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Research Article

Effect of Dietary Orange Peel Essential Oil and Thermotolerance on Histo-morphometry and Serotonin-immunoreactive Endocrine Cell Numbers in the Small Intestines of Heat Stressed Japanese Quails

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Abstract

This study was conducted in order to measure the effects of early period thermal conditioning, feed restriction, supplementation of orange peel essential oil (OEO) into ration or combinations of them on small intestinal morphology and density of serotonin-immunoreactive (IR) endocrine cells (ECs) found in small intestines. 168 7-day-old Japanese quails were divided into six groups of 24-h fasting or thermal conditioning and their subgroups with and without supplementation of OEO (300 ppm) into ration. We determined that fasting and thermal conditioning increased villus height for duodenum in control groups and for jejunum in OEO groups. In addition, we detected that while fasting and thermal conditioning increased villus height/crypt depth (VH/CD) ratio in duodenum and jejunum, these applications did not affect this ratio in ileum. We found that supplementation of OEO into ration increased the number of serotonin-IR ECs in crypts of small intestine. We revealed that early period thermal conditioning increased the number of serotonin-IR ECs in duodenum, jejunum, and ileum especially in groups in which OEO was supplemented into ration. These results indicated that applications of early period thermal conditioning and feed restriction in quails may generally prevent adverse effects, caused by heat stress, on intestinal morphology.

Keywords: *Thermotolerance, Fasting, Orange peel essential oil, Intestinal morphology, Immunohistochemistry, Serotonin*

Yeme Eklenen Portakal Kabuğu Esansiyel Yağının ve Termotoleransın Sıcaklık Stresi Uygulanan Japon Bıldırcınlarında İnce Bağırsak Histo-morfometrisine ve Serotonin-immunoreaktif Endokrin Hücre Sayısına Etkisi

Özet

Bu araştırma erken dönem termal koşullandırmanın, yem kısıtlamasının, rasyona portakal kabuğu esansiyel yağı (PEY) ilavesinin veya bunlarının kombinasyonlarının ince bağırsak morfolojisi ve ince bağırsaklardaki serotonin-immunoreaktif (IR) endokrin hücrelerin yoğunlukları üzerine olan etkilerinin ölçülmesi için yapıldı. 168 adet 7 günlük yaşta Japon bıldırcınları, 24 saatlik açlık veya sıcaklık stresi uygulaması ve bunların rasyona PEY (300 ppm) eklenen veya eklenmeyen alt grupları olacak şekilde altı gruba ayrıldı. Yem kısıtlaması ve termal koşullandırmanın duodenum için kontrol gruplarında, jejunum için ise PEY gruplarında villus yüksekliğini artırdığı saptandı. Ayrıca yem kısıtlaması ve termal koşullandırmanın duodenumda ve jejunumda villus yüksekliği/kript derinliği (VY/KD) oranını artırırken, bu uygulamaların ileumda bu oranı etkilemediği belirlendi. Rasyona PEY ilavesinin tüm ince bağırsak kriptlerindeki serotonin-IR endokrin hücre sayısını arttırtığı tespit edildi. Erken dönem termal koşullandırmanın özellikle rasyona PEY eklenen gruplarda duodenum, jejunum ve ileumdaki serotonin-IR endokrin hücre sayısını arttırdığı gösterildi. Bu bulgular, bıldırcınlarda erken dönem termal koşullandırma ve yem kısıtlaması uygulamalarının sıcaklık stresinin neden olduğu intestinal morfoloji üzerindeki olumsuz etkilerini genel olarak önleyebileceğini göstermektedir.

Anahtar sözcükler: *Termotolerans, Açlık, Portakal kabuğu esansiyel yağı, İntestinal morfoloji, İmmunohistokimya, Serotonin*

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INTRODUCTION

During the past 20 years, animal production has increased particularly in tropical and subtropical regions all over the world [1]. Production of farm animals in tropical and subtropical regions is affected by several factors. Climate of these regions is known as one of primary factors restricting the production efficiency [2].

Heat stress is the major problem to be taken into consideration for production of poultry animals and significantly affects animal health and productivity [3]. Because poultry animals have no sweat gland, they increase their respiratory rate up to 140-170 to stabilize their body temperature at high ambient temperatures, and consequently respiratory alkalosis occur. Depending on these changes in bloodstream, decreases are observed in feed consumption, body weight gain, and feed conversion ratio. For example, in case that ambient temperature exceeds 30°C, feed consumption decreases at the rate of 1-6% per each increase of 1°C [4].

