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RESEARCH ARTICLE

Effect of Vit E on Secretion of HSP-70 in Testes of Broilers Exposed to Heat Stress [1]

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Summary

The objective of this experiment was to investigate the ameliorative effect of vitamin E (Vit E) on tissue damage caused by heat stress in the testes of broilers, and to correlate this effect with the secretion of the HSP-70 protein. In the study totally 30 broilers (breed Ross 308) were used. Animals were divided into three groups, including the control group. Group Control (C); these broilers (24°C) were not administered with Vit E in the diet and not subjected to heat stress. Group Stress (S); these broilers not administered with Vit E in the diet but subjected to heat stress (34°C). Group Stress +Vitamin E (Group S + vit E); broilers subjected to heat stress (34°C) and administered with vitamin E. Histopathological examination demonstrated the presence of degenerative alterations in testes tissue of the group S. However, there has been seen diminishing the severity of degeneration in the group S+Vit E. While secretion of HSP-70 was determined in the testes of all groups in varying degree, the highest secretion level of HSP-70 was in the group C, the lowest secretion level of HSP-70 was in the group S. It is concluded that heat stress reduces the secretion of HSP-70 in testes of broilers, when vitamin E is administered, the secretion of HSP-70 increases again and reduces tissues damage.

Keywords: *Heat stress, Broiler, Testis, Vit E, HSP-70*

Sıcaklık Stresine Maruz Bırakılan Broilerlerin Testislerinde Vit E' in HSP-70 Sekresyonu Üzerine Etkisi

Özet

Bu çalışmanın amacı broilerlerin testislerinde ısı stresinin sebep olduğu doku hasarı üzerine vitamin E (Vit E)'in iyileştirici etkisini araştırmak ve bu etkinin HSP-70 proteininin sekresyonu ile ilişkisini ortaya koymaktır. Çalışmada toplam olarak 30 adet (Ross 308 ırkı) broiler kullanıldı. Hayvanlar kontrol grubu da dahil olmak üzere üç gruba ayrıldı. Grup kontrol (C); sıcaklık stresine maruz bırakılmayan (24°C) ve diyetlerine Vit E eklenmeyen broiler. Grup stress (S); diyetlerine Vit E eklenmeyen ancak sıcaklık stresine maruz bırakılan (34°C) broiler. Grup Stres +Vitamin E (Grup S + vit E); sıcaklık stresine maruz bırakılan (34°C) ve diyetlerine vitamin E eklenen broiler. Histopatolojik inceleme S grubunun testislerinde dejeneratif değişiklikler gösterdi. Ancak S + Vit E grubunda dejenerasyonun şiddetinin azaldığı gözlendi. Grupların tamamının testislerinde farklı seviyede HSP-70 sekresyonu saptanırken, HSP-70'in en yüksek seviyesi grup C'de en düşük seviyesi grup S'deydi. Isı stresinin broilerlerin testislerinde HSP-70 sekresyonunu azaltığı, vitamin E uygulandığında, HSP-70 sekresyonunun yeniden artığı ve doku hasarını azalttığı sonucuna varıldı.

Anahtar sözcükler: *Isı stresi, Broiler, Testis, Vit E, HSP-70*

INTRODUCTION

Heat stress is one of the most challenging environmental conditions affecting commercial poultry production and causes significant economic losses in the poultry industry [1,2]. Compared to other species of domestic animals, poultry

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are more sensitive to high ambient temperatures [3]. Because of the poultry have no sweat glands, a rapid metabolism and high body temperature, heat stress is highly affected this species [4].

Heat stress reduces several physiological and metabolic factors in poultry such as food consumption, growth rate, feeding efficiency, eggshell quality, survivability [5,6] and serum parameters [6]. High stress also causes oxidative stress and thus weakness the *in vivo* antioxidant defence system [2,7]. Dietary manipulations are among the methods to alleviate these negative adverse effects of heat stress. The role of dietary supplements such as vitamins [7-10] and minerals [10,11] for alleviating the effects of heat stress in poultry has been reviewed extensively.

Vitamin E (Vit. E) has been reported an excellent biological functions as a natural antioxidant prevent the oxidation of unsaturated lipid materials within cells, thus protecting the cell membrane oxidative damage [12-15]. Vit. E is not synthesized by poultry, and its availability is through exogenous administration only [10]. Vit E plays an important role in poultry industry because of its beneficial effect on the cell membrane integrity, immune system and reproduction performance [8,12,14,16].

