·Original Article·

Autophagy is activated and might protect neurons from degeneration after traumatic brain injury

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Abstract: Objective To investigate changes of autophagy after traumatic brain injury (TBI) and its possible role. **Methods** Rat TBI model was established by controlled cortical injury system. Autophagic double membrane structure was detected by transmission electronic microscope. Microtubule-associated protein 1 light chain 3 (LC3) and Beclin 1 were also used to investigate the activation of autophagy post-TBI. Double labeling with LC3 and caspase-3, or Beclin 1 and Fluoro-Jade, to show the relationship between autophagy and apoptosis or neuron degeneration after TBI. **Results** An increase of autophagic double membrane structure was observed in early stage (1 h), and the increase lasted for at least 32 d post-TBI. LC3 and Beclin 1 proteins also began to elevate at 1 h time point post-TBI in neurons, 3 d later in astrocytes, and peaked at about 8 d post-TBI. In both cell types, LC3 and Beclin 1 maintained at a high level until 32 d post-TBI. Most LC3 and Beclin 1 positive cells were near the side (including hippocampus), but not in the core of the injury. In addition, in the periphery of the injury site, not all caspase-3 positive (+) cells merged with LC3 (+) cells post-TBI; In hippocampal area, almost all Beclin 1 (+) neurons did not merge with Fluoro-Jade (+) neurons from 1 h to 48 h post-TBI. **Conclusion** Autophagy is activated and might protect neurons from degeneration at early stage post-TBI and play a continuous role afterwards in eliminating aberrant cell components.

Keywords: autophagy; apoptosis; traumatic brain injury; LC3; Beclin1; neurodegeneration

1 Introduction

Traumatic brain injury (TBI) initiates a series of bio-physiological and pathological reactions that result in secondary or multiple brain injuries, which lead to cell death lasting days to weeks and cause brain dysfunction. The pathologic mechanism of nerve cell injuries or death included activation of excitatory amino acids receptor, mitochondrial injury and energy metabolic blockage, production of oxyradical, caspases activation, and activation of inflammatory reaction. Some severely injured cells undergo different self-destruction, while others activate self-protecting processes.

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Autophagy is a major cellular pathway for the degradation of long-lived proteins and cytoplasmic organelles in eukaryotic cells[1-3]. A large number of intracellular/extracellular stimuli, including amino acid starvation and invasion of microorganisms, are able to induce the autophagic response. Under pathological conditions, autophagy and apoptosis interacts each other to determine cells' fate[4,5]. In cells, autophagy both serves as a defense mechanism to resist cell injuries caused by circumstance changes and induces cell death that differs from apoptosis[6]. Autophagy has either deleterious or protective effects depending on the specific situation and stage in the pathological process[7]. Many pathological conditions, including cancer and neurodegenerative disorders, are associated with autophagy[8].

Using closed head injury model, Diskin T *et al*. showed that over expression of Beclin 1 may be important for autophagy at the lesion site and may serve as a mechanism to discard injured cells and reduce damage to cells by disposing of injured components[9,10], and that neuroprotective role

of rapamycin after TBI may involve in autophagy[11]. More recently, Clark's group reported that autophagy is detectable both in mice TBI model and human brain samples[12]. However, the role (protective or damage) of autophagy after TBI is still unknown. We used a controlled cortical impact (CCI) system to establish TBI model on rat to throw a light on the role of autophagy after TBI.

