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Inflammation-Associated HMGB1, α7nAChR, Kir6.2 and Beclin1 Immunoreactivity in PTZ-Kindled Model Seizures in Rats

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Abstract: Epilepsy is a disease with cardiovascular involvementdue to the disruptionof excitatory and inhibitory balance in the ANS (autonomic nervous system). Cardiac pathologies may occur as a result of dysfunction in vagus nerve. The vagus nerve, acetylcholine (ACh) secreted from efferent fibers interacts with α7 nicotinic ACh receptors (α7nAChR) on immune cells, preventing the release of cytokines such as HMGB1 and exerting a protective effect against inflammation. Kir6.2 channels are effective in the regulation of ACh release and inflammation in the vagus nerve to the heart in epilepsy. In addition to taking part in inflammation, HMGB1 regulates autophagy by interacting with Beclin 1, an essential molecule linked to autophagy. In our study, 35 mg/kg pentylenetetrazole (PTZ) agent was administered intraperitoneally to male and female rats and kindling model was created. We examined histologically the immunoreactivity of HMGB1, α7nAChR, Kir6.2 and Beclin 1 proteins in the heart and vagus nerve. Significant increases of HMGB1, α7nAChR, Kir6.2 and Beclin 1 immunoreactivity in the PTZ-kindled group compared to the control.With this study, a new perspective to understand may be provided in terms of inflammation, cardiac pathologies, and dysfunctions in epileptic seizures. It is important to carry out further studies to understand the relationship between the inflammation process and cardiac pathologies.

Keywords: Inflammation, ACh, vagus nerve, heart, epilepsy.

Abbreviations

ACh	Acetylcholine
ANS	Autonomic nervous system
HMGB1	High Mobility Group Box 1
Kir	Inwardly rectifying potassium channels
PTZ	Pentylenetetrazole
RS	Racine scoring
VNS	Vagus nerve stimulation
α7nachr	α7 nicotinic ACh receptors

1. Introduction

Epilepsy is a neurological disease characterized by sudden and uncontrolled electrical discharges in the central nervous system. During epileptic seizures, the ANS (autonomic nervous system) may be affected, causing symptoms in the cardiovascular system [1].

Epileptic seizures can trigger heart rate problems such as sinus tachycardia. Studies show that disruption may occur in the control of ANS activities in patients with epilepsy [2]. Neuronal transmission between the higher central nervous system areas and the autonomic control circuits of the brainstem is carried out by the vagus nerve [3, 4]. The parasympathetic fibers of the vagus nerve provide information transmission between autonomous centers in the brainstem and various organs such as heart and lungs [5, 6]. Increased stimulation of the vagus nerve causes the heart rate to slow down with the release of acetylcholine (ACh) [7]. The vagus nerve can be a potential link in epilepsy to provide the relationship between the brain and the heart.

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The afferent branch of the vagus nerve conveys information to the brain about the inflammation processes occurring in the surrounding tissues [8]. Stimulation of the vagus nerve reduces the levels of High Mobility Group Box 1 (HMGB1), which is an inflammatory cytokine [9].

Increased HMGB1 levels are regulated by the cholinergic anti-inflammatory system [10]. The cholinergic anti-inflammatory system mediates its effect by the interaction of ACh released from the efferent vagus nerve with α7 nicotinic ACh receptors (α7nAChR) on immune cells [11]. HMGB1 and α7nAChR may offer important molecular targets in understanding the mechanism of neuroinflammation in epilepsy.

Potassium channels appear to be associated with epilepsy syndromes through the control of neuronal excitability and ionic homeostasis [12]. In this context, inwardly rectifying potassium channels (Kir) from the family of K^+ channels that mediate electrical stimulation in the vagus nerve may be effective in controlling autonomic functions.

Kir6.x channels are activated as cellular ATP levels decrease and cause membrane hyperpolarization [13, 14]. K_{ATP} channels expressed in the vagus nerve leading to the heart change the heart rate by regulating the release of Ach. K_{ATP} channels affect the heart rate by altering the presynaptic ACh release [15]. In this context, epileptic seizures may regulate the release of ACh by changing Kir6.2 channel expression in the vagus nerve. Accordingly, the change in ACh level due to Kir6.2 channel expression in epilepsy may have an effect on α7nAChR.

Autophagosome formation and maturation, which is a fundamental task in autophagy processes, is regulated by various genes. Among these genes, Beclin 1 can modulate the production and maturation of autophagosomes [16]. Beclin 1 protein levels may increase 12-48 h after epileptic seizure [17]. Examination of Beclin 1 in ANS dysfunction observed in epilepsy may contribute to understanding the effect of autophagy on the mechanism of heart problems accompanying epilepsy.

In this paper, in line with the importance of inflammation and autophagy in seizures, HMGB1, α7nAChR, Kir6.2 channel and Beclin 1 were investigated in the vagus nerve and heart. It was aimed to present the molecular differences related to inflammation and autophagy by examining the immunoreactivity changes of targeted proteins in the rat epilepsy model induced with pentylenetetrazole (PTZ).

2. Material and Method

2.1 Animals

Wistar albino male and female rats (280-380 g, *n*=34) from Kayseri Erciyes University Research Center were used in the experiment. Animals were housed in a controlled environment with a temperature of 24 ± 2 °C and 60% humidity under a 12-hour light/dark cycle. Animals were given free access to tap water and standard food. All procedures were followed by the recommendations in the Guidelines for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (USA) and the Declaration of Helsinki. The experimental protocol of the study was approved by Kayseri Erciyes University Animal Ethics Committee (ethics committee decision number: 2019/027). All efforts have been made to minimize animal suffering by anesthetizing rats with ketamine/xylazine (90/10 mg/kg, intraperitoneal, i.p., respectively).

2.2 Pentylenetetrazol (PTZ) Kindling Model

To induce tonic-clonic epilepsy seizures in the rat, a dose of 35 mg/kg of 1% solution of PTZ is administered i.p. was given as used protocol in our previous paper [18].

2.3 Racine's Scoring System

RS (racine scoring) is one of the paradigms used to assess seizure levels in rodent experimental epilepsy models. Categorizing the five stages of seizures, RS is

based on the behavioral movement of animals during a seizure, including mouth and facial movements (phase 1). In addition, the animal's head shaking (phase 2), the animal's forelimb raising movement (phase 3), clonic seizures (phase 4) and tonic-clonic seizures (phase 5) were defined [19].

2.4 Vagus Nerve and Heart Dissection

For tissue harvest, the chest wall of rats was cleaned with alcohol and removed by cutting towards the sternum [20]. The right and left vagus nerves were released and dissected in the cervical and thoracic region. Later, the chest cavity was opened by cutting the left cartilage at the rib level. Then the heart, aorta, and truncus pulmonalis were dissected by cutting the upper edge.

2.5 Histological Staining

The heart and vagus nerve dissected for histological examination were immediately fixed with 4% formaldehyde solution. The detected tissues were then dehydrated by passing through the graded alcohol series. Tissues transparent with xylol were embedded in paraffin.

2.6 Immunohistochemistry

Avidin-biotin-peroxidase method and immunohistochemical techniques were used to determine protein expression changes in the heart and vagus nerve from experimental epilepsy model rats [21]. Paraffin embedded tissues were cut with a microtome to a size of 5µm. The vagus nerve and heart tissues were evaluated immunohistochemically. In the epilepsy model, the immunoreactive differences of Beclin 1, α7nAChR, HMGB1, Kir6.2 antibodies in the vagus nerve and heart were marked with avidin-biotin-peroxidase method. Immunohistochemistry expression for each marker was examined by evaluating the percentage (prevalence) and staining intensity (intensity) of cells stained with antibodies, including Anti-Beclin 1 (Elabscience, E-AB-70093), anti-α7nAChR (Polyclonal Antibody, Alomone labs, # ANC-007), anti-HMGB1 (Polyclonal Antibody, Elabscience, E-AB-70044) and anti-Kir6.2 (Alomone labs, APPC-020). Immunohistochemistry expression for each marker was examined by evaluating the percentage (prevalence) and staining intensity (density) of the cells stained with antibodies. In our study, the results were evaluated using an Olympus brand microscope at 40×, 100× and 200× magnifications. The closed preparations were analyzed under light microscope and their immunoreactivities were evaluated with Image-J program.The experimental procedure is summarized in a pictorial form in Fig. 1.

