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The histopathological changes of liver and testis of Japanese quail chicks fed different levels of dietary L-valine

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ABSTRACT

This experiment was carried out to investigate the histological changes of liver and testis of Japanese quail fed different levels of dietary valine (Val) in low protein diet. A total of 1000 one-day-old Japanese quail chicks (mixed sex) were assigned to five experimental diets including diets containing 7.5, 8.5, 9.5, 10.5 and 11.5 g digestible (dig.) Val/kg diet in a completely randomized design, with 5 replicates of 40 quail chicks per pen. Experimental diets were formulated to be isoenergetic and isonitrogenous (170 g crude protein/kg) to meet nutrients recommendation of growing quails suggested by Brazilian tables. At d 42, quail chicks were slaughtered, and tissue samples were collected and fixed to evaluate the histological indices of liver and testis. High levels of Val, increased ($P < 0.05$) diameter of liver cell nucleus and liver hepatocytes in both male and female. While 11.5 g Val showed mild hepatosteatosis, bile duct hyperplasia was observed in 10.5 g Val. In 7.5 and 8.5 g Val groups, there was no negative effects on the liver histology. The male quail chicks which fed on diets containing 8.5 g Val had better significant ($P < 0.05$) reproductive indexes [Tubular differentiation (TDI) and spermatic index (SI)]. In conclusion, the use of high levels of Val (≥ 9.5 g dig. Val/kg diet) during d 0 – 42 of age can lead to histological damage in liver and testis of quail chicks.

1. Introduction

High quality protein with adequate amino acid (AA) balance is one of the most important nutrients for quails (Soares et al., 2003). Insufficient levels of AAs can lead to lower performance because deficiency of AAs restricts protein synthesis (Lima et al., 2016). Branched-chain amino acids (BCAAs), including leucine (Leu), isoleucine (Ile), and Valine (Val), are essential AAs for the growth and development of animals, which participate in the synthesis of proteins and precursor of other AAs. The BCAAs have various biological effects, including the promotion of protein synthesis and hepatocyte proliferation, simulation of immune systems, improvement of insulin resistance, inhibition of liver cancer cell proliferation and neovascularization (Tajiri & Shimizu, 2018). Protective effects of BCAAs were shown in previous studies. For example, in a rat model with CCl_4-induced liver injury, supplementation of BCAAs was shown to suppress hepatocyte apoptosis leading to retardation of the progression of the injury (Kuwahata et al., 2012). Interestingly, several studies have reported that fed animals with a BCAAs deficient diet increased lipolysis (Zhang, Zeng, Ren, Mao, & Qiao, 2017). However, the roles of each member of BCAAs' family in diet is an undertreated issue (Sefidabi et al., 2022).

Unlike the other BCAAs, Val is a limiting AA in the corn-soybean diet and must often be supplemented in a low crude protein (CP) diet (Kim, Singh, Wang, & Applegate, 2022). Harms and Russell (2001) reported an improvement in commercial layers' performance by adding Val to a corn-soybean meal diet containing supplemental methionine (Met), lysine (Lys), tryptophan (Trp), Ile and threonine (Thr). They reported that Japanese quail required CP slightly greater than 160 g/ kg with 8.3 g Val/kg in diet. Similarly, 160 g CP/kg diet of laying quail supplemented with Thr, Trp, and Val or their mixture was suitable for better performance, egg quality, lower feeding cost and reduction of nitrogen excretion compared to 200 g CP/kg diet (Alagawany et al., 2014). Jian et al. (2021) reported that dietary supplemented Val enhanced the trypsin activity of duodenum chime and promoted the mRNA expression

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levels of AA transporter, in the jejunum and corresponding serum free Ile, Lys, Phe, Val, and Tyr level in laying hens. Recently, Emadinia, Toghyania, Foroozandeh, Tabeidian, & Ostadsharif, 2020 pointed out the over expression of Val transporter genes with incremental levels of CP and Val supplementation in Japanese quail. More recently, it was suggested that dietary Val supplementation exerts positive effects on performance of the broiler via promoting AA uptake and utilization (Jian et al., 2021).