Heat shock proteins (HSP) are a group of highly conserved proteins that are constitutively expressed in most cells under normal physiological conditions in every organism from bacteria to man. They perform important functions in the folding/unfolding and translocation of proteins as well as in the assembly/disassembly of protein complexes and the refolding of damaged proteins [17,18]. They also involve in both intracellular and extracellular immune functions [19] and apoptosis [20]. The HSP superfamily includes a number of different molecular-weight-class families: HSP110, HSP90, HSP70, HSP60, HSP47, and a group of small HSP ranging from 16 to 40 kDa. They make up approximately 5-10% of the total protein content of cells under conditions of normal, healthy growth [19] and are expressed or increased in response to various biological stressors, such as heat [2,8,9], malignant tumors [21], inflammation [22], viral infections and exposure to toxic chemicals [17,18]. These processes are also involved in the production of ROS, suggesting that oxidative stress is a key mechanism mediating HSP induction [23]. Of all the HSP families, HSP-70 has been most widely studied as a biomarker of stress [24]. However, only a limited number of immunohistochemical studies are available regarding the production of the HSP-70 in the testes of poultry subjected to heat stress.

In this study, our objective is to investigate the relationship between the protective effect of vit E and HSP-70 secretion on the tissue damage occurring in the testes of broilers exposed to heat stress by using histopathological and immunohistochemical methods.

MATERIAL and METHODS

Animals and Experimental Design

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine in Ataturk University (Decision No: 2007/5f). The trial was conducted at the Research and Practice Farm of Atatürk University, Faculty of Veterinary Medicine in 3 groups of animals (broilers of the commercial breed Ross 308). There were 3 cages in total; each cage had 10 broilers. Animals in each group were kept within the cages 100 x 55 x 35 cm in size and fed from day 1 to day 35. The trial groups were designed as follows: Group Control (C); these broilers (24°C) were not administered with antioxidants in the diet and not subjected to heat stress (n=10). Group Stress (S); these broilers not administered with antioxidants in the diet but subjected to heat stress (34°C) (n=10). Group Stress + Vitamin E (Group S + vit E); broilers subjected to heat stress (34°C) and administered with vitamin E (n=10). All groups were exposed to a comfortable temperature until day 14 of the trial. As of day 15, Groups S, and group S+Vit E were subjected to a temperature of 34°C between 8:00 and 16:00 hours, and to a temperature of 24°C for the rest of the 24 h day. Group C subjected to a temperature of 24°C throughout the trial. All groups were exposed to a daily regime of 17 h light and 7 h darkness. While all groups is fed with the basal ration during the study, only group S+ Vit E is given vitamin E (DL α Tocopheryl acetate: 150 mg/kg) in addition to basal ration *(Table 1)*. Dietary nutrient proportions were determined in compliance with the recommendations of the National Research Council [10].

At the end of the trial (Day 35), broilers were killed by cervical dislocation for necropsy and testes tissue were collected for histopathological and immunohistochemical evaluations.

Histopathology

The tissues were kindly removed and half of them were fixed in 10% neutral buffered formalin immediately. After dehydration in a graded ethanol series and clearing with xylene, the samples were embedded in paraffin and 4-µm-thick sections were stained with Hematoxylin-Eosin (H.E) for obser-vation under the light microscope.

HSP-70 Immunostaining

Four µm sections from all the tissue samples were cut and processed for immunohistochemical examination by a standard avidin-biotin-peroxidase method as described by the producer. Mouse monoclonal antibodies that react with human HSP-70 (Clone: BRM-22, Sigma, St. Louis, MO, USA) were used at dilution of 1:5000 for 60 min, respectively. Secondary antibody (horseradish peroxidaseconjugated goat antimouse) was added and incubated for 1 h. 3,3-Diaminobenzidine (DAB) was added an sections were kept in the dark for 10 min. Then, the sections were stained with

Table 1. *Ingredients of crude nutrient proportions in the basal ration*
Tablo 1. *Bazal rasyonda besin içeriklerinin ham madde oranları*

