

## Effects of Chitosan Oligosaccharides Supplementation on the Cell Cycle of Immune Organs in Broilers

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### Abstract

The objective of this study was to investigate effects of COS on cell cycle and relative weights of thymus, bursa of fabricius (BF) and spleen in broilers. Three hundred and sixty 1-day-old healthy Arbor Acres male broilers were randomly divided into control, COS I, COS II and COS III groups, which were respectively fed with diets containing 0, 200, 350 and 500 mg/kg COS for six weeks. The results showed that the relative weights of immune organs were higher in the COS group II than those in the control group at 42 d. When compared with the control group, increased percentages of G<sub>2</sub>/M phase thymocytes in the COS groups II and III at 21 d, and decreased percentage of G<sub>0</sub>/G<sub>1</sub> phase cells, increased percentage of G<sub>2</sub>/M phase cells and PI (proliferation index) of thymus were observed at 42 d. The percentage of G<sub>0</sub>/G<sub>1</sub> phase BF cells was lower, and the percentage of S phase cells and PI of BF were higher in the COS group II at 21 d. The increase of G<sub>2</sub>/M phase splenocytes and PI, decrease of G<sub>0</sub>/G<sub>1</sub> phase splenocytes in the COS group II could be seen at 42 d.

**Keywords:** Chitosan oligosaccharides, Cell cycle phase, Immune organs, Flow cytometer

## Broilerlerde Kitosan Oligosakkarit İlavesinin İmmün Organlarda Hücre Siklusu Üzerine Etkileri

### Özet

Bu çalışmanın amacı Kitosan Oligosakkaritlerin (COS) broiler tavuklarda hücre siklusu ile timus, bursa Fabrisius (BF) ve dalak organ ağırlıklarına etkisini araştırmaktır. Üç yüz altmış adet bir günlük sağlıklı Arbor Acres erkek broiler rastgele olarak kontrol, COS I, COS II ve COS III olmak üzere gruplara ayrıldı ve sırasıyla gruplara 0, 200, 350 ve 500 mg/kg COS altı hafta süresince verildi. İmmün organların orantısal ağırlıkları 42. günde COS II grubunda kontrol grubuna göre daha yüksekti. Kontrol grubu ile karşılaştırıldığında 21. günde COS II ve COS III gruplarında yüzde G<sub>2</sub>/M faz timositler artmıştı. 42. günde COS II grubunda kontrol grubuna göre daha yüksekti. Kontrol grubu ile karşılaştırıldığında 21. günde COS II ve COS III gruplarında G<sub>0</sub>/G<sub>1</sub> faz hücrelerin yüzdesi azalmış, G<sub>2</sub>/M faz hücrelerin yüzdesi ile timusun proliferasyon indeksi (PI) ise artmıştı. 21. günde COS II grubunda G<sub>0</sub>/G<sub>1</sub> faz BF hücrelerin yüzdesi daha düşük ve S faz hücrelerin yüzdesi ile BF'nin PI'sı daha yüksekti. 42. günde COS II grubunda G<sub>2</sub>/M faz splenositlerde ve PI'da artma ile G<sub>0</sub>/G<sub>1</sub> faz splenositlerde azalma görülebilir.

**Anahtar sözcükler:** Kitosan oligosakkaritler, Hücre siklusu fazı, İmmün organlar, Flow sitometre

### INTRODUCTION

Chitosan oligosaccharides (COS) are a type of oligosaccharides, which are obtained by chemical and enzymatic hydrolysis of chitosan <sup>[1,2]</sup>. COS have several biological

functions, including anti-bacterial, anti-tumor, anti-oxidant, anti-inflammatory and immuno-enhancing functions <sup>[3]</sup>. COS could inhibit the proliferation of human lung cancer cells and gastric cancer cells by inducing cell cycle blockage in S or/and G<sub>2</sub>/M phase <sup>[4-6]</sup>. There was a report about the



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effect of COS on the normal Schwann cells, which showed that COS could promote cell proliferation [7].

Previous researches have shown that dietary COS could improve immune functions by stimulating antibody production, but related mechanism has not been explored. Since COS could accelerate proliferation of normal Schwann cells *in vitro*, it is noteworthy that whether COS could promote cell proliferation of immune organs in broilers. Hence, this study was conducted to explore the effects of dietary COS on immune organs of broilers by detecting relative weight, and the cell cycle distribution of thymus, bursa of Fabricius (BF) and spleen.