Nowadays, the use of low-protein diets supplemented with synthetic AAs such as DL-Met, L-Lys, L-Thr, and L-Trp has become very common to provide better AA balance, reduce feed costs and reduce environmental pollution. Since 2009, feed-grade Val has been introduced to the livestock feed industry. Using supplemental L-Val in the diets, may further lower the dietary CP content and consequently reduce the feed costs (Tavernari et al., 2013). However, not only the responses and mechanisms regarding positive/negative effects of high levels of Val were not reported, but also the histology changes in sensitive tissues such as liver and testis were ignored in previous studies. In human studies, high BCAAs levels have been reported in some chronic diseases such as liver failure, obesity, insulin resistance, diabetes, and cardiovascular disease (Burrage et al., 2014). Also, there are concerns about its negative effects on fertility in male athletes who use supplements containing high Val (Sefidabi et al., 2022).

We hypothesized that Val may play a role in the incident failures of the vital organs especially the liver or testes in growing birds. Therefore, our objective was to investigate the effect of supplemental Val under and over recommended levels (7.5, 8.5, 9.5, 10.5 and 11.5 g Val/Kg diet) in low protein diets (170 g CP/kg) on the histology of liver and testes in growing Japanese quails.

2. Materials and methods

2.1. Quails chicks, diets, and management

All experiment procedures used were confirmed by the Animal Welfare Committee of the Department of Animal Science, University of Tehran, Tehran, Iran. A total of 1000 one-day-old Japanese quails' chicks (mixed sex) with an average weight of 7.45 + 0.25 g were randomly allocated to 25 floor pens (100 × 200 cm) covered with wood shavings. Quail chicks were assigned to one of five dietary experimental groups, comprising 5 pen replicates of 40 chicks, and raised under environmentally controlled conditions, following a standard temperature regimen that gradually decreased from 37 to 24°C by 3.5°C weekly. A continuous lighting program was provided on the third day of experiment followed by a 17L: 7D lighting program thereafter. Experimental diets were formulated using standardized ileal digestible (SID) AA values to meet nutrients requirements of growing quails suggested by Rostagno et al. (2011), except for the dig. Val level (Table 1). L-glutamic acid (980 g/kg; Ajinomoto Co. Inc., Japan) was used as filler to complete diet formulations to 1000 g or 1 Kg, and to maintain a similar CP content of experimental diets. Filler was replaced with Val (L-Valine, 980 g/kg; Morghenojan Co., Iran) to produce diets with different levels of SID Val (7.5, 8.5, 9.5, 10.5, and 11.5 g Val/kg diet). Diet and fresh water were provided ad libitum.

2.2. Sampling and tissue preparation

At the end of experiment (d 42), five males and five females quail of each experimental group were randomly selected. The quails were individually weighed and killed by cervical dislocation after 4 h feed deprivation, and then the necropsy was applied to remove the liver and testis. Sections of the liver and testis were dissected and immediately fixed by formalin (10%). After dehydration with ethyl alcohol in increasing concentration (70–100%) and passed in two contents of xylol the samples were embedded in paraffin, sectioned by the rotary microtome at 5 μm. After slides samples were passed through the decreasing concentration (100–70%) of ethylic alcohol and in xylol. The histological slides were stained by Hematoxylin and Eosin stain (Luna, 1968). An ocular micrometer was used to measure the diameter of liver cells nucleus, diameter of liver hepatocytes and volume of sinusoids (as a percentage of the total liver volume).

The counting Sertoli cells (in a radius of 50 μm), the height of the epithelium of seminiferous tubules (μm), and the diameter of testicular capsule was measured for histomorphometric evaluation of testicle, while tubular differentiation index (TDI) and spermatic index (SI) were used to check spermatogenesis and spermiogenesis alterations. Tubular differentiation index included the percentage of spermatogenesis tubules with more than 4 cell lines (TDI positive) compared with seminiferous tubules with less than 4 cell lines (TDI negative). Regarding the spermatic index (SI), the percentage of seminiferous tubules with spermatid to seminiferous tubules without spermatid was included (Asadi, Rahmani, Samadi, & Kalantari Hesari, 2021).