Ingredients	Nutrient Proportions %	
	1-10 day	11-35 day
Corn	56.99	58.74
Corn glutein	20.00	20.00
Wheat short	7.00	7.00
Soybean oil	0.78	3.72
Soybean meal	11.53	7.14
Calcium carbonate	1.36	1.23
Dicalciumphosphate	1.06	0.91
L-lysine	0.40	0.42
Salt	0.26	0.27
Vitamin-mineral premix	0.20	0.20
Toxin binder	0.10	0.10
Anticoccidial	0.10	0.10
Sodium bicarbonate	0.10	0.09
Growth factor	0.05	0.05
Phyzymexptpt	0.03	0.03
DL-Methionine	0.04	-
Nutritional levels		
Metabolic energy kcal/kg	3000	3200
Crude protein %	23	21
Ether extract	3.46	6.37
Crude fiber	2.79	2.68
Methionine	0.5	0.43
Lysine	1.10	1.0
Calcium	1.00	0.90
Phosphorus	0.7	0.65

Mayer's hematoxylin for 5-8 min as counter stain. Finally, the sections were mounted with cover slips using neutral balsam, viewed.

Image Analysis

Tissue sections were evaluated by high power light microscope (Olympus Bx52 with DP72 camera system). All immunohistochemical staining were estimated with an image processing system (Olympus, DP72-BSW). For morphometry of testes, ten tubulus of testes from each broiler (300 tubulus for each group) were chosen randomly. The intensity of staining with HSP-70 immune reactivities were scored semiquantitatively as i) A = non-reactivity, ii) B = weak, individualized cell reactivity in ≤ 25% of tubul, iii) C = mild to moderate reactivity in ≤ 50% of tubul, iv) D = strong reactivity in ≤ 75% of tubul, and v) E = very strong reactivity in >75% of tubul. The average staining intensity was calculated by the formula: [(Ax1) + (Bx2) + (Cx3) + (Dx4) + (Ex5)]/(A + B + C + D + E) and reported, as follows: i) – = 0.00, ii) + = 0.01 – 1.00, iii) ++ = 1.01 – 2.00, iv) +++ = 2.01–3.00, and v) ++++ = 3.01 – 4.00.

Statistical Analysis

Data were presented as means ± standard error (SEM). Values from semi-quantitative analysis of HSP-70 immune positive cells were analyzed separately by using one-way analysis of variance (ANOVA). For determining differences between the groups (C, S and S + Vit E), the Duncan test was used. Differences were considered significant when $P<0.05$.

RESULTS

There was no macroscopic finding in animals of group C, whereas testes were swollen and quite soft consistency in all animals of group S and in 8 animals of group S + Vit E.

Histopathological Findings

Histopathological examination of the control animals (Group C) showed normal testis tissue histology *(Fig. 1a)*. In the S group, thickening, desquamation, degeneration, necrosis in basal layer of some seminiferous tubules, and atrophy in the some seminiferous tubules were observed. It was remarkable of vacuolization in Sertoli cells and spermatogonia in the seminiferous tubules and the loss of spermatogonic cells *(Fig. 1b)*.

Although there were similar findings in testes of animals in group S + Vit E, it has been seen diminishing the severity of these findings, beginning to turn into a normal appearance of seminiferous tubulus, and the presence of spermatogonic cells *(Fig. 1c)*. Whilst there were interstitial connective tissue proliferation and hyperplasia in Leydig cells in most animals of group S, these findings were seen in only 3 animals of group S + Vit E.

Immunohistochemical Findings

In all groups the immunoreactivity of HSP-70 was positive in spermatogonia, spermatocytes, spermatids, elongated spermatids, Leydig cells and also a few Sertoli cells *(Fig. 2a, b,c)*. Although HSP-70 secretion was determined in the testes in all groups, the marked secretion levels were observed in the group C whilst the weak secretion levels in the group S were observed ($P<0.05$). The intensive secretion of HSP-70 was mainly in the cytoplasm of the spermatogenic cells in testes of group C ($P<0.05$), whereas in the group S the most intensive secretion of HSP-70 was in Sertoli cells.

DISCUSSION

Heat stress is one of the most important environmental stressors. For broiler chickens, the environmental thermo-neutral (comfort) (TN) zone covers a temperature range between 18 and 24°C. When environmental temperature increases above the TN zone and exceeds the upper critical temperature, the animals are considered to be heat stressed [1].