2.7 Statistical Analysis

Comparison between groups was made by one-way analysis of variance (ANOVA). Post-hoc Tukey test was used for binary comparisons. In the analysis of the results, a value of $p<0.05$ was considered statistically significant.

3. Results

3.1 HMGB1 Immunoreactivity Was Significantly Increased in the Heart and Vagus Nerve Tissues in the PTZ-kindling Model

Scores of seizure degrees in animals are included in our previous article [18].

The effect of epileptic seizures on expression of HMGB1 molecule in the heart and vagus nerve samplesof rats was evaluated by immunohistochemical staining. It was observed that HMGB1 immunoreactivity increased by 1.64 times ($p<0.05$) and 1.95 times ($p<0.05$), respectively, in the heart tissue of female ($n=10$) and male ($n=10$) rats under the PTZ-kindling model (Fig. 2). HMGB1 staining in the cervical region of the vagus nerve increased 1.17 times ($p<0.05$) in female rats and 1.72 ($p<0.05$) times in male ratsinjected with PTZ(Fig. 3). HMGB1 expression was increased 2.11 times ($p<0.05$) in female rats in the PTZ-kindling group in the samples of the thoracic vagus nerve (Fig. 4).

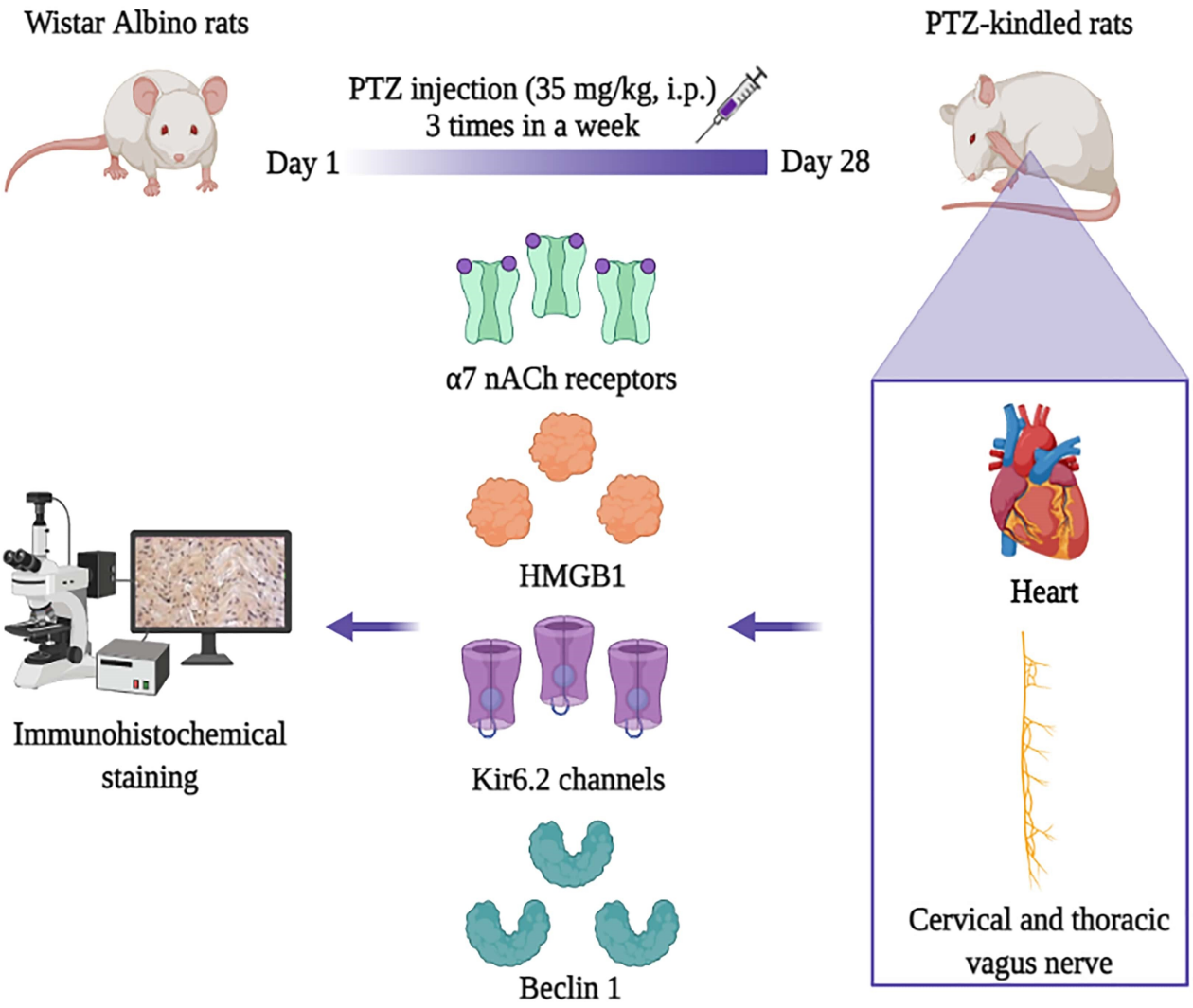

Fig.1 Pictorial representation of the current experiment.

3.2 The Expression of α7nAChR in the Heart and Vagus Nerves of Rats in Which Seizures Were Induced by PTZ Was Significantly Higher

The effect of seizures on the immunoreactivityof α7nAChR in rat heart tissue and vagus nerve was evaluated by immunohistochemical staining. It was observed that the immunoreactivity of the α7nAChR molecule increased 8-fold (p<0.01) and 2.49-fold (p<0.05), respectively, in the heart tissue of epileptic female (n=10) and male (n=10) rats compared to the control group rats (n=7) (Fig. 5). Immunoreactivity of α7nAChR in the cervical region of the vagus nerve increased 6.03-fold (p<0.01) in female rats injected with PTZ compared to control rats, while there was no significant difference in male rats (Fig. 6). In the thoracic vagus nerve samples, α7nAChR expression significantly increased 8-fold (p<0.01) in female rats (p<0.01) and 2.49-fold (p<0.05) in PTZ-kindled male rats (Fig. 7).

