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Research article

Monosodium glutamate induces *tsc1* gene expression in fission yeast

Merve Yilmazer*[1], Damla Kale[1]

[1] *Istanbul University, Faculty of Science, Department of Molecular Biology and Genetics, 34134, Istanbul, Türkiye*

Abstract

Tuberous sclerosis complex (TSC) is a disease of cellular migration and proliferation that produces hamartomas (benign tumors or malignant cancers affecting the brain and skin) and also involves the eyes, lungs, kidneys, and heart in patterns that can vary throughout life. TSC is an autosomal dominantly inherited disease. Alterations in the TSC1 and TSC2 proteins that form the TSC complex are among the factors that cause the emergence of this disease. TSC1 and TSC2 proteins are the suppressors on the mTOR signaling pathway. The health risks of monosodium glutamate, the most commonly used food additive today, are still a controversial issue. However, there are studies revealing that monosodium glutamate has a negative effect on cell proliferation. In the present study, parental and *tsc1*Δ mutant fission yeast cells were used and the effects of monosodium glutamate on *tsc1* gene expression, cell proliferation, and apoptosis were investigated. It was observed that 8 mg/mL monosodium glutamate caused an increase in the expression of the *tsc1* gene. It was concluded that monosodium glutamate may disrupt cell homeostasis and affect cell division and apoptosis processes via the mTOR pathway, depending on the increase in the expression of the *tsc1* gene.

Keywords: *Apoptosis; cell growth; fission yeast; monosodium glutamate; tuberous sclerosis complex*

1. Introduction

A rare autosomal dominantly inherited disease, Tuberous Sclerosis, also known as Tuberous Sclerosis Complex (TSC), is characterized by the development of hamartomatous tumors in various organs such as the brain, heart, skin, eye, kidney, lung, and liver (Curatolo et al., 2022). The main clinical findings of this disease are epileptic seizure, neurodevelopmental delay, kidney tumors, skin tumors, and tumors in the heart and brain. It has been reported that epilepsy is the most common symptom of TSC (Nabbout et al., 2019). TSC occurs due to abnormal cellular differentiation, proliferation, and cell migration processes (Holmes et al., 2007; Kilic, 2021). *TSC1* and *TSC2* genes encode hamartin and tuberin proteins respectively and play a role in the emergence of tuberous sclerosis complex. The *TSC1* and *TSC2* genes are responsible for the regulation of the rapamycin (mTOR) pathway, and the hamartin-tuberin complex represses the mTOR pathway, which controls cell growth and proliferation. Hamartin protein also functions as a tumor suppressor (Slegtenhorst et al., 1997). By interacting with other proteins, Hamartin regulates many cellular activities such as cell division, growth, autophagy, apoptosis, and angiogenesis, which are necessary for the proper functioning of cellular processes (Adhikari et al., 2010; Mallela and Kumar, 2021). Loss-of-function mutations of the *TSC1* and *TSC2* genes cause hyperactivation of the mTOR pathway, which leads to cellular and molecular consequences such as oxidative stress, network imbalance, and inflammation (Curatolo et al., 2024).

Signals such as hypoxia, nutrient availability and growth factors initiate the mTOR pathway and enable the activation of many processes such as transcription and translation control, cell cycle progression, and nutrient uptake (Fingar and Blenis, 2004; Vadysirisack and Ellisen, 2012; Fonseca et al., 2014). In eukaryotic cells, numerous cellular activities such as cell cycle differentiation, progression, and metabolism in response to changing environmental conditions are mediated by protein phosphorylation, and these processes are evolutionarily conserved. Two different multiprotein complexes, TORC1 and

* Corresponding author.
E-mail address: merve.yilmazer@istanbul.edu.tr (M. Yilmazer).
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TORC2, are involved in the mTOR pathway. mTORC1 activity is extremely sensitive to changes in cell growth conditions. The TSC1-TSC2 complex transduces signals from various cellular pathways to regulate mTORC1 activity appropriately. Thus, this complex plays a role as a sensor and regulator of growth conditions (Huang and Mannig, 2008; Nakashima and Tamanoi, 2010).

