



## Evaluation of mTOR signaling pathway proteins in rat gastric mucosa exposed to sulfite and ghrelin

Sevim Ercan<sup>1</sup> , Pınar Şahin<sup>2</sup>, Ceren Kencebay<sup>3</sup> , Narin Derin<sup>3</sup> , Çiler Çelik Özenci<sup>2</sup>

<sup>1</sup>Department of Medical Services and Techniques, Akdeniz University Vocational School of Health Services, Antalya, Turkey

<sup>2</sup>Department of Histology and Embryology, Akdeniz University School of Medicine, Antalya, Turkey

<sup>3</sup>Department of Biophysics, Akdeniz University School of Medicine, Antalya, Turkey

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### ABSTRACT

**Background/Aims:** Mammalian target of rapamycin (mTOR) signaling serves as a central regulator of cell growth, proliferation, and survival. In this study, we planned to evaluate the expressions of mTOR signaling constituents (p-p70S6K, p-mTOR, and p-Tuberin) in rat gastric mucosa and to compare the results in sulfite- and sulfite+ghrelin-exposed groups.

**Materials and Methods:** Rats were divided into three groups: the control group (C), the sodium metabisulfite (Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>) (S) group, and sulfite+ghrelin (SG) group. Sodium metabisulfite at 100 mg/kg/day was administered via gavage, and ghrelin at 20 µg/kg/day was administered intraperitoneally for 35 days. We have used immunohistochemistry for mTOR signaling pathway components.

**Results:** There were no significant differences for p-p70S6K and p-mTOR expression among the C, S, and SG groups. Tuberin expression was significantly increased in the S group compared to the C group. Furthermore, tuberin expression was found to be significantly decreased in the SG group.

**Conclusion:** This study is the first one in the literature that shows the expression of mTOR signaling proteins in gastric mucosa of rats exposed to sulfite and ghrelin. Furthermore, it demonstrates that ghrelin treatment reduces p-Tuberin expression induced by ingested sulfite.

**Keywords:** mTOR proteins, sodium metabisulfite, ghrelin, gastric mucosa

### INTRODUCTION

The mammalian target of rapamycin (mTOR) is a 289-kDa serine threonine kinase, which is a member of kinase phosphatidylinositol-3-kinase (PI3K)-related family, and it has been preserved through evolution (1). mTOR is a component of two functionally different complexes, mTORC1 and mTORC2 (2). It phosphorylates p70 ribosomal S6 kinase 1 (p70S6K1) and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), and it regulates protein synthesis. The phosphorylation of 4E-BP1 prevents its binding to eIF4E, enabling eIF4E to initiate cap-dependent translation (3). Certain cellular processes such as tumor angiogenesis and development, adipogenesis, T-lymphocyte activation, and insulin resistance acti-

vate, whereas some diseases like cancer and type-2 diabetes deregulate mTOR pathway (1).

Positive regulators of mTOR activity include human epidermal growth factor receptors (HER) and related ligands, growth factors, and their receptors (i.e., insulin-like growth factor-1 [IGF-1] and its receptor, vascular endothelial growth factor receptors [VEGFRs] and their ligands) (4). On the other hand, its activity is negatively regulated by tuberous sclerosis complex 1 (TSC1; hamartin) and tuberous sclerosis complex 2 (TSC2; tuberin), and phosphatase and tensin homolog (PTEN) (4). *TSC1* and *TSC2* gene mutations have been identified to contribute equally to the TSC heritability (5). Tuberous sclerosis complex (TSC) is

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**Address for Correspondence:** Sevim Ercan E-mail: sevimercan@akdeniz.edu.tr

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characterized by the presence of hamartomas in many organs, such as, skin, brain, lungs, heart, and kidneys, and it is a dominant disorder that occurs in approximately 1/6000 births (6). The main components in the regulation of mTOR are TSC tumor suppressors (7). The TSC1 and TSC2 form a heterodimeric complex that acts as a GTPase-activating protein (GAP) on the small GTPase Ras homolog-enriched in brain (Rheb) and inhibits mTOR signaling (8). In a GTP-bound state, Rheb potently activates mTOR, and TSC1-TSC2 inhibits mTOR indirectly by reverting Rheb to an inactive GDP-bound state (8). Hereditary TSC deficiency leads to an increased development of several tumor types such as kidney tumors (9). Recent genetic studies have placed TSC2 in the phosphatidylinositol-3-kinase (PI3K)-target of rapamycin (TOR) pathway, whereby the TSC1-TSC2 complex functions downstream of Akt and upstream of TOR to restrict cell growth and cell proliferation (10). TSC2 has been placed in the phosphatidylinositol-3-kinase (PI3K)-target of rapamycin (TOR) pathway in recent genetic studies, by which the TSC1-TSC2 complex functions downstream of Akt and upstream of TOR to restrict cell proliferation and cell growth (10).