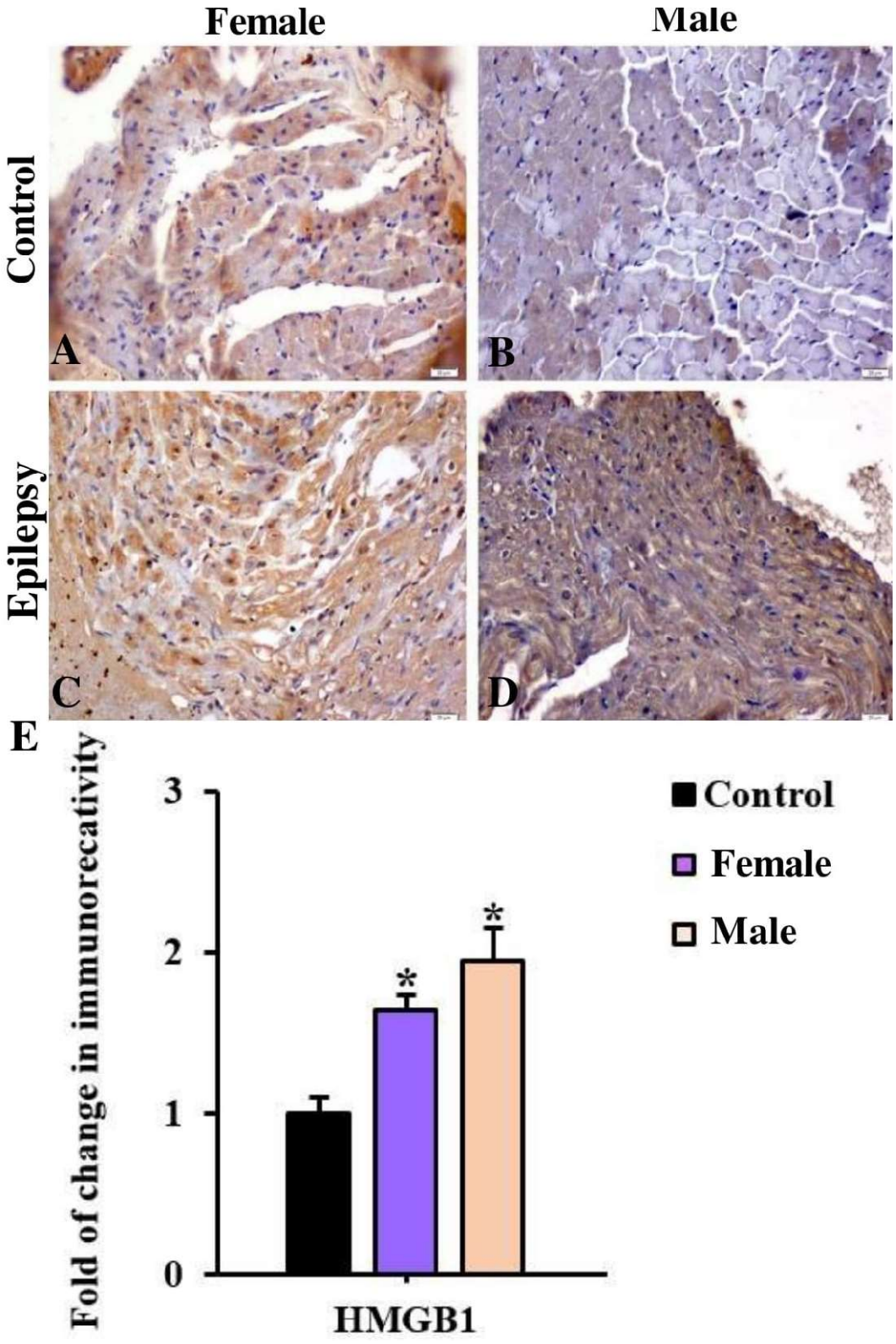

Fig. 2 HMGB1 immunostaining images in heart tissue from female and male control group (A and B) and PTZ-kindled group (C and D) rats. HMGB1 immunoreactivity in heart tissue samples of rats with seizures with control group (E). Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

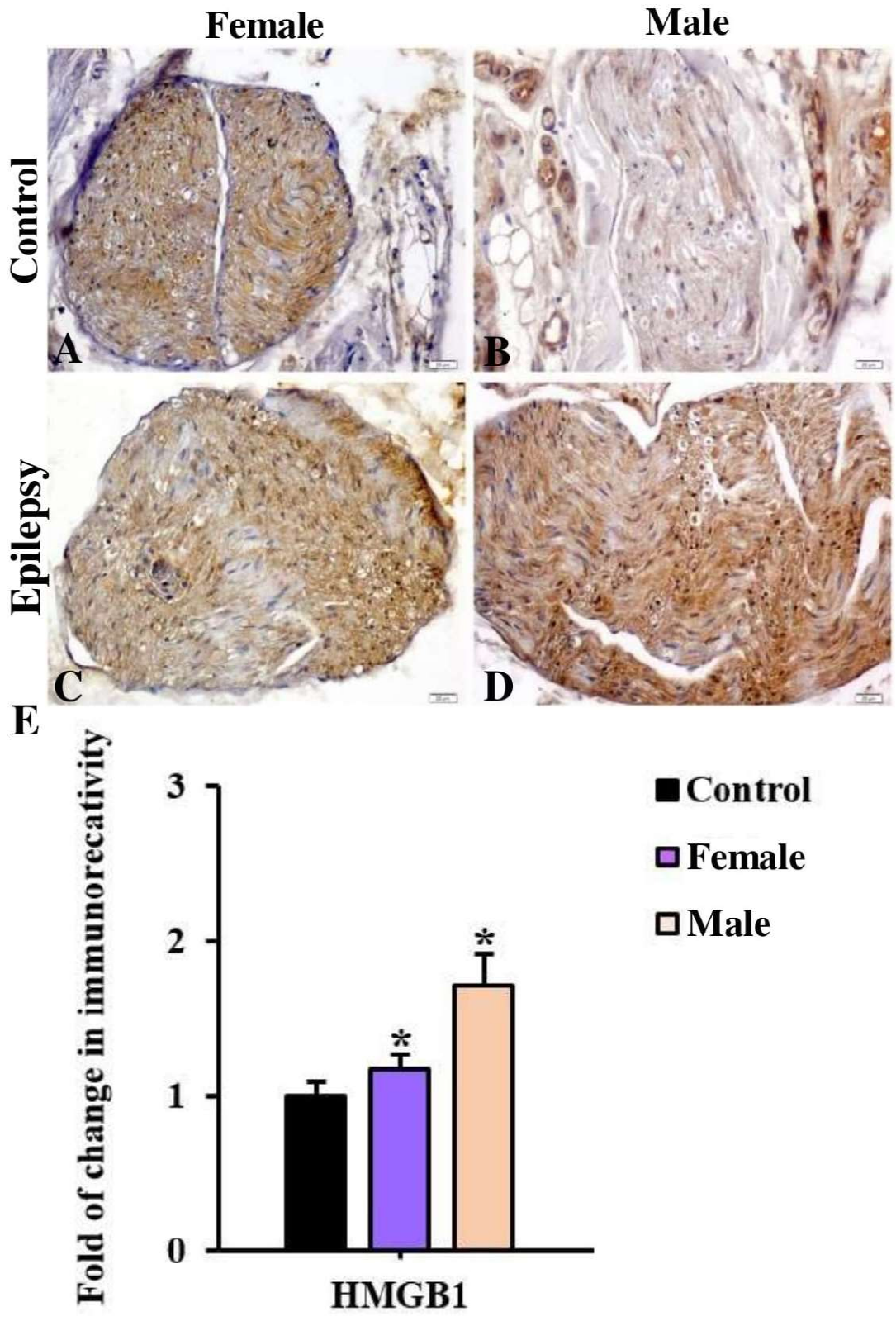

Fig. 3 HMGB1 immunostaining images in the cervical vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. HMGB1 immunoreactivity in cervical vagus nerve samples in rats with seizures with control group and PTZ (E). Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