Epithelium of small intestine consists of continuously renewable clusters of cells. Stem cells are located in crypt region, they form enterocytes by migrating towards villi, and progress to the end points of villi [5,6]. Heat stress also causes some changes specific to gastrointestinal tissue like decrease in intestinal epithelial integrity [7].

Lots of literature information is found concerning possible techniques to eliminate adverse effects caused by heat stress in broiler chicks. One of the practical approaches is that high ambient temperature is applied at early periods of life; promising results were obtained by this way. Thermal conditioning applied at the early age period causes a decrease in weight gain during the first week of life; on the other hand, it leads to faster growing when compared to unconditioned chicks at the following process and enable them to reach higher body weight at marketing age [8-10]. The main purpose of thermal conditioning is to facilitate adaptation of chicks to the ambient in unexpected acute temperature attacks at older ages by activating temperature regulation mechanisms which have not developed yet at the early period of life [8].

One of the methods frequently applied in order to reduce adverse effects of high ambient temperature on performances of poultry animals is dietary changes. Several studies reveal that vitamin A, vitamin E, vitamin C, organic acid, prebiotic, probiotic supplementation to feeding rations of broiler chicks prevent the effects caused by temperature [11-14]. Orange oil (essence), is an essential oil produced by cells in peel of orange fruit *(Citrus sinensis fruit)*. Compared to numerous essential oils, orange oil is extracted as a by-product via centrifugation in production of orange juice and produced as cold pressed oil. A great majority of its components consists of d-limonene (more than 90%) and a very little part of them is β-myrcene (2-2.1%) [15]. In some studies conducted recently, orange peel essential oil (OEO) was used as antiparasitic [16], antifungal [17], antioxidant, antimicrobial, and growth regulator [18] agent.

Japanese quails *(Coturnix coturnix japonica)*, are produced as a source of meat and egg in several countries of the world. They are preferred more as an animal model in biological studies compared to other domesticated poultry animals because they reach sexual maturity in early period and have high egg laying rates, low feed and area requirements, and the most importantly they display high durability against environmental conditions [19].

In previous years, several studies [13,14,18] in which various herbal nutritional supplements were used were conducted in order to decrease numerous adverse effects caused by heat stress, including their effects on morphological structures of small intestines. Upon the deficiency we observed as a result of literature reviews; in this study, it was aimed to measure the effects of early period thermal conditioning, fasting, supplementation of OEO or combinations of them on small intestine morphology and density of serotonin-immunoreactive (IR) endocrine cells (ECs) found in small intestines.

MATERIAL and METHODS

Animal, Feeding and Experimental Design

This study was approved by Fırat University Committee of Animal Welfare and Use. Experimental procedure and protocol of the study was found appropriate by the committee (FUHADEK) (FUHADEK, Approval Number: 04.04.2013/55). 168 7-day-old Japanese quail chicks used for this study were weighed and divided randomly. With a totally random design, 4 replicate clusters (30x80 cm) were formed by dividing them into 6 groups including 7 chicks in each cage. Then, fasting and thermal conditioning among early period stress factors were applied to 4 groups for 24 h. While two of these 4 groups were exposed to fasting, other two were applied thermal conditioning (36±1°C and 70-80%, relative humidity). Accordingly, the groups were formed as follows: 1) The group without stress and supplementation (Negative Control) (NC), 2) Fasting group (F), 3) The thermal conditioning group (TC), 4) The group with supplementation of OEO and without stress (Positive control) (PC+OEO), 5) The group that was subjected to fasting and supplemented with OEO (F+OEO), 6) The group that was applied thermal conditioning and supplemented with OEO (TC+OEO). The temperature stress of 33±1°C was applied to all groups for 6 h on 42th day at the end of application.

Following the early period stress factors applied, conventional production and enterprise procedures were carried out until 42th day (for 35 days). The quails were exposed to continuous light, fed and given water *ad libitum*. The diet was calculated in such a way to meet feed

requirements of Japanese quails (24% HP and 2900 kcal/kg ME for beginning and growth periods) in accordance with recommendation of National Research Council [20]. 1 kg zeolite was supplemented as carrier for each 100 kg basal diet. In essential oil groups, 30 g OEO was mixed with 970 g zeolite in order to provide an essential oil concentration of 300 ppm and added into basal diet. Substances found in diet and composition of the diet were given in *Table 1*. The concentration of the volatile component of OEO was shown in *Table 2*.