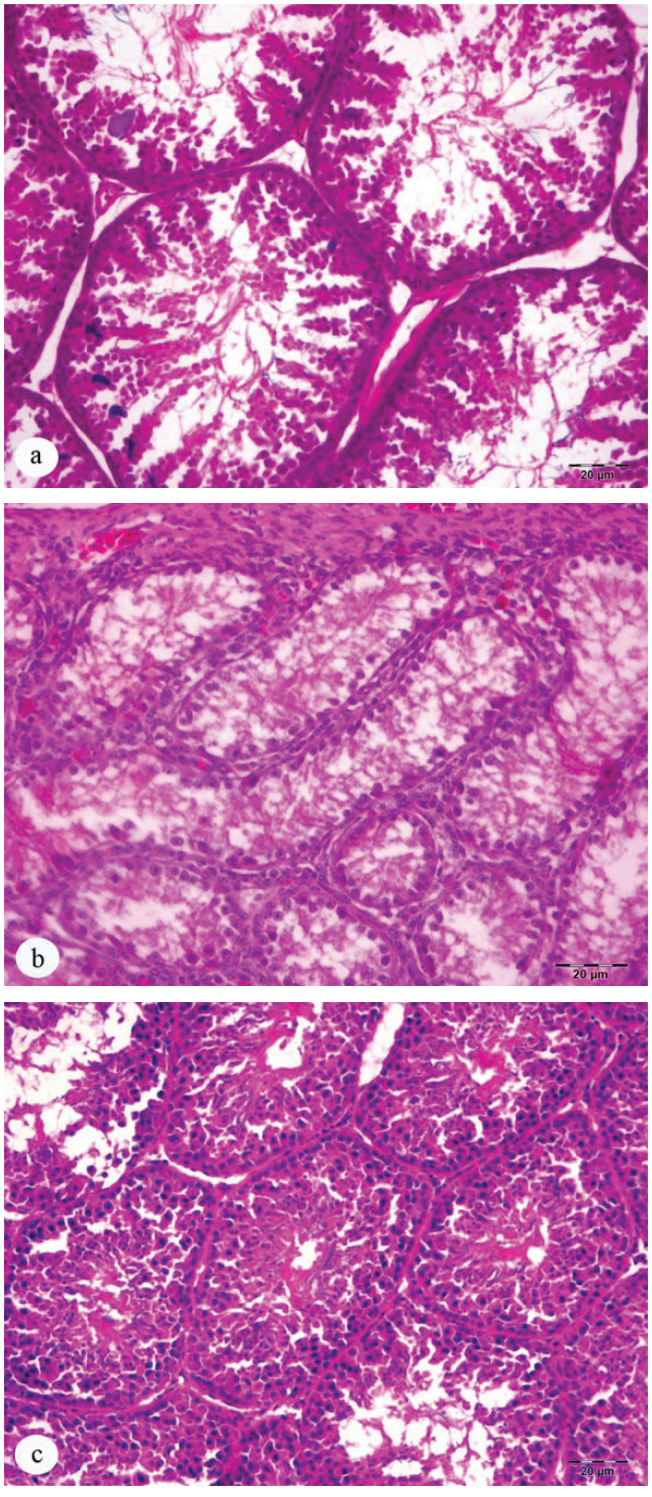

Fig 1. a- Normal appearance of seminiferous tubulus and the presence of spermatogenic cells (Group C), H.E., Bar: 20 µm, **b-** Intensive degenerative alterations in seminiferous tubules and loss of the spermatogenic cells (Group S), H.E., Bar: 20 µm, **c-** Diminishing degenerative alterations in seminiferous tubules and the presence of spermatogenic cells (Group S+Vit E), Bar: 20 µm

Şekil 1. a- Seminifer tubulusların normal görünümü ve spermatojenik hücre varlığı (Group C) H.E., Bar: 20 µm, **b-** Seminifer tubullarda yoğun dejeneratif değişiklikler ve spermatogenik hücre kaybı (Grup S), H.E., Bar: 20 µm, **c-** Seminifer tubullarda azalan dejeneratif değişiklikler ve spermatojenik hücre varlığı (Grup S+Vit E), H.E., Bar: 20 µm