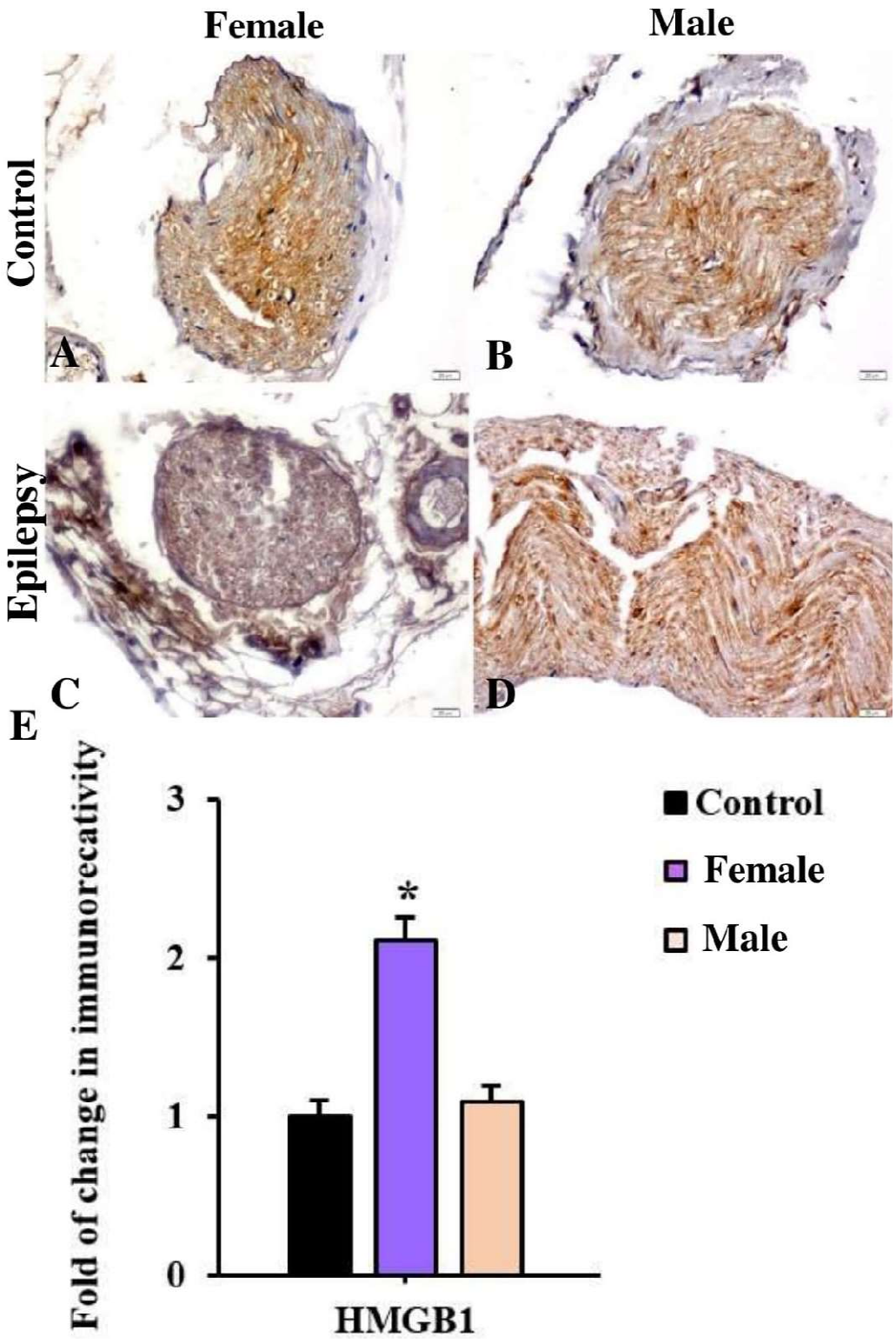

Fig. 4 HMGB1 immunostaining images in the thoracic vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. HMGB1 immunoreactivity (E) in thoracic vagus nerve samples of rats with seizures and control group and PTZ. Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

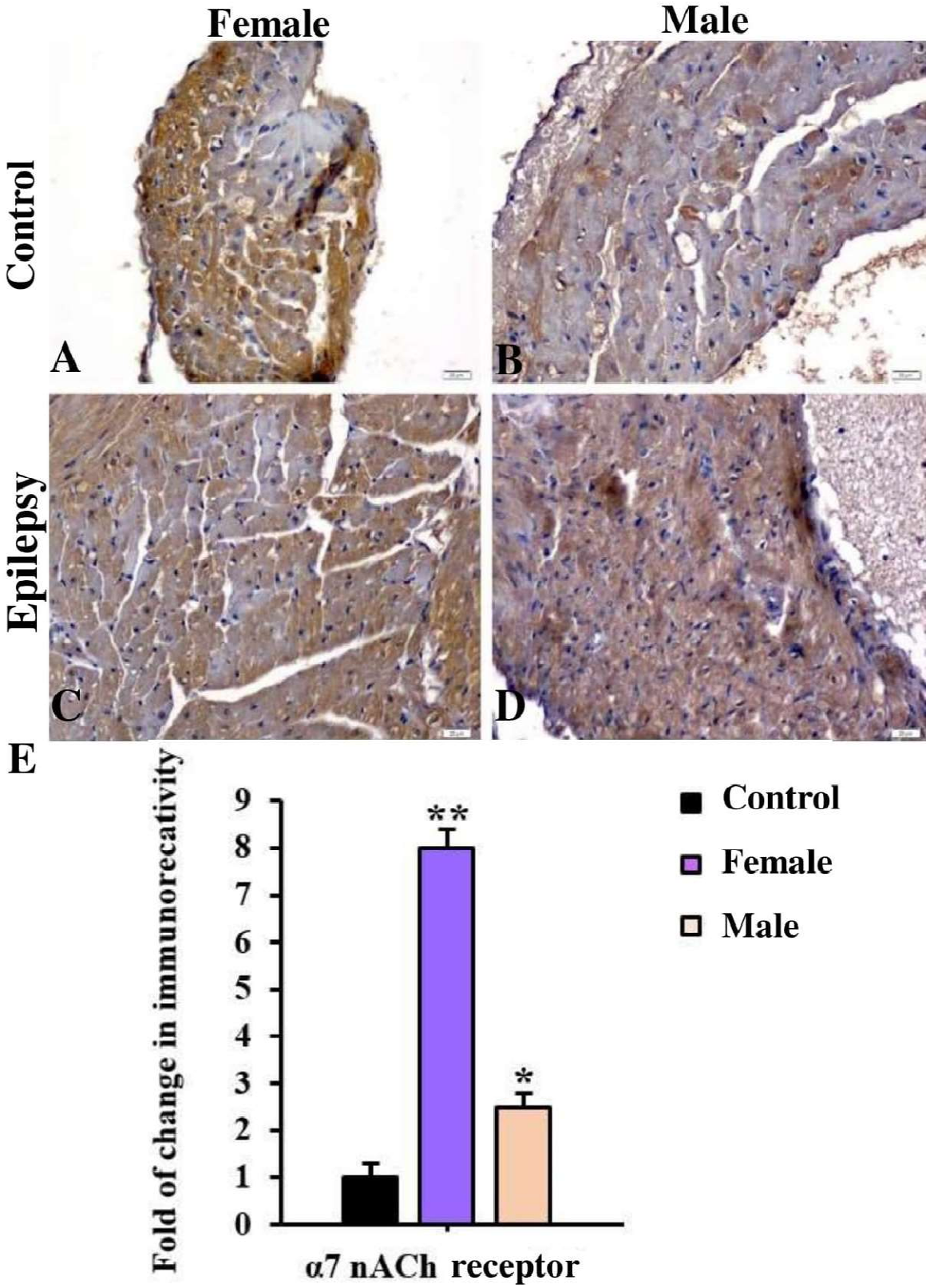

Fig. 5 α7nAChR immunostaining images in heart tissue from female and male control group (A and B) and PTZ-kindled group (C and D) rats. 7nAChR immunoreactivity (E) in heart tissue samples of rats with control group and PTZ model of epilepsy. Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