Chemical composition of food contents (dry matter, crude protein, ash, and ether extract) was analyzed in accordance with AOAC procedures [21] and the amount of crude cellulose was determined according to methods of Crampton and Maynard [22].

Table 1. *Ingredients and chemical composition of standard diet (g/kg)*

Feed Ingredients	Starting and Growing	Calculated Analysis	
Maize	564.3	Crude protein	236.0
Soybean meal	315.0	ME, MJ/kg	12.7
Vegetable oil	30.0	Ether extract	46.5
Fish meal	58.0	Crude cellulose	25.5
Dicalcium phosphate	8.0	Crude ash	63.5
Calcium carbonate	8.0	Calcium	8.1
Salt	2.5	Available Phosphorus	3.6
DL-Methionine	0.5	Methionine + Cystine	8.4
L-Lysine	0.2	Lysine	13.9
Vitamin-Mineral Premix*	2.5		
Zeolite**	10.0		
Total	1000.0		

** Provided per kg of diet: retinol, 2.64 mg; cholecalciferol, 0.04 mg; dl-a-tocopherol-acetate, 11 mg; riboflavin, 9.0 mg; pantothenic acid, 11.0 mg; vitamin B_{12}, 0.013 mg; niacin, 26 mg; choline, 900 mg; vitamin K, 1.5 mg; folic acid, 1.5 mg; biotin, 0.25 mg; iron, 30 mg; zinc, 40 mg; manganese, 60 mg; copper, 8 mg; selenium, 0.2 mg; ** No added orange peel essential oil groups (10 g zeolit); 300 ppm orange peel essential oil added groups (3 g orange oil + 7 g zeolite)*

Table 2. *The concentration of the volatile components in orange peel essential oil (%)*

Analysis	Result*
Limonene	92.3%
Beta Myrcene	3.3%
Alpha Pinen	1.4%
Linalool	0.9%
Sabinen	0.6%
Delta 3 Caren	0.2%
Octanal	0.2%
Undefined	1.1%

** obtained by GC-MS analysis*

Morphometric Analyses

At the end of experiment, 10 quails from each experimental groups that had body weights near to group averages were decapitated by cutting jugular veins. Tissue parts were taken from segments of small intestine through abdominal dissection and divided into 3 segments; duodenum, jejunum, and ileum. 2-cm long tissue parts taken from middle points of each small intestine segments (duodenum, jejunum, and ileum) were washed with cold sterile saline solution. Then, they were fixed with formalin containing 10% neutral buffer. Fixed tissues were washed respectively, dehydrated through series of increasing alcohol concentrations, clearing through series of xylol, and embedded within paraffin blocks. 5-7 µm-thick cross sections taken by using a microtome were placed on glass slides covered with normal and poly-L-lysine. Cross sections placed on normal glass slides were processed according to conventional hematoxylin and eosin staining method [23]. Finally, they were examined by using light microscope (Olympus CX31, Olympus USA) equipped with digital imaging system (Olympus DP20, Olympus USA).

In all morphometric examinations, villus heights, villus bottom widths, villus edge widths and crypt depths related to villi were measured from 7 consecutive villus-crypt complex. Villus heights [AB] was measured form joining point of villi and crypts to top points of villus. Crypt depths [CD] was measured from the middle point of two neighboring villi to base of crypts. While villus edge widths [EF] was measured from just below top point of villus, villus bottom widths [GH] was measured from just above the joining point of villi and crypts *(Fig. 1)*. By using the measured values, ratios of villus height/crypt depth (VH/CD) were also calculated [24].