2 Materials and methods

Male Sprague-Dawley rats weighing 260-280 g were used to develop the TBI model, which closely resemble the clinical manifestations of focal head injury in human[13]. Rats were killed at hour 24 after sham injury or at hour 0, 1, 3, 6, 12, 24, 48, or day 4, 8, 16, 32 following TBI. The trauma protocol complies with the NIH Guide for the Care and Use of Laboratory Animals. Anesthesia was induced in a plexiglass chamber with 4% isoflurane and balanced N_2O/O_2 (2:1), and maintained with 2% isoflurane and balanced N_2O/O_2 (2:1). Anesthetized rat were positioned in a stereotaxic frame and a midline scalp incision was performed. A 5 mm craniotomy was made over the left parieto-temporal cortex using a portable drill and trephine. The bone flap was removed and rats were subjected to CCI using a pneumatic cylinder with a 3-mm flat-tip impounder, 6 m/s velocity, and a 0.5-mm set impact depth. Following CCI, the scalp was sutured closed, and anesthesia was discontinued. The rats recovered in their cages and provided with postoperative care and free access to food and water. Rats in sham operation group were subjected to all aspects of the protocol except for cortical impact, while rats in control group were not suffered CCI or operation. For immunohistochemical analyses of LC3B (Sigma, Cat: L7543), Beclin 1(Abgent, Cat: AP1818a) and Beclin 1 double label with Fluoro-Jade (Chemicon, Cat: AG325) (n = 5-7/group), 8 coronal brain sections (beginning at anterior hippocampus and finish at posterior hippocampus, 12 μm thick, 250 μm apart from each sections) were cut in succession from each animal. For western blot analysis assays, animals were decapitated, and the injured cortex and hippocampus were rapidly dissected separately at the desired time after TBI (n = 5-7/group), protein were extracted and stored at –80ºC. Some tissues were used for transmission electron microscope (TEM) assays.

In immunofluorescence assay, LC3B or Beclin 1 was developed by FITC or Cy3, and the stained brain sections were photographed under 200× using fluoresce microscopy. Six sections per animal and 5 fields per section were selected randomly and were used to count LC3B and Beclin 1 positive cells in cortical region of the injured hemisphere separately. In every stained section, only strong bright dots existing in plasma can be regard as LC3 positive cells. The injured cortex and hippocampus were also homogenized for Beclin 1 Western blot analysis. Semi-quantitative densitometric analysis was carried out on the films obtained from western blot. The relative optical density measurements obtained from the control and injured samples were expressed as a percentage of levels of the load control—β-actin protein. Other agents except mentioned above were purchased from Sigma.

The statistical data were performed by one-way ANOVA marked as mean±SEM, and $P < 0.05$ was considered statistically significant.

3 Results

Compared with control and sham groups, autophagy was increased in all TBI groups. By TEM, double membrane structure containing a mitochondrium in the periphery of injured cortex was observed at hour 1 after TBI, and also at the following different time points, even 32 d after TBI. More lysosomal structures were found at 3 h time-point and last at least 8 d after TBI. At 16 d time-point, a structure of nuclear membrane was found stretching out to enclose cytoplasma (Fig. 1). In immunofluorescence tests, LC3 was activated 1 h after TBI, the amount of LC3 positive cells increased and peaked in 8 d groups, then decreased at 16 d and 32 d time point, but still kept a high level (Fig. 2). The number of Beclin 1 positive cells in the cortex also increased 1 h after TBI, peaked at 8 d time point, and lasted at least 32 d after TBI; Western blot confirmed the cell counts results of Beclin 1 expression both in cortex and hippocampus in our TBI model (Fig. 3 and Fig. 4). LC3B and Beclin 1 positive cells were mainly neurons in early stage (3 d) after TBI, and then glia phenotype afterwards by morphological analysis. Most positive cells mainly observed in periphery of the impact site, but not in the core. Double labeling LC3 immunofluoresced slices by using caspase-3 as an apoptotic marker showed that there were some LC3(+)/caspase-3(+) cells and also LC3(+)/caspase-3 (–) cells around the injury site after TBI (Fig. 5). We also used Fluoro-Jade staining to show neurodegeneration after TBI on the Beclin 1 immunofluorescent sections. In hippocampal area, Beclin 1 (+) neurons do not merge with Fluoro-Jade (+) neurons in early stage (6 h to 48 h) post-TBI (Fig. 6).

