Fish and Shellfish Immunology 86 (2019) 1044–1052

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Glutamine starvation inhibits snakehead vesiculovirus replication via inducing autophagy associated with the disturbance of endogenous glutathione pool

Cheng Li[a,1], Lindan Sun[b,1], Hanzuo Lin[c], Zhendong Qin[a], Jiagang Tu[d], Jun Li[a,e,f], Keping Chen[b], Sarath Babu V[a,**], Li Lin[a,d,f,*]

[a] Department of Core Facility, Guangdong Provincial Water Environment and Aquatic Products Security Engineering Technology Research Center, Guangzhou Key Laboratory of Aquatic Animal Diseases and Waterfowl Breeding, Guangdong Provincial Key Laboratory of Waterfowl Breeding, College of Animal Sciences and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong, 510225, China
[b] Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu, 212000, China
[c] Faculty of Arts, University of British Columbia, Vancouver, British Columbia, V6T1W9, Canada
[d] Department of Aquatic Animal Medicine, College of Fisheries, Huazhong Agricultural University, Wuhan, Hubei, 430070, China
[e] School of Biological Sciences, Lake Superior State University, Sault Ste. Marie, MI, 49783, USA
[f] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao, 266071, PR China

ARTICLE INFO

Keywords:
Glutamine
Glutathione
Autophagy
SHVV
Glutathione pool

ABSTRACT

Autophagy is a degradation cellular process which also plays an important role in virus infection. Glutamine is an essential substrate for the synthesis of glutathione which is the most abundant thiol-containing compound within the cells and plays a key role in the antioxidant defense and intracellular signaling. There is an endogenous cellular glutathione pool which consists of two forms of glutathione, i.e. the reduced form (GSH) and the oxidized form (GSSG). GSH serves as an intracellular antioxidant to maintain cellular redox homeostasis by scavenging free radicals and other reactive oxygen species (ROS) which can lead to autophagy. Under physiological conditions, the concentration of GSSG is only about 1% of total glutathione, while stress condition can result in a transient increase of GSSG. In our previous report, we showed that the replication of snakehead fish vesiculovirus (SHVV) was significant inhibited in SSN-1 cells cultured in the glutamine-starvation medium, however the underlying mechanism remains enigmatic. Here, we revealed that the addition of L-Buthionine-sulfoximine (BSO), a specific inhibitor of the GSH synthesis, could decrease the γ-glutamate-cysteine ligase (GCL) activity and GSH levels, resulting in autophagy and significantly inhibition of the replication of SHVV in SSN-1 cells cultured in the complete medium. On the other hand, the replication of SHVV was rescued and the autophagy was inhibited in the SSN-1 cells cultured in the glutamine-starvation medium supplemented with additional GSH. Furthermore, the inhibition of the synthesis of GSH had not significantly affected the generation of reactive oxygen species (ROS). However, it significantly decreased level of GSH and enhanced the level of GSSG, resulting in the decrease of the value of GSH/GSSG, indicating that it promoted the cellular oxidative stress. Overall, the present study demonstrated that glutamine starvation impaired the replication of SHVV in SSN-1 cells via inducing autophagy associated with the disturbance of the endogenous glutathione pool.

1. Introduction

Autophagy is a homeostatic process that occurs naturally or is activated during infection or nutrient starvation to remove undesirable or injured cells for the maintenance of cellular homeostasis [1]. Previous studies have shown that virus infection can regulate autophagy formation, and in turn, autophagy plays either pro- or antiviral roles in virus replication and survival [2]. In recent years, growing evidence has

* Corresponding author. Department of core facility, Guangdong Provincial Water Environment and Aquatic Products Security Engineering Technology Research Center, Guangzhou Key Laboratory of Aquatic Animal Diseases and Waterfowl Breeding, Guangdong provincial key laboratory of waterfowl breeding, College of Animal Sciences and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong, 510225, China.
** Corresponding author.
E-mail addresses: sarathbabuv@yahoo.com (S. Babu V), linli@zhku.edu.cn (L. Lin).
[1] These two authors contributed equally.

https://doi.org/10.1016/j.fsi.2018.12.041
Received 18 November 2018; Received in revised form 15 December 2018; Accepted 21 December 2018
Available online 24 December 2018
1050-4648/

indicated that the replication of many viruses was associated with the autophagy process, such as Ebola virus [3], influenza virus [4], Newcastle disease virus [5], respiratory syncytial virus [6], Sendai virus [7]. The role of autophagy in viral replication is generally mediated by autophagy-related proteins, which are targeted by viruses during infection [8].

Glutamine (Gln) an important amino acid substrate which is essential for glutathione (GSH) synthesis. It has been reported that the glutamine deprivation regulates many DNA and RNA virus replications, such as vaccinia virus [9], human cytomegalovirus [10], infectious spleen and kidney necrosis virus [11], snakehead fish vesiculovirus [12], red-spotted grouper nervous necrosis virus [13]. Glutamine starvation could reduce the endogenous GSH level. However, viral replications can be regulated by GSH in a dual manner. In porcine circovirus type 2 (PCV2) and dengue virus, additional glutamine inhibited the viral replications by enhancing GSH levels [14,15]. By contrast, additional glutamine promoted ISKNV replication by providing substrates for GSH synthesis [11].

Glutathione, a tripeptide (γ-L-Glutamyl-Cysteinyl-Glycine), is the most abundant low molecular weight, thiol-containing compound in living cells and widely distributed in animals and plants [16]. GSH can be synthesized from glutamate by two successive enzymatic reactions. First, gamma-glutamyl cysteine is synthesized from L-glutamate and cysteine catalyzed by gamma-glutamylcysteine synthetase (γ-GCL). Subsequently, glycine is added to the C-terminal of cysteine catalyzed by glutathione synthetase (GSS) [17]. There is an endogenous cellular glutathione pool which consists of two forms of glutathione, i.e. the reduced form (GSH) and the oxidized form (GSSG). Under physiological conditions, the concentration of GSSG is only about 1% of total glutathione, i.e. the value of GSH/GSSG is around 100. While stress condition can result in a transient increase of GSSG, resulting in the decrease of the GSH/GSHH value [18]. Likewise, GSH serves as an intracellular antioxidant to maintain cellular redox homeostasis by scavenging free radicals and other reactive oxygen species (ROS), which can lead to autophagy [19]. It has been reported that glutamine starvation depletes endogenous anti-oxidant GSH levels and promoted oxidative stress in HuH-7 cells resulted in apoptosis [20].

Snakehead vesiculovirus (SHVV), belongs to the genus *Vesiculovirus,* a member of the rhabdovirus family with a negative-sense ssRNA genome of about 11 kb in length, consists of five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase protein (L, large protein) [21,22]. Recently, it has caused severe disease in snake head fish culture with a great economic loss [12,23].

In our previous report, we showed that the replication of snakehead fish vesiculovirus (SHVV) was significant inhibited in SSN-1 cells cultured in the glutamine-starvation medium [12]. In this study, we investigated the mechanism underlying the glutamine starvation-induced autophagy and the synthesis of glutathione during SHVV infection. The results showed that the inhibition of the GSH synthesis could disturb the endogenous glutathione pool, which induced autophagy in SSN-1 cells resulting in the suppression of SHVV replication. The obtained results will shed a new light on the mechanism underlying the inhibition of viral replication by the starvation of glutamine.

2. Materials and methods

2.1. Cell line and virus

Striped snakehead fry (SSN-1) cell line was kindly provided by the Shenzhen Animal & Plant Inspection and Quarantine Technology Center (Shenzhen, China). The cells were cultured and maintained at 25 °C in MEM medium (GIBCO, USA) with 10% fetal bovine serum (FBS) (GIBCO, USA), penicillin (100 μg/ml), and streptomycin (100 μg/ml). SHVV was isolated from the diseased hybrid snakehead fish from a farm in Guangdong Province, China and kept in our laboratory [24].

