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# Investigation of Inhibition Effect of Oxytocin on Carbonic Anhydrase and Acetylcholinesterase Enzymes in the Heart Tissues of Rats

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**ABSTRACT:** In our study, it was aimed to investigate the effects of oxytocin, oxytocin receptor antagonist atosiban and oxytocin-atosiban injected to rats on carbonic anhydrase (CA) and acetylcholinesterase (AChE) enzyme activities in the heart of rats. For this purpose, four different groups (n = 6) consisting of a total of 24 rats, were formed. (Control group, oxytocin treated group, atosiban treated group, and oxytocin and atosiban combination treated group). The rats were necropsied after 60 minute intraperitoneal injection of the chemicals into the rats. Cardiac tissue of rats was removed. The enzyme activity averages of the groups are statistically compared. According to this, while the activity of carbonic anhydrase enzyme in heart tissue of rats decreases in oxytocin and atosiban given groups, in the combination group with oxytocin and atosiban it was seen that the reduction was relatively less according to the others. Acetylcholinesterase activity decreases in both groups of oxytocin and atosiban which given groups did not show any significant change in the group in which oxytocin and atosiban were given together.

**Keywords:** enzyme activity, carbonic anhydrase, oxytocin, rat

# Sıçanların Kalp Dokusunda Oksitosin'in Karbonik Anhidraz ve Asetilkolinesteraz Enzimleri Üzerine İnhibisyon Etkisinin Araştırılması

**ÖZET:** Çalışmamızda, sıçanlara enjekte edilen oksitosin, oksitosin reseptör antagonisti atosiban ve oksitosin-atosiban hormonlarının, sıçanların kalp dokusunda karbonik anhidraz (CA) ve asetilkolinesteraz (AChE) enzim aktivitelerine etkilerinin incelenmesi amaçlanmıştır.Bu amaçla, toplam 24 adet sıçandan oluşan dört farklı grup (n=6) oluşturuldu. (Kontrol grubu, oksitosin verilen grup, atosiban verilen grup ve oksitosin ile atosiban'ın birlikte verildiği grup). Sıçanlara kimyasallar intraperitoneal olarak enjekte edildikten 60 dakika sonra sıçanlar nekropsiye alındı. Sıçanların kalp dokuları çıkarıldı. Elde edilen her bir enzim için aktivite değerleri istatistiksel olarak hesaplandı.

Buna göre sıçanların kalp dokusunda karbonik anhidraz enzim aktivitesi oksitosin ve atosiban verilen gruplarda azalışa neden olurken, oksitosin ile atosibanın birlikte verildiği grupta ise azalmanın diğerlerine nispeten daha az olduğu görüldü. Oksitosin ve atosiban verilen gruplarda asetilkolinesteraz aktivitesinde görülen azalma, oksitosin ve atosiban'ın birlikte verildiği grupta gözlenmedi.

**Anahtar Kelimeler:**enzim aktivitesi, karbonik anhidraz, oksitosin,rat

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## INTRODUCTION

Carbonic anhydrase (CA, carbonate hydrolase, E.C. 4.2.1.1), which is the most abundant enzyme available in all living species, also this enzyme acts as a pH, water, electrolyte and ion transport regulator in the living systems. It is physiologically catalyzes the conversion of carbon dioxide's hydration and bicarbonate dehydration alternately (Equal 1). The enzyme exist is present in all tissues which perform hydratase activity of $H^+$ and $HCO_3^-$ (Gulçin et al., 2004; Berg et al., 2014; Gocer et al., 2016; Kocyigit et al., 2016; Kucukoglu et al., 2016; Bhatt et al., 2017; Gul et al., 2017).

$$CO_2 + H_2O \overset{CA}{\Leftrightarrow} H_2CO_3^- \Leftrightarrow HCO_3^- + H^+ \quad (1)$$

Acetylcholinesterase (acetylcholine acetylhydrolase, E.C. 3.1.1.7, AChE) is a fundamental enzyme for neurotransmission, and reduces the agglomeration of active neurotransmitters during the synaptic division in extreme cells, and hydrolyzes the neurotransmitter acetylcholine (Akıncıoglu et al., 2014;Gocer et al., 2015, Kose et al., 2015). It has been noted that cholinergic therapeutics for Alzheimer's disease (AD) contain acetylcholinesterase inhibitors (AChEIs), acetylcholine (ACh) precursors, ACh expression facilitators, post-synaptic muscarinic recptor type 1 (M1) receptor agonists (Özbet et al., 2016; Taslimi et al., 2016)

