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CORRESPONDING ERDOSTEINE CHANGES AUTOPHAGY GENES EXPRESSION IN HIPPOCAMPUS ON RHINITIS MEDICAMENTOSA MODEL

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In our study, rats were subjected to Oxymetazoline hydrochloride treatment and Rhinitis medicamentosa (RM) was formed and then autophagy gene expression levels were determined after the application of an antioxidant agent erdosteine (ED). The rats were divided into three groups; Group 1 was the control group. Group 2 (RM) and group 3 (RM+ED) rats received two spray puffs of 0.05% oxymetazoline into the nasal cavities three times daily for eight weeks. After determination of RM in the rats, the RM group were killed. The ED+RM group received 10 mg/kg of an ED suspension. At the end of seven days, these rats were also killed. All groups' hippocampus tissues were obtained for the measurement of autophagy gene expressions. In rhinitis medicamentosa group *Atg5*, *Atg7* and *Atg10* gene expressions in the left hippocampus were reduced as compared to control group ($p=0.01$, $p>0.05$, $p=0.01$, respectively). Also, erdosteine treatments were restored mRNA expression of autophagy genes. In right hippocampus of rhinitis medicamentosa group, *Atg5* and *Atg10* gene expressions was found to be down-regulated as compared to control group ($p>0.05$, $p<0.05$, respectively). Both *BECN1* and *ULK* genes expression were found to be reduced in left hippocampus of rhinitis medicamentosa group. Erdosteine applications was restored the expression of these genes ($p=0.03$, $p=0.03$, respectively). Additionally, in right hippocampus, Erdosteine application was restored the expression of *ULK* gene ($p=0.01$).

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This is the first report that evaluated the expression autophagy genes in RM rat models and the changes observed after erdosteine applications.

Key words: Rhinitis medicamentosa; Autophagy genes; Hippocampus; Erdosteine; Oxymetazoline

INTRODUCTION

Autophagy is a physiological process known as cellular self-digestion that involves the formation of autophagosomes by fusion of lysosomes, cytoplasmic structures and organelles (MEIJER and CODOGNO, 2004). In case of nutrient depletion, autophagy plays a central role in the digestion of intracellular molecules and response to stress occurring in the cell, thus combats against many diseases (MIZUSHIMA *et al*., 2008). Free oxygen radicals are released from cell in stress conditions, and while optimum levels of these free radicals are required for the cell division processes, high levels of them may damages the cellular components (PELICANO *et al*., 2004; CHERUKURI and NELSON, 2008). Moreover, the relationship between these free radicals and autophagy is well known. In particular, the cellular ROS (reactive oxygen species) and autophagy levels advances in case of stress conditions such as starvation and this conditions is related to ATG4 protein which plays a key role in the autophagy (SCHERZ-SHOUVAL, *et al*., 2007). To clear the mechanisms how oxidative stress plays role in autophagy, several hypothesis were reported. Yet, a few of them have been proven to be play role in the regulation of autophagy (FILOMENI, *et al.*, 2015).

In previous studies, it was reported that the free oxygen radicals are generated in brain tissue under certain circumstances (TIEU *et al*., 2003; HEO and CAMPBELL, 2005; LIU *et al*., 2006). Also, a few studies were focused on the autophagic changes occurring in the brain under stress conditions. Oxymetazoline is a selective alpha-1 agonist and partial alpha-2 agonist topical decongestant. It is usually used in the treatment of epistaxis as a vasoconstrictor agent. Besides, it may result in a number of side effects due to the excessive use. Rhinitis medicamentosa is the most commonly observed side effect (TAS *et al*., 2005; SETTIPANE and KALINER, 2013) and the molecular mechnaism of Rhinitis medicamentosa have not been cleared yet (DOKUYUCU *et al*., 2014). It has been known that Oxymetazoline hydrochloride have negative effects on the learning activities of species by an unknown molecular mechanism. However, there is no study showing the effects of Oxymetazoline hydrochloride on the expression of autophagy genes in hippocampus. In our study, rats were subjected to Oxymetazoline hydrochloride treatment and Rhinitis medicamentosa was formed, then autophagy gene expression levels were determined after the application of an antioxidant agent erdosteine.

MATERIALS AND METHODS

The experimental protocol applied in the study was confirmed by the Institutional Animal Care and Ethics Committee of the Mustafa Kemal University (2014-11/8). Twenty-one male Wistar albino rats (12–16 weeks old) weighing 200 to 240 g were used in the study.

The rats were divided into three groups; Group 1 was the control group. This group received two spray puffs (0.05 cc each) of 0.9% saline solution into the nasal cavities three times daily for eight weeks. With the aid of a nasal adapter, the remaining 14 rats received two spray puffs (0.05 cc each) (Iliadin spray, Santa Farma Istanbul, Turkey) of 0.05% oxymetazoline into the nasal cavities three times daily for eight weeks (DOKUYUCU *et al*., 2014).