2.2. Reagents and antibodies

DMEM medium lacking D-glucose, L-glutamine, sodium pyruvate and phenol red (catalog number A14430-01) was purchased from Invitrogen (USA). Complete medium was prepared by adding 1 g/l D-glucose and 2 mM L-glutamine into glucose- and glutamine-free medium [12]. The glutamine-free medium was prepared by adding 1 g/l D-glucose into glucose- and glutamine-free medium. Antibodies against LC3 were raised from rabbit and stored in our laboratory. The antibody against beta-actin (Cat AD-103157) was purchased from Bioss Biotechnology Co., LTD., Beijing, China. The secondary antibody donkey anti-rabbit IgG antibody (Cat 926–68073) was purchased from Gene Co., LTD., Shanghai, China. L-Buthionine-sulfoximine (BSO, CAS 83730-53-4) was purchased from Aladdin Co., LTD., Shanghai, China.

2.3. Cell viability assay

The viability of SSN-1 cells cultured in complete medium with BSO (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2 mM) or glutamine-free medium with GSH (0, 0.1, 0.4, 1, 4, 8, 12 mM) for 24 h was performed according to manufacturer's instruction of Cell Titer 96® Aqueous One Solution Cell Proliferation Assay kit (MTS assay) (Promega, USA). The cell viability was measured by absorbance at OD_{490} nm in an ELISA microplate reader (Infinite M200 Pro, Tecan, Switzerland).

2.4. γ-GCL and GSH/GSSG assays

The BSO is a γ-GCL inhibitor, which prevents the conversion of glutamate to glutathione [25]. SSN-1 cells cultured in glutamine-free medium, with or without 1 mM BSO were collected by centrifugation at 10,000 rpm for 10 min at 4 °C. Later, the cells were washed twice with phosphate buffer solution (PBS, Goodbio Technology Company, Wuhan, China) and determined for γ-GCL activity assay using γ-GCL detection kit (Nanjing Jiancheng Bioengineering Institute Co., Ltd, Nanjing, China), and GSH and GSSG concentration assay kits (Beyotime S0053) following the manufacturers protocols.

2.5. Reactive oxygen species (ROS) determination

The ROS assay was performed as described previously with minor modification [26]. Briefly, the SSN-1 cells were grown in a 96-well tissue culture plate for 24 h with different glutamine concentrations with or without BSO or in glutamine-free mediums with GSH different concentrations. Each well was added with 100 μl of nitroblue tetrazolium (1 mg/ml) (Hi-medium), and the plate was further incubated at 25 °C for 60 min. The plate was centrifuged for 10 min at 540 g and the supernatants were removed from each well. Then, the cells were fixed by adding 100 μl of 70% methanol for 5 min. The cells were washed two times with 70% cold methanol to eliminate the unreduced NBT present in the cells. The NBT crystals were dissolved by adding 120 μl of 2 M potassium hydroxide to each well, followed by the addition of 140 μl of dimethyl sulfoxide (DMSO). The absorbance was measured at 620 nm in a microplate reader (Molecular Devices, USA).

2.6. The effect of BSO in SHVV and LC3 protein

SSN-1 cells were incubated with 1 multiplicity of infection (MOI) of SHVV for 2 h and then fed with complete medium with or without 1 mM BSO for 24 h. The virus mRNA and LC3 protein expression in SHVV-infected cells were determined using qRT-PCR and western blot (WB) assay, and the virus titer in the supernatant was measured by $TCID_{50}$.

2.7. The effect of GSH in SHVV and LC3 protein

SSN-1 cells were incubated with 1 MOI of SHVV for 2 h and then fed

Table 1
Primers used in the experiments.

Gens name	Name	Sequence(5′-3′)	Application
SSN1-β-actin	Actin-F	CACTGTGCCCATCTACGAG	qRT-PCR
	Actin-R	CCATCTCCTGCTCGAAGTC	
SSN1-ATG1	ATG1-F	CCCACCATCCTTGGATCACC	qRT-PCR
	ATG1-R	AGCTCTGCGACCTTCCAAAA.	
SSN1-ATG4	ATG4-F	CTAACAGACGACCGAGAGCA	qRT-PCR
	ATG4-R	TCCACCAAGCACTCCACAAG	
SHVV-N gene	N-FW	CCGCATCGGAAATCAAGCA	qRT-PCR
	N-BW	GTTGACCGCTTGCCCAATT	

with glutamine-free medium, with or without 0.1 mM GSH for 24 h. The virus mRNA and proteins in SHVV-infected cells were determined using qRT-PCR and WB, and the virus titer in the supernatant was measured by $TCID_{50}$. The LC3 of SSN-1 cells were determined by WB assay.

2.8. Quantitative real-time reverse transcription-PCR (qRT-PCR)

The qRT-PCR was performed by a double standard quantitative method as described previously [12]. Specific primers used are listed in Table 1. The reaction cycles were performed in a LightCycler480 system (Hoffmann-La Roche). β-actin was used as the internal control. All the reactions were done in triplicates. The relative expression ratio was calculated using the $2^{-\Delta\Delta}C_T$ method, and all data were given in terms of relative mRNA expression.

2.9. Western blot

Total proteins of SSN-1 cells were extracted by using cell lysis buffer [50 mM Tris-HCl (pH 8), 1 mM EDTA, 150 mM NaCl, 0.1% NP-40, 1 mM DTT, and 1 × protease inhibitor]. The Cell lysates were separated by SDS-PAGE (12% polyacrylamide gels) and then transferred onto a nitrocellulose (NC) membrane (Biosharp, Wuhan, China). The membranes were blocked for 2 h at room temperature (RT) in Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 2% skim milk. Subsequently, the membranes were probed with the primary antibody at RT for 2 h. After washing thrice with TBS with Tween 20 (TBS, 0.05% Tween 20, pH 7.4), the membranes were incubated with a secondary antibody for at RT for 1 h. The primary and secondary antibodies were diluted 1000-fold and 10,000-fold, respectively in 2% skim milk dissolved in TBST. The image was acquired using Odyssey CLx (LI-COR, Inc, USA) and the signal intensity was quantified by Quantity One® Software (Bio-Rad Laboratories).

2.10. Transmission electron microscopy

SSN-1 cells were collected after washing three times with ice-cold 1 × PBS. The cells were fixed with 4% paraformaldehyde at 4 °C overnight, subsequently post-fixed with 1% OsO_4 at RT for 1 h and dehydrated using ethanol. The dehydrated pellets were rinsed with propylene oxide and then embedded in Spurr resin for sectioning. Ultrathin sections of the samples were observed under an H-7000FA transmission electron microscope (Hitachi, Japan) at 160 kV.

