Proceedings of the 4th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/76/2024.19979

Excessive consumption of α-solanine poisons the trophoblast via increasing the concentrations of autophagic marker which induces cell autophagy

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Abstract. There exists a large amount of toxins in the surrounding environment which have the capacity to damage our body's health. α-Solanine, a glycoalkaloid ordinarily extracted from potatoes, possesses potential toxicity to the human body, which may induce cell apoptosis and autophagy. The trophoblast, an embryonic tissue, is crucial in human development. However, how the toxin α-solanine impacts the trophoblast still remains unclear. This endangers many pregnant women and the coming generation nowadays. Hence, this study is aimed at exploring the mechanism of trophoblast poisoning triggered by excessive consumption of α-solanine via varying the concentrations of two proteins, Beclin-1 and LC3, which exert crucial roles in the cell autophagy signaling pathway, using HTR-8/SVneo cells as the sample. By conducting IHC analysis and autophagy staining assay kit with monodansylcadaverine (MDC), the changed concentrations of Beclin-1, LC3 and autophagosome have a relationship between the increasing concentration and duration of α-solanine. Meanwhile, conducting the cell proliferation experiment offers a bidirectional confirmation of the aforementioned results. In conclusion, the mechanism of poisoning is that the excessive consumption of α-solanine promotes the synthesis of Beclin-1 and LC3, which directly stimulates the progress of cell autophagy and decreases cell viability. Therefore, these ultimately cause detriment to the trophoblast.

Keywords: α-Solanine, trophoblast, poisoning, autophagy, mechanism

1. Introduction

α-Solanine ($C_{45}H_{73}NO_{15}$), extracted from the nightshade plant family such as the potato (Solanum tuberosum) and tomato (Solanum lycopersicum), is a steroidal glycoalkaloid and a natural toxin occurring in any part of the aforesaid plant, consisting of leaves, fruit, and tubers [1]. This compound has the capacity to assist the aforesaid plants to defend against invasions from outside enemies, including insects and animals[1]. Recently, several papers have been published to elucidate that α-solanine acts as a crucial role in possessing chemotherapeutic action against various cancers [2]. It has been proved that α-solanine has the potential effects on anti-inflammatory [3,4]. However, while concentrating on conducting research to explore the positive effects of α-solanine, it is necessary to mention that acting as a natural toxin, excessive consumption would induce severe symptoms or even lead to death without suitable storage [5]. So far, it has been concluded that a high intake of α-solanine could trigger nausea, diarrhea, vomiting, stomach cramps, and even worse [6]. Apart from this, α-solanine could also damage

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DOI: 10.54254/2753-8818/76/2024.19979

the reproductive system in an irreversible way [7]. It could cause abortion in mice and cause damage to fetus in rats [8,9].

The trophoblast, a part of the human placenta, is the outer layer of cells, which plays a critical role in embryo implantation and interaction with the decidualized maternal uterus. And malfunctioning of trophoblasts could lead to inflammation, oxidative stress and dysregulated angiogenesis. In brief, whether the trophoblast is normal or not affects the growth and development of the fetus [10]. Unfortunately, α-solanine could influence the function of the HTR-8/SVneo cells which is one type of trophoblast cell via inhibition of cell proliferation and migration, inducing cell apoptosis and autophagy [11]. Previous research has already found the connection between the concentration of α-solanine and each phenomenon. Nevertheless, the mechanism of each process is not clear enough, especially in cell autophagy. In this signaling pathway, Beclin-1 and LC3 are two significant proteins that determine whether to start autophagy or not [12]. It is reasonable to believe that α-solanine has a connection with the synthesis of Beclin – 1 and LC3, and then impacts cell autophagy.

Hence, in this research, experiments have been conducted to investigate the changes in concentrations of Beclin-1, LC3, and autophagosomes in HTR-8/SVneo cells, in response to varying concentrations and treatment durations of α-solanine. This study also aimed to measure the viability of HTR-8/SVneo cells. The above results were eventually used to give a reasonable interpretation of the mechanism of how α-solanine impacts the pathway of trophoblast autophagy.