One of the 14 rats was sacrificed at the end of eight weeks and examined to confirm the development of RM. After determination of RM in the rats, the seven remaining rats (RM group/group 2) were killed, and hippocampus tissues were obtained for the measurement of autophagy gene expressions. The remaining seven rats (ED+RM group/group 3) received 10 mg/kg of an ED suspension (175 mg⁄5 mL) (Sandoz Drug GmBH, Istanbul, Turkey). At the end of seven days, these rats (group 3) were also killed, and hippocampus tissues were obtained for the measurement of autophagy gene expressions. The rats were sacrificed by administering 10 mg/kg of xilazine (Rompun, Bayer, Turkey) and 40 mg/kg of ketamine (Ketalar, Eczacibasi, Turkey), followed by cardiac puncture. They were decapitated, the skin was removed from the heads, and the hippocampus tissues were placed in –80°C for the measurement of autophagy gene expressions.

Gene expression by Real Time PCR

RNA isolation and cDNA preparation

Total RNA samples were isolated using a modified method of Qiagen (Mainz, Germany). They were then reverse - transcribed using a Roche AMV Reverse Transcription Kit according to manufacturer's instructions. Briefly, 10× Buffer RT (2.5 µL), $MgCl_2$ (4 µL), dNTP (2.5 µL), random nanomer (5.25 µL), RNase inhibitory (0.5 µL), AMV reverse transcriptase (0.5 µL), mRNA, and Rnase - free water were mixed to obtain cDNA. The reaction mixture was incubated at 45°C for 45 min for reverse transcription and heated at 94°C for 2 min. to inactivate the AMV reverse transcriptase. The cDNAs obtained were stored at −20°C until testing. The cDNAs were denatured at 94°C for 3 min, annealed at 59°C for 45 s (BMP - 6), at 63°C for 45 s (GDF - 15), at 61°C for 45 s (HJV), and at 59°C for 45 s (beta - actine), before extension at 72ºC for 30 s.

Statistical analysis

The collected data were analyzed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). A chi-square and Fischer's test were used for comparisons between categorical variables. The normal distribution of continuous variables was assessed with the Kolmogorov–Smirnov test. A one-way analysis of variance (ANOVA) and posthoc Tukey's significant difference test were used for comparisons of normally distributed continuous variables between more than two groups. A *P* value less than 0.05 was considered statistically significant for all the statistical data.

RESULTS

At the end of the experiment rats were sacrificed and autophagy gene expressions were determined. Results were presented in Table 1, Table 2, Fig. 1 and Fig. 2. In rhinitis medicamentosa group *Atg5*, *Atg7* and *Atg10* gene expressions in the left hippocampus were reduced as compared to control group ($p=0.01$, $p>0.05$, $p=0.01$, respectively). Also, erdosteine treatments were restored mRNA expression of autophagy genes. Only the *Atg7* expression was remained unchanged. In right hippocampus of rhinitis medicamentosa group, *Atg5* and *Atg10* gene expressions was found to be down-regulated as compared to control group ($p>0.05$, $p<0.05$, respectively). The expression of other genes showed differential expressions. In fig. 1, in the right hippocampus, all of the autophagy genes were found to be increased after erdosteine treatment. However, no significant differences were detected between the right and left hippocampus tissues.

Table 1. Measurements of autophagy genes in the groups. Used One Way Anova and Kruskal Wallis tests, Posthoc student t and Mann Whitney U tests

Genes		Control (1)	Rhinitis (2)	R + ED (3)	P values
Atg5					*1 vs 2 p=0.01
	Left	0.195 ± 0.078	0.062 ± 0.041*	0.170 ± 0.107	*2 vs 3 p=0.04
	Right	0.188 ± 0.052	0.148 ± 0.052	0.228 ± 0.195	NS
Atg7	Left	0.006 ± 0.004	0.003 ± 0.002	0.006 ± 0.004	NS
	Right	0.007 ± 0.003	0.008 ± 0.004	0.012 ± 0.010	NS
Atg10	Left	0.471 ± 0.279	0.085 ± 0.045*	0.291± 0.234	*1 vs 2 p=0.01
	Right	0.438 ± 0.357	0.131 ± 0.028^{+}	0.206 ± 0.146	$^{+}$1 vs 2 p= 0.04
BECN1	Left	0.026 ± 0.016	0.013 ± 0.012*	0.025 ± 0.010	*1 vs 2 p=0.04
	Right	0.021 ± 0.013	0.028 ± 0.018	0.027 ± 0.020	*2 vs 3 p=0.03
					NS
Ulk	Left	0.013 ± 0.007	0.007 ± 0.006*	0.016 ± 0.008	*2 vs 3 p=0.03
	Right	0.010 ± 0.002	0.013 ± 0.006^{+}	0.023 ± 0.014	$^{+}$2 vs 3 p= 0.01

p< 0.05 is significant. R + ED = Rhinitis + Erdosteine

Table 2. Comparison of left and right sides of hippocampus in the groups. Used paired student t and Wilcoxon tests. R + ED = Rhinitis + Erdosteine.

