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The Relationship Between Sod1 And Hsp70 Expression in Broiler Ileum Throughout Post-Hatching Development

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Abstract

Heat shock proteins (Hsps) are molecular chaperones that play critical functions in the survival and development of cells. Hsps influence adaptive and innate immune responses and may promote cross-talk between the two systems. Superoxide dismutases (SODs) are metalloenzymes that play an essential role in the body's defense against oxidative stress by efficiently removing excess reactive oxygen species. This study is an experimental study that was conducted to determine the relationship between SOD1 and Hsp70 expression in the ileum during the post-hatching development of the broiler. In the study, samples were taken from ileum tissue of 0-, 21- and 42-day-old broilers were used as material. While the Hsp70 immunoreactivity observed in the epithelial cells was specific to a few cells on day 0, it was detected in more villus epithelial cells on days 21 and 42. The Hsp70 expression in the ileum increased from the age 0 to up to day 42, especially in villus epithelial cells. In sections stained by SOD1, the ileum's villus epithelial cells and smooth muscle cells showed an intracytoplasmic reaction. From day 21 to day 42, a regular increase in SOD1 expression was detected in the crypt and villus epithelial cells. As a remarkable finding, a more intense intracytoplasmic staining was detected in villus epithelial cells located at the apex of intestinal villi. In conclusion, it was observed that SOD1 and Hsp70 expression increased in the ileum tissue throughout post-hatching development in broilers with a positive correlation with age. Based on the histological findings, it can be concluded that SOD1 and Hsp70 play a critical protective role in the small intestine after hatching and contribute to the rapid development of the intestine.

Keywords: Hsp70, ileum, post-hatching period, SOD1.

Introduction

From birth to adulthood, the gastrointestinal tract gradually achieves its mature structure and function[1]. The ability of intestinal villus epithelial cells to communicate with immune and neurological systems against foreign substances plays an important role in the tissue defense system[2]. Also, the intestinal mucosa's histological layers work together to resist, avoid, and, if required, repair harm[3].

Heat shock proteins (Hsps) are molecular chaperones that play critical functions in the survival and development of cells[4]. Hsps are cellular structural proteins that serve a key role in removing and modifying denatured cellular proteins and preventing aggregation of those proteins[5]. Thus, this chaperone broadly impacts protein homeostasis by regulating protein quality and turnover in both normal and stressful situations[6]. Furthermore, to maintain the cytoskeleton structure, Hsps in cells assist in correctly folding proteins and refolding misfolded proteins[7]. Hsps influence both adaptive and innate immune responses and may promote cross-talk between the two systems[8]. It has also been

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reported that Hsps contribute to the pathogenesis of autoimmune and chronic inflammatory diseases by inducing proinflammatory cytokine production[9]. Hsps consist of many different families differentiated by their molecular weight. The Hsp 70 is one of the well-known chaperones of the Hsp family[10]. Hsp70 plays a protective part in the inflammatory and immune response in case of tissue damage[11].

Reactive oxygen species (ROS) are a product of normal redox reactions in organisms and are important determinants of cellular signaling[12]. On the other hand, excessive ROS production can harm lipids, proteins, and DNA by oxidizing them. As a result, to ensure proper cellular function and survival, a tight balance in ROS levels must be maintained[13]. Superoxide dismutases (SODs) are metalloenzymes that play an essential role in the body's defense against oxidative stress by efficiently removing excess ROS[14]. Enzymatic defense systems such as SOD, catalase, and glutathione peroxidases control the immediate detoxification of intracellular ROS under physiological conditions[15]. SOD, an enzyme system made up of three isozymes: SOD1, SOD2, and SOD3, are one of the most well-known antioxidant defense systems. SOD1 is the most widely distributed in the tissues, accounting for 90% of total SOD activity[16]. SOD1 is required to counteract superoxide generation during mitochondrial respiration and is the first line of defense against oxidative damage. It also offers direct regulation of cellular ROS levels[17].