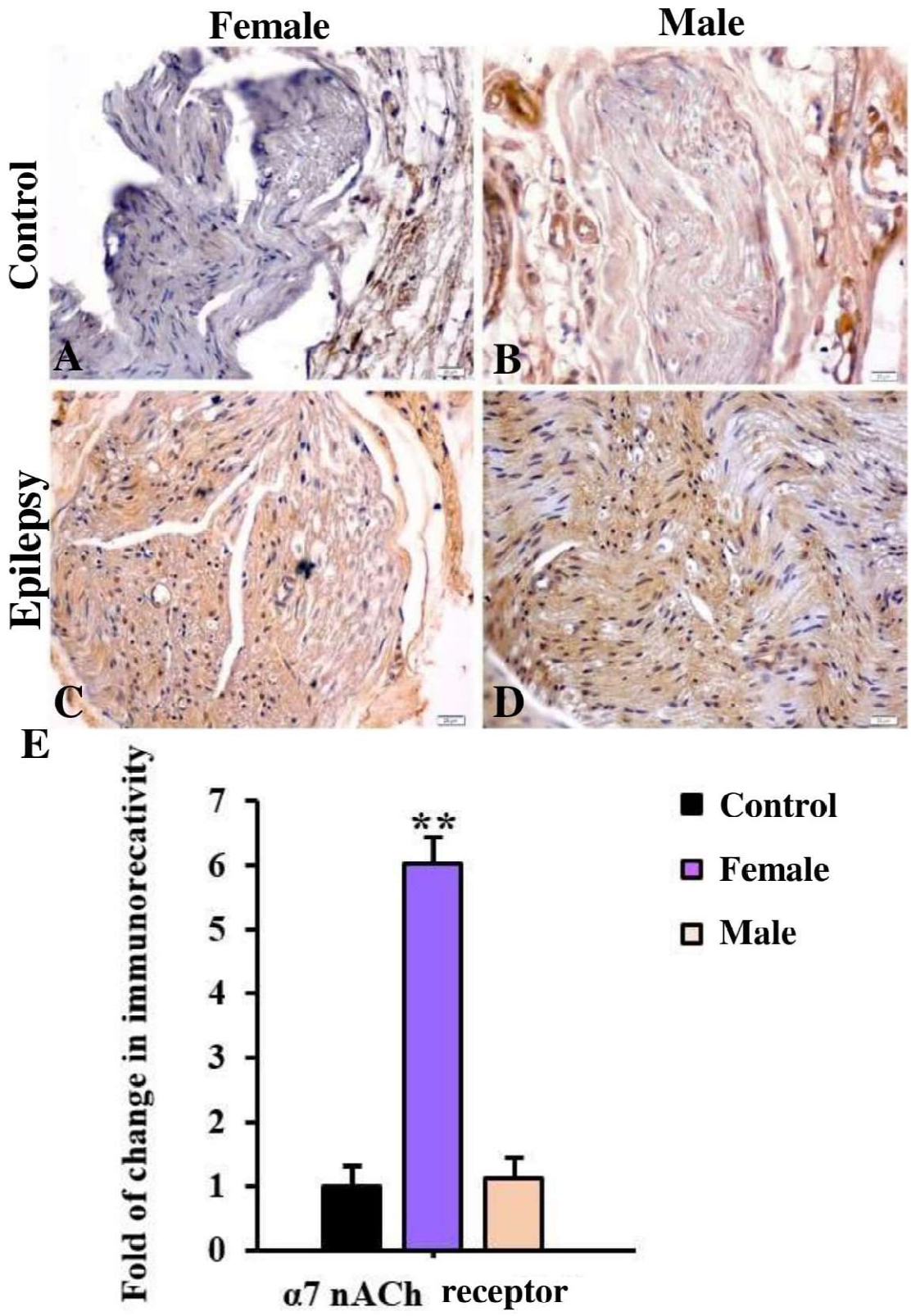

Fig. 6 α7nAChR immunostaining images in the cervical vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. 7nAChR immunoreactivity in cervical vagus nerve samples in rats with seizures with control group and PTZ (E). Images were taken at 40× magnification. Scale bar = 20 μm. **p<0.01.

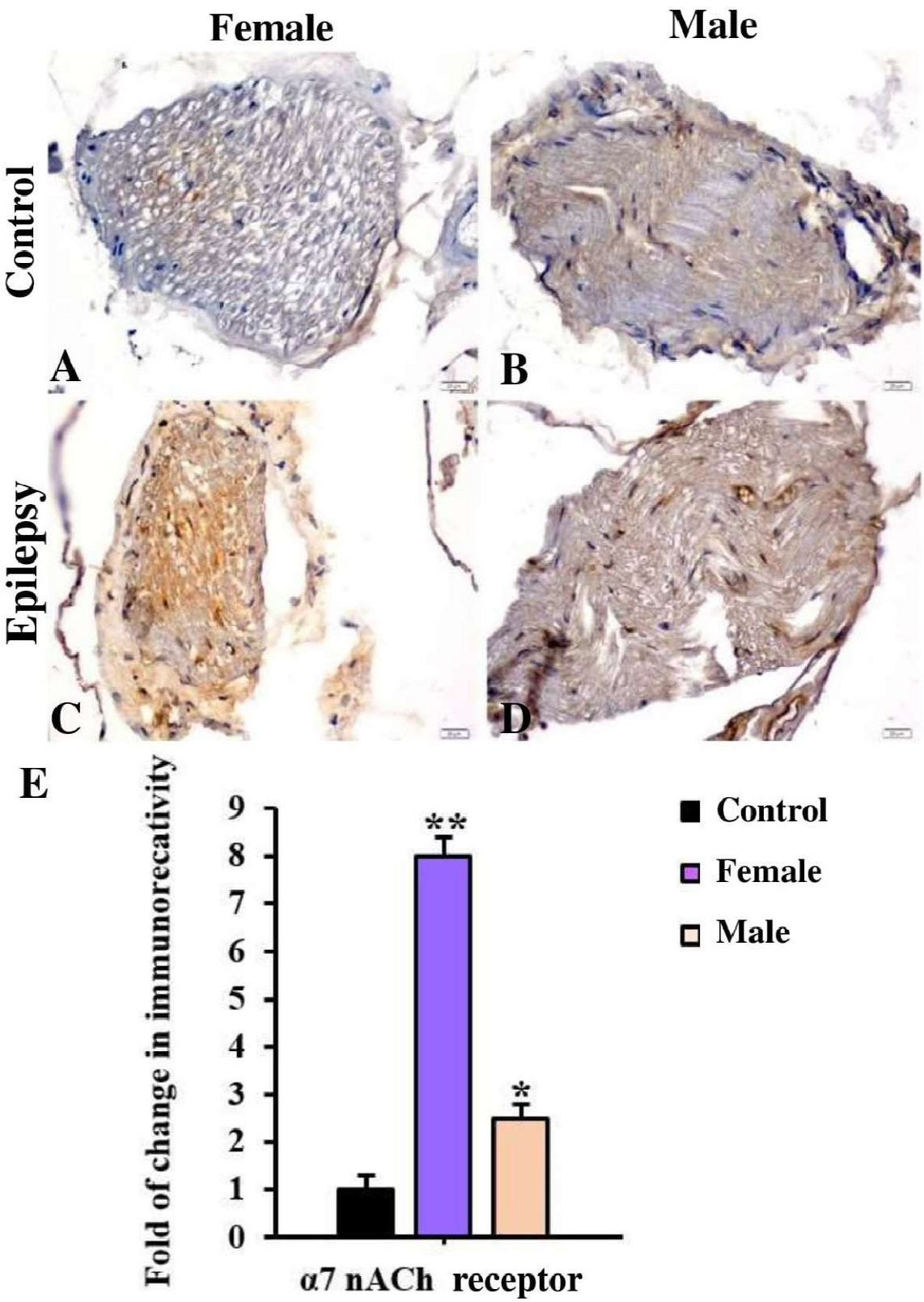

Fig. 7 α7nAChR immunostaining images in the thoracic vagus nerve from female and male control group (A and B) and PTZ-kindled group (C and D) rats. α7nAChR immunoreactivity in thoracic vagus nerve samples in rats with seizures with control group and PTZ (E). Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05 and ** p<0.01.

3.3 Immunoreactivity of Kir6.2 Channel Increased in Cervical and Thoracic Vagus Nerves ofRats with PTZ-Kindling Model

Kir6.2 channel immunoreactivity from the vagus nerve was evaluated by immunohistochemical staining. The immunoreactivity of Kir6.2 channel in the cervicalvagus nerve of epileptic female (n=10) and male (n=10) rats was increased 3.08-fold (p<0.05) and 2.83-fold (p<0.05), respectively, compared to control observed (Fig. 8). In the thoracic vagus nerve samples, Kir6.2 channel expression increased 3.55-fold (p<0.05) in female rats and 7.67-fold (p<0.01) in male rats injected with PTZ compared to control rats (Fig. 9).

3.4 Beclin 1 Immunoreactivity Increased in the Heart and Cervical Vagus Nerve Tissues of PTZ-Kindling Rats

The effect of epileptic seizures on Beclin 1 expression in the heart and vagus nerves was evaluated by immunohistochemical staining. Beclin 1 immunoreactivity increased 2.7-fold (p<0.05) and 1.85-fold (p<0.05), respectively, in the heart of epileptic female (n=10) and male (n=10) rats (Fig.10). Beclin 1 immunoreactivity was foundsignificantly increased 1.57 times (p<0.05) in cervical vagus nerve of PTZ-kindled female rats and 1.78-fold (p<0.05) in male rats (Fig. 11). Beclin 1 immunoreactive staining in the thoracic vagus nerve of epileptic female and male rats significantly increased (Fig. 12).

4. Discussion

To show the role of ACh-related inflammation and autophagy in the molecular mechanism of cardiac dysfunction accompanying epileptic seizures, we examined the immunoreactive changes of HMGB1 protein, α7nAChR, Kir6.2 channel and Beclin 1 molecules. We evaluated the immunoreactivity of these targets in vagus nerve and heart tissue in seizures induced by PTZ.

In our study, we showed that HMGB1 immunoreactivity increased significantly in the heart tissue of PTZ group female and male rats. In a previous clinical study conducted on 53 patients with atrial fibrillation, an increase in HMGB1 expression was found due to impaired cardiac function in the case of atrial fibrillation [22]. The increase in HMGB1 detected in cases such as epilepsy with rhythm disturbances in the heart may be an indicator of an abnormal inflammatory response.

