Immune response following the administration of the anabolic steroid Boldenone Undecylenate in rabbits.

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Abstract: The present study was conducted to clarify the effect of the anabolic steroid boldenone undecylenate (BOL) on some immunological parameters in rabbits. The study was carried out on 60 apparently healthy New Zealand white male rabbits. Rabbits were divided into three groups each of 20 rabbits. The first group was injected with an adjuvant substance (sesame oil) and was considered as control (C). The second group was administered BOL at the recommended dose for rabbits (4.5mg/kg b.w) (group R). The third group was administered BOL at a dose double the recommended dose (9 mg/kg b.w) (group D). All treatments were given three times at three weeks interval. Blood samples were collected three times after the last drug injection at two weeks interval. Evaluated parameters included growth performance parameters [total weight gain (TWG), feed conversion rate (FCR) and feed efficiency (FE)], evaluation of cell-mediated immunity [total (TLC) and differential leukocytic counts (DLC), phagocytic activity of heterophils (PA) and phagocytic index (PI)], and evaluation of humoral immune response [antibody titers by hemagglutination inhibition test (HIT), serum levels of IgA and IgG, in addition serum protein electrophoretic fractionation profile]. The results showed that BOL injection into rabbits resulted in a significant increase in TWG and FE and a significant decrease in FCR particularly with the double dose of the drug. Monitoring the parameters of cell mediated response revealed a significant decrease in lymphocyte percentage, PA and PI. In regard to humoral immunity, the results demonstrated a significant decrease in serum antibody titers measured by HIT, gamma globulins as well as serum concentrations of both IgA and IgG. In most cases these alterations in the immune responses were more significant when the double dose of BOL was administered. This study concludes that administration of BOL to rabbits although, results in a significant improvement in growth rate, it compromises the immune response inhibiting both cell mediated and humoral immune reactions. Nevertheless, the results suggest that the severity of these immunological side effects is dose-dependent.
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1. Introduction

Anabolic-androgenic steroids (AAS) are a class of synthetic steroids usually derived from testosterone and are recognized for their effects on building up muscle (Yesalis and Bahrke, 1995, Urban et al., 1995, Wu, 1997; Meyer, 2001). Testosterone is the primary and most well-known androgen which is the principle hormone in humans and animals that produces male secondary sex characteristics. **(**Zuloaga et al., 2008**)**. AAS interact with androgen receptors in many tissues to produce numerous physiological effects including increased protein synthesis, muscle mass, strength, appetite and bone growth, so they are used as performance-enhancing drugs (Brodsky et al., 1996; Falanga et al., 1998).
However, the new legislation in many countries now is the restriction of use of synthetic AAS as growth promoters due to their undesirable effects on wide variety of physiological systems. (Landry and Primos, 1990, Evans, 2004, Hall, 2005; Nahed et al., 2010).

These effects may be profound and long lasting depending on the dosing regime, types or combinations of AAS used and the extent and duration of AAS abuse (Hall, 2005). While several adverse effects of AAS abuse have been described, their effect on the immune system has not been clearly elucidated. Some studies have suggested AAS to be immunosuppressive agents that is they reduce immune cell number and function. Specifically, several common AAS have been shown to adversely influence lymphocyte differentiation and proliferation, antibody production and natural killer (NK) cytotoxic activity, thereby altering the immune reaction (Burton et al., 1993, Hughes et al., 1995, Angele, et al., 1998; Hughes et al., 1998).

Boldenone undecylenate (BOL) is an injectable synthetic anabolic steroid derived from testosterone, which shows strong anabolic properties. Recently, in the veterinary field BOL is intensively used, exhibiting a pronounced effect in promoting growth rate in animals destined for meat production or as an aid for treating debilitated animals when an improvement in weight or general physical condition is desired (EL-Ghareib, 2003; El-Sayed and Kerdasy, 2007).

As with other steroids, the drug shows a marked ability to induce many adverse effects in

different species and may have some effect on modulating the immune system. Rabbits have since been identified as an economy livestock that could bridge the wide gap in dietary protein intake all over the world (Adeyinka et al., 2007). Nevertheless, there is very little data on the use of anabolic steroids in rabbits for promotion of rabbit production in Egypt and the effects of AAS on the immune system in rabbits remain uncertain. Therefore, the present study aimed to throw light on the effect of BOL as a growth promoting agent on the immune response in rabbits with particular emphasis on the effect of dosage.