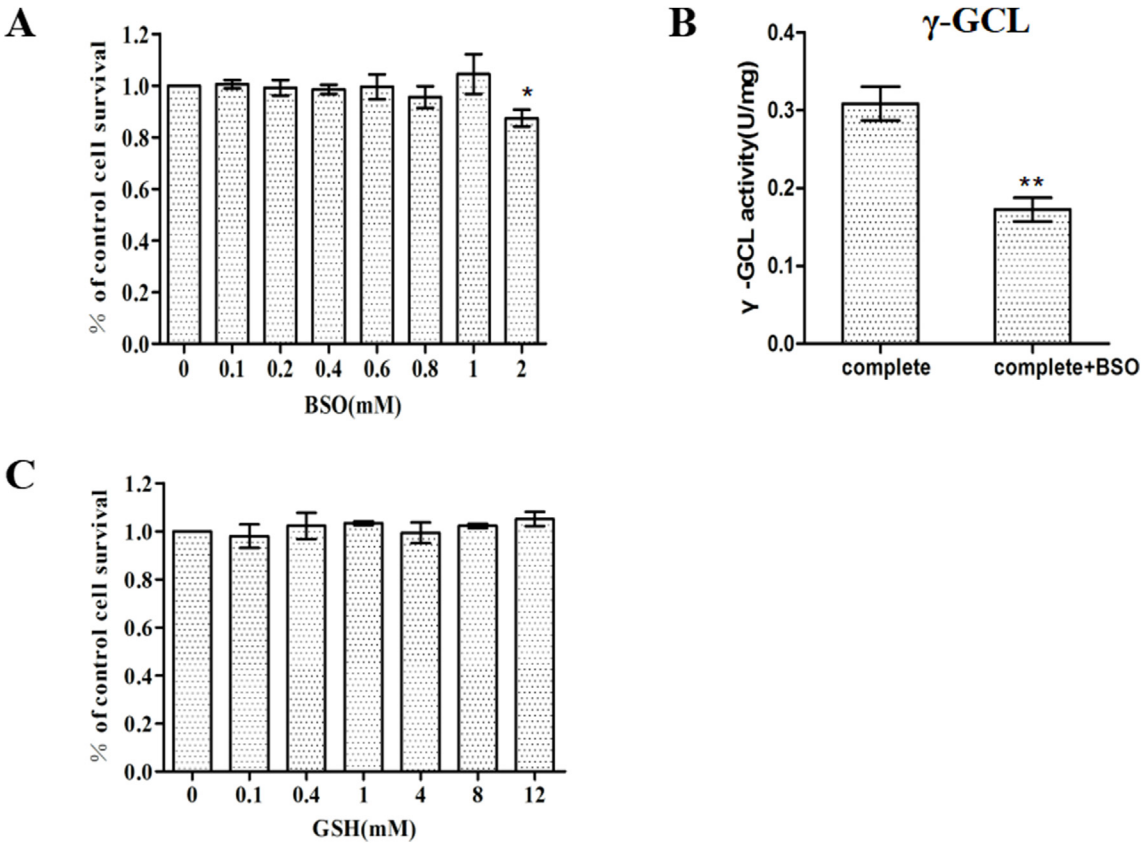

Fig. 1. Cell viability of SSN-1 cultured in medium supplemented with additional BSO or GSH.
(A) Cell viability of SSN-1 cultured in medium supplemented without or with different concentrations of BSO. (B) γ-GCL activities in SSN-1 cells cultured in medium supplemented without or with different concentrations of BSO. (C) Cell viability of SSN-1 cultured in glutamine free medium supplemented without or with different concentrations of GSH. At 24 h post of the treatments, cell viability and γ-GCL activity were measured. Data were analyzed for statistical differences between the groups by one-way analysis of variance (ANOVA). Differences among the groups were considered significant when $P^* < 0.05$ and highly significant when $P^{**} < 0.01$. The error bars were representative of standard deviation (mean ± SD; n = 9).

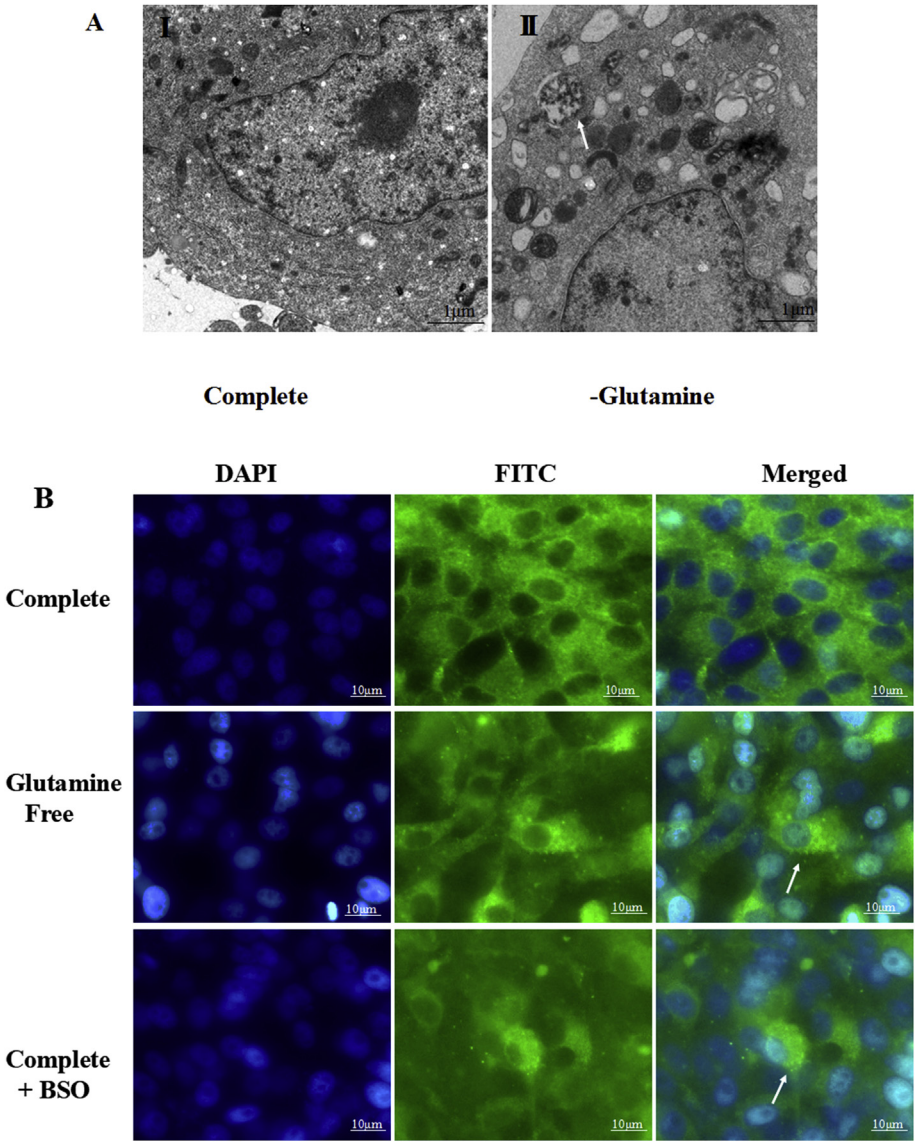

Fig. 2. Lacking of glutamine (glu) or glutathione (GSH) induced autophagy.
(A) Observation of autophagosomes in SSN-1 cells using transmission electron microscopy. The cells were fixed at 24 h post-glutamine deprivation. (I) SSN-1 cells culture in complete medium were used as a control. (II) SSN-1 cells cultured in medium without glutamine. The vesicle with characteristics of autophagosomes was indicated by arrow. (B) The expression of LC3 in SSN-1 cells was revealed by immuno-fluorescence assay (IFA). SSN-1 cells had been cultured in complete medium, glutamine free medium and complete medium supplemented with 1 mM BSO for 24 h, respectively. Thereafter, the expression of LC3 was revealed by FITC (green). The nuclei were stained with DAPI (blue). The LC3 dot was indicated by arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.11. Indirect immunofluorescent assay (IFA)

SSN-1 cells cultured for overnight in 12 well plates were fixed using ice-cold 100% methanol for 15 min at −20 °C. The cells were washed twice with PBS and then blocked with 1% bovine serum albumin (BSA) at RT for 1 h. After blocking, the cells were washed with PBS and then incubated at 28 °C for 1 h with the LC3 (diluted 1:1000 in PBS with 1% BSA) antibody. Subsequently, the cells were washed three times with PBS-Tween 20 (PBST), incubated with a secondary antibody *FITC Goat Anti-Rabbit IgG* (1:200 dilution in PBS with 1% BSA), at 28 °C for 30 min. The cells were counterstained with DAPI (2, 4-diamidino-2-phenylindole) (Beyotime, China) and washed twice with PBST before analyzing through cell photographic imaging multifunctional detection system (Axio imager A2, Germany).

2.12. Statistical analysis

Data were analyzed for statistical differences between the groups by one-way analysis of variance (ANOVA). Differences among the groups were considered significant when $P^* < 0.05$ and highly significant when $P^{**} < 0.01$. The error bars were representative of standard deviation (mean ± SD).