## MATERIAL and METHODS

### Chickens and Diets

Three hundred and sixty 1-day-old healthy Arbor Acres (AA) male broiler chicks (bought from Sichuan Yuguan agriculture Co., Ltd, Sichuan province, China) were randomly divided into four equal groups of 90 each. They were fed on diets as follows: control group (basal diet), COS group I (200 mg/kg COS), COS group II (350 mg/kg COS), and COS group III (500 mg/kg COS) for 42 days. Nutritional requirements were adequate according to the National Research Council (NRC, 1994) and Chinese Feeding Standard of Chicken (NY/T33-2004). The use of animals and all experimental procedures were approved by Sichuan Agricultural University Animal Care and Use Committee.

### Relative Weights of Immune Organs

At 21 and 42 days of age, after the body weight was measured, six birds in each group were euthanized and necropsied. Thymus, BF and spleen were dissected from each chick and weighed after dissecting connective tissue around the organ. Relative weight was calculated by following formula:

Relative weight = Organ weight (g) / Body weight (kg)

### Cell Cycle by Flow Cytometry Method

Six broilers in each group were euthanized at 21 and 42 days of age, the thymus, BF and spleen were removed from each broiler. Single-cell suspension was prepared by dissecting each sample into homogenate and filtering through 300-mesh nylon gauze. The cells were washed and suspended in cold phosphate buffer solution (PBS, pH 7.2-7.4) at a concentration of  $1 \times 10^6$  cells/mL. 500  $\mu$ L cell suspension was transferred into culture tube. And then, 1 mL of 0.25% Triton X-100 was added for perforation. The mixture was incubated for 20 min at 4°C, washed and resuspended with 100  $\mu$ L PBS. Five  $\mu$ L propidium iodide (PI) (BD Pharmingen, USA, 51-66211E) was added into the cell suspension and incubated for 30 min at 4°C in the dark room. Finally, 400  $\mu$ L PBS was added and the cell cycle distribution was assayed by FACSCalibur flow cytometer

(BD Co. Ltd., San Diego, CA, USA) within 45 min. The results were analyzed by ModFit software [8].

Proliferating index value (PI) =  $(S+G_2M) / (G_0G_1+S+G_2M) \times 100\%$

### Statistical Analyses

SPSS 20.0 for Windows was used for statistical analyses. The results were presented as means  $\pm$  standard deviation (M  $\pm$  SD). Statistical analyses were performed using one-way analysis of variance or t-test, and LSD was employed for multiple comparisons. A value of  $P < 0.05$  was considered significant, a value of  $P < 0.01$  was considered extremely significant.

## RESULTS

### Relative Weights of Immune Organs

The relative weight of thymus in the COS group II was significantly higher ( $P < 0.05$ ) than that in the control group at 21 days of age, and significantly higher ( $P < 0.05$  or  $P < 0.01$ ) than those in the COS group I and control group at 42 days of age. No significant difference was observed among the experimental groups at 21 days of age, while the relative weight of BF in the COS group II was higher ( $P < 0.05$ ) than that in the control group at 42 days of age. The relative weight of spleen in the COS group I was higher ( $P < 0.05$ ) than those of the control group and COS group I at 42 days of age, but there was no significance between the four experimental groups at 21 days of age. The results were shown in Table 1.