Numerous researches have been conducted using various animal models, including zebrafish, mice, rats, and non-human primates, in order to examine the disease's natural occurrence, evaluate the effectiveness of potential treatments, and look into the molecular mechanisms underlying TSC (Moavero et al., 2022; Aronica et al., 2023). Although significant progress has been made in understanding the molecular mechanisms in these models, there are also studies using human induced pluripotent stem cells (iPSCs) to investigate the effects of *TSC1* and *TSC2* mutations on human neurodevelopment (Niu et al., 2024). *Schizosaccharomyces pombe*, fission yeast, is frequently used as a model organism for eukaryotic cell studies (Zhao and Lieberman, 1995; Vyas et al., 2021) and is an important model, especially in cell cycle studies (Lee and Nurse, 1988; Hoffman et al., 2015). *S. pombe* has many orthologous genes conserved in vertebrates and the proteins that are the products of these genes (Hoffman et al., 2015). The *TSC1* gene encoding the TSC1 protein in Tuberous Sclerosis Complex (TSC) in human and the *tsc1* gene in *S. pombe* are orthologous genes (Wood et al., 2012). In eukaryotes, the TSC/Rheb/TORC1/S6K/S6 signaling pathway has a crucial role in the regulation of protein synthesis and growth and this pathway is conserved from human to yeast. Rheb, a small G-protein involved in the TORC1 pathway, is found a functional homologous in fission yeast. As in mammals, the TSC1-TSC2 complex and Rhb1 are precursors of the TORC1 pathway in fission yeast (Nakashima and Tamanoi, 2010).

Monosodium glutamate (MSG) is one of the amino acids found abundantly in nature. It is found in food as a flavor enhancer and is used as a food additive (E621) (Kazmi et al., 2017). In addition to ready-to-eat products, fertilizers used for organic agricultural products also contain MSG (Singh et al., 2011; Awang et al., 2020). Clinical trials on human and animal subjects have also revealed several potential health hazards of MSG. Muscular, gastrointestinal, circulatory, neurological, and cardiac disorders are some common samples (Kazmi et al., 2017). It has been shown that MSG can cause genotoxic effects in rats and lead to increased oxidative stress (Farombi and Onyema, 2006). Glutamate is an important stimulant neurotransmitter in the central nervous system, but excess use can lead to excitotoxicity (Kazmi et al., 2017). Excessive activation of the glutamate pathway and excessive influx of calcium ions into neurons are suggested as the biochemical mechanisms behind epileptic seizures. Furthermore, the overactivation of neurons has also been associated with other diseases such as Multiple Sclerosis (MS), Parkinson's, Huntington's, and Alzheimer's Disease. MSG, the most commonly used food additive, increases the level of free glutamate in the brain, putting people at risk of developing these diseases (Singh and Panda, 2024). Studies to understand the possible hepatotoxic, neurotoxic, and genotoxic effects of MSG are limited. Further studies are needed to investigate the molecular and metabolic mechanisms associated with MSG.

This study aimed to investigate the molecular effect of monosodium glutamate on hamartin, which is located in the TSC complex and plays a role in cell growth, in fission yeast. In monosodium glutamate-treated cells (parental and *tsc1Δ* mutant strains), cell division and apoptosis were examined at the molecular level. Additionally, alterations caused by MSG in the expression level of the *tsc1* gene were investigated.

2. Materials and methods

2.1. Yeast strain and culture conditions

S. pombe parental ED666 (*h+/ade6-M210, ura4-D18, leu1-32*) and mutant *tsc1Δ* (*h+/ade6-M210, ura4-D18, leu1-32, SPAC22F3.13::KanMX4*) were obtained from Bioneer Corporation (version 5.0). Deletion of the *tsc1* gene in the *tsc1Δ* strain was confirmed by qPCR. *S. pombe* strains were grown in rich media (YEL; glucose 1%, yeast extract 0.5%) at 30°C. YEL contains the determined amount of MSG in the experimental group.

2.2. Determination of monosodium glutamate concentration and spot assay

To determine the extent of the resistance, serial 10-fold dilutions of the parental and *tsc1*Δ mutant strains which grow in liquid-rich media without MSG or containing an increasing concentration of MSG (0.1-10 mg mL^{-1}) were spotted onto YEA plates (YEL with 2% agar). The plates were incubated for 3 days at 30°C. MSG concentrations were determined by comparing them with their untreated control of strains.

2.3. Growth of the cells

A standard growth curve analysis protocol was followed to determine the growth of the parental and tsc1Δ mutant strain in YEL media without or with monosodium glutamate (8 mg mL^{-1}). The increase in cell growth was measured in a spectrophotometer (EON, Biotek Instruments Inc.) every 2 hours for 40 hours at 600 nm wavelength optical density. Time-dependent graphs of cell growth was obtained according to the standard curve (Petersen and Russell, 2016).

2.4. Gene expression analysis

Firstly, total RNA isolation was performed from parental and *tsc1*Δ mutant cells grown without MSG and containing 8 mg of MSG in rich-media by using "PureLink® RNA Mini Kit (Ambion®by Life Technologies)" according to the manufacturer's instructions. Some modifications were made to

Table 1
Primer sequences used in the study.

