EFFECT OF LARGE DIETARY DOSES OF β-CAROTENE ON PLASMA RETINOID AND β-CAROTENE LEVELS AND ON PROGESTERONE PRODUCTION IN THE GRANULOSA CELLS OF JAPANESE QUAIL

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An experiment was conducted to study the effect of large-dose β-carotene supplementation on blood retinoid and β-carotene levels as well as on the progesterone secretion of the granulosa cells in Japanese quail. Laying quails were assigned to three dietary groups. The control group (Group C) received the basal diet (laying feed containing 9000 IU vitamin A/kg). In the treated groups (Groups BC1 and BC2) the basal diet was supplemented with 10^2 and 10^3 mg/kg β-carotene (BC), respectively. At the end of the two-week feeding period, 10 birds from each group were euthanised. Blood samples were analysed for retinol, retinyl palmitate and β-carotene concentrations. Granulosa cells were isolated from ovarian follicles (F_1 and F_2), and PMSG-induced *in vitro* progesterone (P_4) secretion was measured. Similar retinol concentrations were found in both β-carotene supplemented groups, indicating saturation of the retinol-transporting system. β-carotene supplementation was accompanied by hypercarotenaemia, but did not increase the retinyl palmitate levels in the blood. PMSG-induced P_4 production of the granulosa cells decreased significantly in Groups BC1 and BC2 in a dose-dependent manner.

Key words: β-carotene, retinoids, progesterone, granulosa cell, Japanese quail

Since the 1980s, naturally occurring compounds and a growing number of synthetic compounds with more or less vitamin A activity have been called ret i-noids due to similarities in their structure and effects. The term 'vitamin A' is correct if used as a general term (effect, deficiency, etc.). Wherever possible, the name of the molecule should be given: retinol, retinyl ester, e.g. palmitate, etc. (Goodman, 1987). There are manifold explanations for the metabolic and/or r e-productive symptoms resulting from the deficiency of certain retinoids. These symptoms can be explained by general influences such as effects exerted on the

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synthesis of membrane glycoproteins which participate in the differentiation of the epidermal and connective tissue (Sporn, 1977), by antioxidant functions similar to those of some tocopherols and carotenoids (Livrea and Packer, 1994), and by retinoid influences aimed at the preservation of biological membranes. A number of studies indicate an interrelation between retinoids and the endocrine system (Galdieri and Nistico, 1994; Huang et al., 1985). After binding to rece p-tors, active retinoids (e.g. retinoic acid) directly or indirectly stimulate the e x-pression of specific genes (Moore, 1990; Hashimoto, 1991). They have a specific function in the gene expression of microsomal cytochrome P_{450} oxidases (Hauswirth and Bizuela, 1976), which take part in many steps of the biosynthesis of cholesterol and steroid hormones (Murray et al., 1991). As a result of these discoveries over the last few years, retinoids have been placed in the category of hormones (Wolf, 1990).

The retinoid metabolism of avian species differs from that of mammals in several respects. Rats kept on a vitamin A deficient diet supplemented with ret i-noic acid grow appropriately but become blind and infertile in both sexes. In chicks, appropriate growth can be found, too, and both cocks and hens remain fertile. However, embryonic death will occur already on the second day of inc u-bation (Thompson et al., 1969).

In the ovary, the synthesis of steroid hormones takes place in special cells: the granulosa, external and internal cells of the theca. These cell layers form the wall of the follicle. During follicular maturation, granulosa cells synthesise pr o-gesterone in birds. The primary source of ovarian progesterone is the largest and the second largest preovulatory follicle (F_1 and F_2, respectively).

In this experiment, Japanese quails were fed large doses of β-carotene (BC). The effects of supplementation on the retinol, retinyl palmitate and BC concentration of the blood as well as on progesterone (P_4) secretion of the granulosa cells *in vitro* were studied.