Our study aimed to determine the immunohistochemical expression of SOD1 and Hsp70 throughout post-hatching development in the ileum, a vital tissue in gastrointestinal immunity. It was also to examine the probable relationship between SOD1 and Hsp70 cytokines.

Materials and Methods

Tissue samples

Broiler eggs were incubated in a forced draft poultry incubator at 50-60% relative humidity at 35°C and incubated under appropriate conditions. (Eggs were supplied from Beypiliç A.Ş., Bolu, Turkey). Three groups were formed, each with six animals. Newly hatched (0 days old), 21, and 42-day post-hatching. After the animals were sacrificed under anesthesia, the ileum was sampled for histochemical investigation. A 10 percent formaldehyde solution was used to fix tissue samples for 24 hours. The tissues were kept in a running water bath for 24 hours to remove the formalin, then placed in 70%, 80%, and 96 percent alcohol for 1 hour each. After that, pure alcohol and xylol were applied three times for one hour each. To embed the tissue samples, paraplast was employed. Ankara University, the local animal ethical committee, approved all experimental procedures (2013-5-38).

Immunohistochemistry

Using the Streptavidin biotin complex method, 5µm ileum sections were stained immunohistochemically using rabbit polyclonal Hsp70 (1/200 dilution, Santa Cruz Biotechnology, sc-33575) and rabbit polyclonal SOD1 (1/200 dilution, Bioss Antibodies, bs-10216R) primary antibodies[18]. The secondary antibody was Histostain Plus (Zymed kit: 85-6743, United States). Following deparaffinization, endogenous peroxidase activity was blocked with H2O2 3% in absolute methanol for 15 min. The sections were rinsed with phosphate-buffered salines (PBS, pH:7.2). Sections were heated in a microwave oven at 700 W for antigen retrieval in a citrate buffer (pH:6) solution. The tissues were incubated in a 3% hydrogen peroxide solution to block endogenous peroxidase activity. After washing with phosphate buffer solution, serum was dripped from the kit to prevent non-specific protein binding in sections. The primary antibody was applied, and the samples were kept at +4 ^{0}C overnight. In the negative control group, only the PBS solution was used. After washing, sections were instilled with biotinylated secondary antibody and incubated at streptavidin-horseradish peroxidase complex. The final stage involved using 3,3'-diaminobenzidine (DAP) as a chromogen and covering the slides with entellan after hematoxylin counterstaining.

Immunohistochemical examination

The histological score was determined as follows: Slides were analyzed at a 20 x magnification under the light microscopy (A Nikon digital-sight imaging system was used with a Nikon Eclipse 50i microscope). The intensity of positive staining in the immunohistochemical examination was examined in 10 different areas[19]. Semi-quantitative scoring was used to determine the staining intensity as follows: 0 = no expression; 1 = mild; 2 = moderate; and 3 = intense[20].

Statistical Analysis

All statistical analyses were performed using the IBM SPSS Statistics Version 22.0 statistical software. Comparisons between groups were made with the independent Student's t-test for parametric data. Results were presented as mean ± SEM (standard error of mean) and statistical significance was accepted at $p < 0.05$.

Results

In Hsp 70 immunostaining

Hsp70 protein was mostly found in the cytoplasm of villus epithelial cells, according to immunostaining. While the Hsp70 immunoreactivity observed in the epithelial cells was specific to a few cells on day 0, it was detected in more villus epithelial cells on days 21 and 42. Also, no immunoreaction was observed in goblet cells. It was observed that Hsp70 immunoreactivity was mainly localized to the apical compartment of both villus epithelial and crypt epithelial cells. The Hsp70 expression in the ileum increased from the age 0 to up to day 42. especially villus epithelial cells. In addition, it was seen that the smooth muscle cells forming the tunica muscularis gave a positive reaction (Figure 1). When the effect of the post-hatching period in tunica muscularis, it was determined that the differences amongst study groups were statistically insignificant. Table 1 summarizes and evaluates the immunohistochemical results.