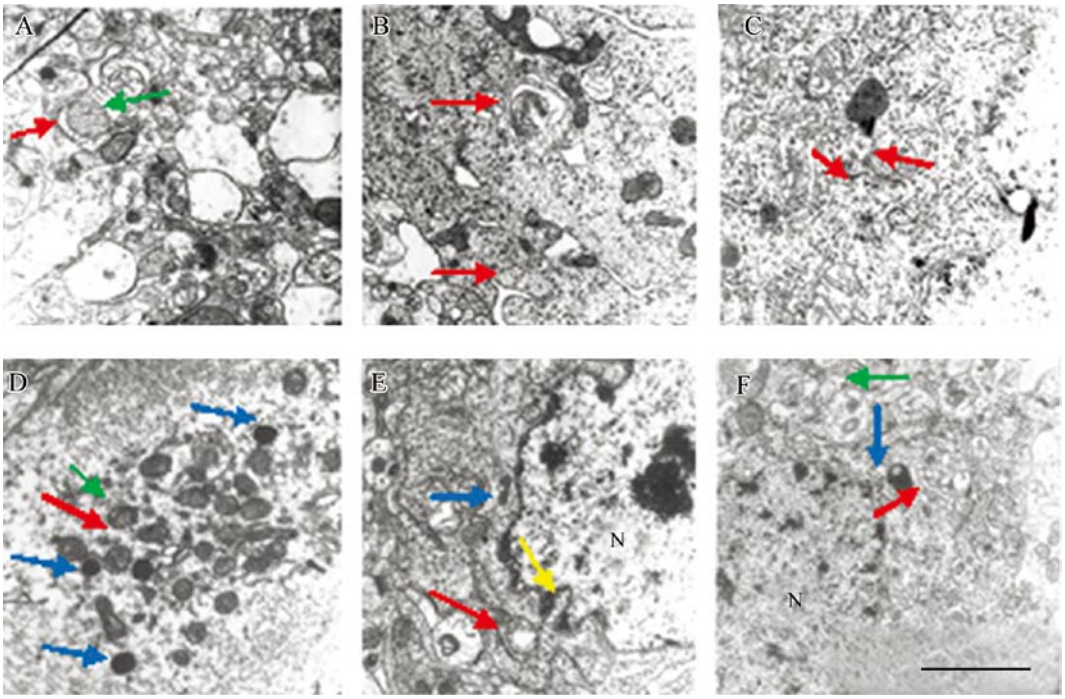

Fig1. Transmission electronic microscopy photomicrographs at different time points after TBI. A, 1 h post-TBI group showed confluence of mitochondria and a double membrane structure containing mitochondrium. B, 3 h post-TBI group showed C-shaped double membrane structure. C, 4 d post-TBI group also showed C-shaped double membrane structure. D, 8 d post-TBI group showed remarkable increase in the number of lysosome. E, 16 d post-TBI group showed intact nuclear membrane stretches out to enclose cytoplasm. F, 32 d post-TBI group showed C-shaped double membrane structure. Red arrows: autophagosome. Green arrows: mitochondria. Blue arrows: lysosome. Yellow arrows: nuclear membrane encasing plasma. N: nucleus. Scale bar, 1 μm.

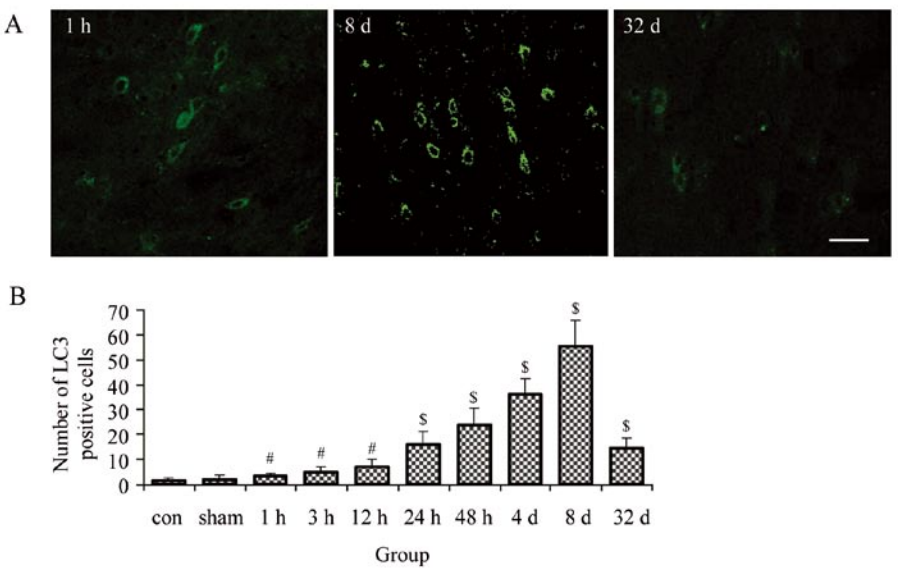

Fig. 2 Immunofluorescent analysis of LC3 expression after TBI. Coronal sections of rat sacrificed at different time points after TBI were analyzed for LC3 expression using rabbit anti-LC3 antibodies followed by FITC-labeled donkey anti rabbit IgG secondary antibodies. A, Representative Immunohistochemistry for LC3; Scale bar, 40 μm. B, Qualitative analysis of the number of LC3 (+) cells. Bar represents mean±SEM from 5 rats in each group. $^{\#}P < 0.05$, *vs* the sham group; $^{\$}P < 0.01$, *vs* the control group.

