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Supplementation with specific branched-chain amino acids (BCAAs) affects mouse sperm parameters and testicular apoptotic gene expression

Roya Kamali[1], Leila Rashki Ghaleno[2], Amin Izadi[2], Zahra-Sadat Ghasemi[3], Vahid Esmaeili[2], Nima Eila[4], Adel Pezeshki[5], Joël R. Drevet[6], Abdolhossein Shahverdi[2,7] and AliReza Alizadeh Moghadam Masouleh[2,7,8,9]

[1]Department of Cellular & Molecular Biology, Faculty of Basic Sciences and Advanced Technologies in Biology, University Science and Culture, Tehran, Iran; [2]Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran; [3]Animal Core Facility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran; [4]Department of Animal Science, Karaj Branch, Islamic Azad University, Karaj, Iran; [5]Department of Animal & Food Sciences, Oklahoma State University, Stillwater, OK 74078, USA; [6]EVALSEM, GReD Institute, CRBC, Faculté de Médecine, Université Clermont Auvergne, Clermont-Ferrand, 63000, France; [7]Department of Basic and Population Based Studies in NCD, Reproductive Epidemiology Research Center, Royan Institute, ACECR, Tehran, Iran; [8]gyn-medicum, Center for Reproductive Medicine, Göttingen, Germany and [9]Institute of Pharmacology & Toxicology, University Medical Center Göttingen, Göttingen, Germany

Research Article

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Corresponding author:
AliReza Alizadeh Moghadam Masouleh;
Emails: alizadehmasouleh@royaninstitute.org; ar.alizadehmasouleh@royan-rc.ac.ir

Summary

In Western diets, high consumption of meat and dairy products, known to be rich in branched-chain amino acids (BCAAs), including leucine (Leu), isoleucine (Ile), and valine (Val), as well as BCAAs supplementation itself, may have unforeseen consequences on sperm quality. In addition, bodybuilders are increasingly resorting to BCAA supplementation to build-up their muscle mass. This study aimed to assess the effect of dietary BCAAs, provided alone or in combination, on semen parameters, apoptotic gene expression, and blood amino acid (AA) profiles. To address this question and determine whether these different BCAAs have a distinct impact on sperm quality and testicular homeostasis, fifty NMRI mature male mice were exposed or not to BCAAs supplementations (control diet: CTR; CTR + Leu supplementation; CTR + Ile supplementation; CTR + Val supplementation; CTR + all three BCAA supplementation). Only valine supplementation resulted in a significant decrease in sperm concentration and viability. In addition, only valine supplementation was associated with a dramatic increase in sperm immotility. The Bax/Bcl2 ratio, an indicator of apoptosis, was found to be significantly higher in the testes of BCAA-supplemented animals when compared with the other groups. *Caspase3* expression was also significantly higher in the testes of BCAA-supplemented and Val-supplemented animals. There were no significant differences in plasma AA profiles between groups. Thus, amongst BCAAs, valine supplementation appears to carry the greatest effect on sperm functional parameters and testicular apoptotic status.

Introduction

Against the backdrop of a pregnant global decline in male fertility, supported by a growing number of reports showing declining sperm qualitative and quantitative parameters, the environment and dietary behaviours are increasingly being singled out for blame (Mann *et al.* 2020). While it is clear that a well-balanced lipid intake is an important factor in the efficiency of spermatogenesis as well as post-testicular sperm maturation (Ferramosca and Zara 2022), little information is available on the effect of protein intake and amino acid (AA) supplementation (Hallak 2020; Ketheeswaran *et al.* 2019; Tottenborg *et al.* 2020). Excessive or/and unbalanced intake of dairy products, red meat and processed foods have been shown to negatively impact male reproductive health (Maldonado-Carceles *et al.* 2019; Nouri-Majd *et al.* 2022) at various sites. One common feature of these protein sources is their richness in branched-chain amino acids (BCAAs) which are therefore suspected to be the detrimental causative agents affecting male reproductive efficiency. For example, bodybuilders are increasingly resorting to BCAA supplementation to build-up their muscle mass (Ketheeswaran *et al.* 2019; Tom and Nair 2006). Given that the vast majority of these consumers are young men of childbearing age, it is important to study the impact of such a choice on their ability to father.

BCAAs (leucine: **Leu**, isoleucine: **Ile** and valine: **Val**), are essential AAs, meaning they must come from our diet. Daily nutritional requirements for humans have been determined for each

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of them, and set at 40 mg/kg, 25 mg/kg and 19 mg/kg for Leu, Val and Ile, respectively (Polis and Samson 2020). In addition to their main role in protein synthesis (S Zhang *et al.* 2017), BCAAs are thought to be involved in other metabolic processes (Arany and Neinast 2018; Holecek 2018). For example, some reports suggest that BCAAs may be involved in insulin resistance and diabetes (Kolahian *et al.* 2015). As another example, Leu has been shown to specifically activate the mTOR (mammalian target of rapamycin) signalling pathway, a central controller of cell growth in response to growth factors and nutrients (Polis and Samson 2020; S Zhang *et al.* 2017). Else, in humans, valine-enriched diets have been associated with a higher risk of hypertension (Mirmiran *et al.* 2019). Intriguingly, lower plasma valine levels were correlated with accelerated cognitive decline, while on the contrary, increased valine concentration in the diet had a protective effect against Alzheimer's disease (Polis and Samson 2020).