The photomicrographs were captured with the aid of a micro camera attached to a microscope (Olympus BX-51) and the images were digitalized on software KS 400.3 (Zeiss 4.3).

2.3. Statistical analysis

All data were subjected to ANOVA using the General Linear Models Procedure of SAS software (version 9.4SAS Institute Inc, 2013). When $p < 0.05$, Tukey's test was performed to determine the significance between mean values. Tukey's test was applied to estimate the possible difference between treatments and finally, statistical differences were declared at $P < 0.05$. Orthogonal polynomial contrasts were used to determine the significance of linear, quadratic, and cubic models to

Table 1
Composition and calculated analysis of basal diet diets (as-fed basis).

	d 0 to 21	d 22 to 42
Ingredients (g/kg)		
Corn Grain	703	692
Soybean Meal-44	233.2	231.5
Wheat Bran	–	30
Dicalcium Phosphate	16	13
Limestone	13	12
Vegetable Oil	6.5	2.0
Mineral mix[1]	2.5	2.5
Vitamin mix[2]	2.5	2.5
Salt	2.5	2.5
L-Lysine HCL	4.64	3.19
DL-Methionine	3.04	2.24
L-Arginine	2.52	0.24
L-Threonine	2.32	1.33
L-Isoleucine	1.18	–
L-Triptophan	2.10	–
L- Glutamic acid [3]	5.0	5.0
Calculated analysis		
AMEn[4] (MJ/kg)	12.1	12.3
CP (g/kg)	177	170
Fat (g/kg)	28.8	29.0
Fiber (g/kg)	2.91	3.17
Calcium (g/kg)	9	8
Available Phosphorus (g/kg)	3.9	3.5
Digestible Lys (g/kg)	12.0	10.8
Digestible Met (g/kg)	5.37	4.57
Digestible Met + Cys (g/kg)	7.6	6.8
Digestible Val(g/kg)	7.5	7.5

[1] Mineral premix supplied per kilogram of diet: Mn, 60 g; Fe, 80 g; Zn, 50 g; Cu, 10 g; Co, 2 g; I, 1 g, Se, 250 mg; and vehicle quantity sufficient to 500 g.
[2] Vitamin premix supplied per kilogram of diet: vitamin A, 15,000,000 IU; vitamin D3, 1500,000 IU; vitamin E, 15,000 IU; vitamin B1, 2.0 g; vitamin B2, 4.0 g; vitamin B6, 3.0 g; vitamin B12, 0.015 g; nicotinic acid, 25 g; pantothenic acid, 10 g; vitamin K3, 3.0 g; and folic acid, 1.0 g.
[3] L-Glutamic acid was replaced with L-Valine to provide diets with different levels of digestible Val (0.85, 0.95, 1.05 and 1.15% of diet).
[4] AMEn: Nitrogen-corrected apparent metabolisable energy.

describe the biological response of the Japanese quail to varying levels of dietary Val. For the analysis of the liver histology, a factorial scheme was used as a statistic design with two factors (valine with five levels and sex with two levels).

3. Results

3.1. Liver histology

As shown in Table 2, the diameter of cell nucleus ($P < 0.05$) and hepatocytes ($P < 0.05$) increased with increasing of dietary Val levels, and the highest amount was at the level of 11.5 g Val/kg (8.47 and 13.32 μm, respectively). Similarly, the highest percentage changes versus control were observed in the 11.5 g Val/kg group (79 and 39% in the diameter of cell nucleus and the diameter of hepatocytes, respectively). However, the difference between control and 8.5 g Val/kg group was not significant. The volume of sinusoids were unaltered by treatments (Table 2).

