Original Article

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# Investigation of Ghrelin Levels in Antimuscarinic Induced Convulsions in Fasted Animals After Food Intake

## Aç Hayvanlara Antimuskarinik Uygulanması ve Yem Verilmesi ile Oluşan Konvulsiyonlarda Ghrelin Seviyelerinin Araştırılması

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### ABSTRACT

**Objective:** Atropine reduces ghrelin secretion and ghrelin inhibits epileptic seizures. It is interesting that atropine treated fasting animals develop clonic convulsions soon after food intake. Present study was designed to investigate relationship between these antimuscarinic induced convulsions and ghrelin levels.

**Methods:** Balb/C mice were fasted for 24 hours, then treated with saline or scopolamine (3 mg/kg, i.p.) and then given food 20 minutes later. All animals were observed for 30 minutes for the incidence and development of convulsions. Then ghrelin levels were measured in blood and brain tissue.

**Results:** Scopolamine treatment caused convulsions in fasted animals after food intake. In saline treated fasted animals, plasma ghrelin concentration was significantly higher than saline treated fed animals. Plasma and tissue ghrelin concentrations were found significantly lower in animals with convulsion than in fasted animals which were given food after saline injection.

**Conclusion:** It was shown that ghrelin levels were reduced in the group with convulsions due to scopolamine administration and food intake. Therefore, it is suggested that ghrelin may have possible role on these convulsions.

**Keywords:** Ghrelin, scopolamine, convulsion, mouse, fasting

### ÖZ

**Amaç:** Atropin ghrelin salınımını azaltmakta, ghrelin ise epileptik nöbetleri baskılamaktadır. Aç hayvanlara atropin uygulanması ve ardından yem verilmesi ile klonik konvülsiyonlar oluştuğunu bildiğimizden bu bilgi dikkat çekicidir. Bu nedenle çalışmamızda, antimuskarinikle indüklenen bu konvülsiyonlar ile ghrelin seviyelerinin ilişkisinin araştırılması amaçlanmıştır.

**Yöntemler:** Balb/C fareler 24 saat aç bırakıldı, bu süre sonunda serum fizyolojik veya skopolamin (3 mg/kg, i.p.) uygulandı ve 20 dakika sonra yem verildi. Konvülsiyon oluşumu ve sıklığını belirleyebilmek için tüm hayvanlar 30 dakika süreyle izlendi. Alınan plazma ve beyin dokularında ghrelin seviyeleri ölçüldü.

**Bulgular:** Skopolamin uygulanan aç farelerde yem yedikten sonra konvulsiyon oluştuğu izlendi. Serum fizyolojik uygulanan aç hayvanlardaki plazma ghrelin seviyesinin, toklara kıyasla anlamlı derecede yüksek olduğu saptandı. Konvülsiyon geçiren hayvanlardaki plazma ve doku ghrelin seviyelerinin, açlık sonrasında serum fizyolojik uygulanan ve ardından yem verilen hayvanlara göre anlamlı derecede düşük olduğu belirlendi.

**Sonuç:** Konvülsiyon geçiren farelerdeki ghrelin düzeylerinin, skopolamin uygulanması ve yem verilmesi sonucunda azaldığı görülmektedir. Çalışmamızdaki bulgular, ghrelinin bu konvülsiyonların oluşumunda rolü olabileceğini düşündürmektedir.

**Anahtar Sözcükler:** Ghrelin, skopolamin, konvülsiyon, fare, açlık

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## Introduction

In previous studies, it was shown that fasted animals treated with antimuscarinic drugs (atropine, scopolamine or biperiden) developed convulsions soon after eating (1-3). It was observed that animals developed convulsions after food deprivation for 2, 3, 12, 18 and 24 h. and it was suggested that stress due to food deprivation, rather than its duration might contribute to the underlying mechanisms of convulsions (4). Prevention of hypoglycemia by glucose intake during fasting had no preventive effect on convulsion development. So, it is suggested that food deprivation itself, but not its hypoglycemic consequence, may have critical role in the development of convulsions (2). Also, it could be argued that convulsions were triggered by oral movements, because convulsions did not occur with fluid or slurry food (5).