	Genes	Left	Right	P values
Atg5	Control	0.195 ± 0.078	0.188 ± 0.052	NS
	Rhinitis	0.062 ± 0.041	0.148 ± 0.052	NS
	R + ED	0.170 ± 0.107	0.228 ± 0.195	NS
Atg7	Control	0.006 ± 0.004	0.007 ± 0.003	NS
	Rhinitis	0.003 ± 0.002	0.008 ± 0.004	NS
	R + ED	0.006 ± 0.004	0.012 ± 0.010	NS
Atg10	Control	0.471 ± 0.279	0.438 ± 0.357	NS
	Rhinitis	0.085 ± 0.045	0.131 ± 0.028	NS
	R + ED	0.291± 0.234	0.206 ± 0.146	NS
BECN1	Control	0.026 ± 0.016	0.021 ± 0.013	NS
	Rhinitis	0.013 ± 0.012	0.028 ± 0.018	NS
	R + ED	0.025 ± 0.010	0.027 ± 0.020	NS
Ulk	Control	0.013 ± 0.007	0.010 ± 0.002	NS
	Rhinitis	0.007 ± 0.006	0.013 ± 0.006	NS
	R + ED	0.016 ± 0.008	0.023 ± 0.014	NS

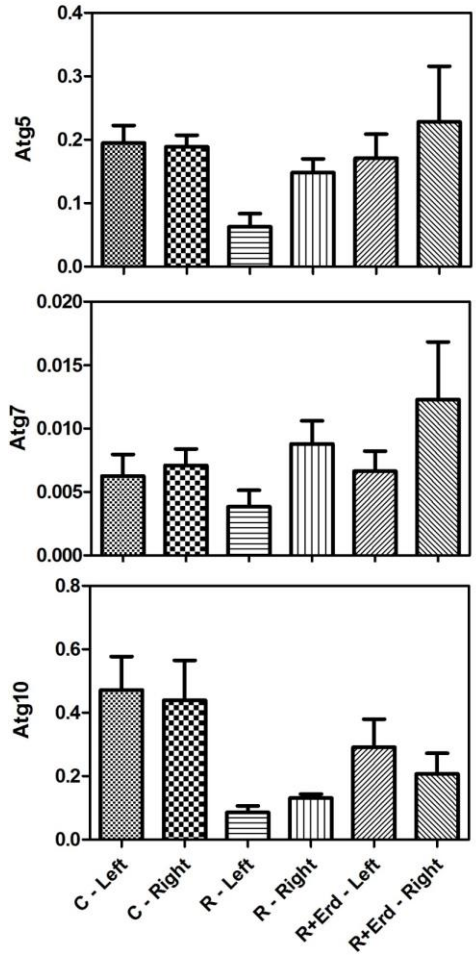

Fig 1. Atg 5-7-10 gene expression levels (C: Control; R: Rhinitis; ED: Erdosteine).

As presented in Table 1, Table 2 and Fig 2, both, *BECN1* and *ULK* gene expression were found to be reduced in left hippocampus of rhinitis medicamentosa group. Erdosteine applications was restored the expression of these genes (p=0.03, p=0.03, respectively). Additionally, in right hippocampus, Erdosteine application was restored the expression of *ULK* gene (p=0.01). No expression changes were observed in other genes. Moreover, *BECN1* expression was remained unchanged in both rhinitis and erdosteine groups.

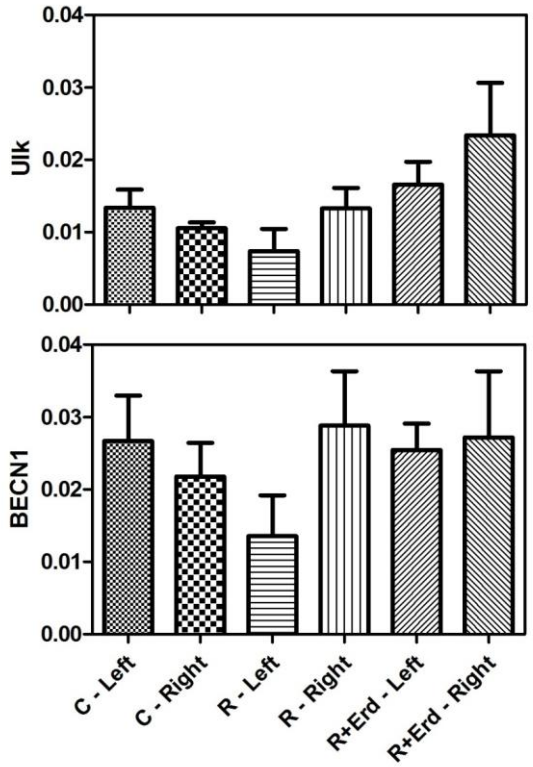

Fig 2. BECN1 and ULK gene expression levels (C: Control; R: Rhinitis; ED: Erdosteine).

