cray, Julia Zaias; Acute Phase Response in Animals: A Review[J], Comp Med. 2009 December; **59(6)**, 517–526 **(2009)**.

[8] M.Komatsu, S.Waguri, T.Ueno'et a1; Impaiment of starvationÿinduced and constitutive autophagy in Atg7-deficient mice[J]ÿJ Cell Biol, **l 69**, 425-434 **(2005)**.

[9] Rao Kai Qing, Xia Dong, Liu Rui; Analysis of the effects of stress on rats in simulated transportation [J], Journal of Southwest University for Nationalities·Natural Science Edition, **12**, **(2007)**.

[10] Bao En-dong, Gong Yuan-ying; Relation Between Pathologic Damages and HSP70 Changes in Acute Heat Stressed Broilers[J], Scientia Agricultura Sinica, **37(2)**, 301-305 **(2004)**.

[11] S.H.Lecker, A.L.Goldberg, W.E.Mitch; Protein degradation by the ubiquitin-proteasome pathway in normal and disease states [J]. J Am Soc Nephrol, **17(7)**, 1807-1819 **(2006)**.

[12] C.W.Wang, D.J.Klionsky; The molecular mechanism of autophagy[J]. Mol Med, **9(3-4)**, 65-76 **(2003)**.

[13] J.D.Rabinowitz, E.White; Autophagy and metabolism[J]. Science, **330(6009)**, 1344–1348 **(2010)**.

[14] I.Tanida, T.Ueno, E.Kominami; LC3 conjugation system in mammalian autophagy[J]. Int J Biochem Cell Biol, **36(12)**, 2503–2518 **(2004)**.

[15] F.Nazio, F.Cecconi; mTOR, AMBRA1, and autophagy: An intricate relationship[J], Cell Cycle. 2013 Aug 15,**12(16)**, **(2013)**.

[16] E.Wirawan, S.Lippens, T.Vanden Berghe; Beclin1: a role in membrane dynamics and beyond. Autophagy[J]. 2012 Jan, **8(1)**, 6-17 **(2012)**.

[17] Maiko Ogata, Shin-ichiro Hino; Autophagy Is Activated for Cell Survival after Endoplasmic Reticulum Stress[J], Molecular And Cellular Biology, 9220–9231 Dec.**(2006)**.

[18] G.H.Peter; Clarke Developmental cell death: morphological diversity and multiple mechanisms[J], Anatomy and Embryology, **181(3)**, 195-213 March **(1990)**.

[19] Yukiko Kabeya, Noboru Mizushima, LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing[J], The EMBO, **19**, 5720-5728 Journal **(2000)**.