Oxytocin is a mammalian peptide hormone that consists of nine amino acids (Sır et al., 2015). Oxytocin (OT) is a well known for its role in birth and breastfeeding which is synthesized from neurons in supraoptic and paraventric nuclei in the hypothalamus (Vireo et al., 2010). In extra-hypothalamic areas determination of these hormones and their receptors; that this hormone can be associated with central functions such as: stress, social, sexuality and maternal behaviors. Oxytocin has been shown by suppressing activate of the hypothalamic-pituitary-adrenal (HPA) axis and cause behavioral and stress-reducing effects (Neumann et al., 2002; Yegen et al., 2010). It has also been demonstrated that this hormone has anti-inflammatory effect by regulating the immunological and inflammatory processes with the inhibition of some interleukin release (Ivell et al.,1995;Yegen et al., 2010). Black and etc. have suggested that the systemic OT has analgesic and anxiolytic effects and may be a very useful agent in chronic pain syndrome by stress (Black et al., 2009).

Oxytocin receptor which is located in the cardiac. Oxytocin, directly produces at the cardiac or atrial natriuretic peptide (ANP) as cardioprotective effects (apoptosis inhibitors) with nitric oxide (NO) stimulation (Paquin et al., 2002; Jankowski et al., 2004)

Oxytocin stimulates differentiation of cardiac stem cells (Favaretto et al., 1997;Soares et al., 1999). It provides via ANP (Petersson et al., 2008). It decreases the systemic blood pressure. Oxytocin also has negative inotropic and chronotropic effects in the cardiac (Houshmand et al., 2009). It is the neuromodulator effective on the parasympathetic system. Treated oxytocin as of birth prevents the development of hypertension in hypertensive rat strain (Ondrejcakova et al, 2009). In myocardial infarction (MI) animal models, oxytocin has been shown to reduce the infarct area and improve cardiac function (Miki et al., 2007;Authier et al., 2009). The IP3 (inositol triphosphate) and IP3K (Inositol triphosphate kinase) systems used by oxytocin have a protective effect against myocardial damage (Jezdinsky et al., 1963). Oxytocin therapy in the rabbit myocardial ischemia-reperfusion model activates the extracellular-signal-regulated kinase ½ (ERK1/2), protein kinase b (AKT) and endothelial nitric oxide synthase (e-NOS) systems(Asadetal.,2001).

An analogue of desamino-oxytocin, atosiban is a nonapeptide, and a vasopressin / oxytocin receptor antagonist (VOTra). Atosiban inhibits the inositol triphosphate release (performed via oxytocin) from the myometrial cell membrane. As a result of this, it reduces the release of intracellular calcium stored in the sarcoplasmic reticulum of myometrial cells and the flow of calcium in the extra cellular liquid through voltage-dependent on calcium channels. In addition, to atosiban suppresses prostaglandin E (PGE) and prostaglandin F (PGF) released from decidua via oxytocin (Sanu et al., 2010).