The prediction has been made that increasing concentrations and treatment durations with α-solanine with HTR-8/SVneo cells will increase the expression of autophagic markers (Beclin-1) and LC3, which promotes autophagy and increase the concentration of autophagosome and eventually decreases cell viability and poisons the trophoblast. The changes of Beclin-1, LC3 and autophagosome kit with monodansylcadaverine (MDC) will be measured by confocal microscopy. Cell viability will be measured by CCK-8 assay. Positive control is Rapamycin, a reagent that can induce cell autophagy. The negative control is DMSO.

2. Methods and Materials

2.1. Cell proliferation assay kit with CCK-8

The trophoblast HTR-8/SVneo cells were seeded at a concentration of 2×10^3 cells/well and separated into three wells. CCK-8 solution (10 μL) was added to each well and the cells were incubated for 2 h at 37 °C. The optical density (OD) was measured by confocal microscopy at a wavelength of 450 nm. The percentage of cell viability was calculated by comparing the OD of the various treated wells to the control well. The positive control is Rapamycin. The negative control is DMSO. The reagents (α-Solanine) from 10 μM, 100 μM to 200 μM are added to each sample and records are taken after 12h ,24h and 48h [11].

2.2. Immunohistochemistry (IHC) analysis

The placenta tissue was fixed with 4 % paraformaldehyde (PFA) and embedded in paraffin. Sections were dewaxed by xylene and rehydrated by ethanol gradient for hematoxylin and eosin (HE) staining for histological analysis, while frozen liver sections were evaluated using an Oilred-O staining kit to visualize and quantify the distribution of Beclin-1 and LC3 in HTR-8/SVneo cells [13].

As for IHC analysis, the serial sections were incubated with the primary antibodies, aniti-Beclin-1 and anti-LC3B, at 4 °C overnight, and images were taken by confocal microscopy. Positive control is Rapamycin. The negative control is DMSO. The reagents (α-Solanine) from 10 μM, 100 μM to 200 μM are added to each sample and records are taken after 12h ,24h and 48h [13].

2.3. Autophagy staining assay kit with mono dansyl cadaverine (MDC)

NTR-8/SVneo cells were incubated in a 100 μmol/L MDC solution at 37 °C for 30 min. Following an experiment assay buffer-wash, NTR-8/SVneo cells were examined under confocal microscopy with 20 × objective lens magnification. The positive control is Rapamycin. Negative control is DMSO. The

DOI: 10.54254/2753-8818/76/2024.19979

reagents (α-Solanine) from 10 μM, 100 μM to 200 μM are added to each sample and records are taken after 12h ,24h and 48h [14].

2.4. Statistical analysis

All experiments were performed at least 3 times in triplicate, and all results data were presented as the mean ± standard deviation (SD). Statistical analyses were performed by using GraphPad Prism 6.0 software. One-way analysis of variance (ANOVA) and student's *t*-test was used for statistical analysis. A p value of < 0.05 was considered statistically significant [11].

3. Results

Table 1. The combination of possible results for four experiments

Combination of possible results (CR)	Increase concentration of Beclin-1 measured by IHC analysis	Increase concentration of LC3 measured by IHC analysis	Increase concentration of autophagosome measured by autophagy staining assay	Decrease HTR-8/SVneo cell viability measured by cell proliferation assay	Whether support the hypothesis or not
CR1	+	+	+	+	**Yes**
CR2	+	+	-	+	**Partially**
CR3	+	-	+	+	**Partially**
CR4	-	+	+	+	**Partially**
CR5	+	+	+	-	**Partially**
CR6	+	-	-	+	**Partially**
CR7	-	+	-	+	**Partially**
CR8	-	-	+	+	**Partially**
CR9	+	-	+	-	**Partially**
CR10	-	+	+	-	**Partially**
CR11	+	+	-	-	**Partially**
CR12	-	-	-	+	**No**
CR13	+	-	-	-	**Partially**
CR14	-	+	-	-	**Partially**
CR15	-	-	+	-	**No**
CR16	-	-	-	-	**No**

The '+' sign means that the phenomenon in each column heading (decrease, increase, increase, increase) is observed in the experiments and is statistically significant compared to the positive controls. The '-' sign means that the phenomenon (decrease, increase, increase, increase) is not observed in the experiments, which may be due to the observation contradicting the hypothesis or being not statistically significant compared to the negative controls.