2. Material and Methods

Rabbits:

Sixty apparently healthy 60 days old male white New Zealand rabbits weighing, 1250-1500g were used in the present study. Rabbits were kept in battery cages, and were administered a prophylactic dose of ivermectin (avemic, avico company) as a safe guard against mange and gastrointestinal nematodes. In addition, a prophylactic dose of inactivated vaccine (Hipra, S.A laboratories) against viral hemorrhagic disease was given at a dose of 0.5 ml/rabbit subcutaneously into the fore back.

Experimental protocol:

After two weeks of adaptation and acclimatization, rabbits were divided uniformly by weight into three equal groups each of 20 rabbits. The first group was administered adjuvant substance (sesame oil) and was considered as control (C). The second group (R) was treated with BOL (Tornel laboratories, Mexico) at the manufactured recommended dose for rabbit (4.5mg/kg b.w) according to Paget and Barnes (1964). The third group was treated with BOL at a dose double the recommended dose (9 mg/kg b.w) and was indicated as group D. All treatments were administered intramuscularly three times at three weeks interval. The rabbits were offered a commercial ration pellets (Atmida Co.). Each buck was fed an amount of pellet ration (60gm/kg b.w/day) according to AOAC that provides normal growth and maintains adult body weight. Fresh tap water was supplied *ad libitum*.

Blood samples:

Blood samples were collected three times following the last injection dose of the drug at two weeks interval, i.e. at 15, 30 and 45 days post injection. Each blood sample was divided into three portions. The first portion was placed in tubes containing disodium ethylene diamine tetracetic acid (EDTA) for total (TLC) and differential (DLC) leukocytic counts. The second portion was placed in tubes containing heparin anticoagulant solution (20 IU/ml blood) for determination of phagocytic activity of heterophils and finally, the third portion was placed in plain centrifuge tubes then, serum samples were separated and stored at -20°C until used for other immunological parameters.

Evaluated parameters:

Growth performance parameters:

Rabbits were weighed weekly early in the morning before feeding. Feed residues, average feed intake (gm) and average body gain (gm) were recorded daily. Total weight gain (TWG) (gm) was determined as the difference between the weights of rabbits at the beginning and end of experiment. Feed conversion rate (FCR) was determined as feed intake (gm) divided by weight gain (gm) according to Degani et al., (1986). Feed efficiency (FE) was determined as daily body gain (gm) divided by daily feed intake (gm) (Degani et al., 1986).

Evaluation of cell-mediated immunity including:

a. Total leukocytic (TLC, x 10^3/µl) and differential leukocytic counts (DLC, %).

b. Phagocytic activity (PA) & phagocytic index (PI) of heterophils using Candida albicans were performed according to the method described by Wilkinson (1981).

Evaluation of humoral immunity:

a. Hemagglutination inhibition test (HIT) was performed to detect the antibody titers (AT) according to the method described by Cruickshank (1968).

b. Serum levels of IgA and IgG were detected by ELISA technique using kits of Hellabio biokits company (USA) and following the manufacturer's instructions.

c. Serum protein electrophoresis was accomplished by Polyaccrylamide Gel Electrophoresis using kits of CobasintegranCompany (Roche, Germany) and following the manufacturer's instructions.

Statistical analysis:

Data were subjected to statistical analysis using one way analysis of variance (ANOVA). All data were presented as mean± standard deviation (SD). Means were compared by the Duncan test at 0.05 level of probability.

3. Results

Growth performance:

Table 1 showed that injection of BOL to male rabbits resulted in significant increase in TWG and FE while FCR was significantly decreased. These changes were more prominent when the double dose of the drug was injected compared to rabbits given the recommended one.

Table 1. Growth performance parameters in rabbits administered BOL at the recommended (R) and double (D) doses compared to the control group (C) (Values are mean ± SD).
Means in the same row followed by different letter superscripts are significantly different at (P< 0.05).