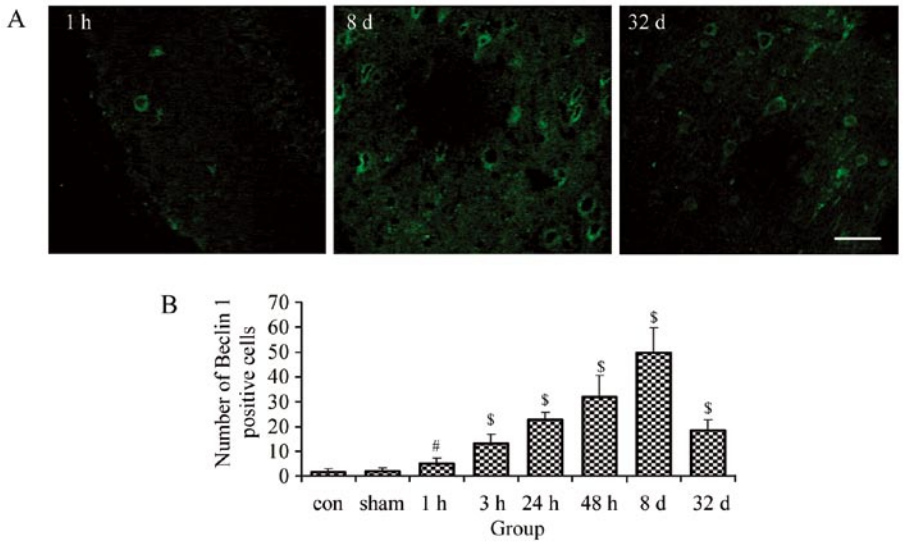

Fig. 3 Immunofluorescent analysis of Beclin 1 expression after TBI. Coronal sections from rat sacrificed at various time points after TBI, were analyzed for Beclin1 expression using rabbit anti-Beclin1 antibodies followed by FITC-labeled donkey anti rabbit IgG secondary antibodies. A, Representative immunohistochemistry for Beclin1; Scale bar, 40 μm. B, Qualitative analysis of the number of Beclin1 (+) cells. Bar represents mean±SEM from 5 rats in each group. $^{\#}P < 0.05$, *vs* sham group; $^{\$}P < 0.01$, *vs* control group.