Amino acids are present in large quantities in seminal plasma and come mainly from the accessory sex glands, in particular the prostate. The importance of the AA profile of seminal plasma has been highlighted (Amirjannati *et al.* 2023; Esmaeili *et al.* 2025; Juyena and Stelletta 2012) and recent findings have shown that AA imbalances in the seminal plasma of patients with severe oligoasthenospermia (Boguenet *et al.* 2020) or teratozoospermia (Mehrparvar *et al.* 2020) were associated with sperm structural and functional changes and in the pathogenesis of male fertility disorders. More recently, we have emphasized that the presence of valine in blood and seminal plasma is correlated with abnormal sperm morphology in patients without metabolic syndrome (Esmaeili *et al.* 2025). However, little is known about the effect of dietary BCAAs on sperm. In male diabetic rats, Leu supplementation has been shown to improve sperm morphology and spermatogenesis (Kolahian *et al.* 2015). A study in obese mice showed BCAAs supplementation significantly increased testosterone levels, testicular weight and germ cell numbers compared to control (Arakawa *et al.* 2011). It seems that BCAA, particularly Leu has beneficial effects on sperm in obese or diabetic males. In zebrafish, leucine was found to increase sperm motility. In mice, BCAA supplementation via gavage at low doses (20 mg/ml) and exercise improved sperm quality and testosterone levels, while at higher doses (60 mg/ml) it had negative effects (Bahadorani *et al.* 2019; J Zhang *et al.* 2017). However, to our knowledge whether dietary supplementation of each member of BCAAs family are associated with sperm parameters has not been addressed. In the work presented here, to better understand the impact of BCAAs on mammalian male reproductive function, we supplemented mice either with Leu or Ileu or Val or a mixture of these three BCAAs. We then assessed conventional sperm parameters as well as testicular apoptotic status.

Material and methods

Animals and study design

Experimental procedures were approved by the ethics committee of the Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.REC. 1399.076). Fifty male NMRI mice (7 weeks old; 27.2 ± 1.55 g) were included in this study. All animals were housed in a controlled environment (12-h light/dark cycle; 21 ± 2°C) for three adaptation days. Animals were randomly divided into five groups (n = 10/group) and fed for 5 weeks on the following diets: basal control diet (**CTR**); diet supplemented with 1.18% leucine (**Leu**); diet supplemented with 0.58% Ile (**Ile**); diet supplemented with 0.65% Val (**Val**) and, diet supplemented with all 3 BCAAs (Leu 1.18%, Ileu 0.58% and Val 0.65% (**BCAA**) [Table 1]. Supplementation levels were chosen with reference to the study by Falavigna et al (2012). Diet formulation for the use of beta-alanine was considered to conform to the Nutrient Requirements of Laboratory Animals, Fourth Revised Edition, 1995 (NRC, 1995). Pelleted feed and water were given *ad libitum*. All AAs were purchased from Supplements Co (Bulk Supplements.com, Henderson, NV, USA). The basal diet, representing 80% of the experimental diet, was obtained from Roshd Daneh Co (Karaj, Iran). For the experimental diets, each AA was added with a constant level of wheat bran, starch and gluten (Table 1). All animals were fed pellets. Feed and water consumption (via graduated bottles) were recorded daily and values were cumulated weekly. At week 5, animals were euthanized by injection of 10% ketamine-xylazine (100 mg/kg body weight) and tissue samples (n = 5/group) were taken from the left testis and stored in liquid nitrogen (-196°C). In addition, blood samples taken from the heart were centrifuged to separate plasma (n = 4/group) at 3,000 g for 12 min at 4°C and samples were stored at −20°C before use.

Table 1. Composition and BCAA levels in experimental diets

	Experimental groups				
	CTR*	Leu	*Ile*	Val	BCAA
Ingredients					
Basal Diet (g)	800	800	800	800	800
Wheat Bran (g)	116	116	116	116	116
Corn Starch (g)	40	40	40	40	40
Wheat Gluten (g)	20	20	20	20	20
Beta-alanine supplementation (g)	24	12.2	18.2	17.5	0
L-Leucine supplementation (g)	0	11.8	0	0	11.8
L-Isoleucine supplementation (g)	0	0	5.8	0	5.8
L-Valine supplementation (g)	0	0	0	6.5	6.5
Total (g)	1000	1000	1000	1000	1000
Composition					
L-Leucine (g/kg diet)	11.8	23.6	11.8	11.8	23.6
L-Isoleucine (g/kg diet)	5.8	5.8	11.6	5.8	11.6
L-Valine (g/kg diet)	6.5	6.5	6.5	13	13
Beta-alanine (g/kg diet)	24.1	12.2	18.2	17.5	0
Total BCAA (g/kg diet)	24.1	36	30	30.5	48.2

*CTR: basic diet; Leu: basic diet + Leucine supplementation; Ile: basic diet + Isoleucine supplementation; Val: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation.