The interaction of different levels of Val and bird sex on the diameter of cell nucleus ($P < 0.05$) and the diameter of hepatocytes ($P < 0.05$) of quail chicks were significant and the highest percentage changes versus control was greater in males (104 and 51%, respectively) than females (66 and 30%, respectively). The diameter of liver cell nucleus (6.64 μm) and the diameter of hepatocytes (12.09 μm) of male were greater than female (6.4 μm and 10.62 μm, respectively) ($P < 0.05$). The volume of sinusoids of female (14.98) was greater than male (10.62) ($P < 0.05$, Table 2).

During microscopic examination, accumulation of inflammatory cells was observed in 9.5 g Val/kg group. While 11.5 g Val showed mild hepatosteatosis, bile duct hyperplasia was observed in 10.5 g Val. (Fig. 1).

3.2. Testis histology

Data in Table 3 showed that tubular differentiation index (TDI) and spermatic index (SI) were altered ($P < 0.05$) by Val inclusion in diet, and the highest amount was at the level of 8.5 g Val/kg. Dietary inclusion of Val did not affect the testicular capsule diameter, epithelium height, and Sertoli cell number (Table 3).

Interestingly, the release of spermatogenic cells into the lumen of the seminiferous tubule was observed in 11.5 g Val/kg group. In the germinal epithelium of the groups fed the levels of 9.5, 10.5 and 11.5 g Val/kg, vacuole formation was observed between the germ cells (Fig. 2).

4. Discussion

The morphological study of the liver, allows to evaluate objectively the main parameters of metabolism, to reveal organ pathology, which develops as a result of the Val supplementation (Fletcher, 2016; Skovorodin et al., 2019). In our experiment, different levels of dietary Val were effective on the histology of quails' liver and testis. With increasing Val level, the highest diameter of liver cell nucleus and hepatocyteswere observed through consumption 10.5 and 11.5 g Val/kg diets in both female and male. Similarly, the percentage of these changes versus control were high which means a decrease in the hepatocytes' functional activity through the reduction of nuclear plasma ratio in high levels of Val in diet. At high levels of dietary Val (10.5 and 11.5 g Val/kg diet), bile duct hyperplasia and mild hepatosteatosis were also observed. In broilers, hyperplasia and fibroplasia of the bile ducts are classified as nonspecific lesions and are associated with changes in hepatic metabolism that occur systematically after lesion of the liver parenchyma (Hochleithner et al., 2005). Altogether, the use of high levels of Val (≥ 9.5 Val/kg) in a low protein diet can lead to histological damage in the liver of quails.

Liver hepatocytes are any of the polygonal epithelial parenchymatous cells of the liver that secrete bile. The BCAAs enhanced hepatocyte regeneration in a rat hepatectomy model (Kim et al., 2011) and were shown to increase the secretion of hepatocyte growth factor (Tomiya et al., 2004). BCAAs has been associated with cell proliferation through activation of mechanistic target of rapamycin complex 1 (mTORC1).

Table 2
The effect of different Val levels and bird sex on liver histology of quail[1].

Item	Diameter of liver cell nucleus (μm)	Changes[2] (%)	Diameter of liver hepatocytes (μm)	Changes[2] (%)	Volume of sinusoids (%)
Sex					
female	6.4^{b}		10.32^{b}		14.98^{a}
male	6.64^{a}		12.09^{a}		10.62^{b}
SEM	0.18		0.259		0.54
Val (g/kg diet)					
7.5	4.72^{c}		9.6^{b}		13.27
8.5	4.68^{c}	−1	8.87^{b}	−8	12.09
9.5	5.95^{b}	26	12.65^{a}	32	11.77
10.5	7.87^{a}	67	12.97^{a}	35	13.46
11.5	8.47^{a}	79	13.32^{a}	39	13.42
SEM	0.285		0.41		0.85
Val level × sex					
7.5 × male	4.49^{d}		9.66^{b}		13.5
8.5 × male	4.27^{d}	−5	8.51^{b}	−12	9.02
9.5 × male	6.73^{bc}	50	14.63^{a}	51	8.73
10.5 × male	8.99^{a}	100	13.96^{a}	45	11.03
11.5 × male	8.71^{a}	104	13.71^{a}	42	10.83
7.5 × female	4.95^{dc}		9.53^{b}		13.04
8.5 × female	5.09^{dc}	3	9.25^{b}	−3	15.16
9.5 × female	5.18^{dc}	5	9.9^{b}	7	14.8
10.5 × female	6.75^{bc}	36	9.98^{b}	1	15.89
11.5 × female	8.23^{ab}	66	12.93^{a}	30	16.0
SEM	0.403		0.580		1.209
p-value					
Sex	0.025		<0.0001		<0.0001
Val level	<0.0001		<0.0001		0.48
Val level × Sex	0.0027		<0.0001		0.057