Gene	Forward Primer	Reverse Primer
act1	TGCTCAATCTTCCTCCCTTG	CAAAGCTGAGGGTTGGAAAA
tsc1	GTGATGAGCAAGAAAGAG	CTTAGCCTCGTAAACAAC
tor1	GAAGCGTGTCTCAAATAAG	ACTACACCATCCTACATAAC
atg14	TCACCCTAGTTTACTCTCAACA	CGGCAAATGTCCATAAAAACTC

use kit and the cells were mechanically homogenized in PBS by using glass beads. Then, isolated RNAs were converted to cDNA by "Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit". Expressions of *tsc1* (hamartin), *tor1* (TORC2 serine/threonine protein kinase Tor1), *act1* (actin) and *atg14* (autophagy associated protein Atg14) genes were analyzed by using the "Thermo Scientific Applied Biosystems PowerUp SYBR™ Green PCR Master Mix". *Act1* gene was used as the reference gene. The primers were designed in the "IDT Primer Quest Tool" program (Table 1). "Roche Light Cycler 480" was used for real-time PCR.

2.5. Imaging of apoptosis with fluorescence microscopy

Dual staining with ethidium bromide and acridine orange was used to examine apoptosis under a fluorescence microscope. The AO/EtBr dual staining experiment was carried out using Agus and colleagues' (2018) modified protocol (Agus et al., 2018). After washing the cells with PBS, 5 μL of AO/EtBr solution (60 µg mL^{-1} AO and 100 µg mL^{-1} EtBr dissolved in PBS) was added. Following a 5-minute incubation period at room temperature, the cells were cleaned with PBS and viewed using an Olympus BX53 fluorescent microscope. All cells were seen as green with AO at λex=500 nm and λem=530 nm, whereas apoptotic cells were seen as orange-red with EtBr at λex=510 nm and λem=595 nm. Cell counting was performed using the "Image J" program.

2.6. Statistical analysis

The qPCR analysis results were evaluated according to the "Pfaffl" method (Pfaffl 2001). Statistical analyses were performed in GraphPad Prism 9 Software with Two-way ANOVA. The graph of apoptosis analysis was obtained by the GraphPad Prism 9 Software according to the results from "Image J" program.

3. Results

3.1. Spot assay and MSG concentration

Cells were cultured in the rich media containing different concentrations of MSG (0,1-10 mg mL^{-1}) for 24 hours. Serial 10-fold dilutions of cells cultured in rich media (YEL) without MSG or increasing concentration of MSG were spotted onto YEA plates. Based on the growth results, 8 mg mL^{-1} concentrations of MSG were selected for the study (Fig. 1).

3.2. MSG reduced cell growth

Cells of parental and *tsc1Δ* mutant strain cultured in YEL media without or with monosodium glutamate (8 mg mL^{-1}) were followed for 40 hours to detect their time-dependent growth. According to the results of the time-dependent growth curve (Fig. 2), MSG caused a decrease and slowdown in cell proliferation.

3.3. Gene expression analysis

The expressions of *atg14*, *tor1*, and *tsc1* genes in parental and *tsc1*Δ mutant cells were comparatively analyzed. When 8 mg/mL MSG was treated to parental cells, a 1.2-fold decrease in the expression of the *atg14* gene was observed, while a 4.3-fold,