Plasma amino acids (AA) analysis

AA profiles were determined in the Massoud Clinical Reference Laboratory (Tehran, Iran) using a HPLC with o-phthalaldehyde and fluorescence detection as developed by Frank and Powers (Frank and Powers 2007). Briefly, plasma samples (20 µL) were

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Table 2. Characteristics of primers used for real-time qPCR, *Bax*; BCL2 antagonist/killer; *Bcl2*, BCL2 apoptosis regulator; *Cas3*, caspase 3

GenBank no.	Gene name	Primer sequence (5′ to 3′)	Product length (bp)
NM_007527.3	*Bax*	F- TTGCTACAGGGTTTCATCCAG R- CCAGTTGAAGTTGCCATCAG	246
NM_009741.3	*Bcl2*	F- GCCTTCTTTGAGTTCGGT R- ATATAGTTCCACAAAGGCATCC	161
NM_001284409.1	*Cas3*	F- AAAGACCATACATGGGAGC R- CGAGATGACATTCCAGTGCT	138
NM_001289726.1	*Gapdh*	F- GACTTCAACAGCAACTCCCAC R- TCCACCACCCTGTTGCTGTA	125

prepared by adding an equal volume (20 µL) of an internal standard and HPLC-grade water for a final volume of 200 µL. Plasma proteins were precipitated by adding 200 µL of 0.5 mol/ L perchloric acid. Samples were then vortexed and centrifuged at 15,000×g for 5 min at room temperature. Supernatants (150µl) were collected and filtered (Spin-X 0.2-µm) by centrifugation at 15,000×g for 1 min. Filtered samples (100µl) were collected and subjected to HPLC analysis. Samples were split between two vials to minimize the time between derivatization and injection into the HPLC system.

Evaluation of semen parameters

For sperm preparation, the dissected epididymis was transferred to 1 ml of pre-warmed T6 culture medium (100 ml distilled water; 473 mg NaCl; 11 mg KCl; 5 mg NaH2 PO4; 10 mg MgCl2,6 H_2O; 26 mg CaCl2,6 H_2O; 210 mg $NaHCO_3$; 200 µl Na-Lactate 100 %; 3 mg Na-pyruvate; 100 mg Glucose; 6 mg Penicillin; 5 mg Streptomycin; 1 mg Phenol red; 0.06 mg EDTA) containing 10% bovine serum albumin (BSA) and incubated at 37°C, 5% CO_2 for 30 min. Sperm count and motility were analysed by computer-assisted sperm analysis (CASA) using a hot plate equipped with the Sperm Class Analyzer® software (SCA, full research version 5.1; Microptic Co., Barcelona, Spain). For sperm concentration, 6 µl of sperm sample was placed on the slide. Then, using a Nikon 10 × 0.25 field negative phase contrast Ph1 BM microscope, at least 400 spermatozoa were counted in each series. To determine sperm viability, sperm samples were mixed with eosin Y (1:1; Merck Chemical Co., Darmstadt, Germany). Sperm showing purple staining under the microscope were considered dead, and sperm showing no staining were counted as alive (200 cells, 100X).

qRT-PCR assay

Real-time qPCR was used to study the expression of apoptotic genes (*Bax*, *Bcl2* and *Cas3*). Briefly, the testis was homogenized in TRIzol for RNA isolation and purification (Qiagen, Dusseldorf, Germany). The DNase I, RNase-free kit (Thermo Fisher Scientific, Carlsbad, CA,USA) was used for DNA digestion. RNA concentration was measured by a NanoDrop ND-2000 spectrophotometer (Thermo Fischer Scientific, MA USA). After assessing RNA integrity, complementary DNA was synthesized using the Takara cDNA synthesis kit (Takara Bio, CA 94043, USA). Expression levels of the three target genes were analysed by the $2^{-\Delta\Delta ct}$ method. *GAPDH* was chosen as the housekeeping gene for quantitative PCR normalization. The sequence of primers used in the present study is shown in Table 2.

Statistical analysis

All statistical analyses were performed using SPSS 20 software (SPSS Inc., Chicago, IL). Data were first assessed for normal distribution using the Kolmogorov-Smirnov test. Comparison between groups was performed by one-way ANOVA with Post hoc Tukey test. *P* values below 0.05 were considered significant. Data are least squares means ± standard deviation.

Results

Plasma AA measurements were first carried out to evaluate the impact of the supplementations. Each individual supplementation (Leu, Ile or Val) for 35-days resulted in a significant increase in the plasma representation of the concerned AA when compared with the control regimen (Table 3) attesting of the enrichments provoked by the regimens. Ileu supplementation did not introduce any change in the representation of other classes of amino acids (AAA = aromatic amino acids, GAA=glucogenic amino acids and KAA= ketogenic amino acids) as the ratios presented in Table 3 were similar. In contrast, both Leu and Val supplementation provoked significant changes in the different ratios. It is of interest to note that Leu supplementation provoked a drop in Val and Ile representation (Table 3), a phenomenon that will be discussed later. Finally, Leu supplementation also provoked changes in the different ratios monitored essentially at the expense of the glucogenic and aromatic amino acids (Table 3). Intriguingly, when the supplementation was carried out with the 3 BCAAs altogether, this was not followed by a plasmatic enrichment in any of the individual BCAAs (Table 3) a point that will be also discussed later.