Immunohistochemical Analysis

Deparaffinization of sections placed on glass slides covered with poly-L-lysine was performed. They were passed through series of alcohol in decreasing concentration beginning from absolute alcohol. The samples were boiled in citrate buffer (10 mM citric acid, pH 6.0) for 20 min in order to enhance antigenity of the ECs. Immunohistochemical analysis was carried out according to protocol of Histostain®-Plus 3rd Gen IHC Detection Kit (Invitrogen: Cat. No. 85-9673). They were incubated with rabbit-anti serotonin (Zymed Lab., 18.0077) antibody at 4°C for over-night in order to determine presence of serotonin-IR ECs in duodenum, jejunum, and ileum of the quails. Immuno-staining was ensured to occur by treating sections with DAB chromogen found in kit for 5 min at the end of protocol. Mayer's hematoxylin was used as counterstain and sections were covered with coverslips by entellan. Sections were examined with Olympus CX31 microscope and photographs were taken. The IR ECs on each section were counted at 10x40 times magnification. The mean numbers of IR ECs in each sample obtained

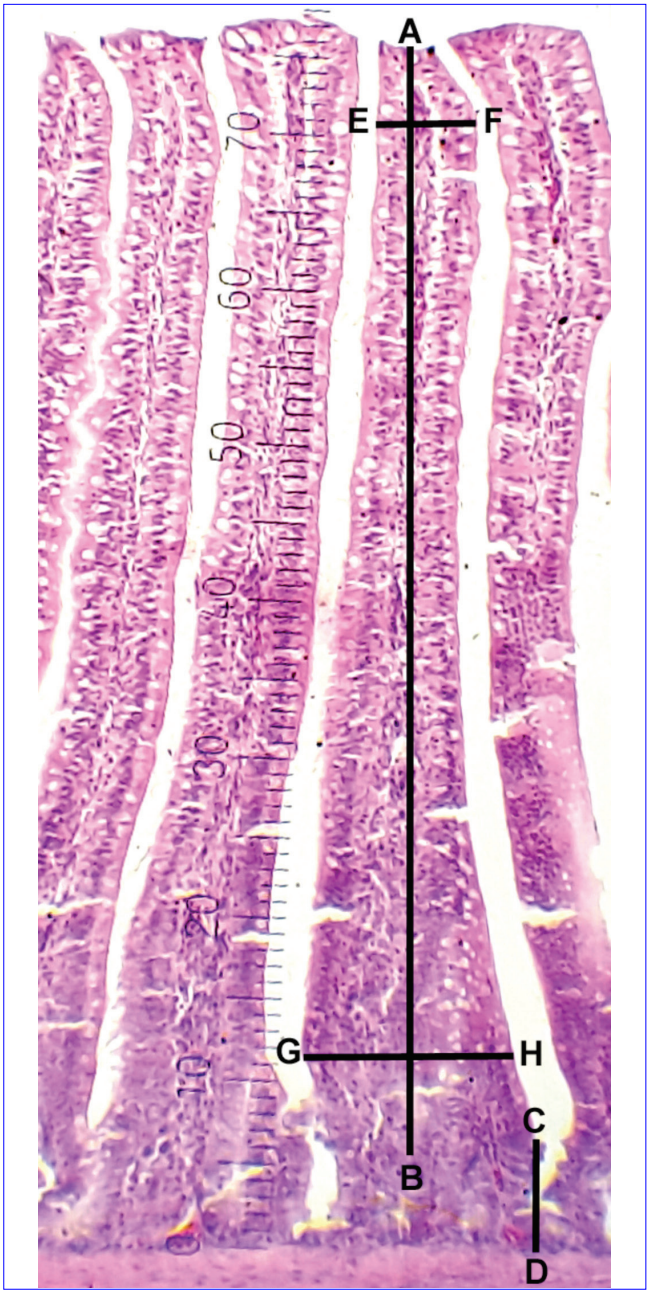

Fig 1. Section of the jejunum (thermal conditioned and OEO supplemented group), haematoxylin-eosin stained, showing the measured parameters. Villus height [AB], crypt depth [CD], villus edge widths [EF] and villus bottom widths [GH], (x 200)

from small intestines were determined by counting the IR ECs in 10 randomly selected microscopic fields with 40x magnification (quantification of IR ECs number/ microscopic field).

Statistical Analysis

The data were subjected two-way anova analysis by using GLM (General Linear Model) procedure. Then, significant differences were subjected to Duncan's multiple range test [25]. The results were accepted as significant if *P* values were < 0.05.

RESULTS

Morphometric Results

Morphometric changes caused by feed restriction, early period thermal conditioning, and OEO supplementation into ration in small intestines of quails were showed in *Table 3*.