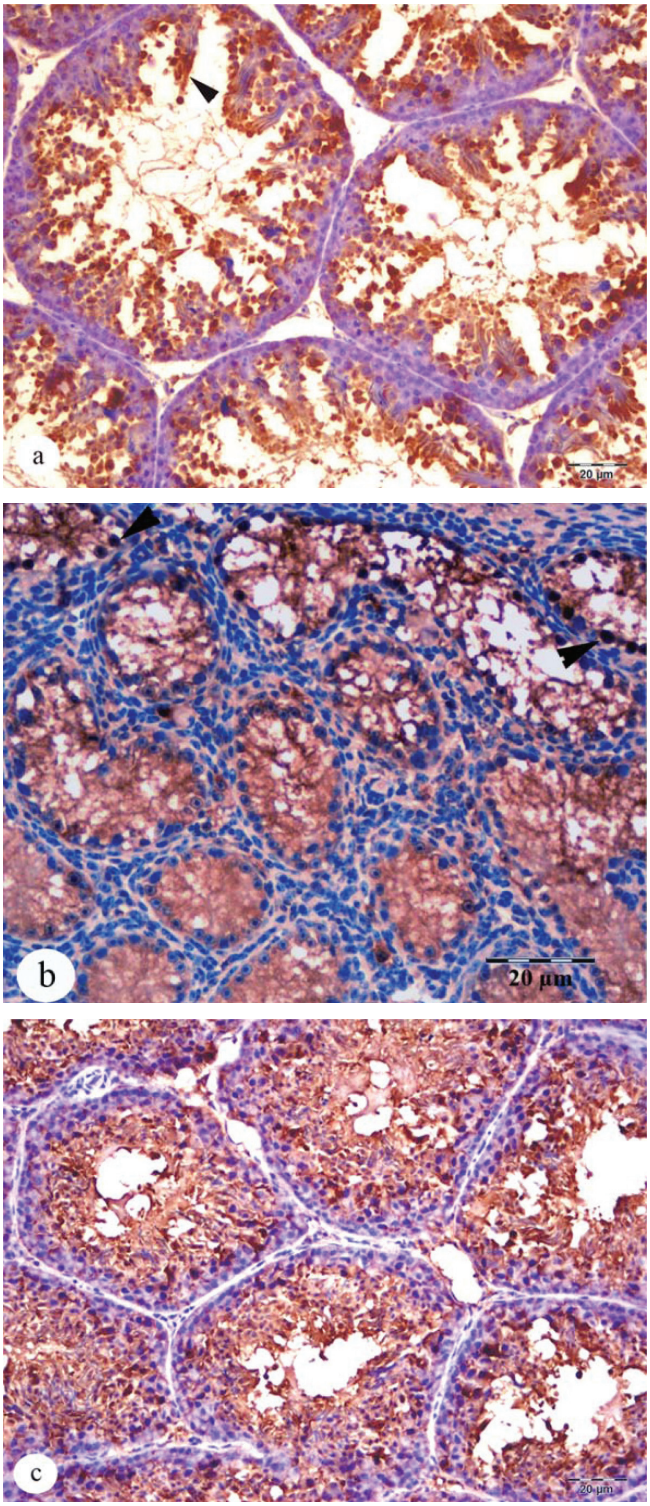

Fig 2. Immunohistochemstry stain, **a-** Intensive secretion of HSP-70 in spermatogenic cells (Group C) *(arrow head)*, Bar: 20 µm, **b-** Secretion of HSP-70 in sertoli cells (Group S), *(arrow head),* Bar: 20 µm, **c-** Moderate secretion of HSP-70 in testis tissue (Group S + Vit E), Bar: 20 µm

Şekil 2. Immunohistokimyasal boyama, **a-** Spermatojenik hücrelerde yoğun HSP-70 sekresyonu (Group C) *(ok başı)*, Bar: 20 µm, **b-** Sertoli hücrlerinde hafif HSP-70 sekresyonu (Grup S) *(ok başı),* Bar: 20 µm, **c-** Spermatojenik hücrelerde orta şiddette HSP-70 sekresyonu (Grup S+Vit E), Bar: 20 µm

For many years, researchers have been investigating the effect of high environmental temperature on the variety of tissues of poultry, such as liver, kidney, brain, heart, and have found that high environmental temperatures have destructive effects on tissues [9,12,25-27]. Previous studies showed that the fertility of male broilers was greatly reduced due to high ambient temperature [28] However, there are a limited number of studies on the effects of heat stress to testis tissues in poultry. There have been some studies regarding the negative effects of heat stress to testis tissue in different kinds of animals. In these studies, it has been reported there was degenerative changes in the seminiferious tubulus and the arrest in spermatogenesis [29-31].