Materials and methods

Experimental animals and design

Three groups (with 10 birds in each group) were formed from 10-week-old Japanese quail hens. The control group (C) was fed basal feed containing 9000 IU vitamin A/kg feed. The basal feed of the treated groups (Groups BC1 and BC2) was supplemented with β-carotene (BC, Table 1). After two weeks of feeding, the birds were killed by bleeding. Retinoid, BC and lipid content of the blood plasma as well as progesterone (P_4) secretion of granulosa cells isolated from the ovarian follicles were determined.

Table 1

Experimental design and treatments

Treatment	Groups[*]		
	C	BC1	BC2
RA (IU/feed kg)	9000	–	–
BC (mg/feed kg)	–	100	1000

[*]Group C = control, basal diet; Groups BC = basal diet supplemented with β-carotene; RA = retinyl acetate; BC = β-carotene

β-carotene used for the experiment

To adjust the required doses of BC, Rovimix-β-carotene 10% (Hoffmann-La Roche, Basle) starch-coated preparation was used for supplementation.

Cell preparations

The granulosa layers were separated from the F_1 and F_2 follicles of the ovaries. After several cycles of washing (MEM Eagle, 20 mM HEPES, 0.1% BSA fraction V, N°A-8022, Sigma, St. Louis, 50 mg/L gentamicin sulphate, 50,000 IU/L penicillin, 50 mg/L streptomycin) the cells were dispersed by digestion with collagenase (Collagenase type IA, N°C-9891, Sigma, St. Louis). This process was followed by several cycles of washing, centrifugation and cell counting (by Trypan Blue exclusion method, no. of live cells > 90%). The final number of cells was adjusted to 10^5 cells/0.8 mL with antibiotic-free MEM. Subsequently the suspension was divided into plastic tubes (0.8 mL/tube) which were treated with 0.5 IU/mL PMSG (Folligon, Intervet) and incubated (37 °C, 5 h).

Analytical methods

ELISA. The progesterone concentrations of the incubates of granulosa cells were measured by an ELISA technique developed at the Agricultural Biotechnology Centre (ABC, Gödöllő, Hungary) (Siklódi et al., 1995).

High-performance liquid chromatography (HPLC). Measurement of plasma retinoid (retinol, Rol; retinyl palmitate, RP) concentrations was carried out by isocratic adsorption HPLC (Biesalski et al., 1986). The modifications and adaptations of the method for BC analysis were published previously (Kerti and Bárdos, 1999).

Statistical analysis

Statistical analysis was performed on individual data obtained from analytical results and presented as means and ± SD. Correlations were calculated by

the Pearson-Braevis formula. Differences between groups and treatments were evaluated by Student's *t*-test and considered as significant if $p < 0.05$.

The animal experiment was carried out with consent of the Animal Experiments Board of the University.

Results

The blood concentration of Rol was significantly higher ($p < 0.001$) in the groups given BC supplementation than in Group C. The RP concentration of the blood increased significantly in Group BC2 ($p < 0.05$), compared with the values measured in Group C (Table 2). Plasma BC level was in close correlation with BC supplementation ($r = +0.7$, $p < 0.05$). PMSG-induced *in vitro* P_4 production of granulosa cells was decreased in the BC-supplemented groups. The decrease seemed to be dose dependent (Table 2).

Table 2

Blood retinoid and β-carotene levels (μg/L)
and *in vitro* progesterone secretion (ng/mL) of granulosa cells ($\bar{x} \pm$ SD)

Parameters[**]	Groups[*]		
	C	BC1	BC2
Rol	692 ± 224	1220 ± 255^c	$1394 \pm 166^{c,x}$
RP	848 ± 880	1200 ± 204	1713 ± 179^a
BC	60 ± 28	551 ± 210^c	$1157 \pm 364^{c,z}$
P_4	79.0 ± 4.1	57.1 ± 33.0	$27.9 \pm 2.7^{c,z}$

[*]Group C = control, basal diet; Groups BC = basal diet supplemented with β-carotene; [**]Rol = retinol, RP = retinyl palmitate, BC = β-carotene, P_4 = progesterone; Values of significance ($p \leq 0.05$; $p \leq 0.01$ and $p \leq 0.001$) [a] and [c] between control and treated groups; [x] and [z] between treated groups