It was determined in the present study that feed restriction and early period thermal conditioning significantly increased the villus heights of duodenum in control feeding group compared to negative control group ($P<0.001$). On the other hand, supplementation of OEO into ration significantly decreased villus heights of duodenum compared to control group ($P<0.001$). The longest villus height of duodenum in groups with OEO supplemented into ration was found in feed restriction group ($P<0.001$). Early period thermal conditioning was also determined to have a reducing effect on duodenum crypt depth in groups with control feeding ($P<0.05$). It was observed that there was an interaction between application and OEO in terms of duodenum crypt depth. Feed restriction and early period thermal conditioning increased duodenum VH/CD ratio significantly ($P<0.001$). OEO supplemented into ration decreased VH/CD ratio significantly ($P<0.001$). A difference was not found between feed restriction and early period thermal conditioning in terms of duodenum VH/CD ratio ($P>0.05$). Interaction between OEO supplemented into ration and applications was found to be significant.

It was determined that feed restriction and early period thermal conditioning increased significantly jejunum villus height in groups with OEO supplemented into ration ($P<0.001$). OEO supplemented into ration also increased villus edge width ($P<0.05$) and crypt depth ($P<0.01$) of jejunum. While feed restriction and early period thermal conditioning significantly increased VH/CD ratio of jejunum ($P<0.01$), OEO supplemented into ration did not affect this ratio ($P>0.05$).

Ileum villus height was found to increase in groups with OEO supplemented into ration ($P<0.01$). The highest ileum villus height in both control feeding and OEO supplemented groups was observed in early period thermal conditioning application ($P<0.001$). It was determined that OEO supplemented into ration decreased villus bottom width ($P<0.01$) and edge width ($P<0.05$) in ileum. The effect of applications on ileum VH/CD ratio was not detected ($P>0.05$).

Immunohistochemical Results

The effect of feed restriction, early period thermal conditioning, and OEO supplemented into ration on serotonin-IR ECs number found in small intestines *(Fig. 2)* of quails was showed in *Table 4*.

It was observed that early period thermal conditioning increased the number of cells in jejunum in control feeding groups (P<0.001), but decreased the number of cells in ileum (P<0.05). Early period thermal conditioning increased the number of serotonin-IR ECs in crypts of duodenum (P<0.001), jejunum (P<0.001), and ileum (P<0.05) in groups with OEO supplemented into ration. OEO supplemented into ration increased the serotonin-IR ECs number in crypts of duodenum (P<0.001), jejunum (P<0.001), and ileum (P<0.05).

Table 3. *Effects of orange peel essential oil supplementation and thermotolerance on histo-morphometric features of small intestines, (μm) Mean±S.E.M., (n=60)*

Histological Parameters	Control			Orange Peel Essential Oil, 300 ppm			P		
	NC	F	TC	PC	F	TC	O	T	O x T
Duodenum									
Villus height	825.71±21.61^{B}	999.95±23.58^{A}	939.04±30.89^{A}	659.53±27.02^{b}	807.47±21.87^{a}	699.42±23.21^{b}	***	***	NS
Villus bottom width	158.85±9.44	152.96±10.06	173.80±13.23	154.63±7.03	160.29±10.56	161.31±12.02	NS	NS	NS
Villus edge width	99.71±5.76	106.88±5.29	112.14±4.46	103.42±4.21	105.30±6.11	102.32±5.26	NS	NS	NS
Crypt depth	147.1±11.56^{A}	105.80±4.46^{AB}	102.14±3.17^{B}	127.49±6.35	122.70±4.22	116.09±5.83	NS	*	*
Villus height/Crypt depth	5.61±0.56^{B}	9.45±0.69^{A}	9.19±0.59^{A}	5.17±0.39^{b}	6.58±0.30^{a}	6.02±0.43^{a}	***	***	*
Jejunum									
Villus height	522.25±18.34	563.94±28.70	541.12±17.98	453.50±16.09^{b}	575.84±17.31^{a}	575.38±16.53^{a}	NS	***	NS
Villus bottom width	107.26±7.56	189.61±7.29	84.16±5.10	107.75±5.64	111.51±5.44	107.32±4.64	NS	NS	NS
Villus edge width	77.49±4.24	73.15±4.16	71.54±2.62	74.74±3.28	82.42±4.22	82.11±3.42	**	NS	NS
Crypt depth	83.80±6.23	86.70±5.80	78.03±3.43	95.39±5.37	83.06±3.17	86.07±4.48	*	NS	NS
Villus height/Crypt depth	6.23±0.59	6.50±0.91	6.93±0.84	4.75±0.36^{b}	6.93±0.35^{a}	6.68±0.38^{a}	NS	**	NS
Ileum									
Villus height	343.04±9.76^{B}	355.91±16.32^{AB}	409.69±14.57^{A}	375.28±12.72^{b}	410.61±12.41^{ab}	436.96±18.10^{a}	**	***	NS
Villus bottom width	98.99±4.56	98.95±6.20	89.12±5.26	86.39±3.31	83.22±4.04	81.28±4.77	**	NS	NS
Villus edge width	76.86±3.77	75.15±4.52	73.92±3.33	71.41±2.95	59.73±2.21	71.6±2.76	*	NS	NS
Crypt depth	50.49±2.69	56.08±3.49	65.67±3.53	64.52±2.61	62.21±2.39	59.54±3.80	NS	NS	NS
Villus height/Crypt depth	6.79±0.41	6.34±0.74	6.23±0.46	5.81±0.53	6.60±0.44	7.33±0.46	NS	NS	NS