Sulfites enter the human body when sulfur dioxide ( $\text{SO}_2$ ), a product of industrial emissions, is inhaled, or they can be ingested as food preservatives (11). Detoxication is required in both the endogenous and exogenous sulfite exposure, as sulfite reaction with various cellular and humoral components may result in toxicity (12). Although sulfite toxic and adverse effects are increasingly reported, the Federation of American Societies for Experimental Biology (FASEB) announced that 30 mg-100 mg of  $\text{SO}_2$  does not have any adverse effects on humans (13). Therefore, expressing the amounts of ingested sulfites as sulfur dioxide equivalents (SDE) has become general practice (13). SDE resulting from sodium metabisulfite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) constituted the allowable daily intake of 0-0.7 mg/kg body weight. It should not be forgotten that the recommended daily sulfite intake is 19 mg. The sulfite oral intake at 163 mg/kg SDE in 99% of population has been reported (14). It has also been argued that sulfite and its derivatives cause DNA damage in numerous organs of mice and rats (15).

The biological activity of ghrelin, a novel 28-amino acid acylated peptide, is ensured via O-n-octanoylation at serine 3 (16). Ghrelin was first discovered in the stomach of rats as an endogenous ligand for growth hormone receptors. In addition, small amounts are also found in the pituitary gland, hypothalamus, jejunum, duodenum, colon, ileum, heart, lungs, kidneys, pancreas, and testes (17). In addition to the stimulation of GH release, ghrelin participates in a variety of physiological activities such as cardiovascular function, food intake, and energy balance, appetite, bone growth, and reproduction (18). It has been shown that ghrelin prevents oxidative damage and apoptosis in different tissues

(19-21). It was also proved that ghrelin protects gastric mucosa against gastric injury caused by stress and ischemia-reperfusion and that it improves gastric motility and gastric emptying (22,23). In a previous study, we have shown that ghrelin treatment decreases the amount of apoptotic cells in the gastric mucosa of rats exposed to  $\text{Na}_2\text{S}_2\text{O}_5$  (24).

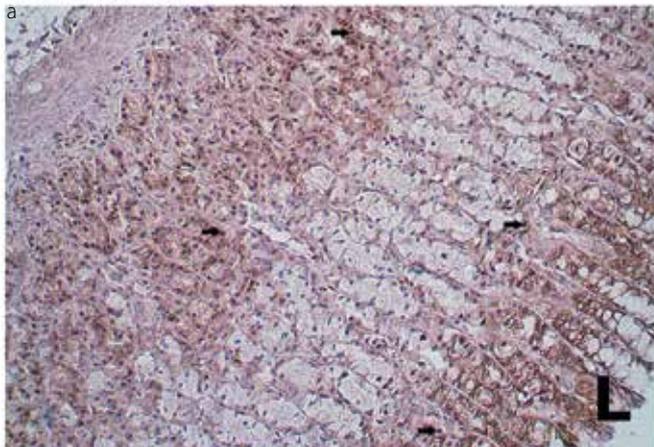
Our aim was to investigate the expressions of mTOR-signaling constituents (p-p70S6K, p-mTOR, and p-Tuberin) in rat gastric mucosa and to compare the results with those from the sulfite- and sulfite+ghrelin-exposed groups.