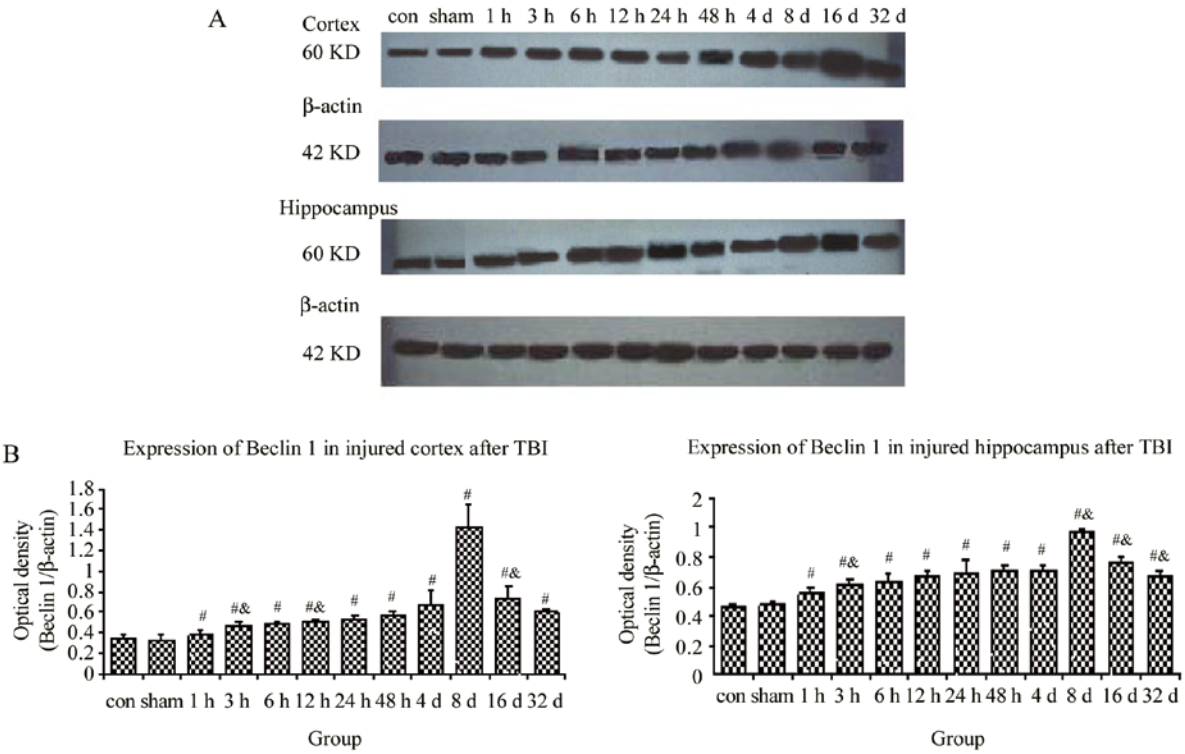

Fig.4 Time-dependent changes of Beclin1 protein in injured cortex and hippocampus after TBI in rats. The expression of Beclin1 in injured cortex and hippocampus was determined by Western blot analysis at 0 h (control group and sham operation group), 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 4 d, 8 d, 16 d and 32 d after TBI. β-actin protein were used as load control. A, Western blot results of Beclin1 protein at different time points. B, Quantitative analysis of changes of Beclin1 protein from five rats at each indicated time with an image analyzer (Sigma pro 5.0). Bar represents mean±SEM from five rats in each group. Staistical analysis was performed using one-way ANOVA. $^{\#}P < 0.05$, *vs* control group, $^{\&}P < 0.05$, *vs* the former time point group.

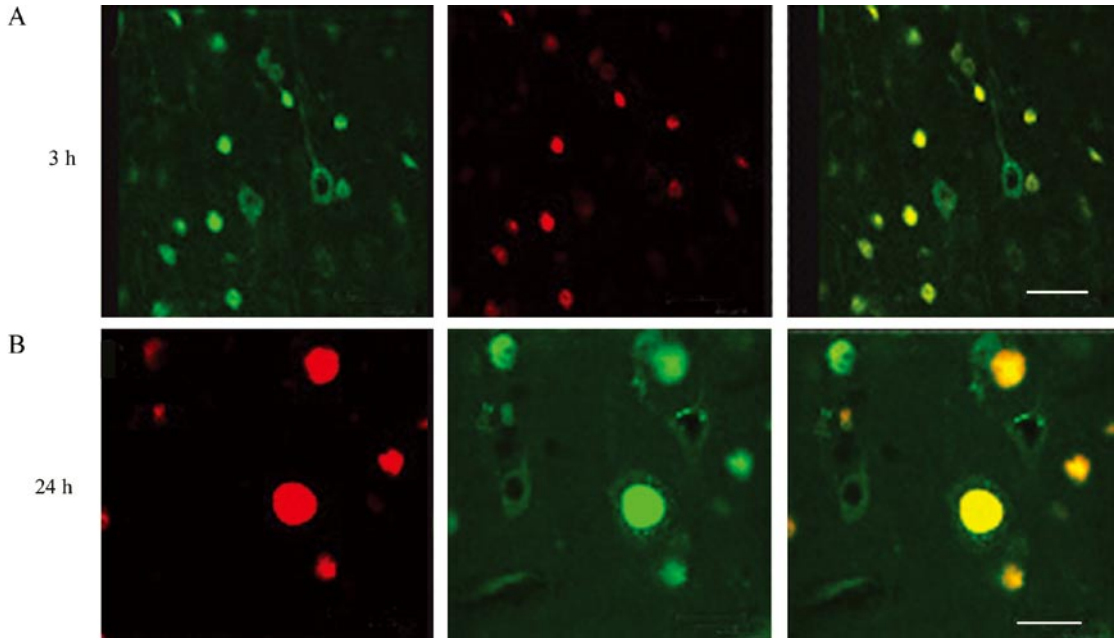

Fig. 5 Caspase-3 and LC3 double label. There were some LC3(+)/caspase-3 (+) and also LC3(+)/caspase-3(–) cells appear around the injury site after TBI. A, 3 h post TBI; B, 24 h post TBI. Red: caspase-3; Green: LC3. Scale bar, 30 μm in A, 20 μm in B.