[a-d]Means in each column with different superscripts are different ($P < 0.05$).
[1] Each value represents the mean of 5 replicates with 3 quail chicks (each sex) per pen selected for liver sample on d 42.
[2] The changes versus 7.5 g Val/kg diet.

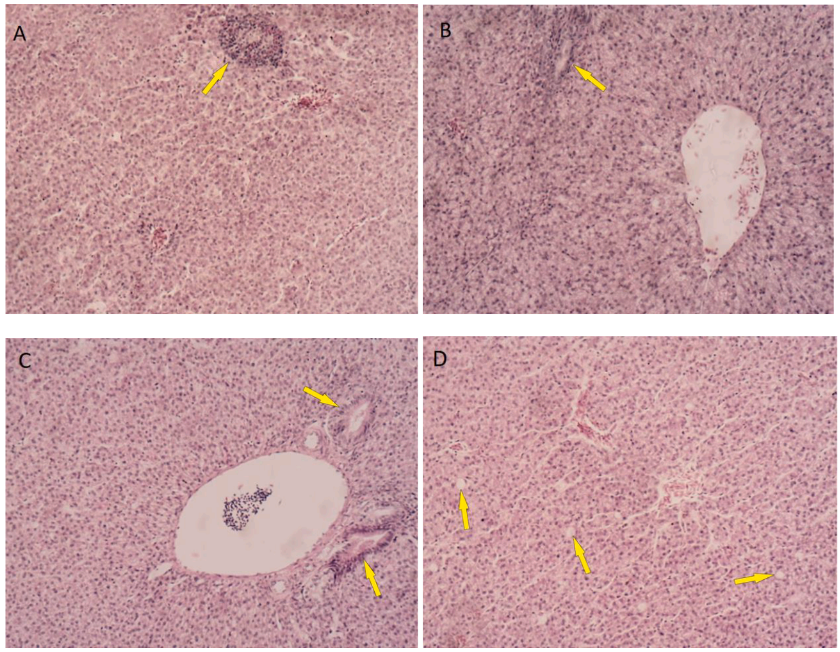

Fig. 1. Section of liver tissue of quails. HXE, 100X. **A.** Accumulation of inflammatory cells in 9.5 g Val/kg group diet. **B, C.** Bile duct hyperplasia in 10.5 g Val/kg diet group. **D.** Mild hepatosteatosis in 11.5 g Val/kg diet group (shown by yellow arrow).

Table 3
The effect of different levels of Val in diet on testis histology of quail[1].

Item	Tubular differentiation index (TDI;%)	Spermatic index (SI;%)	Testicular capsule diameter (μm)	Epithelium height (μm)	Sertoli cells (n)
Val (g/kg diet)					
7.5	51.3^{b}	59.8^{ab}	218.4	54.4	22.7
8.5	59.4^{a}	62.4^{a}	138.5	58.2	24.2
9.5	58.9^{a}	56.8^{b}	136.3	61.4	23.4
10.5	58.1^{ab}	56.4^{b}	152.4	58.2	23.3
11.5	57.4^{ab}	55.5^{b}	145.1	59.3	23.5
SEM	1.73	1.14	34.6	3.74	1.15
p-value					
Val level	0.029	0.002	0.449	0.766	0.925
Linear	0.062	0.001	0.244	0.420	0.853
Quadratic	0.013	0.650	0.226	0.414	0.681
Cubic	0.013	0.032	0.371	0.694	0.473

$^{a-b}$Means in each column with different superscripts are different ($P < 0.05$).
[1] Each value represents the mean of 5 replicates with 3 quail chicks per pen selected for liver sample on d 42.