## MATERIALS AND METHODS

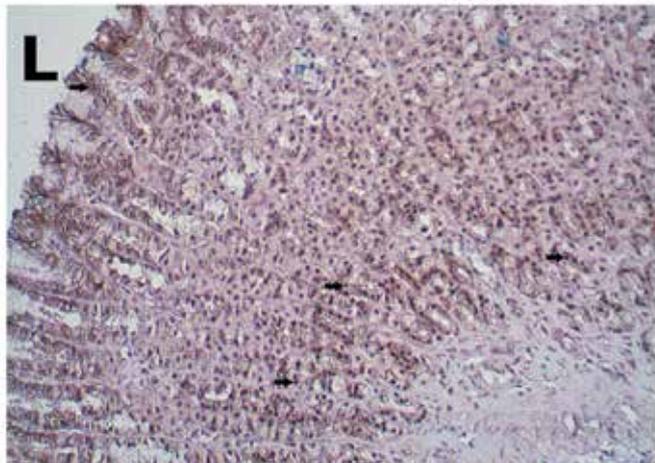
Ethical committee guide for the care and the use of laboratory animals was followed in the study. Male Wistar rats weighing between 250 g and 450 g were used. A temperature of  $23\pm 1^\circ\text{C}$  and a 12-hour light-dark cycle were maintained as the environment. Rats were separated into three groups (n=10 per group): Control (C),  $\text{Na}_2\text{S}_2\text{O}_5$ -treated (S), and  $\text{Na}_2\text{S}_2\text{O}_5$ +ghrelin-treated (SG) groups. Rats in the C group were injected intraperitoneally 1 mL/kg/day saline as vehicle and were given 1 ml/kg/day distilled water through gavage for 35 days. The S group was given  $\text{Na}_2\text{S}_2\text{O}_5$  (100 mg/kg/day) for 5 weeks by gastric gavage (25). Our previous study showed a significant accumulation of S-sulfonate in the S and SG group plasmas, which indicated that the sulfite exposure was successful (24). Rat ghrelin (GenScript, NJ, USA) was dissolved in distilled water and kept at  $-20^\circ\text{C}$  until its application. Ghrelin at 0.1 mg/mL was prepared with 0.9% saline. Ghrelin at 20  $\mu\text{g}/\text{kg}$  had been applied intraperitoneally for 35 days (24).

## Immunohistochemistry

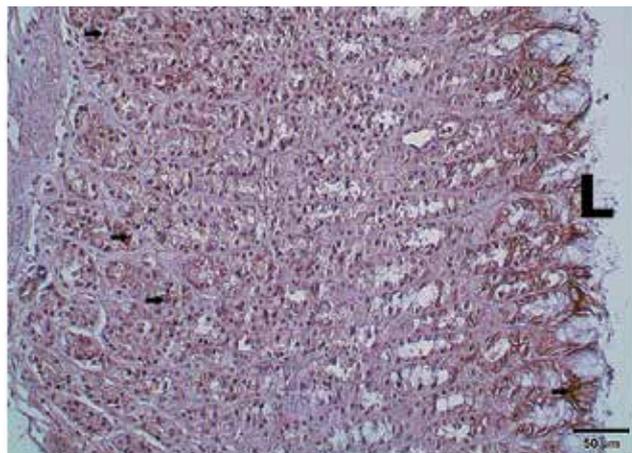
Overnight fixation of stomach tissue samples was performed with 10% neutral buffered formalin. The samples were then embedded in paraffin and cut to 5- $\mu\text{m}$  slices. For immunohistochemical labeling, the 5- $\mu\text{m}$  tissue sections were deparaffinized in xylene and rehydrated through a decreasing gradient of ethanol, and antigen retrieval was performed with citric acid (Ph 6.00). The sections were blocked for endogenous peroxidase activity with methanol containing 3%  $\text{H}_2\text{O}_2$  for 10 min and for nonspecific binding with Ultra V Block (Labvision, Fremont, CA) for 7 min at room temperature (RT). The 1:50 dilution of rabbit polyclonal anti-p-mTOR (Abcam, Ab131538), 1:50 dilution of rabbit monoclonal anti-p-p70S6K (Abcam Ab32359), and 1:100 dilution of rabbit polyclonal anti-p-Tuberin (Abcam Ab59274) primary antibodies were left for overnight incubation in antibody diluent reagent solution (Invitrogen 00-3118). After primary antibody incubations, sections were cleansed in phosphate-buffered saline (PBS). Then, they were incubated with secondary antibody (Biotinylated anti rabbit IgG), (Vector, Burlingame, CA) at 1:500 dilution for 1 hour at RT, and then Vectastain Avidin Biotin Complex (Vector) was applied for 30 min at RT to label the secondary