Among the different supplementations, Ile- and BCAA-supplemented animals had the highest food consumption, while Val- and Leu-supplemented animals had the lowest (Figure 1A). During the 35 days of supplementation, water consumption was highest in the BCAA group and lowest in the CTR group (Figure 1B). There was no significant difference in final body weight between experimental groups (Table 4).

With regard to classical sperm parameters, sperm concentration, total motility and progressive forward motility were significantly reduced in all supplemented groups compared with the control group (Table 5). The Val-supplemented group was the one in which all 3 parameters (total motility, progressive forward motility and sperm concentration) were most affected (Table 5). Logically, the percentage of non-progressive motile sperm decreased in all supplemented groups, while the percentage of immobile sperm increased significantly in all supplemented groups (Table 5). Again, the Val-supplemented group was the most

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Table 3. Plasma amino acid profiles (μM/L)

	Experimental groups (n = 4/group)						
	CTR*	Ile	Leu	Val	BCAA	SEM	p-value
ASP (Aspartate)	28.8	23.7	26.8	15.4	26.0	2.1	0.27
GLU (Glutamate)	100.1	109.9	91.2	97.9	70.4	12.1	0.88
ASN (Asparagine)	60.6	57.2	50.5	63.4	58.2	3.0	0.70
HIS (Histidine)	76.0	78.1	72.0	89.8	70.9	2.9	0.27
SER (Serine)	125.5	119.6	95.0	138.2	106.0	6.4	0.21
GLN (Glutamine)	528.2	459.6	441.4	662.9	515.3	30.8	0.14
ARG (Arginine)	94.3	117.3	125.9	137.0	126.0	9.7	0.69
CIT (Citrulline)	59.8	83.5	67.0	74.5	49.7	5.0	0.23
GLY (Glycine)	238.8	239.6	192.6	239.3	249.8	10.3	0.44
THR (Threonine)	120.9	151.9	147.1	138.0	106.1	10.1	0.67
ALA (Alanine)	472.9	476.2	347.1	588.5	569.6	35.1	0.19
TYR (Tyrosine)	91.9	96.2	91.9	86.9	74.4	5.6	0.79
MET (Methionine)	50.5	55.6	59.1	54.7	39.7	3.4	0.44
VAL (Valine)	273.4	264.4	246.6	285.3	268.4	15.5	0.95
PHE (Phenylalanine)	104.5	103.3	109.3	107.2	104.5	5.6	1.00
ILEU (Isoleucine)	105.9	139.8	96.7	106.3	115.4	6.5	0.24
LEU (Leucine)	186.0	187.5	226.6	188.9	194.1	11.0	0.81
ORN (Ornithine)	168.7	174.5	139.9	133.2	113.5	11.8	0.52
LYS (Lysine)	286.9	293.2	292.1	351.1	288.5	16.8	0.79
TRP (Tryptophan)	88.6	89.1	92.3	76.0	102.0	4.1	0.33
Total BCAA	**565.3**	**591.7**	**569.9**	**580.5**	**577.9**	**29.5**	**1.00**
Total Aromatic AA (AAA) *BCAA/AAA ratio*	285.0 *2.0*	288.5 *2.1*	293.5 *1.9*	270.1 *2.2*	281.0 *2.0*	13.0	0.99
Total Glucogenic AA (GAA) *BCAA/GAA ratio*	1562 *0.362*	1614 *0.367*	1353 *0.421*	1705 *0.340*	1620 *0.357*	74.4	0.63
Total Ketogenic AA (KAA) *BCAA/KAA ratio*	473 *1.2*	481 *1.23*	519 *1.1*	540 *1.08*	483 *1.2*	25.2	0.94

*CTR: basic diet; Leu: basic diet + Leucine supplementation; Ile: basic diet + Isoleucine supplementation; Val: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation.
Total BCAA: VAL + ILE + LEU; **Aromatic AA**: TRP + TYR + PHE; **Glucogenic AA:** ALA + VAL + ILE + GLY + SER + THR + ASP + PHE+ TYR**: Ketogenic AA**: LYS + LEU.