BCAAs are also shown to suppress oxidative stress by stimulating the expression of Peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator (PGC)-α or Sirtuin-1 (SIRT-1), or by activating the genes involved in antioxidant defenses (Tajiri & Shimizu, 2018). These possible mechanisms could also contribute to promote hepatocyte proliferation (Tajiri & Shimizu, 2018). It is surprising to note that among BCAAs family members, Val stimulates the lymphogenesis of granular and agranular lymphocytes as well as increases natural killer cells (Kim, Singh, Wang, & Applegate, 2022). Based on our findings, it seems that the level of 8.5 g Val/kg with 170 g CP/kg diet was suitable for improving the functional activity of the liver, without any negative histological effects. Since BCAAs administration is used in the treatment of hepatic encephalopathy, our experiment paves the way for further exploration of Val in human studies..

In our experiment, differences between the two sexes were also observed. The diameter of liver cell nucleus and the diameter of hepatocytes of male were greater than the female quails. While the volume of sinusoids was not significantly affected by Val inclusion in diet, it was greater in female than male.. Sinusoids, small blood vessels between the radiating rows of hepatocytes, convey oxygen-rich hepatic arterial blood and nutrient-rich portal venous blood to the hepatocytes and eventually drain into the central vein, which drains into the hepatic vein. In adulthood, there is a weight difference between two sexes of quails. Female quail birds attained higher weight than male quails at 4-weeks (Khaldari et al., 2010). Body weight is one of the main factors that affects the birds' maintenance requirements (NRC, 1994). Therefore, it is assumed that the maintenance requirements for males may also be lower than those of females. Thus, the use of similar diets for both sexes could exceed the nutrient needs for males (Retes et al., 2019). Therefore, the difference between the two sexes may be due to the difference in their body size and, as a result, the difference in their maintenance requirements and metabolism.

With increasing Val level in the diet, TDI and SI increased linearly. TDI is the percentage of seminiferous tubules containing at least three differentiated germ cells and SI is the number of spermatozoa per 100 spermatogenic cells. Zamir-Nasta et al. (2021) pointed out that a reduction in the percentage of spermatogenic tubules with TDI, tube replacement (RI) and negative spermatogenesis coefficient (SPI) in testicular tissue, disrupted the process of cell division, which in turn could disrupt the operation of spermatogenesis. Fertilization in avian species depends on the release of spermatozoa from sperm storage tubules (SST), active ciliary movement as well as structural integrity of