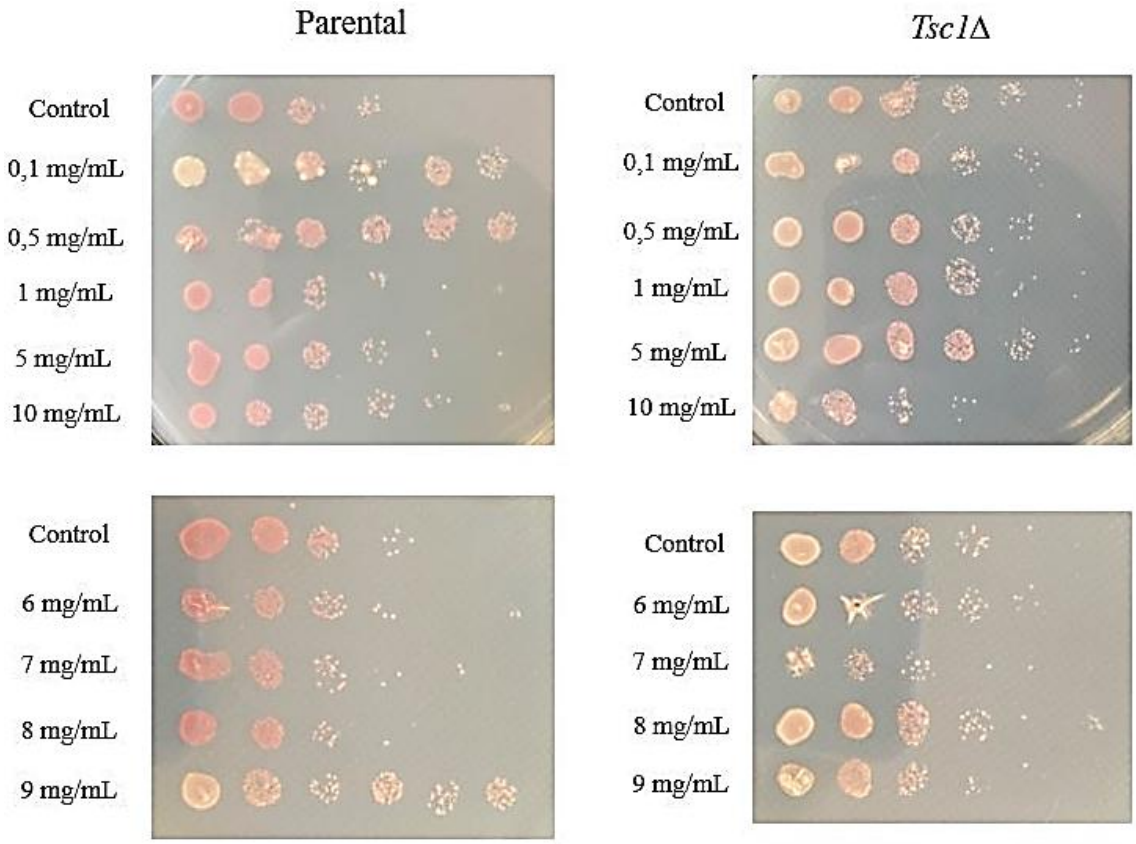
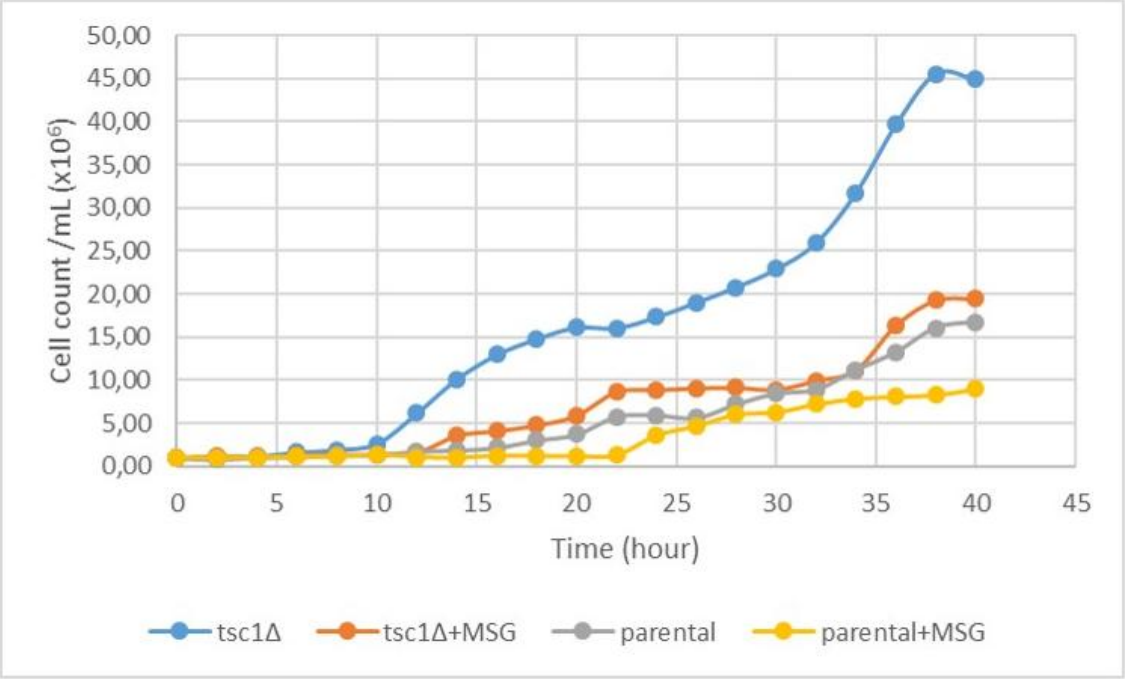


Fig. 1. Spot analyses in the rich media of parental and *tsc1*Δ mutant cells grown in a rich media containing MSG-free and different concentrations of MSG for 24 hours.



Cells	0 h	40 h
*tsc1*Δ	1 x 10^6 cell/mL	44,87 x 10^6 cell/mL
*tsc1*Δ +MSG		19,45 x 10^6 cell/mL
parental		16,74 x 10^6 cell/mL
parental+MSG		9 x 10^6 cell/mL

Fig. 2. Time-dependent growth of the parental and *tsc1Δ* mutant strain in YEL media without or with 8mg/mL monosodium glutamate for 40 hours.