NC: *Negative control,* ***F:*** *Fasted, TC: Thermal conditioned,* ***PC:*** *Positive control,* ***O:*** *Oil,* ***T:*** *Treatment,* ***NS:*** *Not significant, * P<0.05, ** P<0.01, *** P<0.001; -The differences between the mean values with different superscripts (A, B: P<0.05) in the same row within the no added groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests. -The differences between the mean values with different superscripts (a, b: P<0.05) in the same row within the orange peel essential oil groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests*

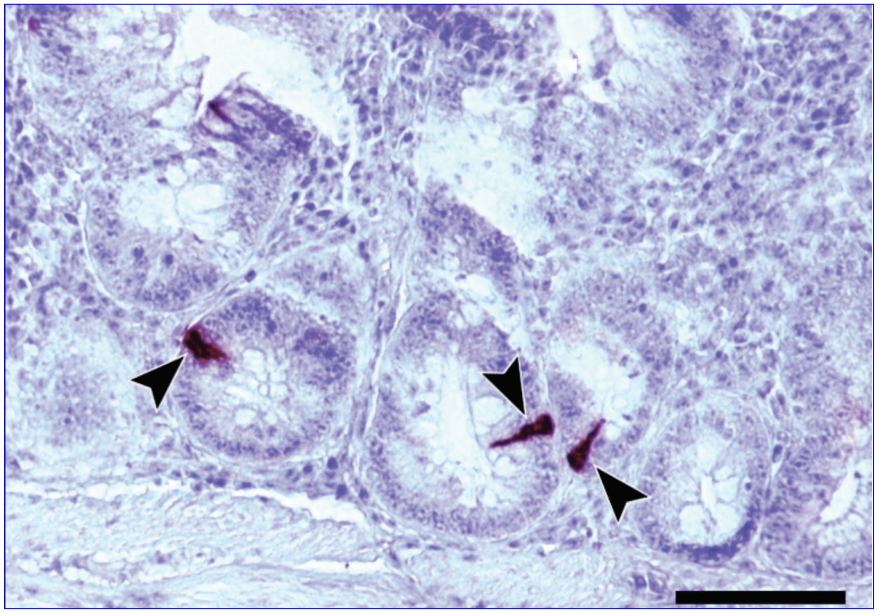

Fig 2. Serotonin-immunoreactive endocrine cells *(arrowheads)* showed in the crypt epithelium of the duodenum (negative control group)

Table 4. *Effects of orange peel essential oil supplementation and thermotolerance on serotonin-releasing endocrine cell numbers of small intestines, (μm) Mean±S.E.M. (n=60)*

Parts of Small Intestine	Control			Orange Peel Essential Oil, 300 ppm			P		
	NC	F	TC	PC	F	TC	O	T	O x T
Duodenum	4.22±0.32	2.96±0.28	3.70±0.31	4.16±0.40^{b}	4.00±0.28^{b}	7.24±0.38^{a}	***	***	***
Jejunum	1.32±0.15^{B}	0.74±0.14^{B}	3.10±0.46^{A}	2.20±0.49^{b}	1.82±0.23^{b}	3.00±0.31^{a}	*	***	NS
Ileum	3.16±0.46^{A}	2.10±0.28^{AB}	1.80±0.24^{B}	2.40±0.28^{b}	2.28±0.18^{b}	4.13±0.51^{a}	*	*	***