### Cell Cycle Determination

Compared with the control group, the percentage of  $G_2/M$

**Table 1.** The relative weight of immune organs in broilers (g/kg)

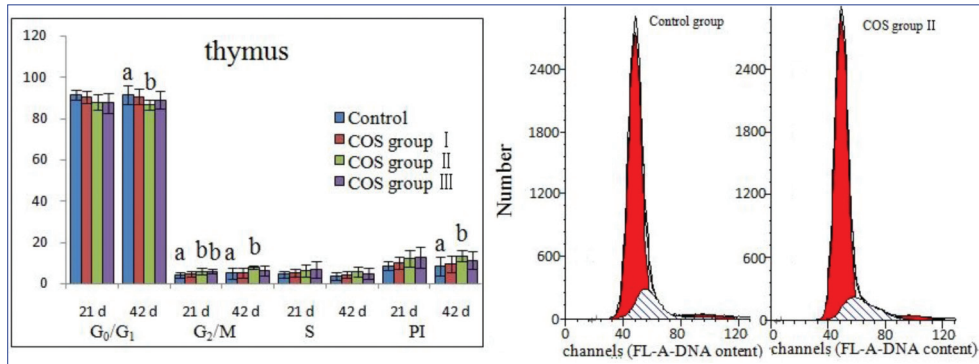
| Time               | Group         | 21 Days of Age                 | 42 Days of Age                  |
|--------------------|---------------|--------------------------------|---------------------------------|
| Thymus             | control       | 3.095 $\pm$ 0.653 <sup>a</sup> | 3.169 $\pm$ 0.591 <sup>A</sup>  |
|                    | COS group I   | 3.764 $\pm$ 1.000              | 3.849 $\pm$ 0.727 <sup>a</sup>  |
|                    | COS group II  | 4.358 $\pm$ 0.876 <sup>b</sup> | 5.094 $\pm$ 1.581 <sup>ab</sup> |
|                    | COS group III | 3.528 $\pm$ 0.976              | 4.113 $\pm$ 0.870               |
| Bursa of Fabricius | control       | 2.306 $\pm$ 0.276              | 1.610 $\pm$ 0.430 <sup>a</sup>  |
|                    | COS group I   | 2.397 $\pm$ 0.329              | 1.739 $\pm$ 0.318               |
|                    | COS group II  | 2.722 $\pm$ 0.430              | 2.051 $\pm$ 0.349 <sup>b</sup>  |
|                    | COS group III | 2.670 $\pm$ 0.391              | 1.839 $\pm$ 0.325               |
| Spleen             | control       | 1.018 $\pm$ 0.132              | 1.127 $\pm$ 0.092 <sup>a</sup>  |
|                    | COS group I   | 1.079 $\pm$ 0.197              | 1.142 $\pm$ 0.121 <sup>a</sup>  |
|                    | COS group II  | 1.112 $\pm$ 0.121              | 1.397 $\pm$ 0.219 <sup>b</sup>  |
|                    | COS group III | 1.151 $\pm$ 0.284              | 1.236 $\pm$ 0.205               |

Data are presented with the means  $\pm$  standard deviation ( $n = 6$ ). Values within a column followed by different capital letters were significantly different ( $P < 0.01$ ) between two groups (A-B). Values within a column followed by different small letters were different ( $P < 0.05$ ) between two groups (a-b)

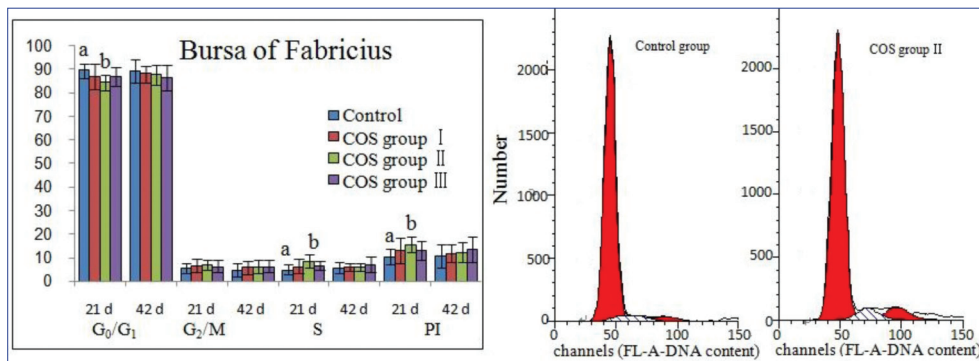
phase thymocytes was increased ( $P < 0.05$ ) in the COS group II and III at 21 days of age. The percentage of thymocytes in  $G_0G_1$  phase was markedly lower ( $P < 0.05$ ) in the COS group II at 42 days of age, and the percentage of  $G_2/M$  phase cells and PI of thymus in the COS group II were evidently higher ( $P < 0.05$ ) than those in the control group at 42 days of age. An arised tendency of thymocytes in S phase was

observed at 21 and 42 days of age, although there was no significant difference. The results are shown in Fig. 1.

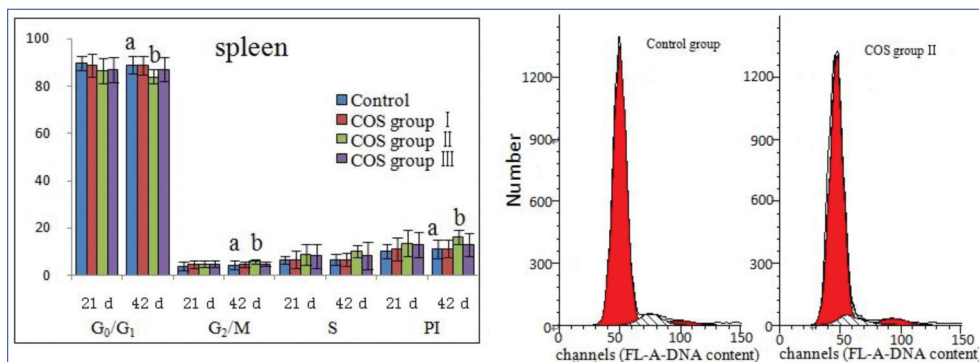
Compared with the control group, the percentage of BF cells in  $G_0G_1$  phase was markedly decreased ( $P < 0.05$ ) in the COS group II at 21 days of age, while the BF cell percentage in S phase and PI value were significantly