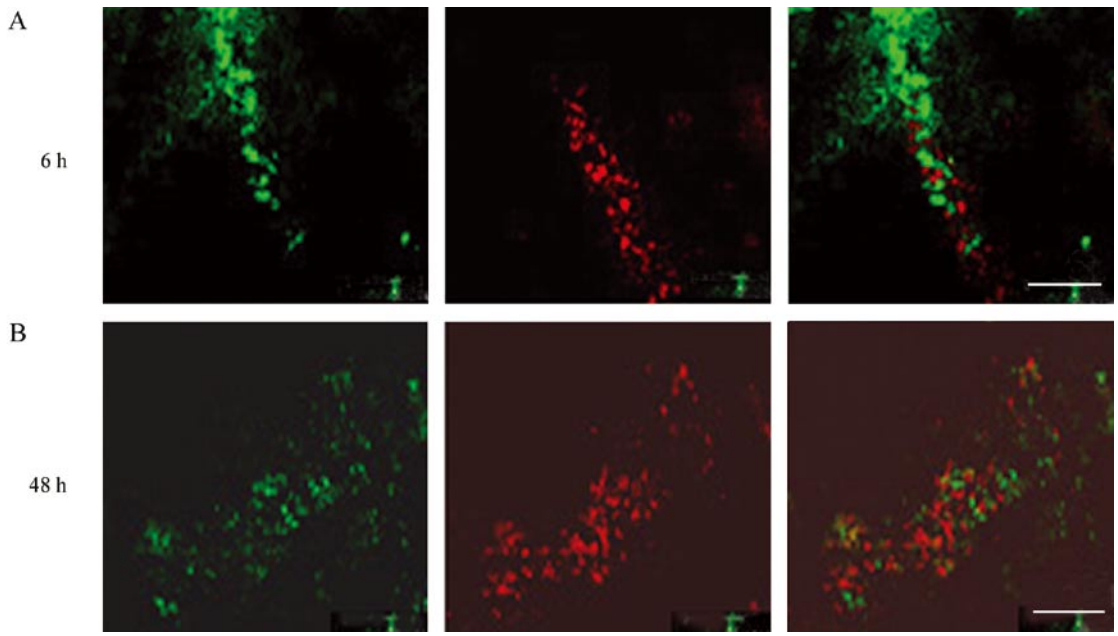

Fig. 6 Beclin 1 and Fluoro-Jade double labeling showed that Beclin 1 (+) hippocampal neurons did not merge with Fluoro-Jade (+) neurons in 6 h to 48 h post-TBI. Red: Beclin 1; Green: Fluoro-Jade; Scale bar, 60 μm.

4 Discussion

Autophagic phenomenon in cells was first found by TEM[14]. So far, TEM is the most reliable method to study autophagy. The sources of autophagosome membrane are not very clear, and multiple membrane sources including endocytoplasmic reticulum, golgiosome, and inner and outer membranes of mitochondria supply the lipids needed for autophagosome formation[15]. When observing the ultrastructure of neurons of 16 d TBI model, we found that nuclear membrane stretch out to encase cytoplasma, which indicate that nuclear membrane might be one of autophagosomal