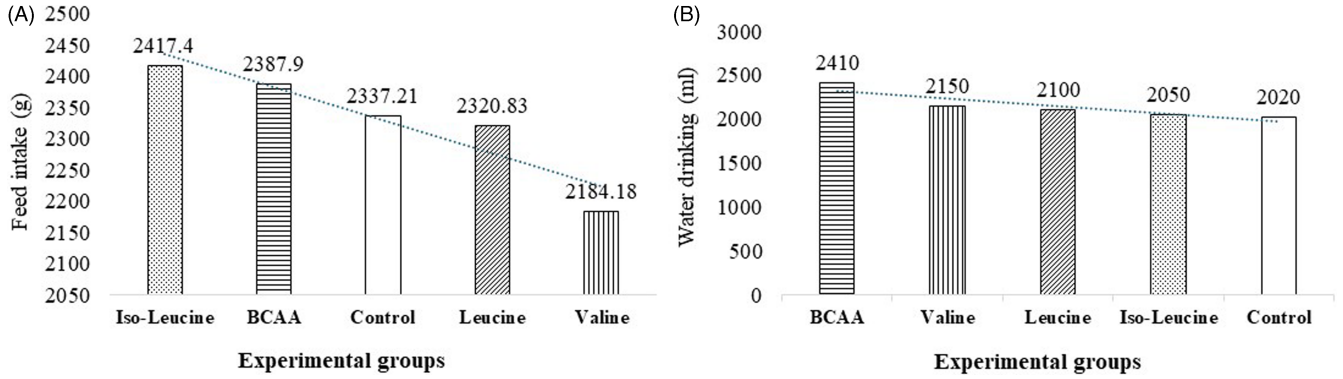


Figure 1. Total feed intake (**A**) and total water drinking (**B**) in 35 days (n = 10/each group). Control: basic diet; Leucine: basic diet + Leucine supplementation; Isoleucine: basic diet + Isoleucine supplementation; Valine: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation.

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Table 4. Initial and final body weights (Mean ± sd)

	Experimental groups (n = 10/group)					
	CTR*	Leu	Ile	Val	BCAA	p-value
Initial Body Weight (g)	26.4 ± 2.13	27.2 ± 2.02	27.3 ± 2.05	26.87 ± 1.84	27.3 ± 1.89	0.845
Final Body Weight (g)	37.3 ± 3.31	40.4 ± 3.09	39.7 ± 3.54	36.6 ± 3.16	37.8 ± 3.4	0.090

*CTR: basic diet; Leu: basic diet + Leucine supplementation; Ile: basic diet + Isoleucine supplementation; Val: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation.

Table 5. Sperm characteristics in experimental groups (Mean ± sd)

	Experimental groups (n = 10/group)					
	CTR*	Leu	Ile	Val	BCAA	p-value
Concentration ($\times 10^6$)	8.4 ± 1.48^{a}	6.3 ± 0.82^{b}	5.0 ± 1.08^{bc}	4.5 ± 0.46^{c}	5.8 ± 1.32^{bc}	**<0.01**
Total Motility (%)	77.7 ± 9.67^{a}	65.1 ± 6.69^{ab}	50.0 ± 16.19^{bc}	36.1 ± 13.07^{c}	48.7 ± 17.14^{bc}	**<0.01**
Progressive Motility (%)	51.5 ± 3.02^{a}	53.4 ± 7.14^{a}	38.2 ± 12.61^{ab}	27.2 ± 13.25^{b}	31.9 ± 19.25^{b}	**<0.01**
Non-Progressive Motility (%)	26.2 ± 11.91^{a}	11.7 ± 4.35^{b}	11.7 ± 9.90^{b}	8.9 ± 4.66^{b}	16.8 ± 7.07^{ab}	**<0.01**
Immotile (%)	23.6 ± 8.14^{c}	34.9 ± 6.73^{bc}	49.9 ± 16.20^{ab}	63.8 ± 13.07^{a}	51.3 ± 17.10^{a}	**<0.01**
Type A (%)	27.4 ± 12.42	27.0 ± 7.41	20.2 ± 11.96	15.6 ± 10.74	20.3 ± 11.92	0.284
Type B (%)	22.7 ± 8.04^{a}	26.3 ± 8.52^{a}	18.0 ± 5.22^{ab}	11.6 ± 7.97^{b}	11.5 ± 8.02^{b}	**<0.01**
VCL (μm/s)**	70.6 ± 15.9^{a}	81.4 ± 19.22^{a}	64.0 ± 12.99^{ab}	50.5 ± 6.43^{b}	62.3 ± 18.86^{ab}	**0.001**
VSL (μm/s)	27.3 ± 8.98	23.5 ± 4.85	19.8 ± 6.22	18.0 ± 6.02	22.4 ± 10.27	0.059
VAP (μm/s)	35.7 ± 11.64	39.0 ± 9.49	30.6 ± 8.37	28.0 ± 6.50	34.9 ± 11.96	0.103
LIN (%)	29.3 ± 6.16	25.1 ± 7.11	25.0 ± 7.25	24.9 ± 6.35	27.3 ± 6.65	0.591
STR (%)	49.2 ± 4.44	48.6 ± 7.86	50.8 ± 7.16	45.3 ± 5.73	52.6 ± 7.77	0.486
WOB (%)	49.2 ± 4.44	45.9 ± 6.53	43.1 ± 8.54	45.3 ± 5.73	47.7 ± 5.79	0.215
ALH (μm)	3.3 ± 0.81^{ab}	4.3 ± 1.00^{a}	3.2 ± 0.61^{b}	3.3 ± 0.69^{ab}	3.3 ± 0.99^{ab}	**0.036**
BCF (Hz)	4.4 ± 0.69	4.2 ± 0.72	3.6 ± 0.85	3.9 ± 1.22	3.7 ± 0.90	0.278
Viability (%)	67 ± 6.04^{a}	61 ± 9.62^{ab}	65 ± 7.68^{ab}	56 ± 5.95^{b}	60 ± 10.79^{ab}	**0.040**