**CONTROL**

b

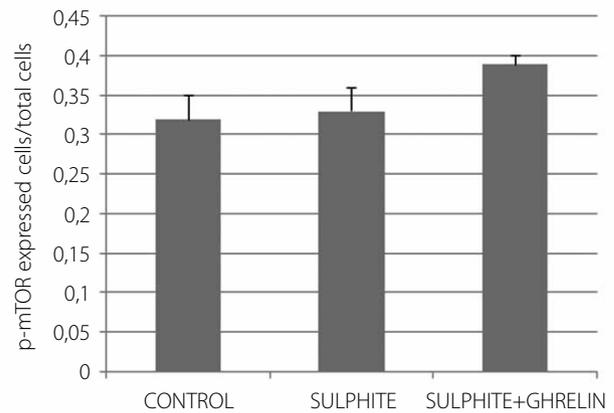
**SULPHITE**

c

**SULPHITE+GHRELIN**

**Figure 1. a-c.** Expression of p-mTOR in the control (a), sulfite (b), and sulfite+ghrelin (c) groups. L: Lumen. p-mTOR was localized in the nuclei and cytoplasm of the gastric mucosal cells. The expression was observed in the surface epithelium and glands in lamina propria. p-mTOR expression was similar in the control, sulfite, and sulfite+ghrelin groups

antibody. Immunohistochemical reactions were visualized with chromogen (diaminobenzidine tetrahydrochloride, DAB) (Bio-Genex, San Ramon, CA). Harris Hematoxylin (Sig-



**Figure 2.** p-mTOR-expressed cells/total cells. There was no significant difference between the groups ( $p < 0.05$ )

ma-Aldrich, St. Louis, MO) was used for counterstaining, and then sections were dehydrated with graded alcohol series and mounted. For each staining, normal rabbit IgG (Vector, I-1000) was used as a negative control. Immunohistochemical reactions were evaluated, and images were taken by Axioplan microscope (Zeiss, Oberkochen, Germany).

**Statistical Analysis**

Results were analyzed with Image J (version 1.47). SigmaStat for Windows, version 3.5 (Jandel Scientific Corp, San Rafael, California) was used for statistical calculations. Statistical significance was determined as  $p < 0.05$ . The differences between the groups were analyzed with one-way analysis of variance (ANOVA) and Holm Sidak test for p-mTOR, p-Tuberin, and p-p70S6K.

**RESULTS****p-mTOR**

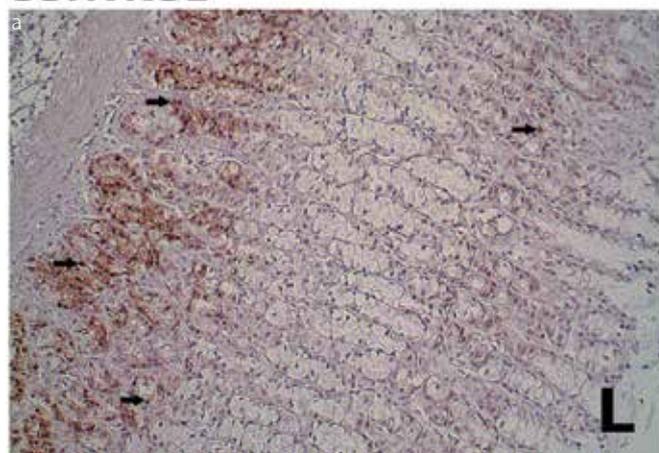
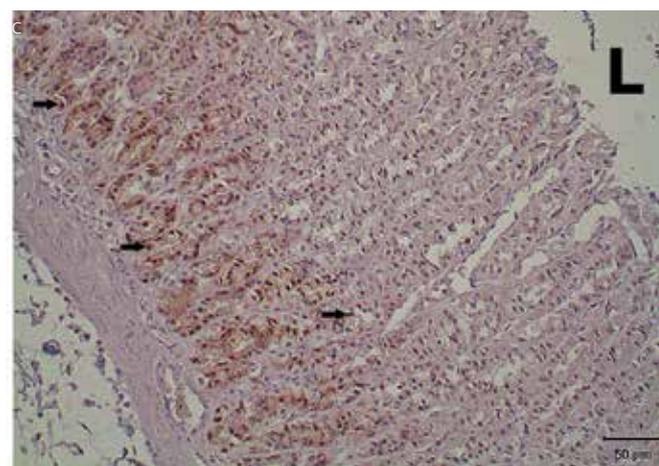
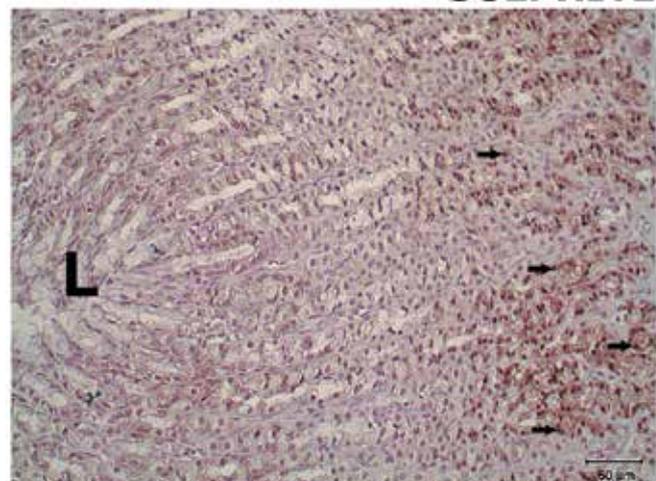
Cytoplasmic and nuclear p-mTOR expression was observed in gastric mucosal cells. The expression of p-mTOR was also observed in glandular cells of lamina propria. p-mTOR expression was similar in all the groups. No significant differences were found for p-mTOR expression between the groups (Figure 1,2).