Table 1. The histological score of HSP70 and SOD1 immunoreactivity in the broiler ileum throughout post-hatching development.

	HSP70			SOD1		
Groups (n=6)	Villous epithelial cell	Crypt epithelial cell	Tunica muscularis	Villous epithelial cell	Crypt epithelial cell	Tunica muscularis
Day 0	1 ± 0.26	0 ± 0	1.33 ± 0.21	0.83 ± 0.17	1.33 ± 0.21	2.17 ± 0.17
Day 21	1.83 ± 0.31	1.17 ± 0.17*	1.33 ± 0.33	1 ± 0	1.37 ± 0.17	2.33 ± 0.21
Day 42	2.83 ± 0.17*,#	2.17 ± 0.17*,$	1.33 ± 0.21	2.17 ± 0.31*,&	2.17 ± 0.31#	2.17 ± 0.31

Data presented as mean ± SEM (Student's t-test).

*p<0.001 when compared to Day 0.

#p<0.05, &p<0.01, and $p<0.001 when compared to Day 21.

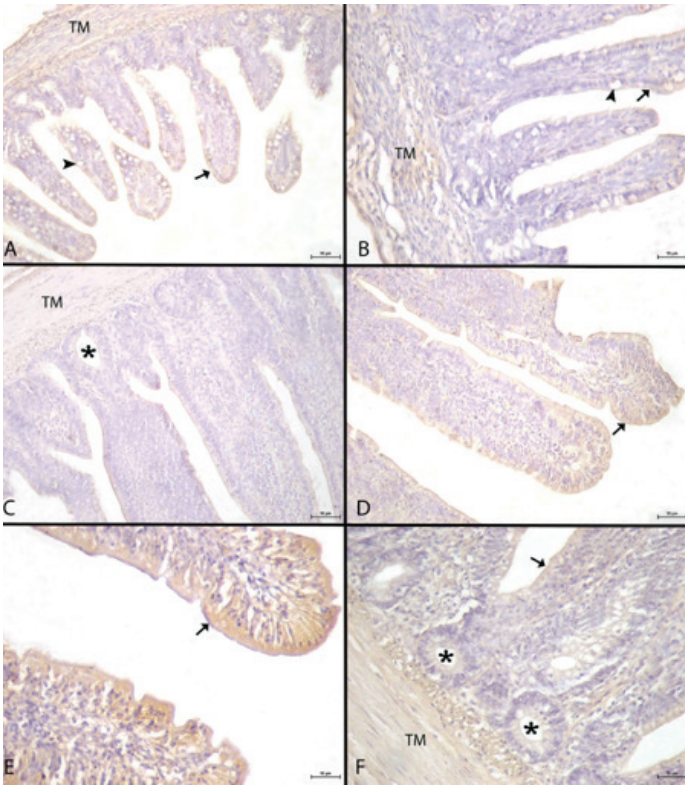

Figure 1. Hsp70 immunostaining in different stages of post-hatching (brown precipitate). A,B: (Day 0), C,D: (Day 21), E,F: (Day 42). Arrow: (villus epithelial cells), arrowhead: (goblet cell), asterix: (intestinal crypt), TM: (tunica muscularis); range bar, 10 µm.

SOD1 immunostaining

In sections stained by SOD1, the ileum's villus epithelial cells and smooth muscle cells showed an intracytoplasmic reaction. On day 0, SOD1 expression in epithelial cells was limited to a few cells; however, by days 21 and 42, it was seen in a more significant number of cells. In addition, there was no SOD1 immunoreactivity found in goblet cells. Furthermore, we found a weak intracytoplasmic SOD1 expression in crypt and villus epithelial cells on days 0 and 21. As a notable finding, after day 21, crypt and villus epithelial cells showed a steady increase in SOD1 expression. Moderate staining was observed on day 42 in SOD1 immunoreactivity. Furthermore, as a remarkable finding, a more intense intracytoplasmic staining was detected in villus epithelial cells located at the apex of intestinal villi (Figure 2). In addition, an increase was observed in the number of stromal cells that reacted positively with SOD1 on the 42nd day. It was determined that smooth muscle cells forming the tunica muscularis were moderate-stained with SOD1 in all groups. In contrast to epithelial cells, smooth muscle cells did not exhibit any differences in SOD1 expression intensity over the post-incubation period. The results of the immunohistochemistry are presented in Table 1.