*CTR: basic diet; Leu: basic diet + Leucine supplementation; Ile: basic diet + Isoleucine supplementation; Val: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation.
** VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; STR = VSL/VAP (straightness); ALH = amplitude of lateral head, and BCF = beat cross frequency. Type A – These are fast-moving spermatozoa that swim rapidly in a straightforward direction. Type B – These are slow-moving spermatozoa that move forward in a disorderly fashion, or even a little more slowly.
^{a,b,c}Values with different superscripts within lines significantly differ. Parameters in bold shows significant differences.

affected (Table 5). Although the percentages of *grade A* motile sperm (very strong activity with forward progression) were not significantly affected in the supplemented groups compared with the controls, there was a clear tendency for it to be lower in the Val-supplemented group (Table 5). This effect was most pronounced for the percentages of *grade B* motile sperm (decent sperm activity with forward progression), which were significantly reduced in both the Val and BCAA-supplemented groups (Table 5). Examining the detailed velocity parameters provided by the CASA analysis (VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF), we observed that the Val-supplemented group had a dramatically lower VCL than the other groups (Table 5). None of the other CASA velocity parameters were significantly altered between groups, with the exception of ALH, which was found to be increased only in Leu-supplemented animals compared with those supplemented with Ile (Table 5). Ultimately, sperm viability was only significantly reduced in the Val-supplemented group compared with CTR animals (Table 5).

As sperm concentration was significantly decreased following BCAA supplementation, we investigated whether this could be linked to the level of testicular apoptosis. Levels of pro-apoptotic *Bax* mRNA in the testes of BCAA-supplemented mice were significantly reduced in BCAA-, Leu- and Ile-supplemented animals compared with control animals, whereas surprisingly, in Val-supplemented animals, *Bax* expression was not altered (Fig. 2A). In contrast, for the *Bcl2* apoptosis inhibitory marker, only Val supplementation led to a significant increase in its mRNA steady-state level when compared to the controls and other supplemented animals (Figure 2B). Looking then at the Bax/Bcl2 ratio, a conventional indicator of survival or death after an apoptotic stimulus, we found that Val, Ile and BCAA supplementations were associated with an increased Bax/Bcl2 ratio, that was significant only for Ile- and BCAA-supplemented animals when compared to controls (Fig 2C). As a downstream executor of apoptosis we also monitored the expression of caspase-3 (*CASP3*) in the different animal groups (Fig. 3). BCAA and Val supplementation were associated with a significant increase in testicular *CASP3* mRNA accumulation compared with control animals and animals supplemented with Leu or Ile (Fig. 3).

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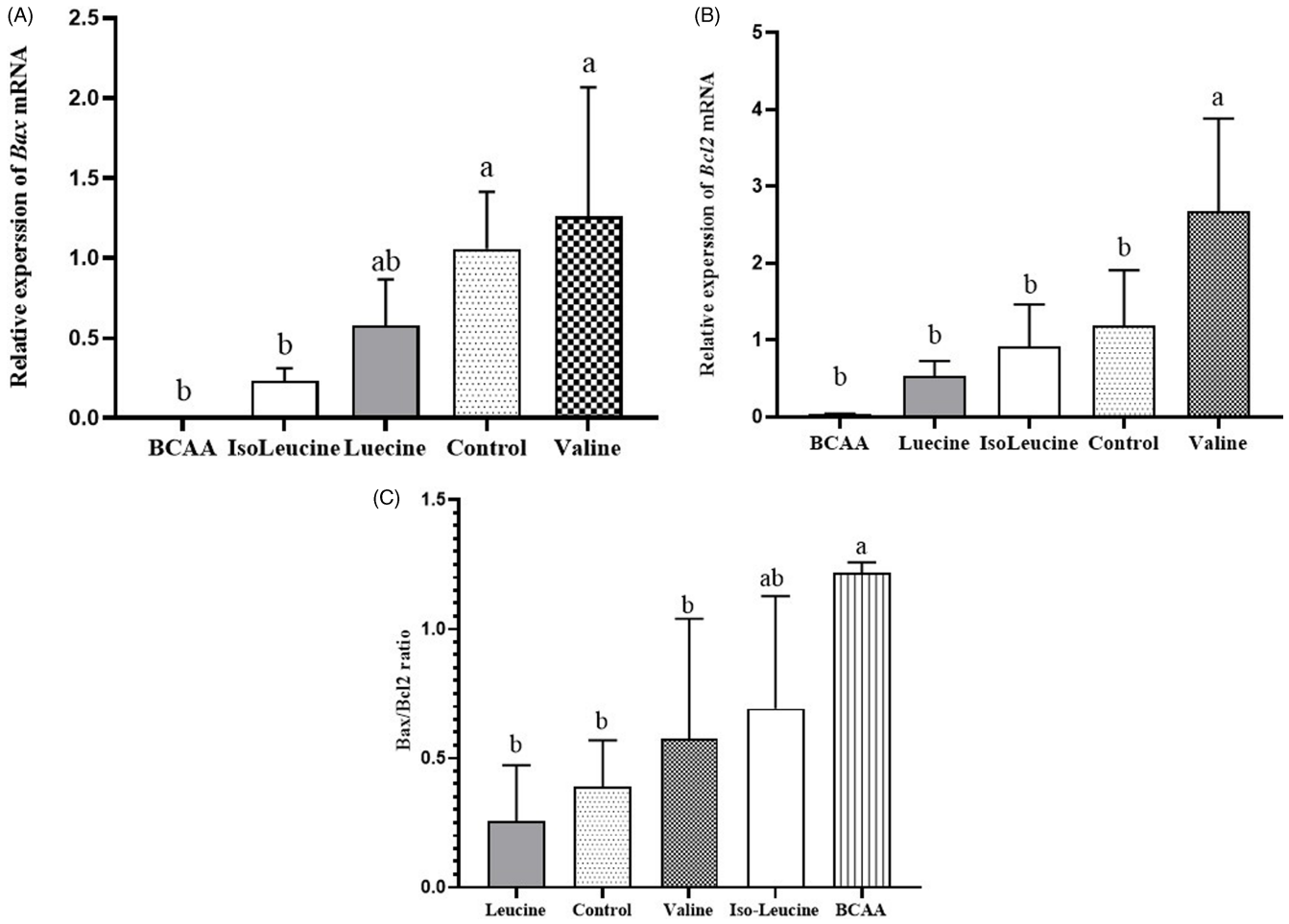