**p-p70S6K**

p-p70S6K expression was observed in gastric mucosal cells. The expression was nuclear and also cytoplasmic. The expression of p-p70S6K was also present in glandular cells. There were no significant differences for p-p70S6K expression among the groups (Figure 3,4).

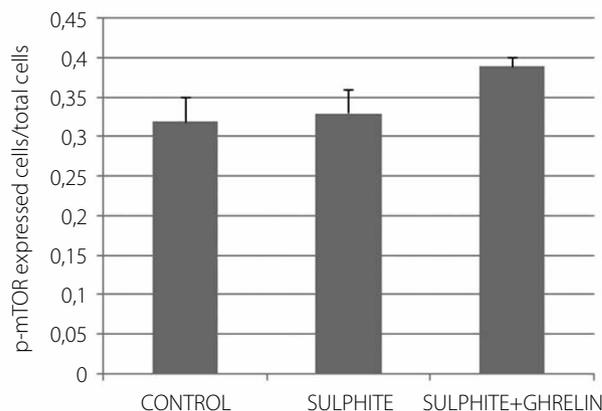
**p-Tuberin**

Nuclear and cytoplasmic p-Tuberin expression was present in gastric mucosal cells. Its expression was also observed in

**CONTROL****SULPHITE****SULPHITE+GHRELIN**

**Figure 3. a-c.** Expression of p-p70S6K in the control (a), sulfite (b), and sulfite+ghrelin (c) groups. L: Lumen. p-p70S6K was localized in the nuclei and cytoplasm of the gastric glandular cells. p-p70S6K expression was similar in the control, sulfite, and sulfite+ghrelin groups

glandular cells of lamina propria. When compared to the C group, p-Tuberin expression was found to be increased in the S group. Furthermore, a significant decrease was found in the SG group (Figure 5,6).



**Figure 4.** p-S6K expressed cells/total cells. There is no significant difference between the groups ( $p < 0.05$ )

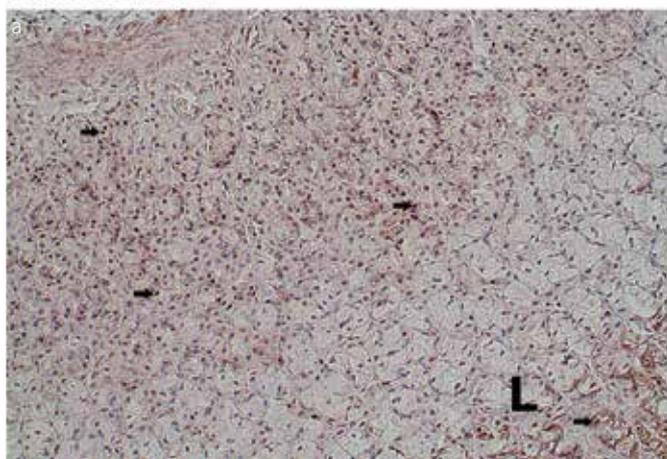
**DISCUSSION**

mTOR pathway regulates cell survival, proliferation, angiogenesis, and apoptosis (26). Velagapudi et al. (27) demonstrated that mTOR pathway is involved in cell death/survival and suggested that mTOR may have a pleiotropic function in cell death regulation.