NC: *Negative control,* ***F:*** *Fasted,* ***TC:*** *Thermal conditioned,* ***PC:*** *Positive control,* ***O:*** *Oil,* ***T:*** *Treatment,* ***NS:*** *Not significant, * P<0.05, ** P<0.01, *** P<0.001; -The differences between the mean values with different superscripts (A, B: P<0.05) in the same row within the no added groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests. -The differences between the mean values with different superscripts (a, b: P<0.05) in the same row within the orange peel essential oil groups are statistically significant according to the ANOVA and post-hoc Duncan's multiple range tests*

DISCUSSION

Intestinal villi are projections that protrude from mucosa of small intestine towards lumen in order to extend the surfaces of digestion and absorption [26,27]. All epithelial cells in villi form by mitotic division of stem cells found in base parts of intestinal crypts and they can reach up to top of villus within a few days by migrating throughout the surface of villus. Such a cellular migration can be altered by the intestinal function [26]. Intestinal crypts are glands found in lamina propia and consist of different cell types including hormone secreting ECs [27]. Intestinal mucosa can activate rapid morphological and functional adaptation mechanisms in response to environmental and nutritional changes [28]. A great majority of intestinal morphology studies conducted in recent years indicate that heat stress decreases villus heights of duodenum [29-32], jejunum [30,32], and ileum [30,32,33], and villus width of duodenum and jejunum [31] in poultry animals. Decreases observed in villus heights and villus widths may be associated with feed intake reduced depending on heat stress [29]. Differently, Burkholder et al.[7] reported that heat stress of 24 h did not change villus height and VH/CD ratio, but decreased crypt depth. On the other hand, Santos et al.[32] determined that heat stress increased villus width in duodenum, decreased VH/CD ratio in duodenum and ileum, and increased crypt depth in jejunum. Sandıkçı et al.[29] showed that heat stress also did not have any effect on villus width of duodenum. Differences between our results and previous studies may be related to starting age and application period of heat stress.

In numerous studies, it was reported that early period thermal conditioning adjusted the body weight gain decreasing due to heat stress [8,34], increased the decreasing slaughter body weight [34,35], caused decreasing plasma T_3 concentration [8,36], decreased the high body temperature [8,37], and decreased the mortality at high temperature applied before slaughtering [8]. When literature information were examined in details, a limited number of study were found about the effects of thermal conditioning and feed restriction on morphology of small intestine. El-Badry et al.[38] showed that early period thermal conditioning increased villus height of jejunum but did not change the crypt depth when compared to control group. Uni et al.[36] determined that early thermal conditioning narrowed villi in jejunum just 24 h after the application and increased the distance between neighboring villi, and accordingly decreased villus volume significantly. However, they reported a rapid increase was observed in villus volume and the highest villus volume was observed 48-72 h after thermal conditioning. The present study reveals that early period thermal conditioning increased the villus height of duodenum and ileum, and did not change the villus height of jejunum in groups with control feeding compared to negative control group. Early period thermal conditioning and feed restriction increased duodenum VH/CD ratio significantly. Early period thermal conditioning increased significantly villus height of jejunum and ileum in groups with OEO supplemented into ration. On the other hand, thermal conditioning had a reducing effect on duodenum crypt depth in groups with control feeding *(Table 3)*. When the results were compared, it was observed that while there was agreement in terms of villus height, a difference was present in terms of crypt depth. This difference may be arising from the age at which thermal conditioning was applied or difference in degree of temperature applied.

