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# Effect of dietary supplementation of omega-3 rich oils on seminal characteristics, oxidative stress and plasma testosterone levels in Beetal bucks

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## ABSTRACT

The objective of the present study was to evaluate the efficacy of dietary supplementation of omega-3 rich oils, chia seed oil (CSO; *Salvia hispanica*) and linseed oil (LSO; *Linum usitatissimum*), on seminal characteristics, and plasma testosterone levels in Beetal bucks. The bucks were fed diet supplemented with either LSO or CSO @1% on dry matter basis or un- supplemented diet for 100 days. Daily intake of omega-3 fatty acids was 0.60 (control diet), 4.04 (diet supplemented with LSO) and 4.57 g (diet supplemented with CSO). Ultrasonography revealed no significant improvement in the testicular size yet the growth rate was much high in animals fed omega-3 supplemented diet. Ejaculate volume, individual motility, viability, sperm concentration, plasma membrane and acrosome integrity were significantly higher in bucks fed CSO supplemented diet as compared to LSO and control diet. The percentage of abnormal spermatozoa was significantly less in bucks fed CSO and LSO diet than control. Melanoaldehyde (MDA, μmole/$10^9$ sperm) concentration was lowest in bucks fed CSO diet followed by LSO and control diet. Activity of superoxide dismutase, glutathione reductase and catalase was significantly higher in spermatozoa of bucks fed CSO and LSO diet as compared to control. Bucks fed CSO and LSO supplemented diet had non-significantly higher plasma testosterone concentrations than control. These findings indicated that CSO supplementation in the diets may be helpful in improving the process of spermatogenesis, sperm output, quality and fertility of bucks.

**Keywords**: Fertility, Omega-3 oils, SDS-PAGE, Semen, Ultrasonography

Omega-3 fatty acids (FA) improve the sperm quality and protect the sperm against stress conditions by maintaining the membrane integrity and sperm viability (Gullivar *et al.* 2012). The vertebrates cannot synthesize omega-3 and omega-6 series of polyunsaturated fatty acids (PUFAs) and hence must be provided in the diet, either in the form of short chain 18 carbon derivatives found in plants or in the form of long chain 20–22 carbon containing 2–6 double bonds derivatives found in animal tissues. Therefore, the omega-3 rich supplements must be included into their feed to fulfil the requirement. Linseed has been extensively investigated for the fertility enhancement in bovine (Gholami *et al.* 2011). The success of a goat flock depends on the number of kids raised, weaned and marketed each year, and production is directly proportional to reproduction. Ultrasonography can be used for andrological evaluation coupled with data from clinical examinations for early diagnosis of disorders of the testes and related structures (Urt *et al.* 2018). Therefore, evaluation of semen parameters becomes highly imperative. *In vitro* evaluation tests measuring the physiological and cytological parameters of spermatozoa, i.e. sperm viability, progressive motility, hypo-osmotic swelling tests, acrosome integrity and morphological abnormalities reduce the economic and time constraints in field conditions. Therefore, aim of the present study was to perceive the effect of supplementation of omega-3 rich oils in the diet of goat bucks on semen attributes, sperm lipid peroxidation and plasma testosterone level.

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## MATERIALS AND METHODS

*Location and ethical compliance:* Study was conducted in the Goat Farm, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India and experimental protocol was approved by Institutional Animal Ethics Committee constituted.

*Animals and feeding:* Eighteen bucks (body weight 16.75 kg) were divided into three equal groups and were fed control diet, diet supplemented with linseed oil (LSO) or chia seed oil (CSO) at 1% on dry matter (DM) for 100 days. The roughage to concentrate (maize-25, barley- 25, soybean meal-10, rice bran-16, groundnut cake-20, mineral mixture-2 and salt and ammonium chloride-1 part each) ratio was 50: 50. The additives (LSO or CSO) were mixed with 100 g of the concentrate mixture just before feeding and offered to the goats at 0900 h. The rest of concentrate mixture and green fodder was offered together at 1,000 h and 1,500 h in two divided doses. The feed samples were dried at 60°C in a hot air oven, ground and analysed for proximate (AOAC 2000) and cell wall constituents (Van Soest *et al.* 1991). Samples of LSO, Chia oil, concentrate and fodder were

analysed for FA content according to method of Ranganna (1986) using FA methyl esters. All animals had free access to water and were kept out in the open area post feeding. The body weight (BW) of animals was recorded fortnightly to modify feeding schedule.