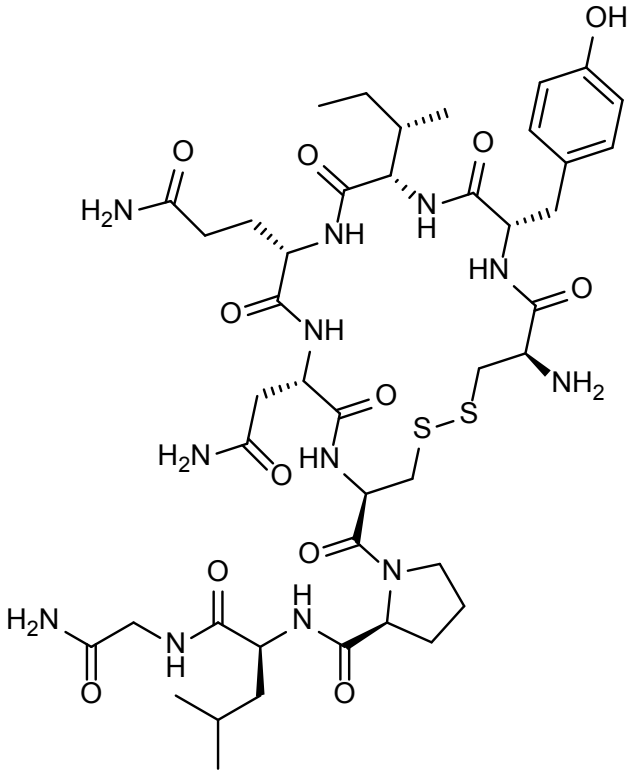

**Figure 1**: Chemical structure of Oxtocin

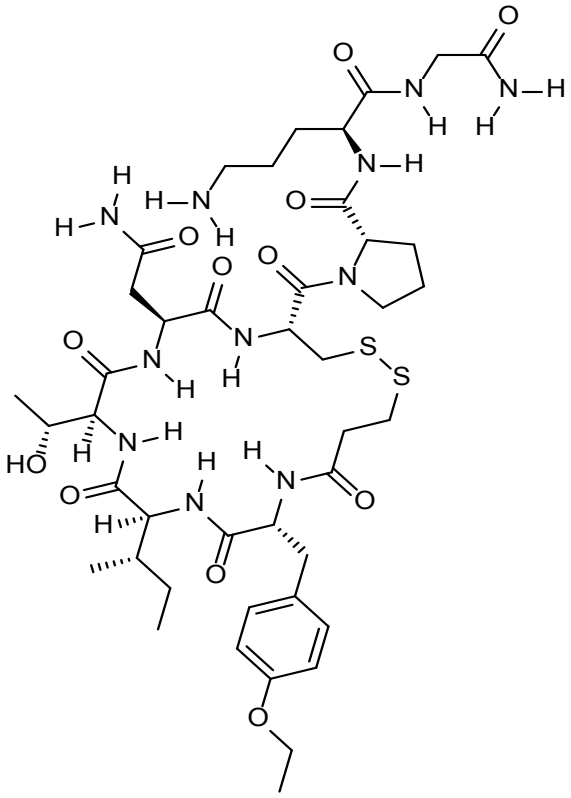

**Figure 2**: Chemical structure of Atosiban

Carbonic anhydrase (CA) and acetylcholinesterase (AChE) are vital enzymes for the living organisms. CA Enzyme has different isoenzymes and different kinetic properties in every living organism and in the tissues. There are thousands of studies on the purification and characterization of CA in each specie and tissue separately in order to determine functions of CA in the living organism and how the enzyme is localized.

The purpose of this study is to investigate the effects of oxytocin, oxytocin receptor antagonist atosiban, and oxytocin-atosiban hormones injected to the rats on the activities of carbonic anhydrase and acetylcholinesterase enzymes in the heart tissues of rats, which has not been studied before. This is the identifying role of oxytocin precisely is complicated due to the pulsatility of oxytocin secretion in live metabolism and difficulty of measuring the level of hormones.

However, it is expected that a contribution is made for fully understanding the role of oxytocin in the light of the data obtained.

## MATERIAL AND METHOD

### Chemicals

*p*-nitrophenyl acetate (PNF), CNBr-activated-Sepharose-4B and protein assay reagent were provided from Sigma-Aldrich Co. (GmbH, Germany). All chemicals for analytical grade were purchased from Merck (Germany).

### Animals and lab.

The research was applied to permission from the Ethics Committee of the Cumhuriyet University on Experimental Animals. 24 adult male Wistar albino rats (weighing approximately 240-260 g) not exposed to the stress and served in cages conforming to the standards were used in the study.

The rats obtained through Cumhuriyet University Animal Laboratory were kept in an insulated room with an 12-hour light / dark cycle at an ambient temperature of 22 ± 2°C and a relative humidity of 53 ± 5%. Also, it was ensured that they fed were adequately and appropriately.

The administration on the experimental animals was performed between 9:00-15:00, and the light and sound levels were kept under consistent control. Experimental animals were selected randomly, and distributed into four groups (n = 6 each) as follows: Group 1 - control, Group 2 - oxytocin (40 IU / kg (160 µg / kg)), Group 3 - atosiban (2.5 mg / kg) and Group 4 - oxytocin (40 IU / kg (160 µg / kg)) & atosiban (2.5 mg / kg) (Erbas et al., 2013; Simsek et al., 2012; Kocyigit et al., 2017)

**Extraction of Tissues and Preparation of Homogenate**

The rats used in the study were necropsied after 60 minutes from the injection of chemicals intraperitoneally. Heart tissues were extracted. Then, the tissues were washed for 3 times with 0.9% NaCl to eliminate the blood and other contaminants. For preparing tissues homogenate, the samples were initially dispersed by using the ultra-turrax device.