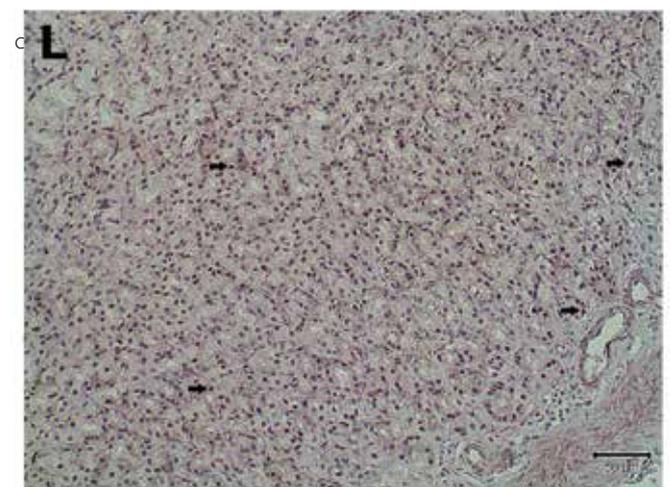
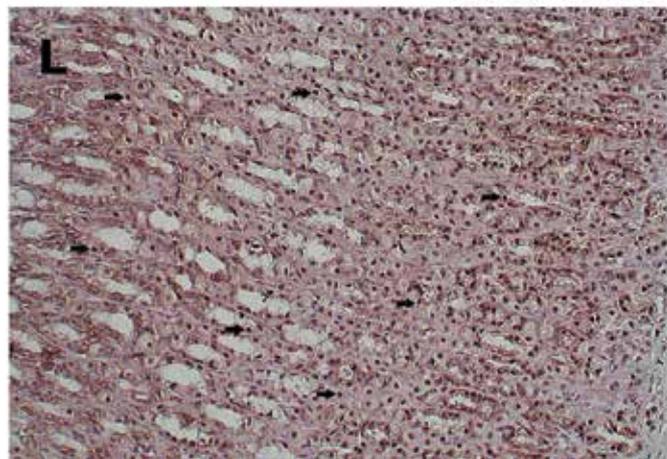
We have previously reported that  $\text{Na}_2\text{S}_2\text{O}_5$  administration markedly increased the amount of apoptotic cells in rat gastric tissue (24). Sulfite dosage was adjusted according to our previous studies, which were designed to investigate the effects of an increased sulfite intake (12,24,25). The ingested sulfite level is usually expressed as  $\text{SO}_2$  equivalents (SDE). Theoretical yield of  $\text{SO}_2$  from  $\text{Na}_2\text{S}_2\text{O}_5$  was reported as 67.39 %, and the dosages were adjusted according to this information (13). As reported by The Joint Expert Committee of the World Health Organization (WHO), 0.7 mg/kg body weight sulfite is acceptable for daily intake (13). On the other hand, it is possible to consume between 180 mg and 200 mg of sulfite in a single day or meal due to its high content in certain foods and beverages (14).

Although Kim et al. (28) demonstrated that an increased gastric epithelial cell migration and wound healing effect of DA6034 treatment may be due to the activation of mTOR-S6K1 pathway, our results demonstrated that there was a statistically non-significant mTOR and p-p70S6K expression in gastric mucosal cells in all the groups. On the other hand, p-Tuberin expression in the S group was significantly increased. Tuberin is a major signaling protein that is intimately involved in protein translation by negative regulation in mTOR pathway. It is a substrate of Akt in human cells and in *Drosophila* (29). Tuberin, a tumor suppressor encoded by TSC-2 gene, inhibits the mTOR signaling pathway when it is activated (27). The inactivation of tuberin through phosphorylation on specific residues or its deficiency activates the mTOR pathway and downstream