Parameter	Group		
	C	R	D
TWG (gm)	775±0.02[b]	868± 0.04[b]	1097±1.25[a]
FCR	10.55±0.02[a]	9.43±0.01[b]	7.31± 0.07[c] 0.136[a]
FE	0.094[c]	0.105[b]	

Effect of BOL on cell-mediated immunity:

a. Total and differential leukocytic counts:

Data presented in (Table 2) revealed that TLC, percents of heterophils (H), eosinophils (E) and monocytes (M) did not show significant changes following BOL administration throughout the experimental period. Meanwhile, BOL injection to rabbits revealed a general trend of reduction in the lymphocytes percent (L) (Figure 1). This reduction was significant (P< 0.05) in both treated groups along the whole evaluated periods. No significant differences in L % between the two groups were observed at any time of the experiment (Table 2).

b. Phagocytic activity &phagocytic index:

Results shown in (Table 3) explored that rabbits given the recommended dose of BOL demonstrated a significant decrease (P< 0.05) in the PA at 30 days post administration while PI significantly decreased at 15 and 30 days. In rabbits administered the double dose of BOL, PA was significantly reduced (P< 0.05) at 15 and 30 days of BOL injection (Figure 1). The decrease in PI in D group was significant (P< 0.05) along the whole evaluated times. Significant differences between the two treated groups were only observed in PI at 45 days following BOL administration.

Effect of BOL on humoral immune response:

a. Antibody titers by (HIT):

BOL treated rabbits revealed a significant decrease (P< 0.05) in the antibodies titer measured by HIT (Figure 2). Significant differences (P< 0.05) from respective control values were seen at 30 days in R group and at 30 and 45 days in D group (Table 4). Significant differences (P< 0.05) between the two doses of BOL were recorded at 30 days of BOL injection.

Table 2. TLC and DLC following BOL administration at the recommended (R) and double (D) doses compared to the control group (C) in rabbits (Values are means ± SD).

Parameter (N=5)	Group	Days post injection		
		15	30	45
TLC ($x 10^3/\mu l$)	C	5.96±0.45[a]	6.93±0.20[a]	6.89±0.19[a]
	R	6.19±0.45[a]	6.67±0.62[a]	6.36±0.11[a]
	D	6.11±0.51[a]	6.67±0.78[a]	6.76±0.81[a]
H (%)	C	30.20±2.19[a]	28.60±1.80[a]	33.55±0.59[a]
	R	34.67±3.06[a]	31.77±0.74[a]	34.57±2.32[a]
	D	36.00±2.52[a]	31.57±1.79[a]	35.60±2.62[a]
E (%)	C	2.00±0.18[a]	2.79±0.37[a]	2.00±0.30[a]
	R	1.57±0.29[a]	2.50±0.20[a]	1.93±0.28[a]
	D	1.85±0.26[a]	2.51±0.28[a]	2.30±0.204[a]
L (%)	C	63.05±3.98[a]	63.29±2.01[a]	60.20±o.94[a]
	R	59.42±3.88[b]	59.50±0.48[b]	57.49±2.32[b]
	D	57.00±2.35[b]	59.21±0.98[b]	55.82±1.27[b]
M (%)	C	4.00±0.57[a]	5.12±0.76[a]	4.14±0.73[a]
	R	4.14±0.79[a]	5.88±0.47[a]	4.87±0.57[a]
	D	5.04±0.67[a]	5.71±0.35[a]	4.81±0.91[a]

Means in the same column followed by different letter superscripts are significantly different at (P< 0.05).

Table 3. PA and PI in rabbits administered BOL at the recommended (R) and double (D) doses compared to the control group (C) (Values are means ± SD).

Parameter Group (N=7)		Days post injection		
		15	30	45
PA (%)	C	80.14±1.18[a]	83.33±0.89[a]	79.04±1.82[b] 85.57±1.17[a]
	R	77.28±1.45[ab]	75.14±0.98[b]	81.14±1.54[b]
	D	74.57±1.34[b]	72.42±0.57[b]	
PI	C	1.90±7.43[a]	1.82±8.71[a]	1.80±7.5[a]
	R	1.70±3.78[b]	1.57±4.20[b]	1.85±7.74[a]
	D	1.65±5.71[b]	1.52±6.53[b]	1.61±5.63[b]

Means in the same column followed by different letter superscripts are significantly different at (P< 0.05).

b. Serum levels of IgA and IgG:

The mean values of serum IgA concentrations were significantly (P< 0.05) lower in both BOL treated groups along the whole experimental times but no significant differences were noticed between the two groups (Table 4). Serum levels of IgG were significantly decreased at 15 and 30 days post injection in R group and at 15, 30 and 45 days in D group (Figure 2). Significant differences (P< 0.05) in IgG values between the two groups were reported at 30 and 45 days after BOL administration (Table 4).