Our examinations of the vagus nerve indicated that HMGB1 immunoreactivity significantly increased in the PTZ-kindled groups in the thoracic and cervical regions. Studies reported that the estrus cycle changes heart rate variability and the expression of inflammatory cytokines [23, 24]. The hormone-dependent variation between the sexes in the vagus is essential to understand our data showing that HMGB1 immunoreactivity is affected at different levels in males and females.

In our study, we showed that there was a significant increase in α7nAChR expression in the heart tissue of female and male rats in the PTZ-kindled group. The effect of ACh on the heart was examined and it was determined that nAChR stimulation has a protective role in vascular damage after vagus nerve stimulation (VNS) application [25]. In this sense, the data we have obtained show that α7nAChR can have a protective effect against cardiac pathology observed in epilepsy patients.

We showed that the immunoreactivity of α7nAChR significantly increased in the cervical and thoracic vagus regions of female PTZ-kindled rats. In this process, the extension of the cervical region of the vagus nerve together with the sympathetic nerve fibers may be effective [26]. A detailed examination of the balance between the sympathetic and parasympathetic nervous systems can contribute to understanding the inflammation mechanisms in epilepsy. As the interaction of ACh and α7nAChR is evaluated, it is important to regulate the level of ACh released from the vagus nerve as a result of epileptic seizures with Kir channels.

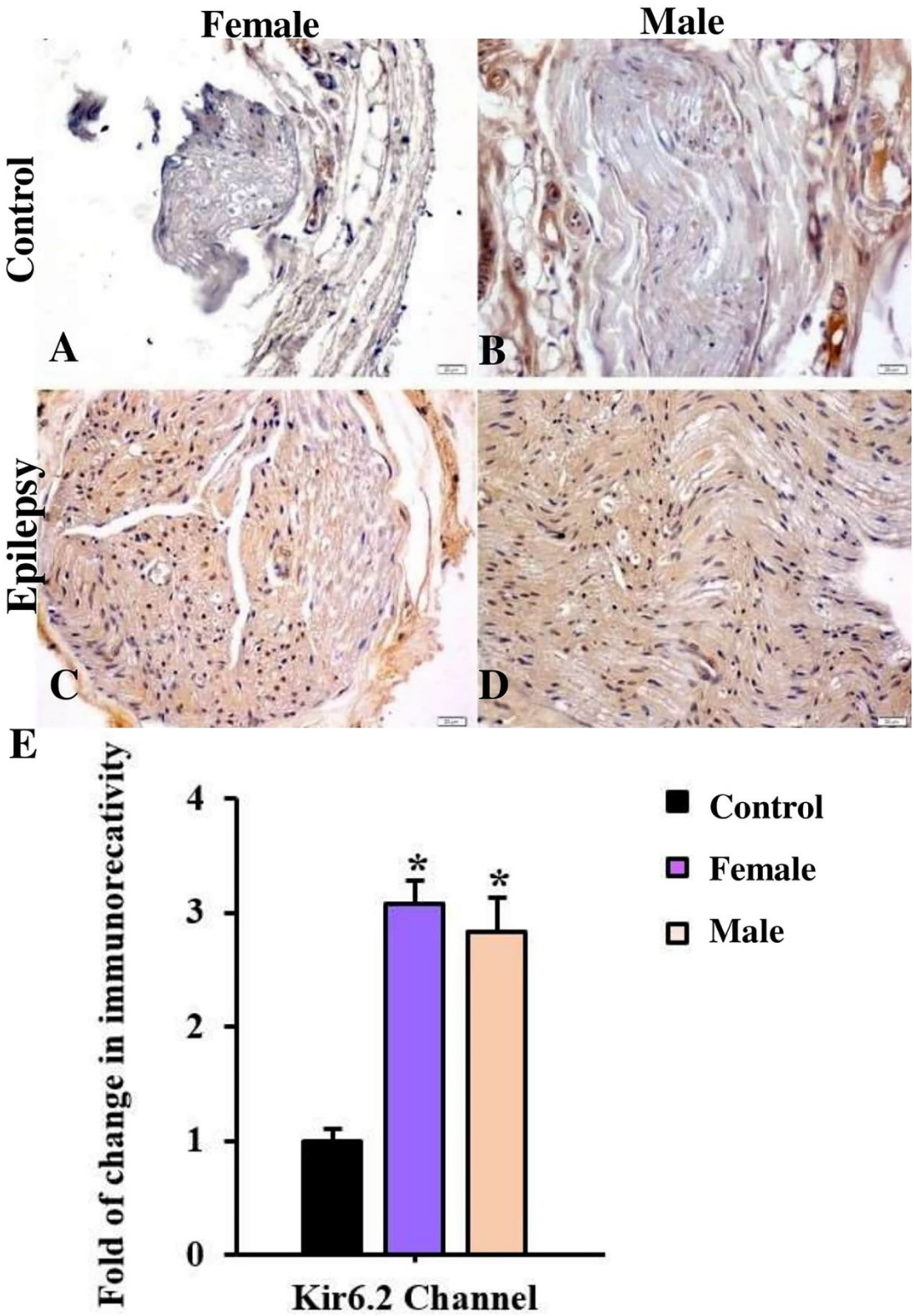

Fig. 8 Immunostaining images of Kir6.2 canal in the cervical vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. Kir6.2 channel immunoreactivity in cervical vagus nerve samples in rats with seizures with control group and PTZ (E). Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

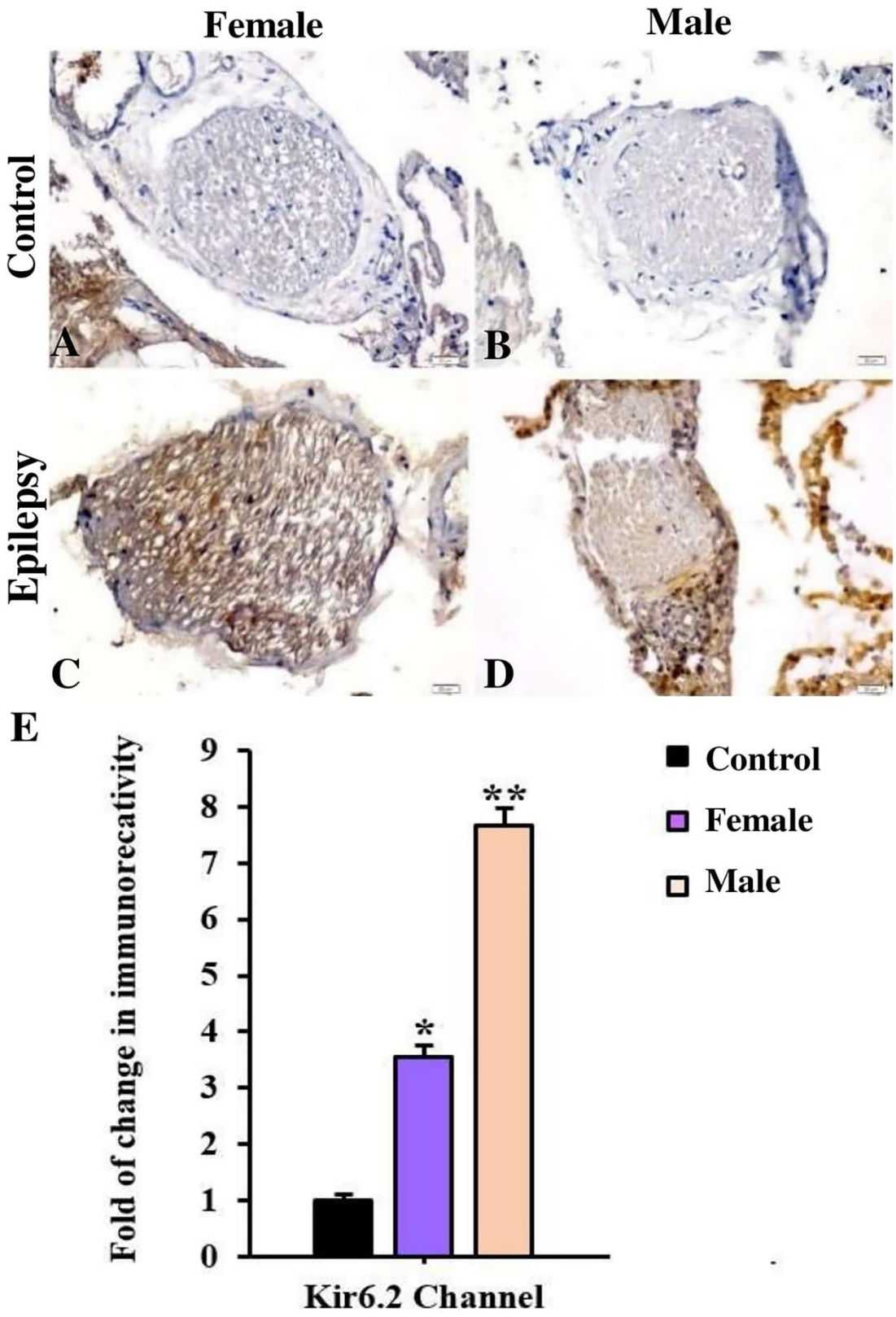

Fig. 9 Immunostaining images of Kir6.2 channels in the thoracic vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. Kir6.2 channel immunoreactivity in thoracic vagus nerve samples in rats with seizures in the control group and PTZ (E). Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05 and ** p<0.01.