**CONTROL**



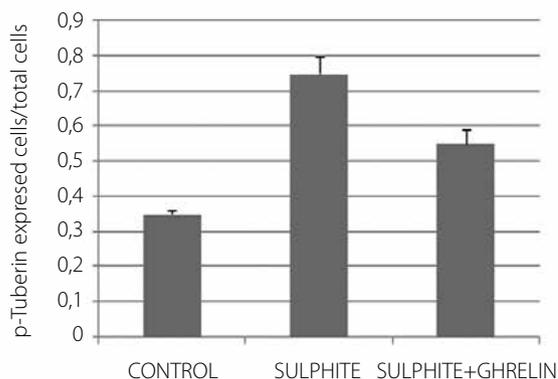
**SULPHITE**



**SULPHITE+GHRELIN**

**Figure 5. a-c.** Expression of p-Tuberin in the control (a), sulfite (b), and sulfite+ghrelin (c) groups. L: Lumen. p-Tuberin was localized in the nuclei and cytoplasm of the gastric mucosal cells. The expression was observed in the surface epithelium and glands in lamina propria

signaling to increase the mRNA translation (30). Additionally, tuberin initiates phosphorylation of Bad on Ser<sup>136</sup> and enhances the relation of BAD/Bcl<sub>2</sub> and BAD/Bcl-X<sub>L</sub> (27). As indicated by Habib et al. (29), deficiency or inactivation



**Figure 6.** p-Tuberin-expressed cells/total cells. p-Tuberin expression was significantly increased in the sulfite group (p<0.05)

of tuberin increases fibronectin expression in the kidney cortex of diabetic rats. It has been shown that tuberin increases tumor cell susceptibility to apoptosis and triggers apoptosis by activating the proapoptotic BAD (27). Although p-Tuberin activation is not a marker of apoptosis, in accordance with our previous findings, the induction of apoptosis by sulfite in gastric cells might be a result of cell proliferation blockage through mTOR suppression by tuberin activation.

Sulfite-induced apoptosis and oxidative stress are inhibited by ghrelin in gastric mucosa of rats, as we have previously reported (24). In addition, ghrelin was reported to stimulate cell proliferation and reduce apoptosis in prepubertal porcine ovarian granulosa and theca interna cells (31). Numerous studies indicated that ghrelin administration reduces caspase 3 levels and TUNEL positivity in spinal cord neurons and endothelial cells, enhances neurogenesis in the hippocampus, and stimulates proliferation, migration, and differentiation of neural progenitor's cells in the subventricular zone and rostral migratory system of interneurons in the olfactory bulb (32-35). There are several studies about the effects of ghrelin on mTOR activity in literature. Martins et al. (36) demonstrated that ghrelin increased food intake by regulating the hypothalamic mTOR pathway, whereas Xu et al. (37) suggested that mTOR signaling mediates the inhibitory effects of ghrelin on GLP-1 production in intestinal L-cells. As demonstrated by Park et al. (38), ghrelin reduces the intestinal apoptosis and increases the crypt cell proliferation in the small intestine. In this study, we demonstrated that ghrelin treatment reduced p-Tuberin expression induced by ingested sulfite. That effect might be due to suppression of mTOR pathway, modulated by sulfite via tuberin activation, by ghrelin, which also supports the increase of cell proliferation by ghrelin administration in our previous study (24). Similarly, a study reported that mTOR/P70S6k signaling pathway mediated ghrelin-induced proliferation of neural stem cells (39).

This study is the first one in literature that shows the expression of mTOR signaling proteins in the gastric mucosa of rats exposed to sulfite and ghrelin. Furthermore, the study shows that the expression of gastric mucosa to either sulfite or sulfite+ghrelin changes tuberin expression, which is a negative regulator for mTOR signaling pathway. Further research is necessary to confirm the role of mTOR pathway in the gastric mucosa.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the Ethics Committee of Akdeniz University School of Medicine (Decision Date: 24.08.2009/Decision Number: 10-42).

**Informed Consent:** N/A.

**Peer-review:** Externally peer-reviewed.

**Author contributions:** Concept - S.E.; Design - S.E, N.D.; Supervision - Ç.Ö.; Fundings - S.E.; Materials - P.Ş.; Data Collection and/or Processing - C.K.; Analysis and/or Interpretation - S.E.; Literature review - N.D.; Writer - S.E.; Critical Review- Ç.Ö.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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