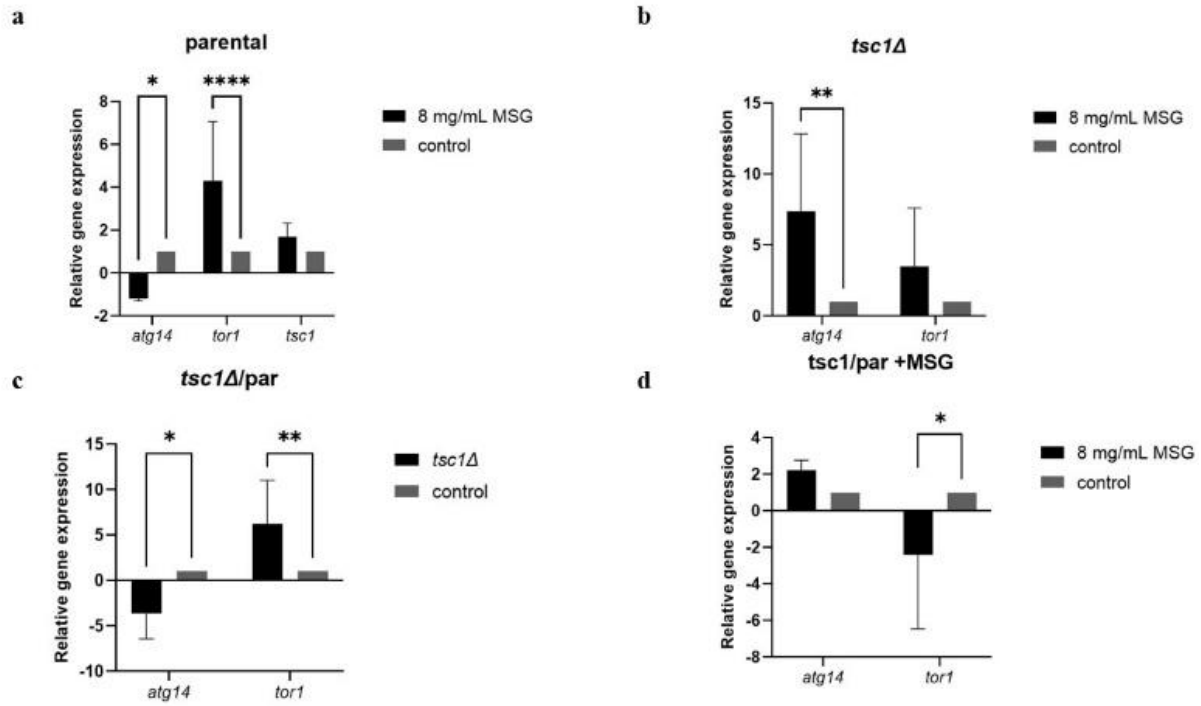


Fig. 3. Relative expression analysis of the *atg14*, *tor1*, and *tsc1* genes in parental and *tsc1*Δ mutant strains treated MSG or not. (a. in parental cells grown in media with or without MSG, b. in *tsc1*Δ mutant cells grown in media with or without MSG, c. *tsc1*Δ mutant cells versus parental cells grown in media without MSG, d. *tsc1*Δ mutant cells versus parental cells grown in media with MSG) Data were analysed using two-way ANOVA (*P= 0.0128, 0.0166, 0.0219; **P=0.0096, 0.0074; ****P<0.0001). The data presented were derived from three independent experiments.

and 1.7-fold increase in the expression of *tor1*, and *tsc1* genes was observed, respectively (Fig. 3a).

After treatment of 8 mg/mL MSG to *tsc1*Δ mutant cells, the expression levels of *atg14* and *tor1* genes increased 7.4 and 3.5 times, respectively (Fig. 3b).

In the absence of MSG, when the gene expression level of *tsc1*Δ cells was compared with the parental cells, the expression of the *atg14* gene was reduced by 3.6-fold, while the expression level of the *tor1* gene increased 6.2-fold. (Fig. 3c).

When MSG treated *tsc1*Δ mutant cells were compared with MSG treated parental cells at the gene expression level, a 2.2-fold increase in the expression of the *atg14* gene was observed. Additionally, the expression of the *tor1* gene decreased 2.4-fold (Fig. 3d). The expression of the *tsc1* gene was also examined in *tsc1*Δ cells for confirmation, and gene expression did not occur as expected.

3.4. Apoptosis under the fluorescence microscope

Apoptosis was detected by ethidium bromide/acridine orange dual staining. After cells were stained with AO/EtBr, they were captured under the fluorescence microscope. While all cells were observed in green, apoptotic cells were in orange due to the uptake of EtBr. The experiment was repeated 3 times and many photos were taken. The examples of pictures for each experimental group are included in Fig. 4. The percentage of apoptosis was calculated by rating the number of red cells to the number of green cells. Cell counting was performed by the "Image J" program and 16 different (8 for AO +8 for EtBr) images for each cell type (total of 64 images) were screened. Only one of these images for each group was given as an example in Figure 5. It was found that apoptosis rates were 23.28%, 29.54%, 10.86%, and 15.24% in parental, MSG-treated parental, tsc1D, and MSG-treated tsc1D cells, respectively (Fig. 5).