Parameter Group N=5		Days post injection		
		15	30	45
AT by HIT	C	5.20±0.37[a]	5.40±0.24[a]	2.80±1.24[a]
	R	4.80±1.20[a]	4.40±1.31[b]	2.80±1.74[a]
	D	4.60±0.37[a]	3.20±0.24[c]	2.00±0.16[b]
IgA (mg/dl)	C	13.60±1.44[a]	17.09±0.46[a]	11.27±0.94[a]
	R	6.70±0.36[b]	4.38±0.35[b]	4.03±0.56[b]
	D	5.83±0.30[b]	3.63±1.10[b]	3.57±0.25[b]
IgG (mg/dl)	C	347.03±37.01[a]	262.07±0.94[a]	393.00±67.08[a]
	R	288.03±19.34[b]	185.47±2.41[b]	392.67±8.14[a]
	D	282.74±6.77[b]	145.74±8.82[c]	255.33±18.00[b]

Table 4. Antibody titers by hemagglutination inhibition test (HIT) and serum levels of IgA and IgG in rabbits following administration of BOL at the recommended (R) and double (D) doses compared to the control group (C). (Values are means ± SD).

Means in the same column followed by different letter superscripts are significantly different at (P< 0.05).

c. Serum protein electrophoretic fractionation pattern:

The major changes observed in protein electrophoretic pattern as shown in Table 5 consisted of a significant increase (P< 0.05) in serum TP at 15 days in rabbits administered the double dose of BOL. Serum concentrations of ALB were not significantly changed following BOL injection in both groups. Compared to the control group, the mean values of serum total GLO showed a significant decrease in both treated groups at 30 and 45 days after BOL administration. Significant differences in total GLO values between the two groups were noticed only at 30 days of BOL injection. At 45 days following BOL administration, serum levels of alpha 1 globulin (α1-g) demonstrated a significant increase (P<0.05) in D group while serum concentrations of alpha 2 globulin (α2-g) showed a significant decrease (P<0.05) in both groups. Significant increase (P<0.05) in serum levels of beta globulin (β-g) was observed in group D at 15 days of BOL treatment. In regard to serum concentrations of gamma globulins (γ-g), the results in Table 5 cleared that there was a significant decrease (P<0.05) in serum levels of γ-g in both treated groups in all evaluated periods (Figure 2). Significant differences (P<0.05) between the two groups were seen at 30 and 45 days of BOL administration.

Table 5. Plasma protein profile (total and electrophoretic pattern) in rabbits administered BOL at the recommended (R) and double (D) doses compared to the control group(C). (Values are means ± SD).

Parameter (N=5)	Group	Days post injection		
		15	30	45
TP (g/dl)	C	6.17±0.05[b]	6.16±0.04[a]	6.41±0.12[a]
	R	6.34±0.04[a]	5.96±0.14[a]	6.25±0.01[a]
	D	6.50±0.20[a]	6.07±0.06[a]	6.30±0.13[a]
ALB (g/dl)	C	3.98±0.02[a]	4.04±0.05[a]	4.17±0.11[a]
	R	4.28±0.14[a]	4.26±0.15[a]	4.35±0.05[a] 4.40±0.17[a]
	D	4.29±0.22[a]	4.17±0.05[a]	
GLO (g/dl)	C	2.20±0.05[a]	2.12±0.03[a]	2.24±0.04[a]
	R	2.02±0.10[a]	1.70±0.09[c]	1.89±0.06[b]
	D	2.20±0.05[a]	1.90±0.10[b]	1.90±0.09[b]
α1-g (g/dl)	C	0.13±0.01[a]	0.13±0.05[a]	0.14±0.05[b]
	R	0.18±0.03[a]	0.14±0.05[a]	0.12±0.04[b] 0.27±0.00[a]
	D	0.17±0.03[a]	0.16±0.04[a]	
α2-g (g/dl)	C	0.54±0.02[a]	0.49±0.03[a]	0.61±0.00[a]
	R	0.57±0.02[a]	0.50±0.02[a]	0.50±0.01[b] 0.49±0.02[b]
	D	0.56±0.02[a]	0.55±0.01[a]	
β-g (g/dl)	C	0.77±0.03[b]	0.78±0.02[a]	0.75±0.04[a]
	R	0.84±0.04[ab]	0.71±0.01[a]	0.81±0.02[a] 0.74±0.08[a]
	D	0.89±0.05[a]	0.83±0.02[a]	
γ-g (g/ dl)	C	0.74±0.01[a]	0.71±0.02[a]	0.74±0.03[a]
	R	0.51±0.01[b]	0.40±0.02[b]	0.46±0.01[b]
	D	0.48±0.02[b]	0.33±0.02[c]	0.40±0.02[c]

Means in the same column followed by different letter superscripts are significantly different at (P< 0.05).