*Ultrasonography, sperm attributes and hormone analysis:* Ultrasonography of the testis was performed by using coupling gel on bucks (30, 45 and 60 days after supplementation of omega-3 rich oils) in standing position and measurements of the mediastinum thickness (transverse plane), epididymis thickness (saggital plane) and pampiniform plexus thickness (frontal plane) in testis were performed. Semen was collected from each of bucks twice a week by artificial vagina and analysed for volume / colour. Sperm concentration (haemocytometer), motility (wet mount method) and viability (eosin-nigrosin stain) were assessed. Plasma membrane integrity was assessed by incubating semen in 125 mosm hypo-osmotic solution (HOS) for 30 min. For acrosome integrity, sperm smears prepared on clean glass slides were stained with Giemsa stain. Spermatozoa with normal and abnormal morphology were evaluated from the eosin- nigrosin stained sperm smears. Lipid peroxidation of neat semen was estimated by MDA concentration. Sperm extract was prepared to get a final sperm concentration of 500 × $10^6$ sperms/ml and stored in aliquots at –20ºC till further use. Antioxidant status was observed by estimating the activities of Superoxide Dismutase (SOD, Nishikimi *et al.* 1972), Catalase (CAT, Goth, 1991) and Gluthathione Reductase (GRE, Krohne-Ehrich *et al.* 1971). The testosterone concentration in blood plasma was conducted on day 15, 45 and 75 after feed supplementation by following the standard protocol supplied along with ELISA kit (Goat T Elisa, Catalog No. EG060066, Biocodon Technologies, USA).

*Qualitative and quantitative analysis of sperm proteins by SDS-PAGE:* Semen was washed twice with PBS, *p*H 7.4 and proteins were extracted by suspending sperm pellet (500 ×$10^6$ spermatozoa) in 1.0 ml of 2% SDS solution in 62.5 mM Tris-HCl (*p*H 6.8) containing protease inhibitors (Cocktail, SERVA). Sperm suspension was sonicated at 20 watts for 3 × 20 sec, centrifuged at 10,000 rpm for 15 min. Pellet was discarded and sperm extract (SE) was concentrated through 3 kDa protein concentrators (Millipore) and stored in aliquots at –20°C till further use. About 100 µg of protein was loaded to each well and discontinuous SDS-PAGE was performed on 12% acrylamide gels (Laemmli 1970). The gel was stained with 0.5% CBB for 2–4 h and de-stained till the blue bands appeared against a clear background. The images were captured by using Gene Snap Image Acquisition software. Images were analysed for molecular weights and quantity by using Gene Tool software (Syngene).

*Statistical Analysis:* Data analysis was performed by using SPSS software (SPSS 23.0 for windows; SPSS, Chicago, IL, USA). The results were analysed by ANOVA with repeated measures, using the General Linear Model (GLM). When ANOVA revealed a significant effect, multiple comparisons between means were tested by Tukey's test. Differences with values P< 0.05 was considered significant.

## RESULTS AND DISCUSSION

The chemical composition revealed that the organic matter, ash, CP, fat, cellulose, NDF and ADF content of green fodder and concentrate mixture were 90.3 and 92.9%, 9.7 and 7.1%, 10.3 and 20.3%, 2.25 an 3.75%, 27.9 and 7.2%, 61.3 and 34.9%, 39.0 and 11.8%, respectively on DM basis.