Discussion

The plasma vitamin A level (both Rol and RP) of Japanese quails fed high dietary doses of BC showed a significant increase by the 2nd week of treatment. In Group BC1 no increase of RP level was found, but in Group BC2 a slight increase did occur (Table 2). The retinyl ester fraction (RP) indicates intensive absorption from the intestine (Goodman and Blaner, 1984). Elevation of the level of retinyl esters in the blood is a clinical laboratory diagnostic indicator of hypervitaminosis A (Smith and Goodman, 1976). In this experiment no clinical symptoms of hypervitaminosis A were detected, probably due to the short period of supplementation. From these results it can be concluded that the transform a-

tion of BC into vitamin A was intensive in Japanese quail. When increasing the dose of BC the efficiency of transformation decreased in the same manner as had been found in chicks (Brubacher and Weiser, 1985). This is one of the possible explanations why excess BC causes only carotenaemia and does not pose a risk of hypervitaminosis A.

Ovarian granulosa cells have several types of ion channels, e.g. potassium, chloride and calcium. The ion milieu is a very important factor during *in vitro* studies. In the hen, luteinizing hormone induced P_4 production of ovarian granulosa cells is inhibited when chloride ions are removed from the medium (Chiang et al., 1997). In the present experiment all ions in the MEM were within the recommended physiological range.

The results of this experiment indicate that the ovarian steroid metabolism of Japanese quails is affected by BC treatment. This influence was detected in *in vitro* P_4 production occurring as a response to PMSG stimulation of granulosa cells. Vitamin A stimulates the P_4 secretion of granulosa cells *in vitro* (Talavera and Chew, 1988). Taking into account that steroid hormones are secreted at the rate of their production, it seems reasonable to suppose that the increased P_4 secretion was the result of an increased synthesis. According to some hypotheses, retinol plays a part in the transformation of pregnenolone into P_4 (Ganguly et al., 1980). At that step the enzymes $\Delta5$-3β-hydroxysteroid-dehydrogenase and $\Delta5$-3β-isomerase are the most important in birds.

In certain mammals BC is known to have a retinoid-independent role in reproduction, in addition to its antioxidant and provitamin functions. There are reports in the literature that BC affects the reproductive functions of cows in a manner independent of vitamin A (Lotthammer and Ahlswede, 1977; Lotthammer, 1978). In cows, the increase of dietary BC was found to stimulate *in vitro* P_4 production of isolated luteal cells (Pethes et al., 1985).

The pattern of *in vitro* steroidogenesis in Japanese quail granulosa cells is similar to that described for the chicken (Etches and Cunningham, 1976) and turkey (Hammond et al., 1981). Quantitative differences in the capacity of steroidogenesis between developing and mature follicles are more striking in the quail (Asem et al., 1985). In this *in vitro* experiment, PMSG-stimulated granulosa cells isolated from Japanese quails having different blood retinoid and/or BC levels as a result of dietary BC supplementation showed differences in P_4 production (Table 2). Although the efficiency and thus the vitamin A potential of BC were depressed (Brubacher and Weiser, 1985), extremely large doses of BC resulted in an unexpected influence on *in vitro* P_4 secretion of the granulosa cells. The rate of P_4 secretion was inversely proportional to the dietary doses and the plasma BC level, respectively. Both levels of BC supplementation resulted in an approximately equal plasma Rol level, suggesting that the RBP-TTR transport protein system was fully saturated. BC is known to have only a single transport mechanism which is of low specificity and dependent on the metabolism of plasma

lipoproteins (Schweigert et al., 1993). This is supported by previous results, i.e. that an increase in the level of RP belonging to the fraction of plasma lipopr o- teins caused a decrease in plasma BC (Kerti and Bárdos, 1999), and that different BC doses resulted in different plasma BC levels (Kerti and Bárdos, 1997) in Japanese quail.

Although BC as a vitamin A precursor has never been reported to exert any harmful effect on the functioning of the animal organism, the results of this study show that extreme hypercarotenaemia can depress the P_4 production of granulosa cells.

Acknowledgements

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