3.1. Combination of possible results 1 (CR1)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. And both the concentrations of Beclin-1 and LC3 measured by IHC analysis have increased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result entirely supports the hypothesis.

3.2. Combination of possible results 2 (CR2)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. And both the concentrations of Beclin-1 and LC3 measured by IHC analysis have increased. But the concentration of autophagosome measured by autophagy attaining assay has decreased. This result partially supports the hypothesis.

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3.3. Combination of possible results 3 (CR3)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. But only the concentration of Beclin-1 measured by IHC analysis has increased, while the concentration of LC3 measured by the same assay has decreased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result partially supports the hypothesis.

3.4. Combination of possible results 4 (CR4)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. But only the concentration of LC3 measured by IHC analysis has increased, while the concentration of Beclin-1 measured by the same assay has decreased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result partially supports the hypothesis.

3.5. Combination of possible results 5 (CR5)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. Both the concentrations of Beclin-1 and LC3 measured by IHC analysis have increased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result partially supports the hypothesis.

3.6. Combination of possible results 6 (CR6)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. But only the concentration of Beclin-1 measured by IHC analysis has increased, while the concentration of LC3 measured by the same assay has decreased. Meanwhile, the concentration of autophagosome measured by autophagy attaining assay has decreased. This result partially supports the hypothesis.

3.7. Combination of possible results 7 (CR7)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. But only the concentration of LC3 measured by IHC analysis has increased, while the concentration of Beclin-1 measured by the same assay has decreased. Meanwhile, the concentration of autophagosome measured by autophagy attaining assay has decreased. This result partially supports the hypothesis.

3.8. Combination of possible results 8 (CR8)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. But both the concentrations of Beclin-1 and LC3 measured by IHC analysis have decreased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result partially supports the hypothesis.

3.9. Combination of possible results 9 (CR9)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. And only the concentration of Beclin-1 measured by IHC analysis has increased, while the concentration of LC3 measured by the same assay has decreased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result partially supports the hypothesis.

3.10. Combination of possible results 10 (CR10)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. And only the concentration of LC3 measured by IHC analysis has increased, while the concentration of Beclin-1 measured by the same assay has decreased. The concentration of autophagosome measured by autophagy attaining assay has increased. This result partially supports the hypothesis.

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3.11. Combination of possible results 11 (CR11)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. Both the concentrations of Beclin-1 and LC3 measured by IHC analysis have increased. But the concentration of autophagosome measured by autophagy attaining assay has decreased. This result partially supports this.

3.12. Combination of possible results 12 (CR12)

The HTR-8/SVneo cell viability measured by cell proliferation assay has decreased. However, both the concentrations of Beclin-1 and LC3 measured by IHC analysis have decreased. Meanwhile, the concentration of autophagosome measured by autophagy attaining assay has decreased. This result entirely rejects the hypothesis.

3.13. Combination of possible results 13 (CR13)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. And only the concentration of Beclin-1 measured by IHC analysis has increased, while the concentration of LC3 measured by the same assay has decreased. Meanwhile, the concentration of autophagosome measured by autophagy attaining assay has decreased. This result partially supports the hypothesis.

3.14. Combination of possible results 14 (CR14)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. And only the concentration of LC3 measured by IHC analysis has increased, while the concentration of Beclin-1 measured by the same assay has decreased. Meanwhile, the concentration of autophagosome measured by autophagy attaining assay has decreased. This result partially supports the hypothesis.