[$^3$H]glutamate binding kinetics significantly changed after fasting for 48 h and this change was partly antagonized by scopolamine treatment and eating (6). Typical epileptiform discharges were shown in cortical electroencephalography recordings in animals with convulsion (7). Tizanidine, clonidine, chlorpromazine, haloperidol and MK-801 (noncompetitive N-methyl-D-aspartate antagonist) provided effective treatments in these convulsions (1,3,6). However, most of the major antiepileptics drugs (AEDs) and new AEDs were ineffective to suppress convulsions (2,8).

The manifestations of the seizures and the triggering factors of convulsions in antimuscarinic treated fasted animals beared some similarities with patients who had eating-evoked epilepsy (9). So, these convulsions in fasting animals may help to explain the mechanisms of this rare form of reflex epilepsy.

Ghrelin, an orexigenic peptide, regulates appetite and meal initiation. While its level increased during hunger and it sent signals to brain to eat (10), exogenous ghrelin administration caused body weight gain and induced food intake (11-13). Ghrelin secretion was under cholinergic control and a muscarinic receptor antagonist, atropine abolished its secretion (14).

It was shown that serum ghrelin levels were increased in epileptic patients (15). Exogeneous ghrelin administration inhibited development of seizures, reduced severity of seizures, and neuronal cell loss in hippocampus (16-18). Antiepileptic effects of ghrelin were shown in animals with pentylenetetrazole-(PTZ)-induced epilepsy (16). In another study, it was determined that ghrelin concentration was reduced in animals with PTZ-induced seizures (19).

Seizure development, fasting and cholinergic system all interact with each other in scopolamine induced convulsions in fasting mice after food intake. Also, based on recent data, ghrelin secretion was under cholinergic control and it was associated with convulsions and fasting state. There was no study in the literature that investigated the relationship between these mechanisms. So, we aimed to investigate the ghrelin levels in plasma and brain tissue for understanding relationship between mechanisms in these convulsions.

## Methods

The present study was approved by the Istanbul University Local Ethics Committee on Animal Experiments (63/29.04.2010). All studies were in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

### Drugs

Scopolamine hydrobromide (Sigma, St Louis, MO) 3 mg/kg (1-4) was dissolved in saline and injected intraperitoneally (i.p.).

### Procedure

In this study, male Balb/C mice, weighing between 25-30 g were used. Animals were housed under standard laboratory conditions until experimentation. After weighing, mice were divided into two main groups as fed and fasted mice. Fasted animals were deprived of food for 24 hours. During the fasting period, animals could access to water.

Half of fed animals were injected 4 mL/kg saline (fed+sal group) and the other half 3 mg/kg scopolamine i.p. (fed+scop group).

Fasted mice were reweighed and injected saline or scopolamine i.p. and placed in wire mesh cages individually. Twenty minutes later, half of fasted animals were given 2 grams of food pellets and allowed to eat (fasted + sal + food group and fasted + scop + food group). The other half of fasted animals were not given food (fasted + sal group and fasted + scop group). All mice were observed for determining the incidence and onset of convulsions for 30 minutes after feeding. At the end of observing period, all animals were decapitated and tissue and blood samples were collected.

Stages of seizure activity were scaled as; no difference (stage 0); freezing and gustatory movements (stage 1); forelimb clonus (stage 2); forelimb clonus with rearing (stage 3); forelimb clonus with rearing and/or falling down (stage 4); generalized convulsions with rearing, falling down and jumping (stage 5).

Stage 3, 4 and 5 were assessed as a convulsive response. Onset of convulsions was defined as the time between re-feeding and the stage 3 activity. The incidence of convulsions was expressed as the percentage of animals displaying either stage 3, 4 or 5 activity.

Experiments were carried out in a temperature-controlled (21±2 ºC) room, between 08:00 and 22:00. Observers were blind to the groups.

### Plasma and Tissue Ghrelin Levels

Blood samples of animals were collected in plastic ethylenediaminetetraacetic acid (EDTA) tubes soon after decapitation and for tissue processing, all brains were removed on ice.