DISCUSSION

Erdosteine (N-karboksimetiltioasetil-homosistein tiolakton) is a mucolytic agent developed for the treatment of chronic pulmonary diseases and exhibits antioxidant properties due to its sulfhydryl groups (MORETTI and MARCHIONI, 2007; RAHMAN, 2008). Erdosteine was determined to have neuroprotective properties in rat ischemia reperfusion models (OZEROL *et al*., 2009). It is reported that Erdosteine shows rapid antioxidant response to ROS (MORETTI and MARCHIONI, 2007; DOKUYUCU *et al*., 2014).

In the present study, expression levels of *Atg5*, *Atg7*, *Atg10*, *Ulk* and *BECN1* autophagy genes were evaluated in RM rat model before and after application of erdosteine. As presented in Figure 1 and 2, the expression levels of autophagy genes were found to be decreased in the left hippocampus of RM groups ($p<0.05$). In addition, the expression these genes were restored after erdosteine applications. Likewise, expression of the genes was found to be down-regulated in right hippocampus and erdosteine applications were restored the expression of these genes. However, the results were insignificant as compared to control group.

It has been reported that many of the antioxidants block the autophagy (UNDERWOOD *et al*., 2010). Application of the agents that stimulates the release of ROS increases the expression

of antioxidant stress response genes (DEWAELE *et al*., 2011; LISANTI *et al*., 2011). Also, autophagy acts as a cellular defence mechanism against ROS damage (DEWAELE *et al*., 2011; LISANTI *et al*., 2011). It is hypothesized that widespread dissemination of oxidative stress may reduce the activation of autophagy in neurons (GIORDANO *et al*., 2014).

In our study, expressions of the autophagy genes were down-regulated as a result of Oxymetazoline hydrochloride applications and antioxidant application were restored the gene expressions. This can be linked to blocking activity of erdosteine on ROS.

In conclusion, this is the first report that evaluated the expression autophagy genes in RM rat models and the changes observed after erdosteine applications. This study will shed light on the further studies. Also, subsequent histopathological examination of tissues, determining the ROS contents, and western blot studies to confirm expression results will be a great of interest in the further studies.

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PROMENE EKSPRESIJE AUOTFAG – GENA ERDOSTEINOM U HIPOKAMPUSU

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Izvod

U ispitivanjima pacovi su izloženi tretmanu oksimetazolin hidrohlorida i uspostavljen je medikamentozni model lečenja rinitisa (RM). Nivoi ekspresije autofag gena su determinisani posle primene antioksidansa Erdosteina (ED). Pacovi su podeljeni u tri grupe: Grupa br. 1 – kontrolna grupa, Grupa 2 (RM) i grupa 3 (RM+ED). Pacovi u grupi br.2 (RM) i u grupi br. 3 (RM+ED) su bili tretirani 0.05% oxymetazoline sprejom u nosnim kanalima tri puta dnevno u toku 8 nedelja. Posle determinacije RM kod pacova grupa 2 je ubijena. Pacovi u grupi tretiranoj erdosteinom (ED+RM) je primila 10 mg ED suspenzije. Posle tretmana od sedam dana pacovi su takođe ubijeni. Tkivo hipokampusa je uzeto od pacova svih grupa i vršeno je merenje ekspresije autofag gena

.. U grupi lečenoj od rinitisa, ekspresija *Atg5*, *Atg7* i *Atg10* gena je bila redukovana u levoj strani hipokampusa u poređenju sa kontrolnom grupom ($p=0.01$, $p>0.05$, $p=0.01$). Tretmanom erdosteinom izvršena je restoracija mRNK ekspresije autofag gena. U desnom hipokampusu lečenih pacova ekspresija *Atg5* i *Atg10* gena je bila smanjena u poređenju sa kontrolnom grupom ($p>0.05$ i $p<0.05$). Ekspresija *BECN1* i *ULK* gena je bila redukovana u levom kampusu lečenih pacova. Tretmanom erdosteinom izvršeno je obnavljanje ekspresije tih gena ($p=0.03$ I , $p=0.03$). Pore toga u desnom hipokampusu primenom erdosteina je izvršena restoracija ekspresije *ULK* gena ($p=0.01$).

Ovo su prvi objavljeni rezultati koji su potvrdili ekspresiju autofag gena u lečenju rinitisa kod pacova, kao i o promenama dobijenim posle primene erdosteina.

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