3. Results

3.1. Glutamine or glutathione deprivation lead to autophagy of SSN-1 cells

To investigate whether BSO or GSH were toxic for the growth of SSN-1 cells, first of all, we evaluated the viability of SSN-1 cells grown in complete medium with different concentrations of BSO or GSH. As showed in Fig. 1A, the cell viability was not affected in complete medium containing BSO ranged from 0.1 to 1 mM but a significant decrease in the survival of the cell could be observed in the medium with 2 mM BSO. Hence, 1 mM BSO was preferred for our supplementary studies. We further evaluated the activity of γ-GCL in SSN-1 cells cultured for 24 h in glutamine-free medium with or without 1 mM BSO. The results showed that the activity of γ-GCL in the cells cultured with and without BSO was 1.7U/mg and 3.0 U/mg, respectively (Fig. 1B). No changes were seen in the cells when cultured with various GSH (0–12 mM) concentrations (Fig. 1C). Next, we wanted to know whether the glutamine or glutathione deprivation could lead to autophagy in cells. The SSN-1 cells was determined by transmission electron microscopy (TEM) after culturing for 24 h in medium with or without glutamine. The TEM photomicrographs showed the formation of typical autophagosome vesicles around the nucleus in SSN-1 cells cultured in medium without glutamine for 24 h, which was not observed in the

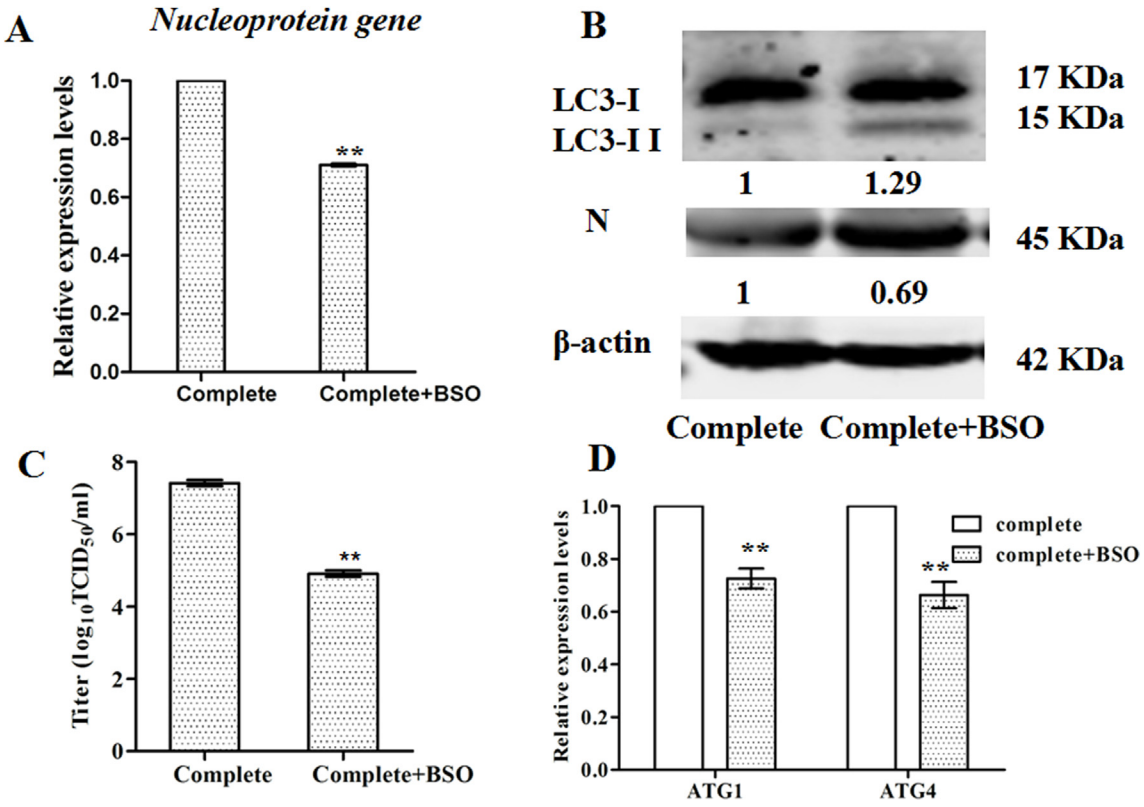

Fig. 3. Inhibition the synthesis of GSH induced autophagy and suppressed viral replication.
The SHVV infected SSN-1 cells were cultured in medium in the presence or absence of BSO. (A) The mRNA of nucleoprotein gene of SHVV was determined by qRT-PCR. (B) Nucleoprotein of SHVV and LC3 of SSN-1 were determined by Western blot (WB). (C) The replication of SHVV was determined by viral titer. (D) The mRNA levels of ATG1 and ATG4 were determined by qRT -PCR. Data were analyzed for statistical differences between the groups by one-way analysis of variance (ANOVA). Differences among the groups were considered significant when P* < 0.05 and highly significant when P** < 0.01. The error bars were representative of standard deviation (mean ± SD; n = 9).

cells cultured with glutamine (Fig. 2A). To determine whether the autophagy was also triggered by glutathione starvation, an indirect immunofluorescent assay was performed using LC3 antibody on SSN-1 cells cultured in complete medium with or without 1 mM BSO. The green fluorescence was observed profusely in SSN-1 cells cultured in complete medium. Whereas, sparse green dots were seen in the cells cultured in medium without glutamine or in complete medium supplemented with 1 mM BSO, indicating that the autophagy was induced by glutathione starvation (Fig. 2B). In summary, lack of glutamine or glutathione could cause autophagy in SSN-1 cells.

3.2. Inhibition of GSH induced autophagy to suppress viral replication

To study the effect of GSH starvation could induce the autophagy on SSN-1 cells and its impact on SHVV replication. SSN-1 cells were infected with 1 MOI of SHVV and then cultured in complete medium, with or without 1 mM BSO. At 24 h post of infection, the N gene mRNA and N protein were significantly reduced to 70% and 69%, respectively (Fig. 3A and B). LC3 is an important indicator of autophagy, the conversion of LC3I to LC3II protein ratio represents the activation and the level of autophagy. Our western blot results showed that the addition of BSO could increase the conversion ratio of LC3II/LC3I protein level than without BSO containing medium (Fig. 3B). The virus titer of SHVV cultured in BSO was significantly reduced to 66% when compared to complete medium (Fig. 3C). Autophagy-related genes (ATGs) are group of genes which are usually used as markers of autophagy. Among them, ATG1 protein and ATG4 protein could inhibit the autophagy process. ATG1 protein inhibits the autophagosome formation, while ATG4 inhibits both at the initial processing of LC3 (priming) and at the delipidation step. When the SSN-1 cultured in the complete medium supplemented with 1 mM of BSO, the mRNA levels of ATG1 and ATG4 was significantly decreased to 72% and 66%, respectively (Fig. 3D), indicating that BSO could inhibit the autophagy process associated with ATG1 and ATG4 proteins in SSN-1 cells.

3.3. Adding GSH inhibited autophagy and promote viral replication

To determine the effects of GSH on the autophagy and SHVV replication, SSN-1 cells were infected with 1 MOI of SHVV for 2 h and then fed with glutamine-free medium with or without 0.1 mM GSH for 24 h. In contrast to GSH starvation, an addition of GSH has significantly increased the SHVV N gene mRNA and protein level to 3.8 and 1.46 times, respectively (Fig. 4A and B), an addition of GSH has decreased the conversion of LC3I to LC3II (Fig. 4B). The virus titer increased was increased to 134% compared to that in complete medium (Fig. 4C) and the mRNA of ATG1 and ATG4 were significantly increased to 122% and 177% compared to those in complete medium, respectively (Fig. 4D). All the data above suggested that the GSH could inhibit autophagy and promoted the SHVV viral replication in SSN-1 cells.

3.4. Glutamine was used in the synthesis of GSH to promote viral replication

To determine the effect of glutamine and GSH on the replication of SHVV, we measured the expression of N protein of SHVV during the infection in cells cultured in medium with various concentration of additional glutamine with or without BSO (Fig. 5A and B). With the addition of glutamine, the level of SHVV N protein has increased from 1 to 1.19. However, with the addition of BSO, it decreased the N protein from 1 to 0.76 (without additional glutamine) and from 1.19 to 1.07(with additional 1 mM glutamine).