3.15. Combination of possible results 15 (CR15)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. And both the concentrations of Beclin-1 and LC3 measured by IHC analysis have decreased. But the concentration of autophagosome measured by autophagy attaining assay has increased. This result entirely rejects the hypothesis.

3.16. Combination of possible results 16 (CR16)

The HTR-8/SVneo cell viability measured by cell proliferation assay has increased inversely. And both the concentrations of Beclin-1 and LC3 measured by IHC analysis have decreased. Meanwhile, the concentration of autophagosome measured by autophagy attaining assay has decreased. This result entirely rejects the hypothesis.

3.17. Possible results for the variables of concentrations and treatment durations

The concentrations of α-solanine are in a positive correlation with the concentrations of Beclin-1, LC3 and autophagosome, but in a negative correlation with the cell viability.

The concentrations of α-solanine are in no correlation with the concentrations of Beclin-1, LC3, autophagosome and cell viability.

The concentrations of α-solanine are in a negative correlation with the concentrations of Beclin-1, LC3, and autophagosome but in a positive correlation with the cell viability.

The treatment durations of α-solanine are in a positive correlation with the concentrations of Beclin-1, LC3 and autophagosome, but in a negative correlation with the cell viability.

The treatment durations of α-solanine are in no correlation with the concentrations of Beclin-1, LC3, autophagosome and cell viability.

The treatment durations of α-solanine are negatively correlated with the concentrations of Beclin-1, LC3, and autophagosome but positively correlate with cell viability.

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4. Discussion

The experimental results from the concentrations of Beclin-1, LC3 and autophagosome with the changed concentrations and treatment durations of α-solanine present a complex and nuanced understanding and elucidation of the role of α-solanine in trophoblast cell autophagy and placenta tissue poisoning. However, it is still effortless to divide them into 8 groups that support or reject the distinguished logical chains of the hypothesis (Figure 1). The hypothesis can be split into 5 logical chains. The first chain is the increasing concentration of α-solanine increases the concentration of Beclin-1. The second is the increasing concentration of α-solanine increases the concentration of LC3.The third is the increasing concentration of Beclin-1 promotes autophagy and increases the concentration of autophagosome. The fourth is the increasing concentration of LC3 promotes autophagy and increases the concentration of autophagosome. And the fifth is the promotion of autophagy and increasing concentration of autophagosome decreases the viability of the HTR-8/SVneo cells.

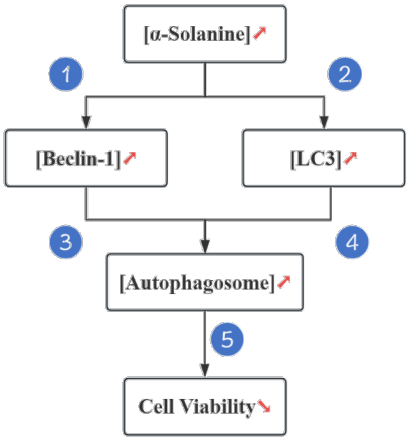

Figure 1. The logical chain of the hypothesis

Table 2. The combination of possible results whether support or reject each chain

Combination of possible results (CR)	First chain Increasing concentration of α-solanine increases the concentration of Beclin-1	Second chain Increasing concentration of α-solanine increases the concentration of LC3	Third chain Increasing concentration of Beclin-1 promotes autophagy and increases the concentration of autophagosome	Fourth chain Increasing concentration of LC3 promotes autophagy and increases the concentration of autophagosome	Fifth chain Promotion of autophagy and increasing concentration of autophagosome decreases the viability of HTR-8/SVneo cell
CR1	✓	✓	✓	✓	✓
CR2	✓	✓	×	×	×
CR3	✓	×	✓	×	✓
CR4	×	✓	×	✓	✓
CR5	✓	✓	✓	✓	×
CR6	✓	×	×	×	×
CR7	×	✓	×	×	×

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Table 2. (continued).