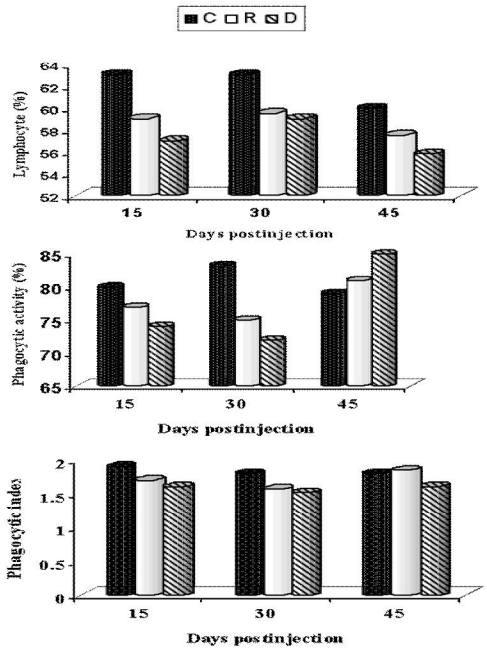

Fig. 1: cell-mediated immune response following administration of BOL at the recommended (R) and double (D) doses compared to the control group (C) (Values are means ± SD). Values at (P< 0.05) were significant.

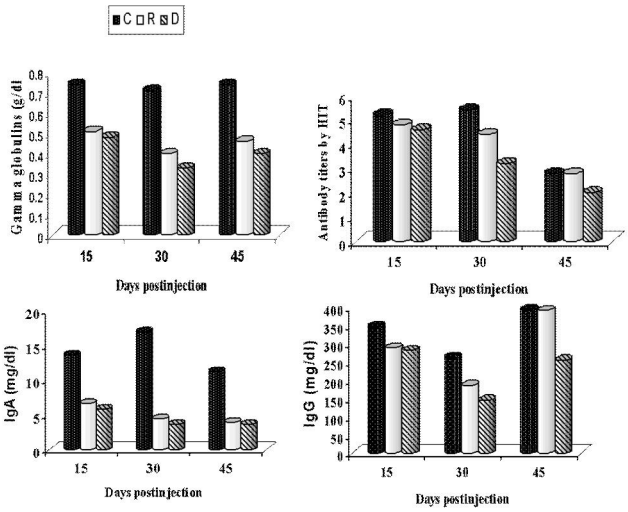

Fig. 2. Humoral immune response following administration of BOL at the recommended (R) and double (D) doses compared to the control group (C) (Values are means ± SD). Values at (P< 0.05) were significant.

4. Discussions

Anabolic-androgenic steroids are derivatives of testosterone whose development was centered on the need for agents that exhibited different characteristics than did testosterone (Meyer, 2001). In general, the goal was to develop agents that were more anabolic and less androgenic than testosterone (Landry and Primos1990). Therefore, these agents are often used to enhance physical performance and promote muscle growth (Brodsky et al., 1996; Evans, 2004) but there is no enough available information on their use in rabbits to make firm recommendation for their use in these animals. The present results revealed that injection of the anabolic steroid BOL to male rabbits evoked a significant increase in growth rate as indicated by a significant increase in TWG and FE as well as the improved FCR particularly in rabbits treated with the double dose of the drug (Table 1). Similar findings were reported by Nahed et al., (2010). This effect could be attributed to BOL acting upon the androgen receptors in anabolic-responsive tissues promoting the body tissue building processes due to increased protein synthesis. This is achieved by converting the negative nitrogen balance into a positive one through improving the use of ingested proteins and increasing nitrogen retention and animal's appetite (Falanga et al., 1998). Others reported that anabolic hormones can increase the cellular protein biosynthesis indirectly via stimulation of growth hormone and insulin like growth factor secretion (Arnold et al., 1996).

Furthermore, it was also assumed that AAS reduce recovery time by blocking the effects of stress hormone cortisol on muscle tissue, so that catabolism of muscle is greatly reduced (Sauerwein, 1991). Total protein significantly increased with the double dose of BOL at 15 days postinjection due as mentioned previously to the multifactorial effects of BOL on the protein metabolism (Abdelatif et al., 1993; El-Ghareib and Ashry, 2003; El-Sayed and Kerdasy, 2007).