Tissues were homogenized and supernatants were collected for procedure. Total ghrelin levels were determined in supernatants and plasma, by an enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc. EK-031-31, Burlingame, USA) according to manufacturer's guidelines. Absorbance

data were collected by using a microplate reader (ELX-800 spectrophotometer, Vertmont, USA).

### Statistical Analysis

Body weight loss was evaluated by using paired samples t-test. The data of time to onset of convulsions were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test. For the evaluation of the convulsion incidence, we used Fisher's exact test. Ghrelin levels in tissue and in plasma were investigated with One-way ANOVA followed by Tukey's test. Spearman correlation analyze was applied to investigate relationship between convulsion stages and ghrelin levels. A p-value less than 0.05 was considered statistically significant. All values were presented as mean ± SEM.

## Results

### Convulsions

The body weights of the animals fell to 91.3% of their initial body weights after food deprivation for 24 h. Only scopolamine treated fasted mice showed convulsions after food intake with an incidence of 83.3%. When compared with the saline-treated control group, the difference was statistically significant ($p<0.05$). The time to onset of convulsions was found 6.3±1.7 min.

### Plasma Ghrelin Levels

Total plasma ghrelin levels are shown in Figure 1.

Total plasma ghrelin level was significantly higher in 24-hour fasted+sal group (5.6±0.5 nmol/mL) than fed+sal (2.9±0.3 nmol/mL) group ($p<0.05$). Ghrelin level in fasted+sal+food group (7.0±0.9 nmol/mL) was significantly higher than fed+sal group ($p<0.01$), and fasted+scop+food (3.8±0.9 nmol/mL) group ($p<0.05$).

### Tissue Ghrelin Levels

Total tissue ghrelin levels are shown in Figure 2.

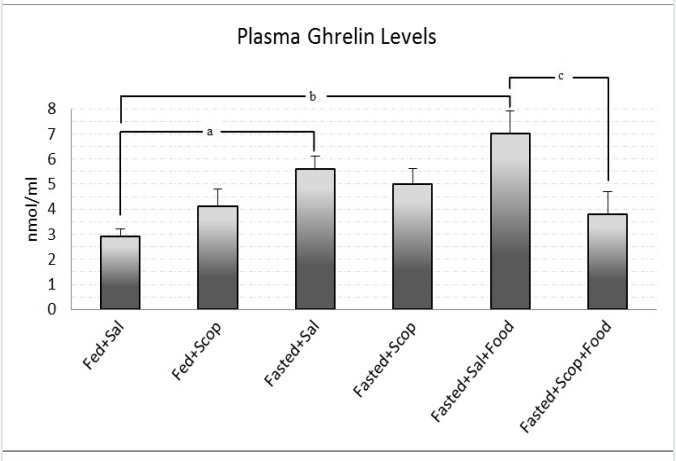


**Figure 1.** Total plasma ghrelin levels in all groups. Bars are presented as mean ± SEM

[a]$p<0.05$, [b]$p<0.01$ when compared to fed+saline group.
[c]$p<0.05$, when compared to fasted+scopolamine+food group.

Total ghrelin level in brain tissue was higher in fed + scop (1.5±0.6 nmol/mL) group than fed + sal (1.4±0.3 nmol/mL) group. Ghrelin level in fasted+scop (0.8±0.1 nmol/mL) group was lower than fasted + sal (1.8±0.4 nmol/mL) group. Animals in fasted + sal + food (3.8±0.6 nmol/mL) group had significantly increased tissue ghrelin levels when compared to fasted + sal group ($p<0.05$) and fed + sal, fasted + scop + food (0.9±0.1 nmol/mL) groups ($p<0.01$).

### Convulsion Stage and Ghrelin Levels

Fasted mice developed convulsions after scopolamine treatment and food intake. Stage and time to onset of convulsions and ghrelin levels are shown in Table 1.

When we investigated the relationship between stage of convulsions and plasma and tissue ghrelin levels; weak positive correlation was found (Spearman's rho=0.29, $p<0.58$).