CR8	×	×	×	×	✓
CR9	✓	×	✓	×	×
CR10	×	✓	×	✓	×
CR11	✓	✓	×	×	×
CR12	×	×	×	×	×
CR13	✓	×	×	×	×
CR14	×	✓	×	×	×
CR15	×	×	×	×	×
CR16	×	×	×	×	×

The '✓' sign means that the combination of possible results supports the logical chain in each column heading compared to the positive controls. The '×' sign means that the combination of possible result rejects the logical chain in each column heading compared to the negative controls.

4.1. Group A (CR1)

The outcome of CR1 perfectly follows all logical chains of the hypothesis, so it entirely supports the hypothesis. It could nearly be the expected mechanism. Further research will concentrate more on the other proteins in the autophagy pathway.

4.2. Group B (CR12, CR15 and CR16)

The outcomes of CR12, CR15 and CR16 do not follow any logical chains of the hypothesis, so they entirely reject the hypothesis.

The probable reason for CR12 is that the changed concentration of α-solanine is independent of the cell autophagy signaling pathway, such that the concentrations of Beclin-1, LC3, and autophagosome have not changed yet. However, the changed concentration of α-solanine impacts the other pathway, which eventually decreases cell viability, such as cell apoptosis. Further research will concentrate more on the apoptosis pathway.

The probable reason for CR15 is that the changed concentration of α-solanine is independent of the synthesis of Beclin-1 and LC3, but impacts the synthesis of other proteins which are significant on the cell autophagy signaling pathway such that it promotes cell autophagy and increases the concentration of autophagosome. However, the autophagic cell has not died yet, or other pathways have been activated, which eventually increases cell viability. Further research will concentrate more on the other proteins and pathways.

The probable reason for CR16 is the changed concentration of α-solanine is independent of the entire process. It is possibly nontoxic.

4.3. Group C (CR2 and CR11)

The outcomes of CR2 and CR11 follow the same two logical chains of the hypothesis, so they partially support the hypothesis.

The probable reason for CR2 and CR11 is that the changed concentration of α-solanine could impact the synthesis of other proteins which are significant to the cell autophagy signaling pathway, such that they resist the influence of the increasing concentrations of Beclin-1 and LC3. However, the difference is in CR2, the changed concentration of α-solanine impacts the other pathways, which eventually decreases cell viability, such as cell apoptosis, but in CR11, the autophagic cell has not died yet, or other

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pathways have been activated, which eventually increases cell viability. Further research will concentrate more on the other proteins and other pathways.

4.4. Group D (CR5 and CR8)

The outcomes of CR5 and CR8 follow the complementary logical chains of the hypothesis, so they partially support the hypothesis.

The probable reason for CR5 is that the autophagic cell has not died yet or other pathways have been activated which eventually increases cell viability. Further research will concentrate more on the other processes of autophagy and other pathways.

The probable reason for CR8 is that the changed concentration of α-solanine is independent of the synthesis of Beclin-1 and LC3, but impacts the synthesis of other proteins which are significant to the cell autophagy signaling pathway such that it promotes cell autophagy and increases the concentration of autophagosome. Further research will concentrate more on the other proteins.

4.5. Group E (CR3 and CR4)

The outcomes of CR3 and CR4 follow the same three logical chains of the hypothesis, so they partially support the hypothesis.

The probable reason for CR3 and CR4 is that the changed concentration of α-solanine is independent of the synthesis of Beclin-1 or LC3, but impacts the synthesis of other proteins which are significant on the cell autophagy signaling pathway such that it promotes cell autophagy and increases the concentration of autophagosome. The difference between them is in CR3, α-solanine is independent of LC3, but in CR4, α-solanine is independent of Beclin-1. Further research will concentrate more on the other proteins.

4.6. Group F (CR6 and CR7)

The outcomes of CR6 and CR7 follow the same three logical chains of the hypothesis, so they partially support the hypothesis.

The probable reason for CR6 and CR7 is that the changed concentration of α-solanine is independent of the synthesis of Beclin-1 or LC3 but impacts the synthesis of other proteins which are significant to the cell autophagy signaling pathway such that it resists the influence of the increasing concentrations of Beclin-1 and LC3. However, the changed concentration of α-solanine impacts the other pathway, which eventually decreases cell viability, such as cell apoptosis. The difference between them is in CR6, α-solanine is independent of LC3, but in CR7, α-solanine is independent of Beclin-1. Further research will concentrate more on the other proteins and other pathways.