membrane sources. The number of lysosome structure was also increased in this TBI model, which was in concordance with the results of our cathepsins expression research before[16]. LC3, the mammalian orthologue of Atg8, which exists on the autophagosome membrane and isolated membranes, is targeted to the autophagosomal membranes in an Atg5-dependent manner and remains on it even after Atg12-Atg5 dissociates[17-19]. Thus, LC3 is the only credible marker of the autophagosome in mammalian cells[20-23]. There are three human isoforms of LC3 (LC3A, LC3B and LC3C) that undergo post-translational modifications during autophagy[23-26]. Another important protein involved in autophagy is Beclin1, which has been shown to interact with Bcl-2—a protein involved in the control of apoptosis[27,28]. Beclin 1 is required for vacuolar transport and autophagy, and acts as a suppressor of tumorgenesis. Beclin 1 expression in a closed brain injury model studied by immunohistochemical staining, showed that Beclin 1 expression may last at least 21 d after TBI, and autophagy might be activated in TBI model[9]. In our research, in addition to using these two autophagic indicators, which provided evidence consisted with the current consensus on TBI model, we also double labeled caspase-3 with LC3 and Fluoro-Jade with Beclin 1 to examine the relationship between autophagy and apoptosis or neurodegeneration. Before 24 h time point, or even 48 h time point of TBI, autophagy positive cells remained "healthy", as there were few caspase-3(+)/LC3(+) or Beclin 1(+)/Fluoro-Jade(+) overlapped cells, while the other cells showed caspase-3(+) or Fluoro-Jade(+) which means suggestive of undergoing apoptosis or neurodegenerative. After 24 h time point of TBI, the number of caspase-3(+)/LC3(+) overlapped cells increased and last to 32 d after TBI, which indicated that autophagy was also activated in apoptotic cells and might be act as a cleaner to maintain the homeostasis of the brain during the latter time after TBI.

Our results indicate that TBI activates autophagy, and the activation may begin at 1 h or earlier, and last at least 32 d after TBI. The autophagosomes appeared ahead of apoptosis and the LC3(+) cells did not overlap with caspase-3(+) cells, which suggest that autophagy activation could protect neurons from apoptosis during early stage after TBI. Autophagy also plays a continuous role in the bulk elimination of aberrant cell components afterwards, which contributes to the cellular homeostasis of the brain. The number of LC3(+) and Beclin 1(+) cells peaked at day 8 following TBI, suggesting that autophagic cell death occurs during the second wave of neuronal loss following injury[9], because autophagy is also involved in elimination of impaired cells in a non-apoptotic cell death pathway. Another explanation of the day 8 peak of autophagy after TBI is that autophagy has a repair function to eliminate waste materials from brain. Autophagy also serves as a mechanism to discard injured cells or recycle cellular components in injured cells, thus reducing the damage to surviving cells by disposing injured components and protecting cells from apoptosis and necrosis[29,30]. In conclusion, autophagy acts as a double-edged sword after TBI, as during long term of repair after TBI, autophagy protects nerve cells injured slightly from death by eliminating noxious agents such as injured cellular organ and excitatory amino acids, and in the meanwhile is also responsible for unrecoverable nerve cells death.

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大鼠脑外伤后自噬被激活并在早期对受损神经元起保护作用

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摘要：目的 研究大鼠脑外伤后自噬是否被激活并探讨其在脑外伤后神经细胞损伤和修复中的作用。**方法** 建立大鼠定量脑外伤模型，于脑外伤后不同时间点处死动物并取脑；应用透射电镜检测脑组织自噬双层膜结构以及次级溶酶体的形成情况；应用自噬标记抗体LC3B和Beclin-1对脑外伤后不同时间点的脑组织进行免疫荧光和Western blot检测；LC3和caspase-3或Beclin 1和Fluoro-Jade双标记检测。**结果** 脑外伤后1 h在损伤区周围即检测到双层膜结构，并且一直持续到脑外伤后32天。脑外伤后1 h，脑组织中LC3和Beclin-1表达增加，损伤后3天内阳性细胞以神经元为主，之后阳性胶质细胞增加，第8天达到高峰，并可持续至脑外伤后32天仍维持高表达。大多数阳性细胞分布在损伤区周围(包括海马)而不是损伤区。此外，脑外伤后24小时以前，在损伤区周围不是所有的LC3阳性细胞都与caspase-3阳性细胞重叠。同样脑外伤后6 h至48 h，Beclin 1阳性海马神经元与Fluoro-Jade染色不重叠。**结论** 脑外伤后自噬被激活，在损伤后早期保护损伤区周围神经细胞免于凋亡和退行性变，并对神经细胞损伤与修复发挥长期作用。

关键词：自噬；脑外伤；LC3；Beclin 1；神经退行性变