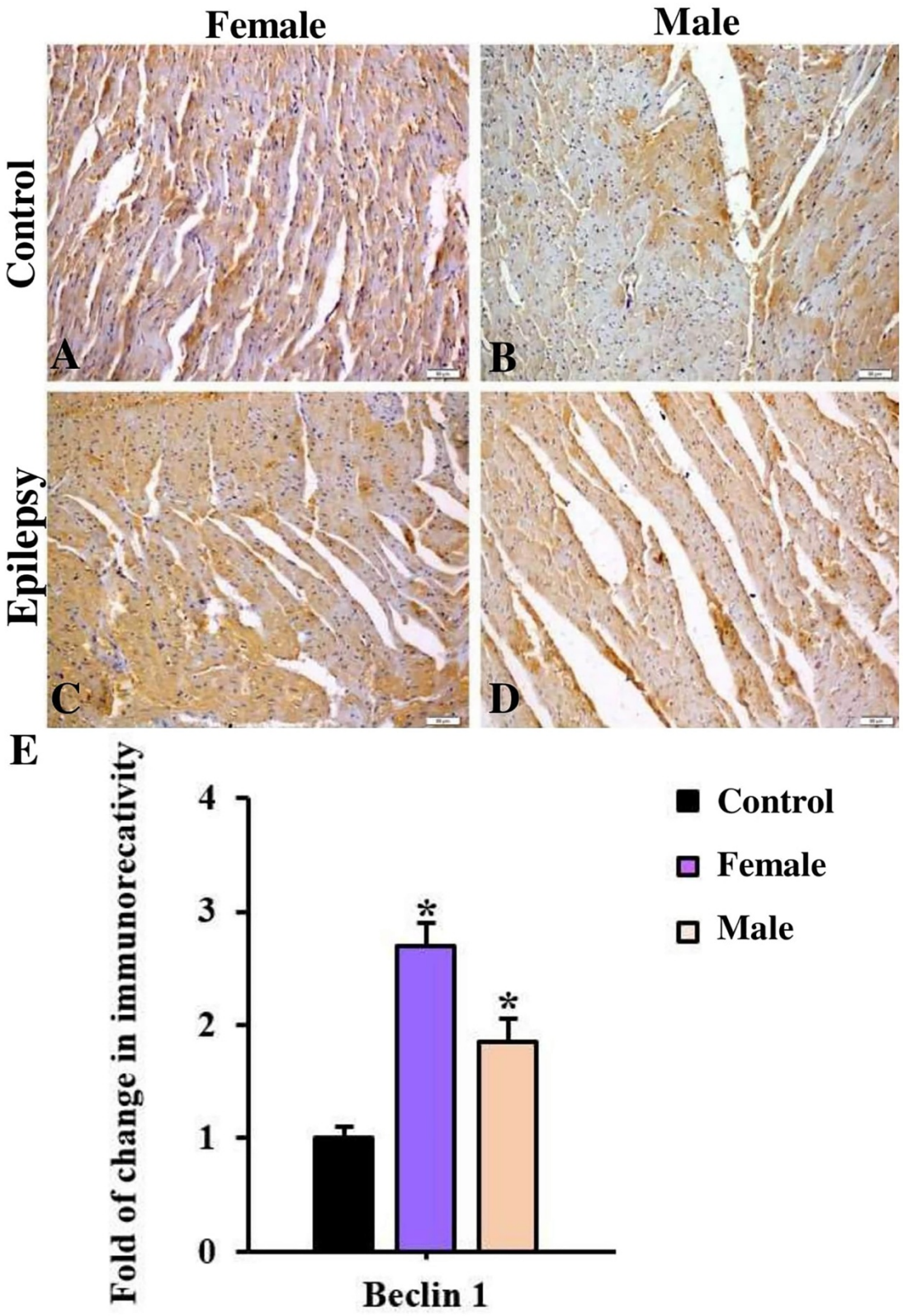

Fig. 10 Beclin 1 immunostaining images in heart tissue taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. Beclin 1 immunoreactivity in heart samples of rats with seizures with control group and PTZ (E). Images were taken at 40× magnification. Scale bar = 20 μm. * p<0.05 and ** p<0.01.

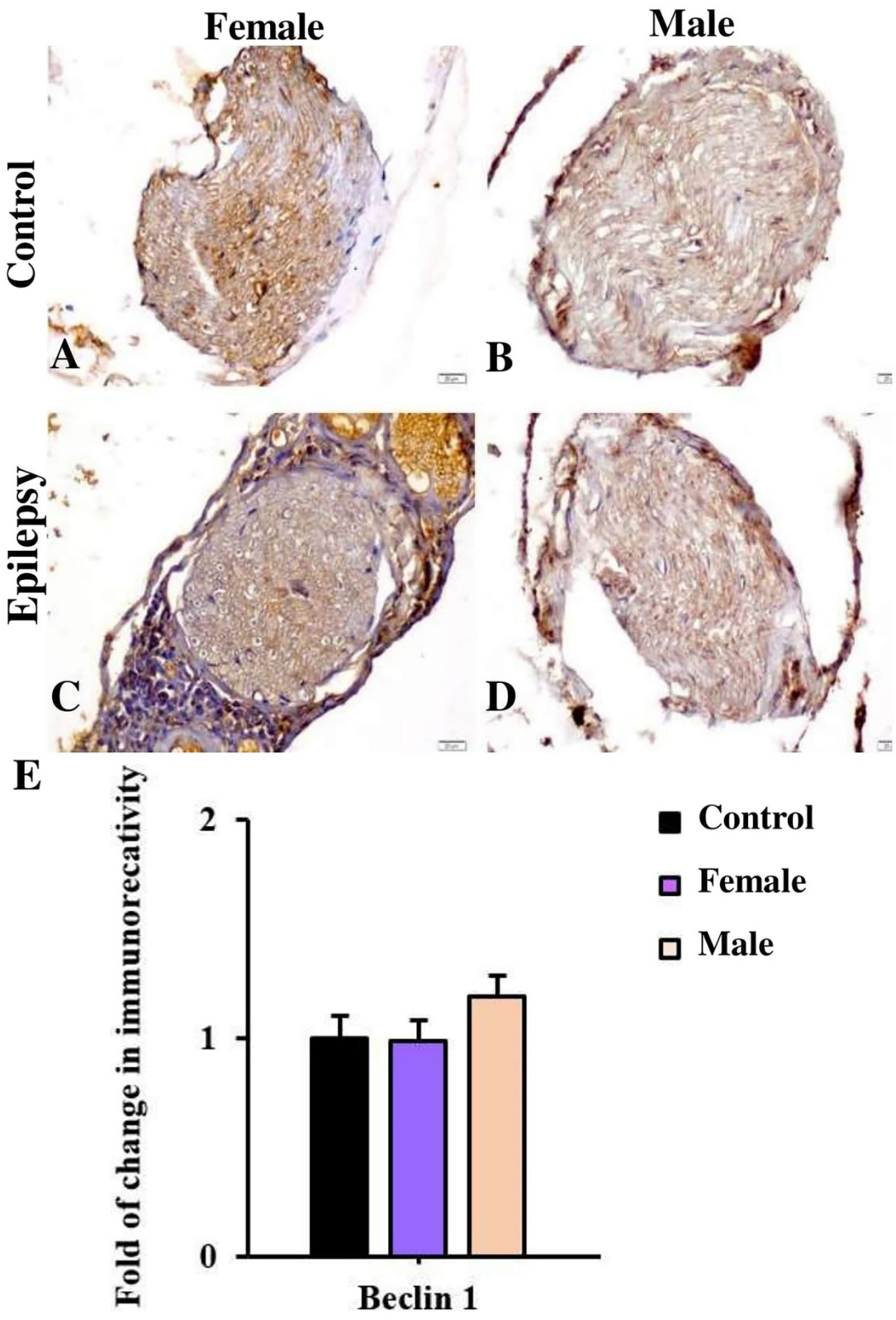

Fig. 11 Beclin 1 immunostaining images of the cervical vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. Beclin 1 immunoreactivity (E) in cervical vagus nerve samples in control group and PTZ rats with seizures. Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