Thus, tissues were dispersed into extremely small pieces. After that, they were dispersed in liquid nitrogen, and homogenized in 3 mL/g with 25 mM Tris HCl/0.1 M $Na_2SO_4$ (pH= 8.7) buffer solution.

This suspension was centrifuged 2 times at 20000 rpm for 60 minutes, and used for supernatant analyses (Pullan et el.,1985;Wistrand et al., 2002; Kocyigit et al., 2017).

**Measurement of carbonic Anhydrase Activity**

**Hydratase Activity**

Hydratase activity was used to determine the activities of CA enzymes Wilbur et al., 1948, which was modified later by Rickli *et al*., 1964 This method is based on the principle that the pH change caused by the $H^+$ ion resulting from the hydration of $CO_2$ is identified by the bromine thymol blue indicator and the time elapsed is measured.

To this end, 1 mL vernal buffer (0.025 M, pH: 8.2), 0.1 mL 0.04% bromine thymol blue, 0.6 mL purified water and 2.5 mL $CO_2$ solution were added into a blind tube. Thus, the total volume in the activity measurement medium was 4.2 mL. Then, the time elapsed for the blue color of the indicator to turn into the yellowish green (pH: 6.3), starting from the moment the $CO_2$ solution was added and measured via a chronometer. 0.05 mL water was removed and instead, enzyme solution added into the sample tube, and finally, the time elapsed for the blue color of the indicator to turn into the yellowish green (pH: 6.3) as soon as $CO_2$ solution was added ($t_C$) was measured via a chronometer (Kocyigit et al., 2017).

According to this method of relative activity, an enzyme unit (EU) is defined as the amount of enzyme that reduces the time compared to $CO_2$ hydration without the enzyme.

$$EU = \frac{t_o - t_c}{t_c} \qquad (2)$$

The enzyme unit per the volume of enzyme solution was calculated by the formula above (Equal 2) (Maren, 1960; Huyut et al., 2016a; Huyut et al., 2016b ). The same procedures were applied to determine activity in hemolysate, pure enzyme solution and inhibition studies (Kocyigit et al., 2017).

**Esterase Activity**

The esterase activity method was applied to kinetic and inhibition studies. This method is based on the fact that CA has the esterase activity. Shortly, the principle of method is to hydrolyze the *p*-nitrophenylacetate (PNA), used as a carbonic anhydrase enzyme substrate, to *p*-nitrophenol or *p*-nitrophenolate, providing absorption at 348 nm. The reaction mechanism is as provided below:

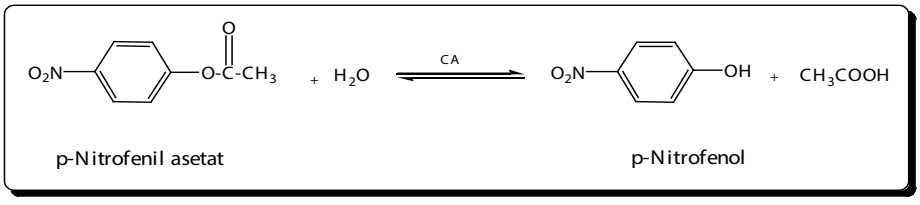


**Figure 3**. *p*-nitrophenylacetate - *p*-nitrophenol conversion mechanism

*Both p*-nitro phenol and *p*-nitrophenolate display the same absorbance at 348 nm. Hence, whether the $H^+$ ion in the phenol group is decomposed does not affect the measurement (Armstrong et al., 1966; Verpoorte, 1967). Since *p*-nitrophenylacetate has very little absorption at this wavelength, it is used as blind .

The procedure below was observed for determination of activity measured by using a 1 mL quartz vessel per sequence of involvement of the substances forming the reaction mixture into the treatments (Table 1).

**Table 1**. The solutions used at each 1 mL quartz vessel for esterase activity

| **Materials Used** | **Control Tube (µL)** | **Sample Tube (µl)** |
|---|---|---|
| Tris-$SO_4$ (0.05 M; pH:7,4) | 467 | 467 |
| *P*-Nitro phenol acetate | 333 | 333 |
| Pure Water | 200 | 167 |
| Enzyme Solution | - | 33 |
| Total Volume | 1000 | 1000 |

After preparing the reaction mixture in Table 1, the amount of absorbance was checked every 15 seconds and the absorbance difference was calculated by using the absorbance at 348 nm at 25°C after 3 minutes. The spectrophotometer was set to zero with the absorbance of the mixture obtained by replacing the enzyme with pure water after 3 minutes.