However, there is a wide apprehension that ASS administration is often associated with various adverse effects resulting in a variety of health problems that are generally are dose related (Hall, 2005). There is a large amount of data indicating that anabolic steroids may have some effects on modulating the immune system (Hughes et al., 1995). Considering the past demonstrations on the actions of normal steroids on immune response, it was apparent that AAS may be immunosuppressive leading to a decreased effectiveness of the defense system that is they reduce immune cell number and function (Ferrández et al., 1996). But such data are not available for rabbits. In this respect we evaluated the effect of BOL on some immunological parameters related to both cellular and immune response. In regard to the cellular immune response, the present study revealed a significant reduction in the lymphocytes percent in both treated groups along the whole evaluated periods. Also, monitoring of phagocytic activity and phagocytic index indicated that there was a significant decrease in both phagocytic activity percent and phagocytic index during most of the experimental period particularly when the double dose of BOL was administered. The modulation of the immune system by androgens and estrogens has been well studied in different animal species. In veal calf production anabolic androgenic and estrogenic steroids were used as growth promoters in various combinations to enhance performance of the calves. Besides growth stimulation, these anabolic hormones were found to affect the function of the immune system and compromise the health of these animals (Burton et al., 1993, Biolatti et al., 2005).

Ferrández et al., (1996) studied the effect of high doses of anabolic steroids on activity of immune cells in cultures of rat spleen and thymus lymphocytes. They reported impaired lymphocyte mobility and an inhibition of mitogen-induced proliferative response of 90 percent.

This immunosuppressive effect of AAS on cellular immunity could be related as mentioned in previous reports to two mechanisms. First, androgens have the ability to bind to the reticuloendothelial cell receptors on the thymus, altering the release of thymic factors that could affect the function of effectors T-lymphocytes (Guarda et al., 1990). Second, androgens can directly affect T-lymphocytes by binding to their androgen receptors on the plasma membrane or cell nucleus (Olsen and Kovacs, 1996, Benten et al., 1999; Olsen and Kovacs, 2001). Other mechanisms may include increased suppressor T-cell populations and reduced T-helper cell function (Wunderlich et al., 2002). Testosterone also could induce a rapid influx in intracellular free Ca concentration in activated T cells which can alter T-cell function (Benten et al., 1999; Wunderlich et al., 2002).

The expression of nitric oxide synthase in macrophages has been reported to be inhibited by testosterone which resulted in impaired NK cell activity and the phagocytic capacity of macrophages (Friedl et al., 2000; Zhang et al., 2001). Other studies have implicated that AAS adversely influence cellular immunity by reducing the production of inflammatory chemicals in body tissues and certain cytokines, thereby reducing complement components and altering NK cytotoxic activity, chemotaxis and opsonization of particles which leads to defective phagocytosis and by all these manners these agents can suppress the cellular immunity. (Burton et al., 1993, Friedl et al., 2000, Zhang et al., 2001; Hedger

and Meinhardt, 2003). To evaluate the humeral immunity in BOL treated rabbits, serum protein electrophoretic fractionation, antibody titers by HIT and serum levels of IgA and IgG were determined. The most important changes in serum protein electrophoresis were the significant decrease in serum concentrations of gamma globulins particularly with larger dose of BOL. In addition, antibody titers measured by HIT and serum levels of IgA and IgG showed a significant decrease. These decreases indicate lowered humoral immunity and could be attributed to the decreased percent of lymphocytes that secrete antibodies. These results tend to support the previous findings in that the immunosuppressive action of BOL on humoral immunity could be attributed to BOL reduces B-cell lymphopoiesis thus, decreases antibody secreting cells, affecting antibody production and lowering the circulating levels of immunoglobulins. (Lucas et al., 1985, Burton et al., 1993; Olsen and Kovacs, 2001, Braude et al., 1999; Cuesta et al., 2007). Others found that testosterone can suppress the humoral immunity indirectly by reducing IL-6 production of monocytes and thus inhibiting B cells (Kanda et al., 1996).

In conclusion, the present study suggests that although administration of boldenone undecylenate into rabbits induces a significant improvement in the growth rate, the drug produces marked immunosuppressive effects as indicated by inhibition of both cell-mediated and humoral immune responses which may not recommend BOL for use as a growth promoter in rabbits. Nevertheless, the results support previous reports that the severity of these immunosuppressive effects is dose-dependent.

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