4. Discussion

Tuberous sclerosis complex (TSC) is a multisystemic genetic disorder that results in benign tumors in various organs as a result of excessive activation of the mTOR pathway. The TSC protein complex consists of *TSC1/2* gene products, which are crucial for the PI3K/AKT/mTOR (PAM) signaling pathway. Mutations in the *TSC1* and *TSC2* genes are the primary cause of TSC, an autosomal dominant disorder. (Islam, 2021; Fu et al., 2024).

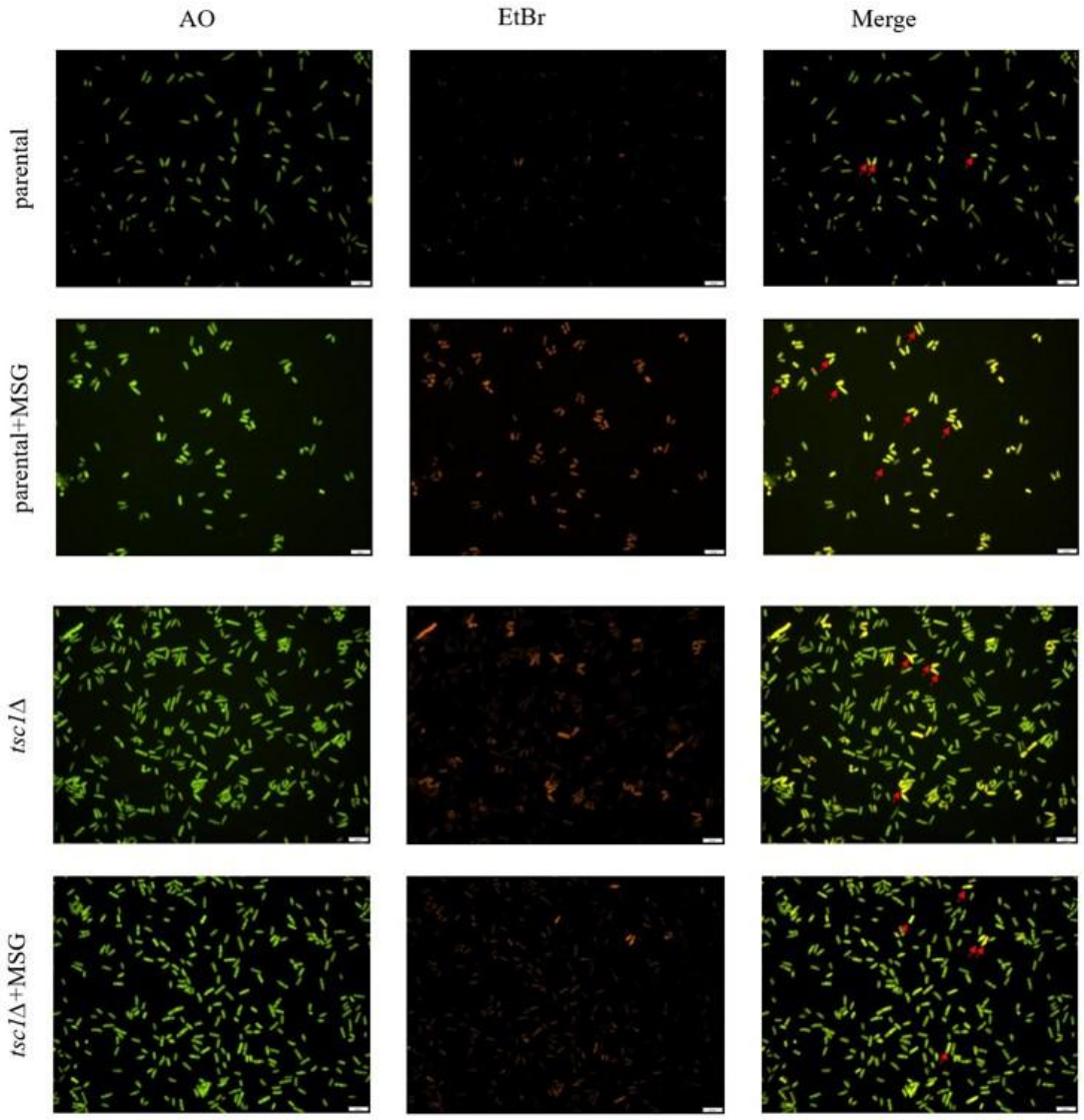


Fig. 4. After cells were stained with AO/EtBr to evaluate apoptosis, they were captured under the fluorescence microscope. (Red arrows indicate examples of apoptotic cells).