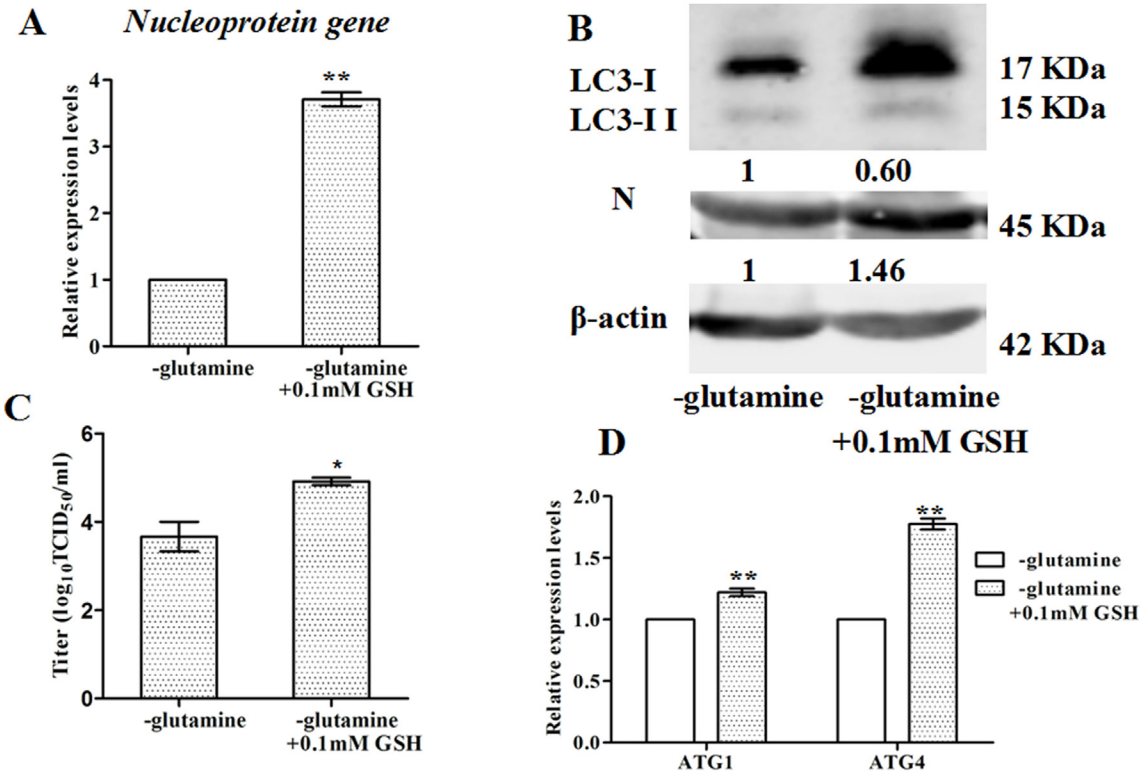

Fig. 4. Addition of GSH inhibited autophagy and promoted viral replication.
SHVV infected SSN-1 cells were cultured in medium in the absence of glutamine with or without GSH (0.1 mM). (A) The mRNA of nucleoprotein gene of SHVV was determined by qRT- PCR. (B) Nucleoprotein of SHVV and LC3 of SSN-1 were determined by WB. (C) The replication of SHVV was determined by viral titer. The mRNA levels of ATG1 and ATG4 were determined by qRT -PCR. Data were analyzed for statistical differences between the groups by one-way analysis of variance (ANOVA). Differences among the groups were considered significant when P* < 0.05 and highly significant when P** < 0.01. The error bars were representative of standard deviation (mean ± SD; n = 9).

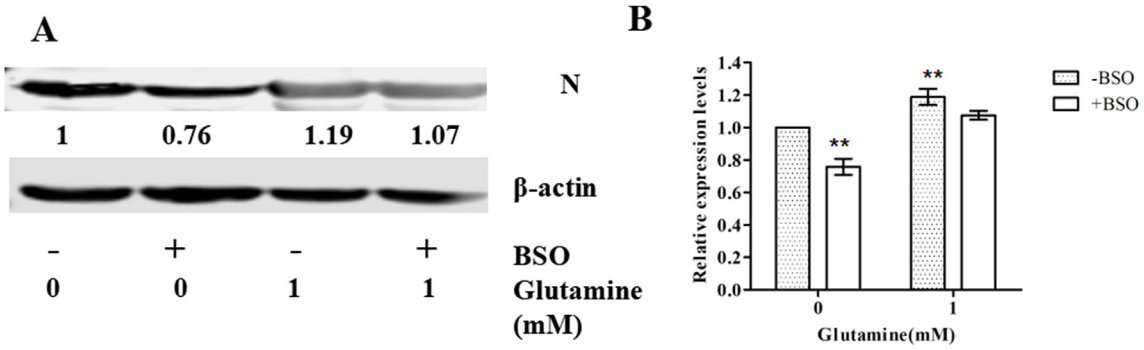

Fig. 5. Glutamine was used in the synthesis of GSH to promote viral replication.
SHVV infected SSN-1 cells were cultured in medium with or without glutamine in the presence or absence of BSO (1 mM). (A) Nucleoprotein of SHVV was determined by WB. (B) The statistic results of the WB results. Data were analyzed for statistical differences between the groups by one-way analysis of variance (ANOVA). Differences among the groups were considered significant when P* < 0.05 and highly significant when P** < 0.01. The error bars were representative of standard deviation (mean ± SD; n = 9).

3.5. GSH deprivation induced autophagy by disturbing GSH/GSSG ratio

To better understand the regulation of GSH starvation response induce autophagy, glutamine, BSO or GSH were added in the glutamine-free medium. The results showed that the GSH starvation or addition of GSH did not have any impact on ROS production in SSN-1 cells (Fig. 6A and B). To assess the effect on the ratio of GSH/GSSG in the exposed cells, the GSH and GSSG level and the ratio of GSH/GSSG were measured. Under physiological condition (cells cultured in complete medium without SHVV infection), the concentration of GSH and GSSG was 3.86 and 0.043, respectively, with the ratio of GSH/GSSG was 89.8 (Fig. 6C–E). However, under SHVV infection, the concentration of GSH and GSSG was 1.86 and 0.14, respectively, with the ratio of GSH/GSSG was 13.3 (Fig. 6C–E). While, under SHVV infection with addition of BSO, the concentration of GSH and GSSG was 0.03 and 0.12, respectively, with the ratio of GSH/GSSG was 0.25 (Fig. 6C–E). Apparently, the addition of BSO not only inhibited the synthesis of GSH, but also disturbed the balance of the GSH and GSSG, resulting in the promotion of cellular oxidative stress (Fig. 6E).