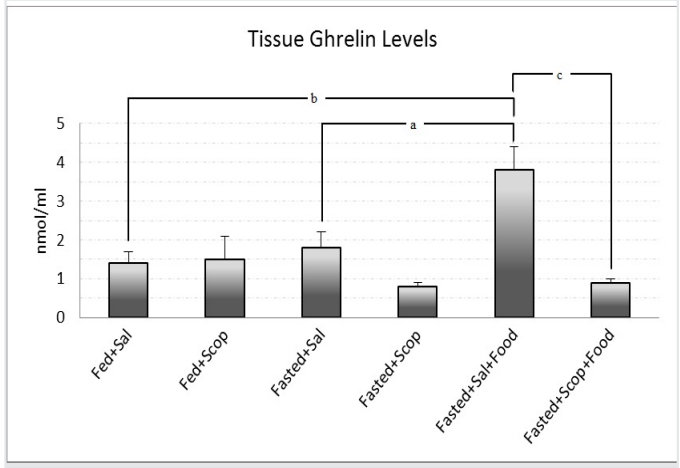


**Figure 2.** Total tissue ghrelin levels in all groups. Bars are presented as mean ± SEM

[a]$p<0.05$ when compared to fasted+saline group.
[b]$p<0.01$ when compared to fed+saline group.
[c]$p<0.01$ when compared to fasted+scopolamine+food group.

**Table 1.** Stage and onset of convulsions in each animal in fasted + scopolamine + food group. Also, the plasma and tissue ghrelin levels of each mouse are presented below

| Animals | Plasma ghrelin levels (nmol/mL) | Tissue ghrelin levels (nmol/mL) | Stage of convulsion | Time to onset of convulsion (minute) |
|---|---|---|---|---|
| 1 | 3.4 | 0.8 | 5 | 11 |
| 2 | 4.1 | 0.7 | 3 | 3 |
| 3 | 6.9 | 0.7 | 2 | 4 |
| 4 | 1.3 | 1.5 | 2.5 | 5 |
| 5 | 2.1 | 0.8 | 4 | 4 |
| 6 | 5.7 | 0.8 | 4 | 12 |

## Discussion

In this study, we investigated the relationship between ghrelin levels and scopolamine-induced convulsions for the first time. In accordance with previous studies (1-3), scopolamine induced convulsions in 24-hour fasted mice (83.3%) after food intake. Recent studies showed that, animals had convulsions (50%) after fasting for 2 hours. So, it can be assumed that development of convulsions are not related to duration of fasting and thus, ghrelin concentration. However, the stress due to food deprivation and eating solid food can be related to ghrelin levels. Because, mRNA expression of plasma ghrelin increased significantly following acute tail pinch stress (20). Plasma ghrelin concentrations also increase in cases of calorie restriction, prolonged energy deficit and reduced feed intake (such as anorexia). Then, higher ghrelin levels in fasted groups due to stress of food deprivation may be expected . Considering that ghrelin concentration falls significantly after atropine administration to fasted animals, scopolamine as another antimuscarinic drug, may be expected to reduce ghrelin concentration in fasted animals. As we expected, ghrelin levels in plasma and tissue were higher in saline treated fasted animals, whereas they were lower in scopolamine treated fasted animals. But, this difference was not statistically significant.

Elevation of ghrelin levels with exogenous ghrelin administration, shortened time to onset of seizures and increased the incidence of seizures (16,18). It could be argued that, ghrelin did not show its protective effect against convulsions in fasted + scop + food group, because of low ghrelin levels in this group. In another study, it was shown that plasma ghrelin levels were decreased in rats with pentylenetetrazol induced seizures (21). Similar to this finding, plasma ghrelin levels of animals with convulsion reduced in our study.

Ghrelin plasma concentration elevated in fasting, and decreased to normal levels after meals (11,22). In our study, ghrelin levels in 24-hour fasted animals significantly elevated when compared to controls. Interestingly, plasma ghrelin levels in scopolamine-treated fed animals elevated too. It was known that, ghrelin secretion was modulated by cholinergic, dopaminergic and adrenergic systems (14,23). Previous studies showed that, ghrelin secretion increased with erythromycin, which was a cholinergic prokinetic agent (23) and decreased acutely after vagotomy (14). Also, there were studies showing changes in both muscarinic (24), glutamatergic (1) and GABAergic receptors (25) in different brain regions, after 24-hour or longer fasting.