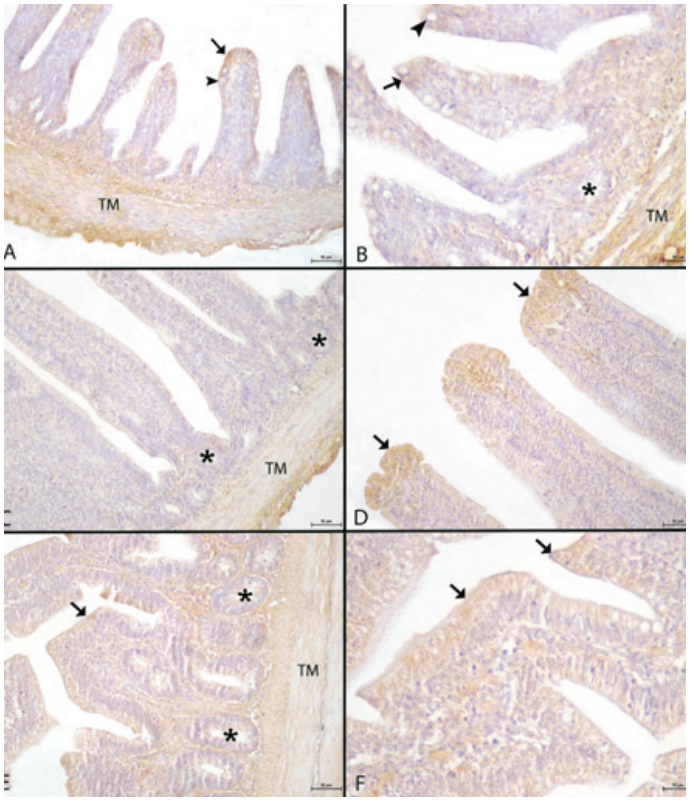

Figure 2. SOD1 immunostaining in different stages of post-hatching (brown precipitate). A,B: (Day 0), C,D: (Day 21), E,F: (Day 42). Arrow: (villus epithelial cells), arrowhead: (goblet cell), asterix: (intestinal crypt), TM: (tunica muscularis); range bar, 10 µm.

Discussion and Conclusion

Cytokines/chemokines are soluble polypeptides with a low molecular weight secreted by immune cells and other cell types. They are produced in response to microorganisms and other antigens, regulate immunological and inflammatory responses, and function in paracrine and autocrine ways[21]. In addition to aiding the transport, digestion, and absorption of nutrients, the small intestines' large surface area acts as a regulatory barrier for microbes and exogenous pathogens[22]. Also, It is known that intestinal epithelial cells can produce proinflammatory cytokines and function as antigen-presenting cells[23].

It is well known that oxidative stress increases the expression of Hsp70 and SOD1 and that the regulation of these two cytokines is positively correlated[24]. There are studies in the literature examining the interaction between SOD1 and Hsp70. For example, it was shown that Hsp70 and SOD1 expression increase in retinal ganglion cells when intraocular pressure is applied to rats[25]. Another study has revealed that decreased Hsp70 and SOD1 levels due to gastric mucosal damage were increased with aucubin administration in ethanol-induced gastric mucosal injury in mice[26].