4.7. Group G (CR9 and CR10)

The outcomes of CR9 and CR10 follow the same two logical chains of hypothesis, so they partially support the hypothesis.

The probable reason for CR9 and CR10 is that the changed concentration of α-solanine is independent of the synthesis of Beclin-1 or LC3, but impacts the synthesis of other proteins which are significant to the cell autophagy signaling pathway such that it promotes cell autophagy and increases the concentration of autophagosome. However, the autophagic cell has not died yet, or other pathways have been activated, which eventually increases cell viability. The difference between them is in CR9, α-solanine is independent of LC3, but in CR10, α-solanine is independent of Beclin-1. Further research will concentrate more on the other proteins and other pathways.

4.8. Group H (CR13 and CR14)

The outcomes of CR13 and CR14 follow the same logical chain of hypothesis, so they partially support the hypothesis.

The probable reason for CR13 and CR14 is that the changed concentration of α-solanine is independent of the synthesis of Beclin-1 or LC3 but impacts the synthesis of other proteins which are

DOI: 10.54254/2753-8818/76/2024.19979

significant to the cell autophagy signaling pathway such that it resists the influence of the increasing concentrations of Beclin-1 and LC3. However, the autophagic cell has not died yet, or other pathways have been activated, which eventually increases cell viability. The difference between them is in CR13, α-solanine is independent of LC3, but in CR14, α-solanine is independent of Beclin-1. Further research will concentrate more on the other proteins and other pathways.

4.9. Discussion of the possible results for the variables of concentrations and treatment durations

The possible results are that three of the factors are positively correlated, and one is negatively correlated due to the increasing concentrations, which can increase the synthesis of proteins Beclin-1 and LC3 and promote autophagy, which increases autophagosome. The higher the concentrations, the higher the yields. Therefore, the cell viability decreases.

The possible result that four of the factors are not correlated due to the increasing concentrations are independent of increasing the synthesis of proteins Beclin-1 and LC3, promoting autophagy such that the concentration of autophagosome does not change and decreasing the cell viability.

The possible results are that three of the factors are negatively correlated, and one is positively correlated due to the increasing concentrations, which can decrease the synthesis of proteins Beclin-1 and LC3 and inhibit autophagy, which decreases autophagosome. The higher the concentrations, the lower the yields. Therefore, the cell viability increases.

The possible results are that three of the factors are positively correlated, and one is negatively correlated due to the increasing treatment durations, which can increase the synthesis of proteins Beclin-1 and LC3 and promote autophagy, which increases autophagosome. The higher the treatment durations, the higher the yields. Therefore, the cell viability decreases.

The possible result that four of the factors are not correlated due to the increasing treatment durations is independent of increasing the synthesis of proteins Beclin-1 and LC3, promoting autophagy such that the concentration of autophagosome does not change and decreasing the cell viability decreases.

The possible results are that three of the factors are negatively correlated, and one is positively correlated due to the increasing treatment durations, which can decrease the synthesis of proteins Beclin-1 and LC3 and inhibit autophagy, which decreases autophagosome. The higher the treatment durations, the lower the yields. Therefore, the cell viability increases.

5. Conclusion

This study investigates the varied concentrations of Beclin-1, LC3 and autophagosome with the changed concentrations and treatment durations of α-solanine with HTR-8/SVneo cell. The results and discussion have demonstrated whether the proposed mechanism is involved. Further experimental validation and in-depth mechanistic studies are warranted to ascertain the precise role of α-solanine and its regulatory impact on increasing the concentrations of Beclin-1 and LC3. This will offer valuable insights into the cognition that excessive consumption of α-solanine may poison the reproductive system, especially the trophoblast on the placenta. However, further experiments are required to verify this conclusion.

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