4. Discussion

In our previous study, we demonstrated that infection of glutamine depleted SSN-1 cells with SHVV, reduced the production of SHVV viral

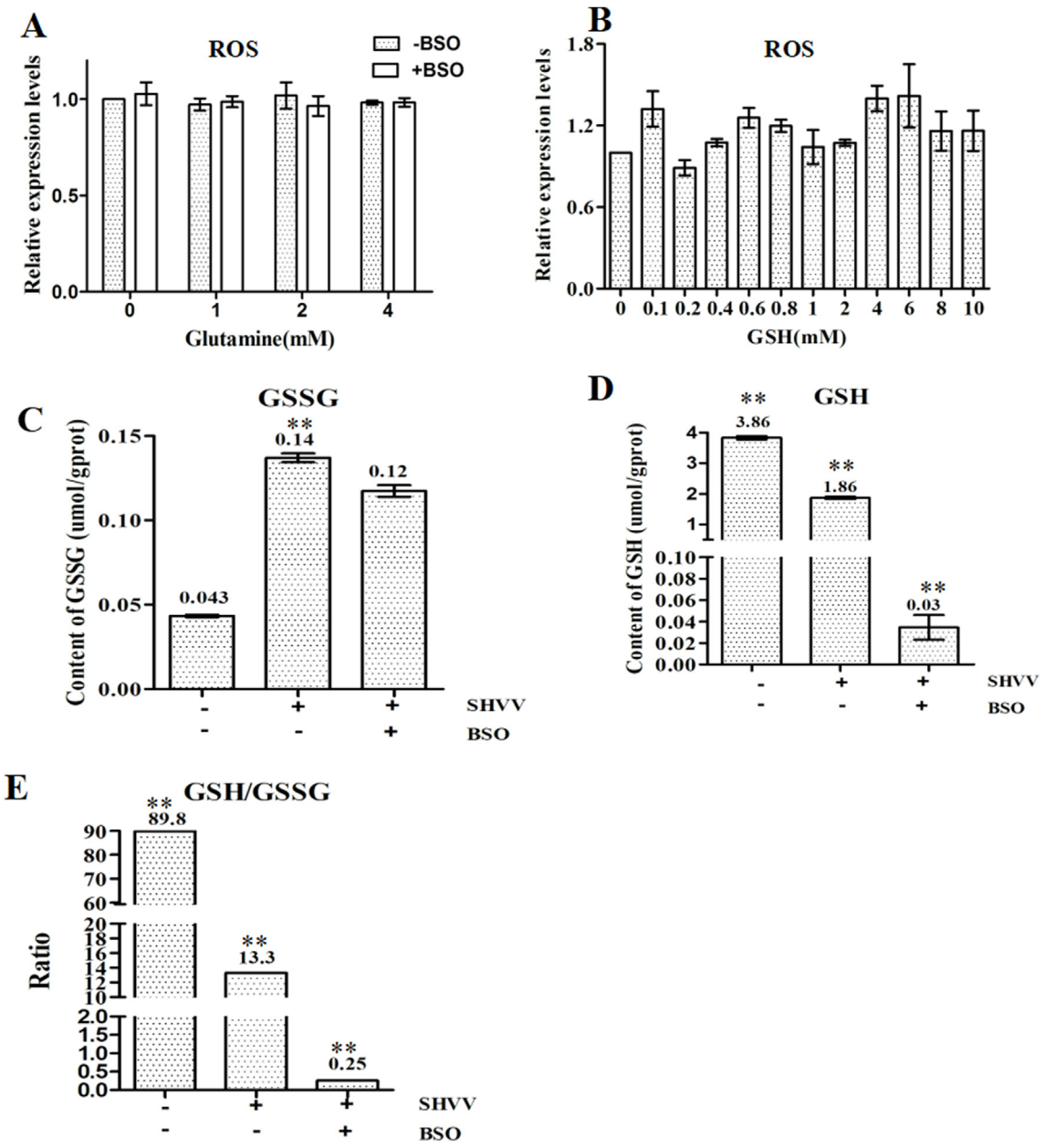

Fig. 6. GSH deprivation disturbed the glutathione pool.

(A) ROS level of SSN-1 cells cultured in medium supplemented with different concentrations of glutamine with or without 1 mM BSO. (B) ROS level in SSN-1 cells cultured in glutamine free medium without or without GSH. (C): GSSG levels in SSN-1 cells without SHVV infection, with SHVV infection in the presence or absence of BSO. (D): GSH levels in SSN-1 cells without SHVV infection, with SHVV infection in the presence or absence of BSO. (E). The ratio of GSH/GSSG in SSN-1 cells without SHVV infection, with SHVV infection in the presence or absence of BSO. Data were analyzed for statistical differences between the groups by one-way analysis of variance (ANOVA). Differences among the groups were considered significant when $P^* < 0.05$ and highly significant when $P^{**} < 0.01$. The error bars were representative of standard deviation (mean ± SD; n = 9).

particles [12]. However, the participation of glutamine starvation could induce autophagy in piscine cells and its impact on virus replication is unknown. In this study, glutamine starvation-induced autophagy pathway was profoundly analyzed in SSN-1 cells and the influence of starvation-induced autophagy in the replication of SHVV in SSN-1.

Autophagy is a self-degradative process that is indispensable for the cell in response to nutrient starvation and other types of stressful conditions, such as pathogen infection [27,28]. Depletion of GSH also enhances autophagy [18,29,30]. In the current study, GSH was successfully depleted in SSN-1 cells by using γ-GCL inhibitor BSO, as reported previously [31]. It has been reported that GSH depletion has been reported earlier in the range from 0.1 to 10 mM BSO [32,33]. Our study showed that addition of various concentrations of BSO or glutamine did not alter much in the survival of cells (Fig. 1). Considering this we used an average volume of BSO (1 mM) and GSH (0.1 mM) to avoid overloading the cells to induce the autophagy. The results showed the evidences of autophagy characterized by autophagic vacuoles within the cell by TEM and the accumulation of LC3 on the autophagosomal membranes by indirect immunofluorescence in 1 mM BSO treated cells (Fig. 2) and the fluctuation ratio of LC3II/LC3I (Fig. 3B), indicating that incorporation of BSO supports the autophagy as reported by Refs. [31,34]. However, the addition of GSH in glutamine free medium withdrawn the autophagy marker levels (LC3II/LC3I) (Fig. 4B). Similar reports have been stated that the addition of glutamine had increased the expression of autophagy markers in fibroblasts [35], rat intestinal epithelial cells and human colonic epithelial cells [36] and in

dopaminergic cells [34]. On one hand, we tested the N protein by WB in glutamine free medium and 1 mM glutamine medium with or without BSO to verify the glutamine (Fig. 5A and B) has a same role with GSH (Figs. 3 and 4) on the effect of virus replication because glutamine could be used for GSH synthesis. Therefore it is reasonable to believe that glutamine depletion could disturb the GSH synthesis. On the other hand, to confirm the autophagy formation we quantified the mRNA level of ATG1 and ATG4, the results showed both ATG1 and ATG4 were decreased during GSH depletion (Fig. 3D), while they were increased during the addition of GSH (Fig. 4D). This may be due to the starvation inhibit the mTOR activity and repress the activity of ATG1, which participates in the formation of complete autophagosomal structure [37]. In addition, ATG4 is a target for redox regulation under starvation and the accumulation of lipidatation LC3 (LC3-II) might suppress the ATG4 activity [38]. These results suggest that deprivation of GSH in SSN-1 cells could decrease the ATG1 and ATG4 activity, resulting in the enhancement of LC3-II level and autophagy.

It has also been demonstrated that ROS are essential for starvation-induced autophagy and specifically target ATG4 [38]. Hence, we evaluated the ROS level and redox regulation levels of SSN-1 cells treated with BSO. Our current results demonstrate that supplementation of BSO had significantly decreased the levels of GSH (reduced form of glutathione), and GSH/GSSG ratio was < 1 (Fig. 6C–E), without fluctuating the ROS production (Fig. 6A and B). The reduced GSH could be oxidized to GSSG with the conversion of H_2O_2 into water [39]. In a resting cell, the molar GSH: GSSG ratio is about 100:1, while in various models of oxidative stress, this ratio has been demonstrated to decrease rapidly to values of about 1:1 [40]. The ratio of GSH/GSSG represents a reliable measure of oxidative stress which can lead to autophagy [41–43]. Above all, addition of GSH decreased the value of GSH/GSSG and promoted cellular oxidative stress and autophagy [18].

It has been shown that deprivation of glutamine broke the balance of GSH and GSSG during ISKNV replication [11]. However, the oxidative stress including the synthesis of ROS had not been characterized. It has been reported that generation of ROS through oxidative stress induces autophagy [44]. Recently, there is growing evidence that BSO enhances autophagy with no correlation with the generation of ROS [18,30,31]. The present study clearly demonstrated the BSO and GSH starvations could induce autophagy in SSN-1 cells without changing the ROS levels.