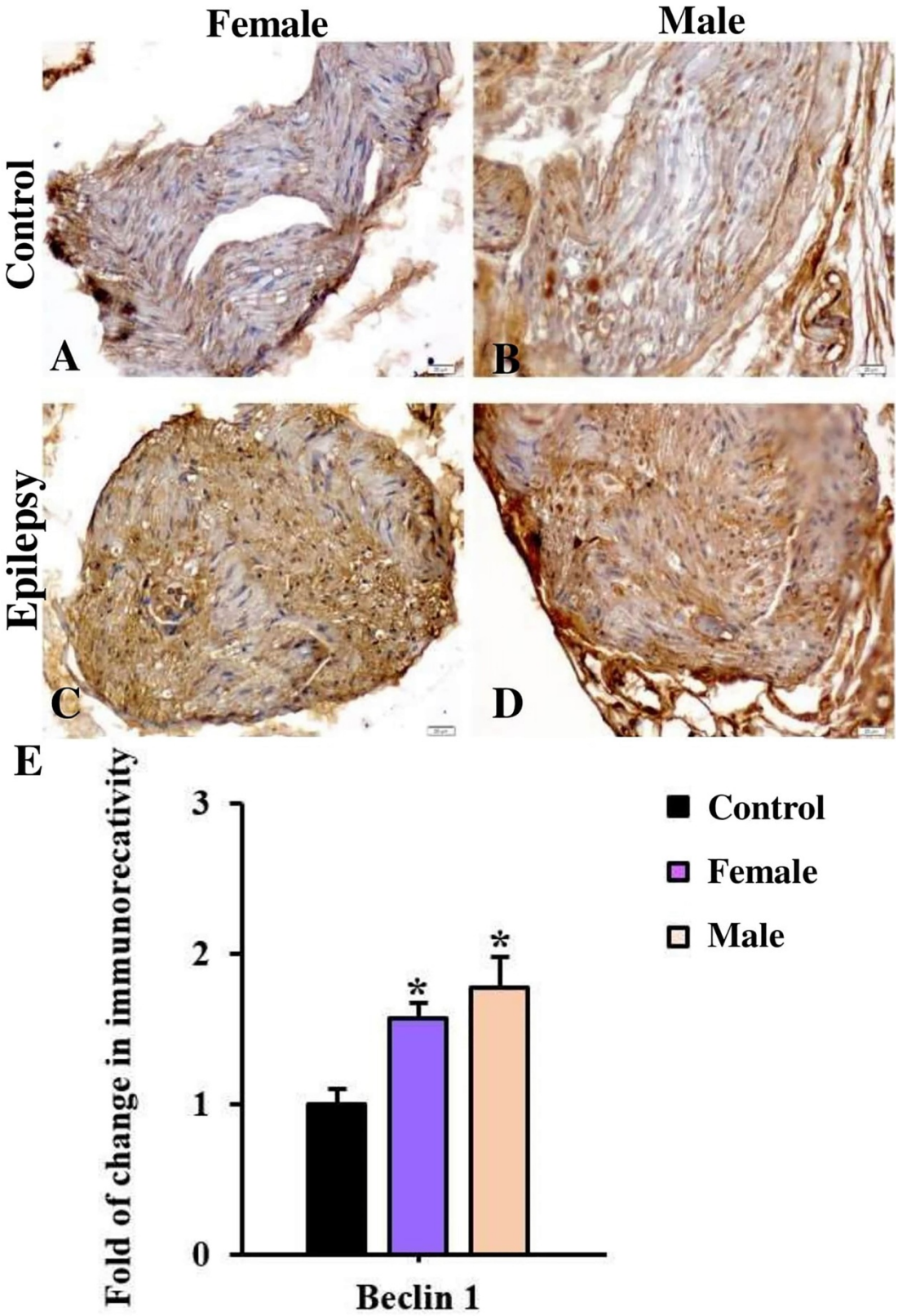

Fig. 12 Beclin 1 immunostaining images of the thoracic vagus nerve taken from female and male control group (A and B) and PTZ-kindled group (C and D) rats. Beclin 1 immunoreactivity (E) in thoracic vagus nerve samples in control group and PTZ-induced seizure rats. Images were taken at 40× magnification. Scale bar = 20 μm. *p<0.05.

In our study, the expression of Kir6.2 channel in the cervical and thoracic vagus nerve significantly increased in PTZ-kindled rats. This increase might be suggested decreasing the release of ACh from the vagus nerve. This claim is supported and consistent with audiogenic seizures induced in DBA/1 mice by suppressing efferent fibers of the vagus nerve, impairing respiratory and cardiac function [27]. In our previous study, we determined that the mRNA level of Kir6.2 channels in the heart tissue of rats showing electrocardiographic changes as a result of epileptic seizures significantly decreased [28]. Accordingly, our data may present a new approach to suppressing the parasympathetic effect in the heart as a result of epileptic seizures in terms of vagal Kir6.2 channel associated ACh release.

The change in the ACh receptor levels in the heart may reflect the activity of the vagus nerve [29]. Our previous data reveal that the muscarinic ACh receptor 2immunoreactivity increased in the atrial tissue of epileptic rats [30]. Supporting these data, we showed that immunoreactivity of α7nACh receptor increased in the heart of PTZ-group rats compared to control rats. High levels of ACh receptor immunoreactivity in the heart may indicate a decrease in ACh level due to increased vagal Kir6.2 channel immunoreactivity. In addition, chronic nicotine administration to chromaffin cells for 7 days in culture has been reported to result in high Kir6.2 channel expression. Nicotine-mediated increase in Kir6.2 channel production was inhibited due to the suppression of α7nACh receptors by the α-bungarotoxin agent [31, 32]. In this context, the high Kir6.2 channel immunoreactivity in the vagus nerve of PTZ-kindled rats may also be associated with increased vagal α7nAChR expression of female rats. In addition, increased Kir6.2 immunoreactivity by regulating ACh release from the vagus nerve may also affect autophagy mechanisms.

In the PTZ-kindled groups, there was a significant increase in Beclin 1 protein immunoreactivty in the cervical vagus nerve of rats.Increased Beclin 1 immunoreactivity observed in the cervical vagus nerve may cause cellular stress in the stimuli to the heart. Autophagic markers were significantly decreased after VNS in the ischemic heart model [33]. In our study, Beclin 1 levels were observed as significantly increased in the heart tissue in the PTZ-kindled group. Cell damage due to seizures in the cardiovascular system may result from the coordinated activation of inflammatory and autophagic pathways.

In summary, we found significant increases in the immunoreactivity of HMGB1, α7nAChR, Beclin 1 and Kir6.2 in the heart tissue of male and female rats kindling with PTZ. In this direction, our data may help to indicate HMGB1 an important target in understanding the relationship between cardiac pathology and inflammation observed in seizures. Thismay also provide a new perspective for understanding the mechanism of action of vagus nerve stimulation. In addition, we anticipate that the high Kir6.2 channel immunoreactivity in the vagus nerve of epileptic rats may be related to the increased expression of vagal α7nAChR. In addition, our Beclin 1 data, which have increased in addition to HMGB1, could provide evidence that the increased inflammation process in epilepsy can trigger autophagy. However, the data we have obtained should be supported by Western-Blot analysis and advanced functional research.

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