It was known that dopamine secretion increased after re-feeding (26), dopamine inhibited acetylcholine secretion presynaptically (27) and acetylcholine increased ghrelin secretion (23). Also, it was shown that atropine, an antimuscarinic drug as scopolamine, reduced the plasma ghrelin levels in fasted animals (14). We found similar results in our study: Scopolamine-treated fasted animals had lower ghrelin levels than saline-treated fasted animals. It could be speculated that ghrelin secretion reduced in scopolamine-treated fasted animals after food intake for two reasons; firstly, increased dopamine suppressed acetylcholine and secondly, an antimuscarinic drug was administered. It is known that the convulsions in scopolamine-treated re-fed animals are not affected by the administration of physostigmine (1), but suppressed by administration of clonidine and tizanidine. In light of these findings, the effects of fasting, re-feeding and cholinergic system interactions on ghrelin secretion and convulsion development should be elucidated.

The ghrelin concentration declines to normal values, after 1 hour of eating (11,21). When we compared the fasted + sal group and fasted + sal + food group, we observed that ghrelin level increased after feeding. This finding was interesting, because ghrelin level after meal was expected to decrease. When 24-hour fasted animals were allowed to eat, they did not start to eat immediately and ate little amount of pellets. Considering this finding, it could be argued that there was no satiety in animals and therefore ghrelin concentration did not decrease. Also we could argue that, blood and tissue samples were taken 30 minutes after eating and ghrelin levels had not returned to normal values yet. These suggestions could explain why ghrelin level was found high in fasted + sal + food group.

Ghrelin suppressed apoptosis in hypothalamic neurons in the absence of oxygen and glucose, and reduced toxicity caused by kainic acid (28). In ischemic conditions, ghrelin was found to protect cortical neurons against cell death (29); while in other tissues it also had protective effect by reducing ischemia/reperfusion injury (30). Also, it was recently shown that oxidative stress markers were reduced by ghrelin administration in PTZ-induced seizures in rats, and seizures were reduced in accordance with high ghrelin levels (31). Considering all these findings, it may be thought that ghrelin suppresses the seizures by reducing degeneration. In a different study, it was shown that the plasma level of ghrelin decreased soon after PTZ-induced seizures in rats (19). Similar to this finding, fasted + scop + food group, in which all mice had convulsions, had significantly decreased tissue and plasma ghrelin levels compared with the fasted + sal + food group.

According to the findings of our unpublished studies, c-fos expression was suppressed in scopolamine-treated fasted animals. When evaluating this finding together with plasma and tissue ghrelin levels in this study, it seemed that decrease in ghrelin levels might be parallel with suppressed c-fos expression. The increase in ghrelin levels may be thought to contribute to the convulsions by increasing neuronal activity.

In this study, the relationship between ghrelin levels and antimuscarinic induced convulsions in fasted mice after food intake was investigated for the first time. As expected, plasma concentration of ghrelin was found elevated in fasted animals compared to fed animals. Tissue and plasma ghrelin concentrations were found to be low because scopolamine administration suppressed ghrelin secretion. Since ghrelin was shown to be protective in development of seizures; it could be argued that the protective effect of ghrelin was not observed in this study due to the low ghrelin level in animals with convulsion.

## Conclusion

Present study suggested for the first time that ghrelin could have a possible role in antimuscarinic induced convulsions. Since there are many mechanisms effective on development of convulsions and regulation of fasting, further studies are needed to fully explain the mechanism of antimuscarinic induced convulsions in fasted mice after food intake.

### Ethics

**Ethics Committee Approval:** The present study was approved by the Istanbul University Local Ethics Committee on Animal Experiments (63/29.04.2010).

**Informed Consent:** Is animal work.

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

### Authorship Contributions

Concept: A.Z.T., A.N., Design: A.Z.T., A.N., Data Collection or Processing: A.Z.T., A.N., Analysis or Interpretation: A.Z.T., A.N., Literature Search: A.Z.T., A.N., Writing: A.Z.T., A.N.

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

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