Figure 2. (A. B. C). Expression of testis apoptotic markers *Bax, Bcl2*, following BCAA supplementation. **A:** *BAX* mRNA expression levels in the testes of mice fed experimental diets. **B:** *BCL-2* mRNA expression levels in the testis of mice fed experimental diets. **C:** BAX/BCL2 ratio. Control: basic diet; Leucine: basic diet + Leucine supplementation; Isoleucine: basic diet + Isoleucine supplementation; Valine: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation. Data are presented as mean ± SD. [a,b] Bars with distinct superscripts are significantly different ($p < 0.05$).

Discussion

The BCAA supplementations reported in the present work clearly had an impact on the reproductive function of male mice, as evidenced by significant changes in sperm concentration, motility and viability as well as in testis apoptosis status. With regard to sperm parameters, Val supplementation had the greatest effect, followed by supplementation with the 3 BCAA mix (Val, Leu, Ile). Val supplementation caused a marked decrease in sperm concentration, total motility and progressive motility, as well as a sharp increase in the percentage of immobile spermatozoa. In addition, Val and BCAA supplementation were associated with a significant increase in the steady-state level of testicular caspase3 mRNA, suggesting testicular apoptosis. This powerful effect of Val supplementation on spermatogenesis efficiency and conventional sperm parameters can be tentatively explained.

It has been reported that Val catabolism generates intermediates such as methylmalonic acid (MMA), which in turn is associated with mitochondria-mediated generation of reactive oxygen species (ROS) (Caioli *et al.* 2016; Liu *et al.* 2022; Stepien *et al.* 2017; Wang *et al.* 2020). Moreover, Val catabolism product accumulation, including propionyl CoA and succinyl CoA, that may lead to chronic hyperinsulinemia and consequently cell dysfunction and insulin resistance, contributes to incomplete lipid oxidation (Newgard 2012). Thus, Valine-induced ROS generation could explain the reduced spermatogenetic efficiency via testicular apoptosis. Reactive oxygen species (ROS) play an essential role in defining the functional competence of spermatozoa. While moderate amounts of ROS promote sperm optimal maturation and capacitation, excessive ROS generation and/or compromised antioxidant defences in the reproductive system can induce a state of oxidative stress (OS) that impairs sperm fertilizing capacity and the structural integrity of sperm cells (Drevet & Aitken, 2020; Aitken & Drevet, 2020). In the context of future work, additional tests could be carried out to reinforce this hypothesis by examining classic markers of oxidative stress such as the level of malondialdehyde and the level of 4-hydroxynonenal (4-HNE) in the protein extracts of testicular, epididymal or spermatic cells of animals supplemented with BCAA. Monitoring mitochondrial function by measuring JC-1 could also provide indications of the state of spermatozoa mitochondria.

Ile is also a precursor of MMA, but at a lower level than Val. This may therefore explain why, in BCAA-supplemented animals, Val plus Ile have a synergistic effect, whereas alone (Ile-supplemented animals), the effect recorded on testicular function and spermatozoa is weaker. It could also explain the effects on sperm motility observed in both Valine-supplemented animals and