Histopathological examination performed in the present study demonstrated the loss of spermatogonic cells and degenerative alterations in testes tissue in broilers subjected to heat stress. These findings were in compliance with previous studies.

During heat stress, the antioxidant defence of reproductive system downplays and generates an oxidative stress that may impair testis function and affect negatively semen characteristics. Because of high content of polyunsaturated membrane lipids, testicular tissue becomes one of the targets for oxidative stress [13]. Dietary manipulations are therefore among the methods used to alleviate these negative adverse effects of environmental stress. Several studies have shown that antioxidant nutrient supplementation is effective in preventing the deleterious effects of heat stress and that these types of nutrients could be included in diets to alleviate the negative effects of heat stress [2,8,9,12]. Vitamin E has been shown to be curative for reproductive tissues in several species. Those study reported that the supplementation of vitamin E during the devastating effects facilitated the recovery of the structure of the seminiferous tubules indicating normal epithelium with a regular arrangement of germinal cells with most phases of spermatogenesis process included differentiation phase of spermatids [13,15,32].

The results demonstrated that the tissue damage observed in testes such as degenerative alterations and sperm loss caused by heat stress could be minimized with the administration of Vit E.

As living organisms, chickens have protective measures against environmental challenges. HSPs are either constitutively expressed or their expression is induced by heat shock and other stresses which suggest their role in repair and protective mechanisms in various tissues [8,9,22,33,34]. The HSP-70 family is the most conserved and the most important constituent of the HSP proteins [24]. HSP-70 is involved in cellular repair and protective mechanisms. Previous studies have shown that heat stress is associated with HSP-70 induction, and an increase in the expression of HSP-70 occurred when poultry were kept at high ambient temperatures [8,9,25,26].

Recently, several HSPs have been demonstrated to be present and have important functions in the male chickens testis. HSPs are constitutively expressed in specific spermatogenic cell types during spermatogenesis [33,34].

In this study, it has been determined that HSP-70 was secreted in all groups. While secretion of HSP-70 was at the highest level in the group C, it was at the lowest level in the group S. It has been also seen that secretion of HSP-70 was at the moderate level in Group S + Vit E due to use of vit E. It is found that loss of spermatogenic cells in group S is directly proportional to decrease of secretion of HSP-70. This is the first study that importance of HSP-70 for spermatogenesis in testes of the poultry has been shown immunohistochemically.

Several studies demonstrated that heat stress causes an increased free radical production [7,11,14] and lowers the concentrations of antioxidant vitamins and minerals such as E, C, A, and Zn in serum. A strong relationship is now recognized between free radical production and HSP-70 synthesis in stressed cells [23]. Previous studies have demonstrated that levels of vit E are correlated with the secretion of HSP [35,36].

In another study, Sahin et al.[8] reported that serum HSP-70 levels increased in poultry exposed to heat stress, but also indicated that the HSP-70 levels of the groups administered with antioxidants did not differ from the levels measured in the stress group.

In the study performed by Terim et al.[9] it has been shown immunohistochemically that alpha- lipoic and ascorbic acid added to ration of the broilers exposed to heat stress decrease tissue damage in the liver and kidney and increase secretion of HSP-70.

In the present study, it has been observed that an increase secretion of HSP-70 in testes of broilers administered to vit E and a decrease in tissue damage occurred in heat stress.

Previous studies have reported that with increasing temperature increased secretion of HSP proteins in poultry. However, it has not been explored this increase and relationship with the vitamin E by using histopathological and immunohistochemical methods in the testes of poultry. This study has indicated the relationship between healing effect of vitamin E in tissue damage occured in testes due to heat stress and secretion of HSP-70.

In conclusion, today, HSP proteins used in cancer immunotherapy owing to the nature of biological adjuvant will take an important place in preventing of bacterial and viral disease in poultry, in supporting of immune system against to stress, in vaccine producing, and in poultry producing.

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