**Fig 1.** Cell cycle distribution of thymocytes in the broilers fed on the control and COS supplementation diets. Bar graph indicates the percentages of thymocytes in  $G_0G_1$ ,  $G_2/M$  and S phases and PI value (a-b:  $P < 0.05$ ,  $n = 6$ ). The DNA histogram by FCM shows that the first red peak is in  $G_0G_1$  phase, the second red peak is in  $G_2/M$  phase, and the white peak with blue stripe is in S phase. At 42 days of age, the percentage of thymocytes in  $G_2/M$  phase is higher in the COS group II than those in the control group



**Fig 2.** Cell cycle distribution of BF cell in the broilers fed on the control and COS supplementation diets. Bar graph indicates the percentages of BF cells in  $G_0G_1$ ,  $G_2/M$  and S phases and PI value. (a-b:  $P < 0.05$ ,  $n = 6$ ). The DNA histogram by FCM shows the percentage of BF cells in S phase is higher in the COS group II than those in the control group at 21 days of age



**Fig 3.** Cell cycle distribution of splenocytes in the broilers fed on the control and COS supplementation diets. Bar graph indicates the percentages of splenocytes in  $G_0G_1$ ,  $G_2/M$  and S phases and PI value (a-b:  $P < 0.05$ ,  $n = 6$ ). The DNA histogram by FCM shows the percentage of splenocytes in  $G_2/M$  phase is higher in the COS group II than those in the control group at 42 days of age

increased ( $P < 0.05$ ). An arised tendency of BF cells in  $G_2/M$  phase was observed at 21 and 42 days of age, although there was no significant difference. The results are shown in Fig. 2.

No significant differences were observed among the four groups at 21 days of age ( $P > 0.05$ ). The percentage of splenocytes in  $G_0G_1$  phase was decreased ( $P < 0.05$ ) in the COS group II compared with that in the control group at 42 days of age, and the percentage of  $G_2/M$  phase splenocytes and PI of spleen in the COS group II were significantly higher ( $P < 0.05$ ) than that in the control group at 42 days of age. An arised tendency of splenocytes in S phase was observed at 21 and 42 days of age, although there was no significant difference. The results are shown in Fig. 3.

## DISCUSSION

Thymus and BF of avian species are known as the central immune organs for diversification and maintenance of T cell and B cell respectively. The spleen, a crucial non-specific peripheral lymphoid organ, has a dominant role in the generation of immune responses [9]. In the present study, it was observed that the relative weights of thymus, BF and spleen in the COS group II were increased when compared with those in the control group. These results suggested that dietary COS supplements with 350 mg/kg could accelerate development of immune organs in broilers. Previous studies [10,11] showed that 50 mg/kg and 100 mg/kg dietary COS could increase the relative weights of thymus, BF and spleen in chicks. However, another research suggested that the relative weight of thymus and BF was increased and that of spleen was decreased in the chickens fed with 50 mg/kg dietary COS [12]. According to previous studies and this research, there were no consistent results about optimal COS level existing enhancing-effects on the relative weight of immune organs, and opposite results were found about the relative weight of spleen in chickens fed on the same dosage of COS. The possible reasons could be related to several factors, including the species and, age of bird, the dosage and duration of feeding COS.

In this study, the percentages of thymocytes, BF cells and splenocytes in  $G_2/M$  phase or/and S phase were increased, and those in  $G_0G_1$  phase were decreased in some degree, when compared with those in the control group. At the same time, the increased proliferation index of thymus, BF, and spleen was seen at 42 days of age. As we know,  $G_0G_1$  phase is a resting phase before it enters the process of synthesizing new DNA (S phase) in preparation for another round of mitosis, and  $G_2/M$  phase is a division stage [13]. This results suggested that when 350 mg/kg COS were supplemented into the broiler's diets, more cells in the three immune organs entered into the division stage, which might result in the increase of relative weights of these immune organs, and might finally induce the improvement of immune function. As far the effects of

COS on cell cycle of normal cells, there was only one in vitro research, which showed that the proliferation index and the expression of cyclin  $D_1$  of normal Schwann cells treated with 0.25, 0.5 and 1.0 mg/mL COS were increased when compared with those of control [7]. The increased proliferation index observed in our study was in agreement with the result of this research, which hints at the possibility that appropriate levels of COS could improve the proliferation of normal cells *in vivo* and *in vitro*. Its mechanisms probably based on the activation of signaling pathways related to DNA and protein syntheses, which need to be furtherly studied.

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