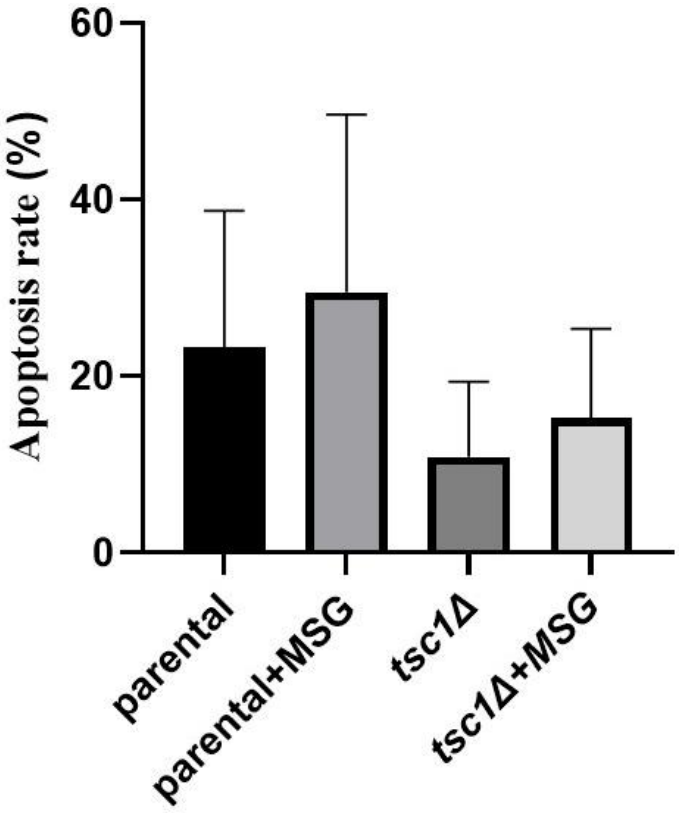


Fig. 5. The apoptosis rate of cells under the fluorescence microscope.

The TSC1 (hamartin) and TSC2 (tuberin) proteins form mTORC1, a TSC protein complex that functions as a tumor suppressor. A key component of the mTORC1 signaling pathway, the TSC complex integrates extracellular signals. The TSC complex suppresses tumor growth by adversely regulating the mTORC1 complex's activity (Rehbein et al., 2021). Pathogenic variants in genes encoding proteins of the mTORC1 signaling pathway lead to mTORopathies, which involve hyperactivity of this pathway. (Kim and Lee, 2019; Crino, 2020). Inactivation of either gene results in overactivation of mTOR signaling (Islam, 2021).

As part of a heterodimeric complex with tuberous sclerosis complex 2 (Tsc2), tuberous sclerosis complex 1 (Tsc1) controls several important processes including autophagy, cell survival and proliferation, protein synthesis, and lipid synthesis (Kim and Guan, 2019). Deletion of Tsc1 led to a pro-apoptotic phenotype in natural killer cells (Yang et al., 2016).

The PI3K/AKT/mTOR (PAM) signaling pathway is highly conserved in eukaryotic cells. As a result of TSC1/TSC2 mutation in TSC pathogenesis, the TSC protein complex is inactive, mTOR inhibition is lost, and the cell cycle and cell growth are disrupted (Mizuguchi et al., 2021). The cell cycle is disrupted by decreased inhibition of the mTOR pathway and this situation leads to abnormal cell proliferation and migration, which results in symptoms of TSC (Northrup et al., 2021; Fu et al., 2024). mTORC1 controls cell growth by controlling protein, lipid, and nucleotide synthesis, while also inhibiting autophagy (Ballesteros-Álvarez and Andersen, 2021). mTOR inhibition increases the activity of the autophagy-lysosomal pathway, which results in the degradation of damaged macromolecules and organelles (Johnson et al., 2015). In the present study, the *tsc1*Δ mutant cells showed more proliferation compared to other cells in both growth curve and spot analysis. These findings support that *tsc1* gene deletion causes an increase in cell proliferation. At the level of gene expression, compared to the parental cells, *tor1* gene expression is higher in the *tsc1*Δ mutant cells, while *atg14* gene expression is lower. As expected, in the absence of hamartin, the product of the *tsc1* gene, which is a repressor of the mTOR complex, inhibition on mTOR1 was removed and an increase in the expression of the *tor1* gene was observed. Depending on this signaling pathway, a decrease in the expression of the autophagy-related *atg14* gene was observed, similar to the findings of Johnson et al. (Johnson et al., 2015).

Monosodium glutamate (MSG) is a globally used food additive found in many commercially processed foods. MSG use has increased significantly over the last 30 years. However, studies have identified MSG consumption as a significant contributing factor to the development and progression of syndromes such as hypertension, diabetes mellitus, cancer, and obesity. Additionally, Alzheimer's disease, brain damage, depression, addiction, anxiety, epilepsy, Parkinson's disease, and stroke are pathological disorders that can occur due to the neurotoxic effects of MSG. MSG has both positive and negative effects, depending on the amount consumed. Low doses of MSG have the potential to increase energy balance and homeostasis, while excessive consumption may cause genotoxic and cytotoxic effects that lead to metabolic disorders (Kesherwani et al., 2024). Despite the controversy over the risks of MSG, its global consumption is still very high (Kayode et al., 2023).