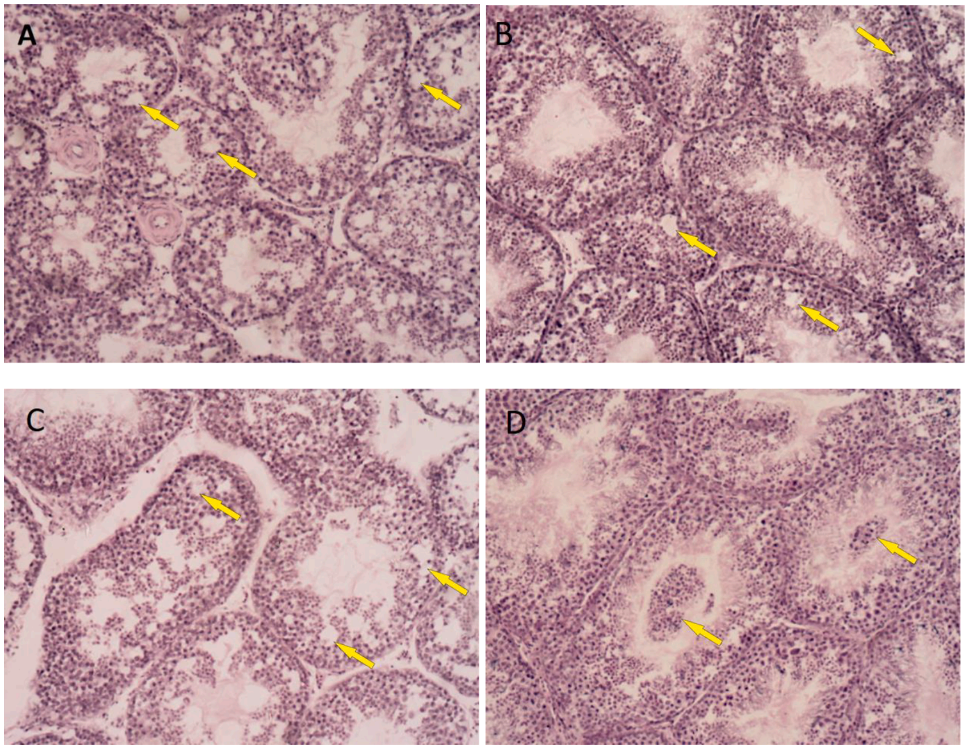

Fig. 2. Optical photomicrograph of seminiferous tubules of male quails. HXE, 100X. Formation of vacuoles between germ cells in the germinal epithelium in 9.5 g Val/kg (**A**), 10.5 g Val/kg (**B**), and 11.5 g Val/kg (**C**) diet groups, respectively (shown by yellow arrow). **D.** The release of spermatogenic cells into the lumen of the seminiferous tubule in 11.5 g Val/kg diet group (shown by yellow arrow).

glandular cells (Kimaro, 2016). Release of spermatogenic cells into the lumen of the seminiferous tubule was observed in 11.5 g Val/kg group and in the germinal epithelium of the groups with the levels of 9.5, 10.5 and 11.5 g Val/kg diet, vacuole formation was observed between the germ cells (Fig. 2). Uniquely, these vacuoles can indicate the loss of cell connections or the reduction of adhesive molecules such as cadherins and can be considered as one of the signs of apoptosis (MohamadGhasemi et al., 2010). Altogether, the use of high levels of Val (9.5, 10.5 and 11.5 g Val/kg diet) with 170 g CP/kg in the diet lead to histological damage in the testis of quails.

In our experiment, testicular capsule diameter, epithelium height as well as Sertoli cell number were not influenced either. Retes et al. (2022) reported that the Sertoli cell umber of quail at d 60 increased linearly with increasing dietary CP. Increases in the number of Sertoli cells and spermatogonia were not associated with increases in the sperm concentration of the birds (Retes et al., 2022). While the number of Leydig cells decreased, they showed that the testis size, seminiferous tubular area, number of spermatogonia, and germinal epithelial height at 35 days of age increased linearly with increasing dietary CP. Hanafy and Attia (2018) stated that 180 g CP/kg with 2 g Val/kg diet were suitable for breeder quails at 14–28 weeks of age. They showed that cloacal gland area and semen ejaculate volume of male quail were significantly improved by 180 g CP/kg diet. It was suggested that dietary protein concentration affected body and testicular development in male Japanese quails without affect reproductive efficiency (Retes et al., 2022). During the growth phase, rapid body development is directly related to reproductive organ development (Sarabia Fragoso et al., 2013). Thus, the supply of AAs during this phase may affect bird growth. Our findings confirmed that supplementation of low protein diet (170 g CP/kg) with Val (8.5 g Val/kg diet) may improve testis histology characteristics through increasing TDI and SI without any negative effect on testicular histology.

5. Conclusion

Our study demonstrated that feeding quail (1–42 days) with diets containing Val up to 8.5 g/kg diet does not hurt the histological indexes of the liver and testes. But feeding high levels of Val (9.5, 10.5, and 11.5 g Val/kg diet), lead to negative effects on both liver and testis histology which warrants further studies.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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