In this study, we further assessed autophagy pathway in SSN-1 cells during SHVV infection. Under the GSH starvation conditions, virus titer, N gene, and protein levels were decreased revealing that the SHVV replication was repressed (Fig. 3), while the addition of GSH restored SHVV replication (Fig. 4). There have reported that influenza virus and ISKNV replication were both suppressed by GSH within the range of 1–30 mM [11,45]. This suggests that viral multiplication benefits from a low GSH concentration. Mechanistic investigation of EF25-(GSH)2 (double glutathione with curcumin) revealed that low concentration of the compound induced autophagy, while high concentrations activated a reversal of the autophagy process [46]. Maybe GSH also has the similar phenomenon of autophagy that reflects on virus replication in a dual way.

Acknowledgments

This work was jointly supported by the National Natural Science Foundation of China (31872606, 31572657, U1701233); "Innovation and Strong Universities" special fund from the Department of Education of Guangdong Province (KA170500G, TK222001G, KA18058B3, KA1819604); Fund from the Department of Science and Technology of Guangdong Province (KA1810312); Fund from Chinese Postdoctoral Science Foundation (2018M642185), V Sarath Babu was supported by Chinese Postdoctoral Science Foundation (189103).

References

[1] Y. Wang, K. Jiang, Q. Zhang, S. Meng, C. Ding, Autophagy in negative-strand RNA virus infection, Front. Microbiol. 9 (2018) 206, https://doi.org/10.3389/fmicb.2018.00206.
[2] Y. Wang, N. Chen, A.M. Hegazy, X. Liu, Z. Wu, X. Liu, L. Zhao, Q. Qin, J. Lan, L. Lin, Autophagy induced by snakehead fish vesiculovirus inhibited its replication in SSN-1 cell line, Fish Shellfish Immunol. 55 (2016) 415–422, https://doi.org/10.1016/j.fsi.2016.06.019.
[3] A.I. Chiramel, J.D. Dougherty, V. Nair, S.J. Robertson, S.M. Best, FAM134B, the selective autophagy receptor for endoplasmic reticulum turnover, inhibits replication of Ebola virus strains makona and mayinga, J. Infect. Dis. 214 (2016) S319–s325, https://doi.org/10.1093/infdis/jiw270.
[4] Y. Sun, C. Li, Y. Shu, X. Ju, Z. Zou, H. Wang, S. Rao, F. Guo, H. Liu, W. Nan, Y. Zhao, Y. Yan, J. Tang, C. Zhao, P. Yang, K. Liu, S. Wang, H. Lu, X. Li, L. Tan, R. Gao, J. Song, X. Gao, X. Tian, Y. Qin, K.F. Xu, D. Li, N. Jin, C. Jiang, Inhibition of autophagy ameliorates acute lung injury caused by avian influenza A H5N1 infection, Sci. Signal. 5 (2012) ra16, https://doi.org/10.1126/scisignal.2001931.
[5] C. Meng, Z. Zhou, K. Jiang, S. Yu, L. Jia, Y. Wu, Y. Liu, S. Meng, C. Ding, Newcastle disease virus triggers autophagy in U251 glioma cells to enhance virus replication, Arch. Virol. 157 (2012) 1011–1018, https://doi.org/10.1007/s00705-012-1270-6.
[6] M. Reed, S.H. Morris, S. Jang, S. Mukherjee, Z. Yue, N.W. Lukacs, Autophagy-inducing protein beclin-1 in dendritic cells regulates CD4 T cell responses and disease severity during respiratory syncytial virus infection, J. Immunol. 191 (2013) 2526–2537, https://doi.org/10.4049/jimmunol.1300477.
[7] Q. Zhang, H. Zhu, X. Xu, L. Li, H. Tan, X. Cai, Inactivated Sendai virus induces apoptosis and autophagy via the PI3K/Akt/mTOR/p70S6K pathway in human non-small cell lung cancer cells, Biochem. Biophys. Res. Commun. 465 (2015) 64–70, https://doi.org/10.1016/j.bbrc.2015.07.130.
[8] I.P. Gregoire, C. Rabourdin-Combe, M. Faure, Autophagy and RNA virus interactomes reveal IRGM as a common target, Autophagy 8 (2012) 1136–1137, https://doi.org/10.4161/auto.20339.
[9] K.A. Fontaine, R. Camarda, M. Lagunoff, Vaccinia virus requires glutamine but not glucose for efficient replication, J. Virol. 88 (2014) 4366–4374, https://doi.org/10.1128/JVI.03134-13.
[10] J.W. Chambers, T.G. Maguire, J.C. Alwine, Glutamine metabolism is essential for human cytomegalovirus infection, J. Virol. 84 (2010) 1867–1873, https://doi.org/10.1128/JVI.02123-09.
[11] X. Fu, X. Hu, N. Li, F. Zheng, X. Dong, J. Duan, Q. Lin, J. Tu, L. Zhao, Z. Huang, J. Su, L. Lin, Glutamine and glutaminolysis are required for efficient replication of infectious spleen and kidney necrosis virus in Chinese perch brain cells, Oncotarget 8 (2017) 2400–2412, https://doi.org/10.18632/oncotarget.13681.
[12] L. Sun, L. Yi, C. Zhang, X. Liu, S. Feng, W. Chen, J. Lan, L. Zhao, J. Tu, L. Lin, Glutamine is required for snakehead fish vesiculovirus propagation via replenishing the tricarboxylic acid cycle, J. Gen. Virol. 97 (2016) 2849–2855, https://doi.org/10.1099/jgv.0.000597.
[13] M. Asim, S. Jiang, L. Yi, W. Chen, L. Sun, L. Zhao, M.N. Khan Khattak, J. Tu, L. Lin, Glutamine is required for red-spotted grouper nervous necrosis virus replication via replenishing the tricarboxylic acid cycle, Virus Res. 227 (2017) 245–248, https://doi.org/10.1016/j.virusres.2016.11.007.
[14] X. Chen, X. Shi, F. Gan, D. Huang, K. Huang, Glutamine starvation enhances PCV2 replication via the phosphorylation of p38 MAPK, as promoted by reducing glutathione levels, Vet. Res. 46 (2015), https://doi.org/10.1186/s13567-015-0168-1.
[15] Y. Tian, W. Jiang, N. Gao, J. Zhang, W. Chen, D. Fan, D. Zhou, J. An, Inhibitory effects of glutathione on dengue virus production, Biochem. Biophys. Res. Commun. 397 (2010) 420–424, https://doi.org/10.1016/j.bbrc.2010.05.108.
[16] B.A. Kumar, K. Amandeep, Glutathione degradation, Antioxidants Redox Signal. 27 (2017) 1200–1216, https://doi.org/10.1089/ars.2017.7136.
[17] D.R. Sappington, E.R. Siegel, G. Hiatt, A. Desai, R.B. Penney, A. Jamshidi-Parsian, R.J. Griffin, G. Boysen, Glutamine drives glutathione synthesis and contributes to radiation sensitivity of A549 and H460 lung cancer cell lines, Biochim. Biophys. Acta 1860 (2016) 836–843, https://doi.org/10.1016/j.bbagen.2016.01.021.
[18] E. Desideri, G. Filomeni, M.R. Ciriolo, Glutathione participates in the modulation of starvation-induced autophagy in carcinoma cells, Autophagy 8 (2012) 1769–1781, https://doi.org/10.4161/auto.22037.
[19] G. Wu, Y.-Z. Fang, S. Yang, J.R. Lupton, N.D. Turner, Glutathione metabolism and its implications for health, J. Nutr. 134 (2004) 489–492, https://doi.org/10.1093/jn/134.3.489.
[20] Y. Xu, Q. Nguyen, D.C. Lo, M.J. Czaja, c-myc-Dependent hepatoma cell apoptosis results from oxidative stress and not a deficiency of growth factors, J. Cell. Physiol. 170 (1997) 192–199, https://doi.org/10.1002/(SICI)1097-4652 (199702) 170:2 < 192:AID-JCP11 > 3.0.CO;2-K.
[21] W. Zeng, Q. Wang, Y. Wang, C. Liu, H. Liang, X. Fang, S. Wu, Genomic characterization and taxonomic position of a rhabdovirus from a hybrid snakehead, Arch. Virol. 159 (2014) 2469–2473, https://doi.org/10.1007/s00705-014-2061-z.
[22] C. Zhang, L. Yi, S. Feng, X. Liu, J. Su, L. Lin, J. Tu, MicroRNA miR-214 inhibits snakehead vesiculovirus replication by targeting the coding regions of viral N and P, J. Gen. Virol. 98 (2017) 1611–1619, https://doi.org/10.1099/jgv.0.000854.
[23] L. Sun, J. Tu, L. Yi, W. Chen, L. Zhao, Y. Huang, R. Liang, J. Li, M. Zhou, L. Lin, Pathogenicity of snakehead vesiculovirus in rice field eels (Monopterus albus), Microb. Pathog. 110 (2017) 578–585, https://doi.org/10.1016/j.micpath.2017.07.042.
[24] X. Liu, Y. Wen, X. Hu, W. Wang, X. Liang, J. Li, V. Vakharia, L. Lin, Breaking the host range: Mandarin fish is susceptible to a vesiculovirus derived from snakehead fish, J. Gen. Virol. 96 (2015) 775–781, https://doi.org/10.1099/vir.0.000037.