The *p*-nitrophenylacetosubstrate solution used in the experiments was prepared daily: 27.2 mg PNA, dissolved in 1 mL acetone, was added gradually to 49 mL distilled water and stirred rapidly. This solution is 3 mM, and it is not possible to prepare more concentrated version due to the limited solubility of ester. Acetone was preferred because it was relatively the least inhibiting solvent for the hydrolysis reaction among other organic solvents (Verpoorte, 1967). Activity measurements during kinetic studies were performed with the esterase activity of the enzyme (Kocyigit et al., 2017).

### Acetylcholinesterase Activity Measurement Method

The basis of this method can be described as follows; per observations from the previous studies, cholinesterases catalyze the reaction decomposing acetylcholine into thiocolchine and acetate. Yellow colored 5-thio-2-nitrobenzoic acid is formed by the reaction of thiocolchine and 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) which are obtained from as product. Resulting color is measured at 412 nm (Ellman et al., 1961). The absorbance of the sample and blind vessels was measured at 412 nm wavelength for 5 minutes (Table 2) (Kocyigit et al., 2017).

**Table 2**. Vessel content in the study performed via acetylcholinesterase method

| **Materials Used** | **Control Tube (µL)** | **Sample Tube (µL)** |
|---|---|---|
| Tris-HCl | 100 | 100 |
| Pure Water | 790 | 780 |
| Sample | - | 10 |
| DTNB | 50 | 50 |
| Enzyme Solution | 10 | 10 |
| Acetylcholinethioiodide | 50 | 50 |

### Statistical Assessment

All the experimental res208

ults were provided in the mean SD (mean standard deviation).

Since the data was compatible with the normal range, it was tested via variance analysis (ANOVA) and then, significance was determined by the Tukey test.

Statistical significance was identified to be $p < 0.05$ (Kocyigit et al., 2017).

## RESULTS AND DISCUSSION

The results show that the effect of hormones on the activity of carbonic anhydrase and acetylcholinesterase enzymes in the heart tissue are indicated in Table 3.

**Table 3.** The results of hormone effects on the activity of carbonic anhydrase and acetylcholinesterase enzymes in the heart tissue.

| Variable | | Control $X^- \pm SD$ | Oxytocin $X^- \pm SD$ | Atosiban $X^- \pm SD$ | Oxytocin +Atosiban $X^- \pm SD$ | ANOVA | Comparison Group | Post hoc p-value |
|---|---|---|---|---|---|---|---|---|
| **CA Activity (EU/mL)** | Esterase | 8.99±0.86 | 2.56±0.41 | 3.73±0.25 | 4.40±0.45 | F= 134.864 P=0.001 | 1 Vs 2 | 0.001 |
| | | | | | | | 1 Vs 3 | 0.001 |
| | | | | | | | 1 Vs 4 | 0.001 |
| | | | | | | | 2 Vs 3 | 0.017 |
| | | | | | | | 2 Vs 4 | 0.001 |
| | Hydratase | 492.11±55.42 | 142.96±6.34 | 188.10±9.68 | 223.87±17.6 | F= 140.285 P=0.001 | 1 Vs 2 | 0.001 |
| | | | | | | | 1 Vs 3 | 0.001 |
| | | | | | | | 1 Vs 4 | 0.001 |
| | | | | | | | 2 Vs 3 | 0.116 |
| | | | | | | | 2 Vs 4 | 0.001 |
| **AChE Activity (EU/mL)** | Acetylcholiniodide | 0.18±.0.012 | 0.18±0.014 | 0.13±0.023 | 0.18±0.015 | F= 12.822 P=0.001 | 1 Vs 2 | 1.000 |
| | | | | | | | 1 Vs 3 | 0.001 |
| | | | | | | | 1 Vs 4 | 0.996 |
| | | | | | | | 2 Vs 3 | 0.001 |
| | | | | | | | 2 Vs 4 | 0.997 |

When the activities of CA are evaluated according to the esterase method in the cardiac tissue; comparing to control group became a statistically significant difference between oxytocin treated group ($p < 0.05$), according to the control group a statistically significant difference between atosiban treated group ($p < 0.05$), comparing to the control group have become a statistically significant difference in combination treated with the oxytocin and atosiban.