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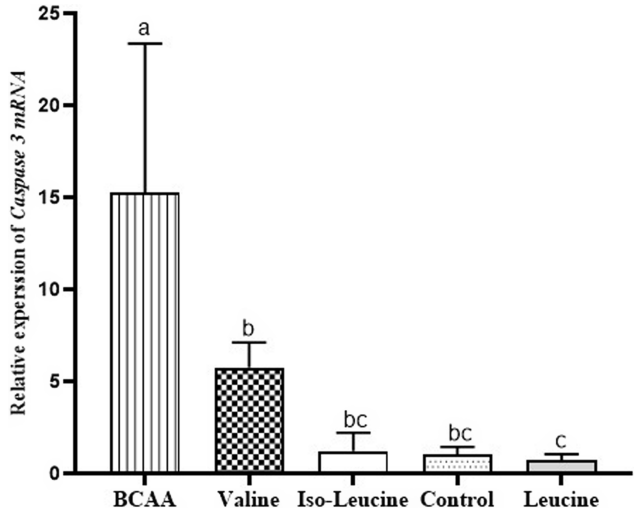


Figure 3. CASP-3 mRNA levels in mice testis fed experimental diets (mean ± SD). Control: basic diet; Leucine: basic diet + Leucine supplementation; Isoleucine: basic diet + Isoleucine supplementation; Valine: basic diet + Valine supplementation and BCAA: basic diet + BCAA supplementation. [a,b] Bars with distinct superscripts are significantly different ($p < 0.05$).

those supplemented with all three BCAAs, via mitochondrial malfunction and ROS-mediated damage (Amaral *et al.* 2013). The weakest effects recorded here, on both testicular apoptosis status and sperm parameters, were observed in Leu-supplemented animals. This makes sense, as it has been reported that excessive Leu intake can decrease plasma levels of the other branched-chain essential AAs, Ile and Val (Holecek 2018), which is exactly what we observed (see Table 3). It should be noted, however, that with regard to Leu supplementation, our observations are not in agreement with a previous report (Kolahian *et al.* 2015) indicating that Leu supplementation provided in drinking water increased the percentage of sperm with normal morphology, improved spermatogenesis and testicular histological parameters in diabetic rats. The configuration of these two experiments (diabetic and non-diabetic animals) being very different, it is difficult to explain the reasons for this discrepancy.

It therefore appears that BCAAs, and valine in particular, have a powerful effect on male reproductive function. Extrapolation of these results to men will require further study. However, it is interesting to note that studies have already reported significant differences in the seminal plasma AA profiles of fertile and infertile oligozoospermic men (Silvestroni *et al.* 1979). We also found in our infertility clinic that the AA profiles of blood and seminal plasma of men with and without metabolic syndrome were significantly different, with particular changes in valine concentration (Esmaeili *et al.* 2025). On the contrary, no differences in blood BCAA levels were observed elsewhere when comparing azoospermic and oligozoospermic men (Sugiyama *et al.* 2021). Whether seminal plasma and blood plasma behave differently with regard to AA content will need to be investigated. This is one of the limitations of our study, in which only the AA profiles of blood plasma were examined. A complementary study should follow in parallel the AA content of blood plasma and seminal plasma after BCAA supplementation. A second limitation of our work is that only one dose of each BCAA was used in the supplementations. This was an arbitrary choice, as our main objective in this pioneering study was to test the effect of BCAAs (including the 3 members of the Leu, Ileu and Val family) on mammalian sperm physiology. The doses chosen were based on previous internal work, work already published elsewhere and the transposition to mice of the human doses found in commercially available food supplements. For practical reasons, including the costs associated with the animals, the costs of animal caretakers and facilities and compliance with the 3Rs (Replace/Reduce/Refine) rules in animal experimentation, as applied by our local ethics committee, we used a single-dose approach. Now that we have recorded certain effects with certain BCAAs, a rigorous approach would be to test increasing doses to see what happens. In this regard, Rashidi et al (2023) pointed out recently that the use of a wider range of Val doses (≥ 9.5 g Val/kg diet) during d0 to d42 of age can lead to histological damage in testis of quail chicks which open the road for more investigation on Val levels and testis function. More recently, our team pointed out the negative effects of high valine levels in diet on testis and semen parameters in mature quail (Dinari *et al.* 2025).

The unrestricted use of muscle building supplements (MBS) to improve performance is a growing public health concern, particularly among athletes. In addition to improving athletes' performance, the growing use of MBS among the general population stems from the social pressure exerted on adolescents and young men of reproductive age to conform to an idealized muscular body (Girardi *et al.* 2022). One study reported that 87.5% of high-level athletes take dietary supplements (Girardi *et al.* 2022). Recently, Bajool et al. pointed out that protein and dietary supplements containing AA were the most common dietary supplements worldwide (Bajool *et al.* 2024). Although there has been a lot of media hype suggesting that BCAA consumption stimulates muscle protein synthesis (MPS) and produces an anabolic response in men, these claims are poorly substantiated (Boccanegra *et al.* 2020; Holecek 2022; Wolfe 2017). Conversely, negative effects of BCAA on the health of athletes, in particular BCAA-rich supplements, have been factually reported (Girardi *et al.* 2022; Manuel and Heckman 2011). Overall, our present findings suggest that further studies are warranted to examine the roles of BCAA supplements particularly in young male body-builders suffering from subfertility/infertility.

In conclusion, this report illustrates quite clearly that certain BCAA supplements, particularly Val, can have adverse effects on male fertility. Although it is preliminary and has not yet been transposed to men, it suggests that a principle of caution should be considered when BCAA supplementation is used.

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