[25] R. Babu, S. Eaton, D.P. Drake, L. Spitz, A. Pierro, Glutamine and glutathione counteract the inhibitory effects of mediators of sepsis in neonatal hepatocytes, J. Pediatr. Surg. 36 (2001) 282–286, https://doi.org/10.1053/jpsu.2001.20690.
[26] K. Nakhro, T.B. Devi, D. Kamilya, In vitro immunopathogenesis of Edwardsiella tarda in catla Catla catla (Hamilton), Fish Shellfish Immunol. 35 (2013) 175–179, https://doi.org/10.1016/j.fsi.2013.04.002.
[27] K.I. Jung, C.W. Pyo, S.Y. Choi, Influenza A virus-induced autophagy contributes to enhancement of virus infectivity by SOD1 downregulation in alveolar epithelial cells, Biochem. Biophys. Res. Commun. 498 (2018) 960–966, https://doi.org/10.1016/j.bbrc.2018.03.089.
[28] P. Sun, S. Zhang, X. Qin, X. Chang, X. Cui, H. Li, S. Zhang, H. Gao, P. Wang, Z. Zhang, J. Luo, Z. Li, Foot-and-mouth disease virus capsid protein VP2 activates the cellular EIF2S1-ATF4 pathway and induces autophagy via HSPB1, Autophagy 14 (2018) 336–346, https://doi.org/10.1080/15548627.2017.1405187.
[29] M. Deffieu, I. Bhatia-Kissova, B. Salin, A. Galinier, S. Manon, N. Camougrand, Glutathione participates in the regulation of mitophagy in yeast, J. Biol. Chem. 284 (2009) 14828–14837, https://doi.org/10.1074/jbc.M109.005181.
[30] D.L. Pereira, A.C. Dos Santos Ferreira, G.P. de Faria, J.K. Kwee, Autophagy interplays with apoptosis and cell cycle regulation in the growth inhibiting effect of Trisenox in HEP-2, a laryngeal squamous cancer, Pathol. Oncol. Res. 21 (2015) 103–111, https://doi.org/10.1007/s12253-014-9794-6.
[31] H. Mancilla, R. Maldonado, K. Cereceda, F. Villarroel-Espindola, M. Montes de Oca, C. Angulo, M.A. Castro, J.C. Slebe, J.C. Vera, S. Lavandero, Concha II, glutathione depletion induces spermatogonial cell autophagy, J. Cell. Biochem. 116 (2015) 2283–2292, https://doi.org/10.1002/jcb.25178.
[32] E.P. Clark, E.R. Epp, M. Morse-Gaudio, J.E. Biaglow, The role of glutathione in the aerobic radioresponse. I. Sensitization and recovery in the absence of intracellular glutathione, Radiat. Res. 108 (1986) 238–250.
[33] M.A. Fath, I.M. Ahmad, C.J. Smith, J. Spence, D.R. Spitz, Enhancement of carboplatin-mediated lung cancer cell killing by simultaneous disruption of glutathione and thioredoxin metabolism, Clin. Canc. Res. 17 (2011) 6206–6217, https://doi.org/10.1158/1078-0432.CCR-11-0736.
[34] P. Chandramani Shivalingappa, H. Jin, V. Anantharam, A. Kanthasamy, A. Kanthasamy, N-acetyl cysteine protects against methamphetamine-induced dopaminergic neurodegeneration via modulation of redox status and autophagy in dopaminergic cells, Parkinsons. Dis. 2012 (2012) 424285, https://doi.org/10.1155/2012/424285.
[35] Y.-H. Ko, Z. Lin, N. Flomenberg, R.G. Pestell, A. Howell, F. Sotgia, M.P. Lisanti, U.E. Martinez-Outschoorn, Glutamine fuels a vicious cycle of autophagy in the tumor stroma and oxidative mitochondrial metabolism in epithelial cancer cells: implications for preventing chemotherapy resistance, Cancer Biol. Ther. 12 (2011) 1085–1097, https://doi.org/10.4161/cbt.12.12.18671.
[36] T. Sakiyama, M.W. Musch, M.J. Ropeleski, H. Tsubouchi, E.B. Chang, Glutamine increases autophagy under Basal and stressed conditions in intestinal epithelial cells, Gastroenterology 136 (2009) 924–932, https://doi.org/10.1053/j.gastro.2008.12.002.
[37] J. Lee, S. Giordano, J. Zhang, Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling, Biochem. J. 441 (2012) 523–540, https://doi.org/10.1042/BJ20111451.
[38] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, Z. Elazar, Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4, EMBO J. 26 (2007) 1749–1760, https://doi.org/10.1038/sj.emboj.7601623.
[39] M. Valko, C.J. Rhodes, J. Moncol, M. Izakovic, M. Mazur, Free radicals, metals and antioxidants in oxidative stress-induced cancer, Chem. Biol. Interact. 160 (2006) 1–40, https://doi.org/10.1016/j.cbi.2005.12.009.
[40] Y.C. Chai, S.S. Ashraf, K. Rokutan, R.B.J. Johnston, J.A. Thomas, S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide, Arch. Biochem. Biophys. 310 (1994) 273–281, https://doi.org/10.1006/abbi.1994.1167.
[41] C. Hwang, A.J. Sinskey, H.F. Lodish, Oxidized redox state of glutathione in the endoplasmic reticulum, Science 257 (1992) 1496–1502.
[42] R. Exner, B. Wessner, N. Manhart, E. Roth, Therapeutic potential of glutathione, Wien Klin. Wochenschr. 112 (2000) 610–616.
[43] H.-S. Chiang, M. Maric, Lysosomal thiol reductase negatively regulates autophagy by altering glutathione synthesis and oxidation, Free Radic. Biol. Med. 51 (2011) 688–699, https://doi.org/10.1016/j.freeradbiomed.2011.05.015.
[44] Y. Chen, E. McMillan-Ward, J. Kong, S.J. Israels, S.B. Gibson, Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells, Cell Death Differ. 15 (2008) 171–182, https://doi.org/10.1038/sj.cdd.4402233.
[45] J. Cai, Y. Chen, S. Seth, S. Furukawa, R.W. Compans, D.P. Jones, Inhibition of influenza infection by glutathione, Free Radic. Biol. Med. 34 (2003) 928–936.
[46] J.Z. Zhao, L.M. Xu, M. Liu, Z.Y. Zhang, J.S. Yin, H.B. Liu, T.Y. Lu, Autophagy induced by infectious hematopoietic necrosis virus inhibits intracellular viral replication and extracellular viral yields in epithelioma papulosum cyprini cell line, Dev. Comp. Immunol. 77 (2017) 88–94, https://doi.org/10.1016/j.dci.2017.07.025.