In a study conducted on broiler chicks, it was reported that body weight was suppressed at first depending on feed restriction, it was balanced with control group as a result of rapid compensatory growth observed in following process, plasma T_3 concentration was suppressed, mortality occurring due to being exposed to temperature on 42th day decreased [10]. Numerous light microscopy studies also reported that fasting applications caused a decrease in intestinal villus heights. However, the decrease in villus height observed based on fasting was stated to recover depending on nutrition 1 day after re-feeding [26,39]. Yamauchi et al.[41] determined that 24-h feed restriction at early age period decreased significantly villus heights of duodenum and gradually those of jejunum, but a significant decrease was not observed in ileum. In the same study, they reported that a distinct increase was seen for all intestinal villus heights just 24 h after re-feeding started following the feed restriction. They observed that recovery in villus heights occurred rapidly in duodenum but slowly

in ileum. Slow recovery of ileum villus height may indicate that ileum could be less effective in absorptive function. In accordance with the results in these studies, we determined that feed restriction increased villus heights of duodenum significantly in control feeding groups compared to negative control group and significantly villus heights of jejunum in groups with OEO supplemented into ration, but did not cause any change in ileum. In previous studies, feed restriction was reported to decrease significantly mitotic division in intestinal cells. Transition to re-feeding also increases mitotic division, thus activates cell renewal and makes it to reach control values. According to these researchers, the change in villus height occurred as a result of the increase in number of epithelial cells depending on increased mitosis in cells [40,41].

In a recent study conducted on broilers fed under high ambient temperature; it was reported that extracts of orange and lemon peels supplemented into ration did not affect villus height, villus depth, crypt width, and VH/CD ratio [42]. On the other hand, in the present study, we determined that OEO supplemented into ration decreased villus heights of duodenum compared to control group and increased villus heights of ileum. We also determined that OEO supplemented into ration increased villus end width and crypt depth of jejunum, but decreased both villus edge width and bottom width in ileum. We observed there was interaction between application and OEO in terms of duodenum crypt depth. While supplementation of OEO into ration decreased VH/CD ratio in duodenum, VH/CD ratio did not change in jejunum. Differences between results of these two studies may be associated with the amount of herbal supplement added into feed, different starting age, and different time of supplementation. It was reported in another study that a mixture of oregano, cinnamon, and pepper essential oils (200 mg/kg) increased villus height [43].

Serotonin (5-hydroxytryptamine, 5-HT), is a monoamine neurotransmitter. It is released from serotonergic neurons of central nervous system and enterochromaffin cells of gastrointestinal tissue. Approximately 95% of peripheral serotonin is released and stored by enterochromaffin cells found in crypts in gastrointestinal tissue. In addition to the other functions of serotonin, it also has functions related to cardiovascular and gastrointestinal tract smooth muscles, and cell growth and differentiation. Serotonin activates neural reflexes and plays a vital role in intestinal secretion, sensitivity, and peristaltic movements [44]. In rats, it was stated that while feed restriction of 24 and 48 h caused a decrease in brain serotonin level, it led to an increase in gastrointestinal serotonin level [45]. When we reviewed the literature, we did not encounter any study concerning the effects of early period thermal conditioning and supplementation of OEO into ration on serotonin-IR ECs in intestinal tract. In the present study, it was determined that thermal conditioning increased the number of serotonin-IR ECs in jejunum, but decreased in ileum in groups with control feeding. It was found that supplementation of OEO into ration increased the number of serotonin-IR ECs in all crypts of small intestine. Early period thermal conditioning increased the number of serotonin-IR ECs in duodenum, jejunum, and ileum especially in groups with OEO supplemented into ration. Şimşek et al.[46] reported that prebiotics/probiotics and/or organic acids and antibiotics supplemented into feeds of quails caused a significant decrease in the number of serotonin-IR ECs in all intestinal segments compared to control group. The difference between results is likely resulted from difference of supplements supplemented into ration.

Consequently, in this study, we examined the effects of thermal conditioning, feed restriction, supplementation of OEO into ration or combinations of them on morphology of small intestine and the number of serotonin-IR ECs in small intestine. We determined that feed restriction and thermal conditioning increased villus height in control groups for duodenum and in OEO groups for jejunum. We also found that while feed restriction and thermal conditioning increased VH/CD ration in duodenum and jejunum, these applications did not affect this ratio in ileum. Early period thermal conditioning, particularly supplementation of OEO into ration increased the number of serotonin-IR ECs in all crypts of small intestine. We showed that supplementation of OEO into ration increased the number of serotonin-IR ECs in duodenum, jejunum, and ileum in groups with OEO supplemented into ration. In addition, the effects of early period thermal conditioning, feed restriction, and herbal extract essential oils especially on serotonin-IR ECs should be supported by new results in future further studies.

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