Hsps expression is stimulated during the development and differentiation of tissues to protect against physiological stress[27]. Hsps have critical functions in embryogenesis, tissue maintenance, and injury response. The Hsp70 protein is essential for cell viability[28]. Its important function is to aid in protein folding and cycling. Quality control systems in normal cells prevent the accumulation of toxic misfolded protein species[29]. Hsp70 is known to protect intestinal epithelial cells, which are easily injured by numerous stressors, from toxic chemicals and ulcerogenic conditions in the gastrointestinal mucosa[30]. Upregulation of Hsps, particularly Hsp70, is also thought to be a protective mechanism because it can inhibit the expression of pro-inflammatory cytokines[31]. Heat shock factors (Hsf) regulate Hsp expression and mediate the multifaceted response to stress. Stress is known to initiate Hsf phosphorylation and trimerization, and these Hsf trimers bind to heat shock elements. Up-regulated Hsf increases Hsp70 expression in the ileum, contributing to cell functions under stress conditions[32]. Hsp70 is primarily known for its cytoprotective function, but it has also been proposed that it regulates cell growth via modulating certain signaling pathways. Under normal conditions, one of the leading roles of Hsps is to maintain the appropriate embryonic and postnatal development of different tissue and organ systems, particularly the neurological system[10]. It is well known that age-related processes or disease conditions can disrupt cellular protein homeostasis. During aging, the accumulation of misfolded proteins increases, possibly leading to elevated Hsp levels[33]. During the development of skeletal muscle myoblasts, the Hsp70 protein is also involved in the formation of myosin thick filaments[34]. Zhong et al.[35] reported that Hsp70 expression appeared predominantly localized in the epithelial cell cytoplasm in the ileum. Our work agrees with previous reports that Hsp70 expression in the ileum. In addition, we also observed that age might have an effect on Hsp70 expression in the ileum. Hsp70 expression in the ileum gradually increased from the age 0 to up to day 42 in villus epithelial cells. We thought that Hsp70, which is known to play an important role in protein folding and cycling, may be necessary for the functional integrity of cells in the broiler ileum during the post-incubation period.

SOD1 plays an important role in protecting tissues against oxidative stress by catalyzing the conversion of superoxide radicals to hydrogen peroxide, which can be reduced to water[36]. By generating cytokines or creating direct cell-cell contact, intestinal immune cells can influence mucosal barriers[37]. SOD1 is thought to regulate the coordinated balance between inflammation, mucosal immune cells, and various soluble factors[38]. In a study on chick embryos, SOD1 immunoreactivity was observed in ectodermal origin tissues and the cauda mesoderm during the gastrulation to neurulation. Also, it has been reported that SOD1 might indirectly regulate cell proliferation during gastrulation[39]. It was found in the study conducted by van der Loo et al.[40] that there was an increase in SOD1 expression in the aorta depending on age in rats. Of this find, they suggested that this increase may be an essential determinant for overcoming oxidative stress as a critical factor in a series of events that appear to be a physiological consequence of aging. A previous study shows that SOD1 expression is observed in the epithelial layer, submucosa, and muscle layer of the ileum tissue. Furthermore, it has been reported that SOD1 was highly expressed in mature epithelial cells compared to immature cells present in the intestinal crypt[41]. In our study, we observed SOD1 expression in smooth muscle cells, crypt and villus epithelial cells in accordance with previous findings. Furthermore, we noted that SOD1 expression in villus epithelial cells was limited to a few cells, it was seen in more cells on days 21 and 42. Also, the expression of SOD1 in the crypt and villus epithelial cells increased steadily from day 0 to day 42. We speculated that SOD1 might be necessary for the ileum's antioxidant defense systems and immune system in the post-hatching period.

In conclusion, we tried to determine the expression of SOD1 metalloenzyme and Hsp70 chaperone in the developmental stage of ileum tissue after post-hatching in broilers. The expression of SOD1 and Hsp70 was observed to increase in a positive correlation with age. Based on the histological findings, it can be concluded that SOD1 and Hsp70 play a critical protective role in the small intestine after hatching and contribute to the rapid development of the intestine.

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