When the activities of carbonic anhydrase enzyme according to the hydratase method are evaluated in the cardiac tissue; comparing to the control group become a statistically significant difference between oxytocin terated group ($p < 0.05$), according to the control group a statistically significant difference between atosiban treated group ($p < 0.05$), according to the control group have become a statistically significant difference in combination treated with oxytocin and atosiban ($p < 0.05$).

When the activities of AChE enzyme in cardiac tissue are evaluated comparing to the control group a statistically significant difference between atosiban treated group ($p < 0.05$), according to oxytocin become a statistically significant difference between atosiban treated group ($p < 0.05$), according to the atosiban has become a statistically significant difference in combination treated with oxytocin and atosiban ($p < 0.05$).

In the previous study, the effects of oxytocin and atosiban on enzyme activities in liver and kidney tissues of rats were investigated and similar results were obtained (Kocyigit et al., 2017).

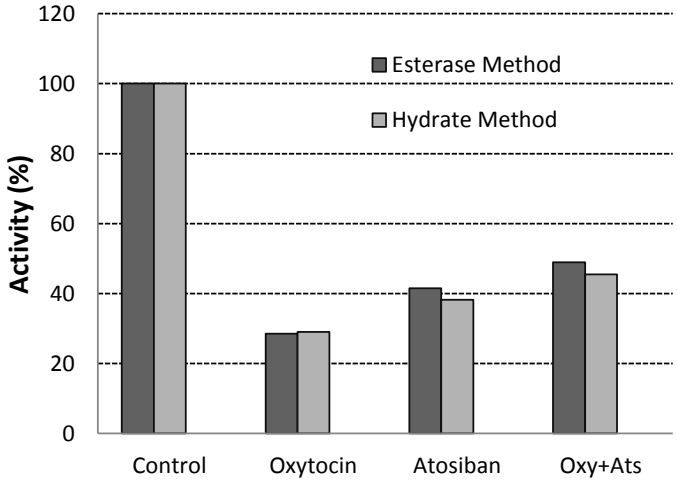


**Figure 1.** The effect of hormones in the heart tissue of a rat on the Carbonic anhydrase enzyme

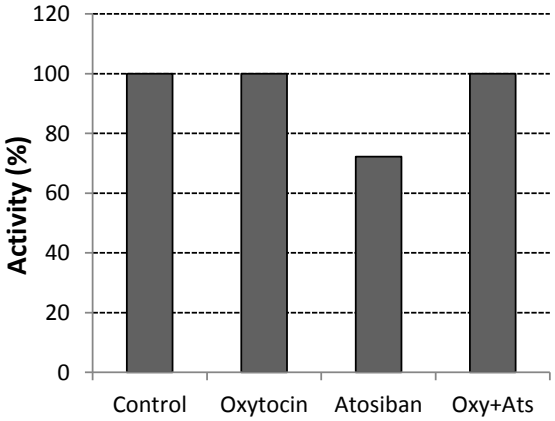


**Figure 2.** The effect of hormones in the heart tissue of a rat on the acetylcholinesterase enzyme

The carbonic anhydrase enzyme has both hydratase and esterase activity. However, hydratase activity is important in physiological terms. Also hydratase activity is important due to the regulation of acid-base balance of the live organism. In this research, the activity of CA enzyme was measured via both methods and consistent results were obtained. Considering the chemical structures of the oxytocin hormone and oxytocin antagonist atosiban (Figure 1-2), it might be considered that there is a reduction in activities of CA and AChE due to -OH and -$NH_2$ groups in their structures. We have achieved similar results in our previous work (Kocyigit et al., 2017). For this reason the results support each other.

Absence of a significant change in the activities of enzymes in the group where oxytocin and atosiban were administered together might be considered as a result of having antagonistic effects (Figure 2).

## CONCLUSION

According to this, the carbonic anhydrase enzyme activity in cardiac tissue of rats lead to a decrease in the groups with the oxitocin or atosiban. However, this decrease was relatively less in the groups in which oxytocin and atosiban were given together. In addition, decreasing acetycholinesterase activity was observed in the atosiban-treated group, while this situation did not show in oxytocin or oxytocin+atosiban-treated groups

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