Monosodium glutamate causes genotoxic effects *in vitro* and *in vivo* through both direct and indirect mechanisms. MSG can directly cause chromosomal aberrations, clumping, and stickiness of chromosomes. It indirectly causes oxidative stress in cells. Reactive free radicals cause functional and structural defects in genes. Molecular mechanisms such as changes in the levels of p53, TNF-α, gadd45, NF-kB, Bcl-2, and Bax have been associated with the genotoxic effects of MSG (Imam, 2019).

Oxidative stress due to reactive oxygen species causes apoptosis. Apoptosis is the normal physiological response of the cell to aging and cell damage and can be triggered by various factors such as oxidative stress (Li et al., 2021). High levels of Bax and low levels of Bcl-2 were seen in the kidneys and livers of MSG-treated rats, indicating significant induction of apoptosis (Kassab et al., 2022). It has been reported that MSG treatment induces apoptosis by causing downregulation of Bcl-2 protein in the thymus glands (Rezzani et al., 2003).

Many studies have supported the findings that MSG causes cell death (González-Burgos et al., 2001; Akataobi, 2020). MSG dose-dependently decreased thymocyte proliferation and increased cytotoxicity (Pavlovic, 2006). A study on human hepatoblastoma cell lines has shown that MSG causes cell damage and death by causing ROS accumulation. Additionally, mRNA upregulation of genes related to apoptosis and autophagy has been reported (Kakade et al., 2024). It has been shown that MSG increased DNA fragmentation and apoptosis in *Chlorella vulgaris* and *Spirulina platensis*. The study reported that MSG caused upregulation of caspase-3 and *Bax* genes and downregulation of *Bcl-2* gene expression. Oxidative stress and hepatic cell damage were triggered (Umbuzeiro et al., 2017).

In our fluorescence microscope examination, when MSG was treated, an increase in the apoptosis rate was observed in both parental and *tsc1*Δ mutant cells compared to the untreated control groups. In addition, it was observed under a fluorescence microscope that the cell density was higher in the *tsc1*Δ mutant cells than in the other cells cultured for the same time and initial concentrations. An increase in the expression of the *tsc1* gene

and *tor1* gene was observed when 8 mg/mL MSG was treated in the parental cells. There was a decrease in the expression of the *atg14* gene associated with autophagy. When MSG was treated to *tsc1*Δ mutant cells, a lesser increase in the *tor1* gene was observed compared to the parental cells, and a significant increase in the expression of the *atg14* gene was observed. The *tsc1* mutation results in the devoid of *tor1* inhibition, and when these cells were treated with MSG, there was a decrease in the increase of the *tor1* gene, independent of the absence of the *tsc1* gene. When MSG-treated *tsc1*Δ mutant cells were compared with MSG-treated parental cells, MSG treatment caused a decrease in the expression of the *tor1* gene in cells lacking the *tsc1* gene, and an increase in the expression of the autophagy-related *atg14* gene was observed, consistent with the flow of the signaling pathway. Despite the negative effects revealed in previous studies, there is no scientific evidence that requires a prohibition on MSG use. However, the ongoing debate about the negative effects of MSG and the lack of complete evidence about its harmlessness indicate that MSG may pose a health risk (Ataseven et al., 2016). Depending on the dosage used, MSG can be both beneficial and harmful. Considering the effects on cell viability, correct dosage adjustment is of great importance. In this study, the proliferation of cells treated with MSG (8 mg mL^{-1}) increased less than in the control group. Additionally, it was seen that MSG caused an increase in the expression of the *tsc1* gene, and an increase in autophagy in conditions where the *tsc1* gene was deleted. The findings obtained in the study suggest that MSG acts via the mTOR signaling pathway. MSG may be disrupting cell homeostasis by affecting the TSC complex.

5. Conclusion

The results of the studies on MSG are controversial. Considering that too much of anything is harmful, it can be concluded that glutamate is a nutritious amino acid and therefore no adverse effects are seen when used up to a certain level (Kazmi et al., 2017). In addition, it should not be ignored that excessive consumption causes serious health problems such as circulatory, muscular, gastrointestinal, cardiac, and neurological disorders. It has also been reported that MSG exhibits mild cytotoxic and DNA damage activity in tumor cells (Merinas-Amo et al., 2024). To understand the variable clinical picture of TSC characterized by tumorigenesis, the roles of TSC1 and TSC2 in the mTOR pathway and the genotype-phenotype correlations and molecular mechanisms of associated genetic variants are still poorly understood and need to be further investigated (Man et al., 2024). The direct effect of MSG on TSC and the mTOR pathway has not been investigated previously. In this regard, this study is the first to investigate the effects of MSG treatment on TSC and mTOR pathways using a yeast model. According to our findings, MSG may disturb cell proliferation via the mTOR signaling pathway by affecting the TSC complex.

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Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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