*Fatty acid profile:* The fatty acid profile fodder, concentrate, LSO and CSO and daily intake of FA by different experimental group are presented in (Table 1). Supplementation of oil sources in the diet led to higher (P<0.01) intake of FA in LSO and CSO supplemented diet as compared to control diet. The daily intake of omega-3 fatty acids was observed to be 0.6 (control), 4.04 (diet supplemented with LSO) and 4.57 g/animal (diet supplemented with CSO). Glasser *et al.* (2008) and Morsy *et al.* (2015) also reported that linseed oil contained high level of omega 3 FA, particularly α-linolenic acid (ALA; 50% to 60% of total FA). Similarly, Chia oil has high content of α-linolenic acid (about 60%), linoleic acid, oleic acid and stearic acid (Neetika *et al.* 2019).

*Ultrasonography:* Results revealed (Table 2) that mean mediastinum (MT) and cauda epididymal thickness (CET)

Table 1. Fatty acid profile (feedstuffs and oils) and daily intake of major FA by different experimental groups

| Parameter | FA, mg/g DM | | FA, mg/g oil | | Daily intake, (g/100 g FA) | | | P value |
|---|---|---|---|---|---|---|---|---|
| | Fodder | CON | LSO | CSO | Control | LSO | CSO | |
| Σ SFA | 7.19 | 13.31 | 132.20 | 155.60 | 7.05$^a$ | 7.49$^b$ | 7.75$^b$ | 0.000 |
| Σ MUFA | 13.10 | 13.00 | 196.50 | 232.10 | 8.99$^a$ | 9.73$^b$ | 10.10$^c$ | 0.000 |
| Σ PUFA | 7.60 | 14.30 | 667.40 | 612.30 | 7.55$^a$ | 11.42$^b$ | 11.21$^b$ | 0.000 |
| Σ Trans FA | 0.22 | 0.05 | 3.90 | ND | 0.04$^a$ | 0.07$^b$ | 0.04$^a$ | 0.001 |
| $^1$n–3 FA | 0.18 | 1.58 | 536.90 | 609.60 | 0.61 | 4.04 | 4.57 | 0.269 |
| $^2$n–6 FA | 7.42 | 12.70 | 131.00 | 2.90 | 6.94$^b$ | 7.37$^c$ | 6.64$^a$ | 0.001 |

$^1$n-3 FA: C18: 3n-3 + C20: 3n-6 + C20: 5n-3 + C22: 6n-3; $^2$n-6 FA: C18: 2n-6 + C18: 3n-6 + C20: 4n-6 + C20: 2n-6 + C20: 3n-6+ C22: 2n-6; ND, Not detectable; CON, Concentrate mixture; FA- Fatty acid; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids; LSO, Linseed oil; CSO, Chia seed oil; Values within a row with different superscripts differ significantly at P<0.01 for daily intake of FA.

Table 2. Effect of supplementation of omega-3 rich oils on testicular parameters and back fat thickness as observed by Ultrasonography

| Parameter | Thickness, cm | | | |
|---|---|---|---|---|
| | Mediastinum | Cauda epididymal | Pampini formplexus | Back fat |
| *Effect of supplementation*[1] | | | | |
| Control | 1.91[a] | 1.62[a] | 1.25[a] | 0.59 |
| Chia oil | 1.58[b] | 1.37[b] | 1.05[b] | 0.64 |
| LSO | 1.56[b] | 1.29[b] | 1.21[a] | 0.58 |
| SEM | 0.05 | 0.05 | 0.05 | 0.04 |
| P-value | 0.00 | 0.004 | 0.004 | 0.621 |
| *Effect of duration of supplementation, days*[2] | | | | |
| 15 | 1.41[b] | 1.31 | 0.93[b] | 0.31[b] |
| 30 | 1.56[ab] | 1.40 | 1.18[a] | 0.66[a] |
| 45 | 1.66[ab] | 1.43 | 1.27[a] | 0.78[a] |
| 60 | 1.76[a] | 1.46 | 1.31[a] | 0.66[a] |
| P-value | 0.008 | 0.731 | 0.00 | 0.00 |

[1]Irrespective of duration of supplementation; [2]irrespective of supplementation; [a,b]Values within a row with different superscripts differ significantly at P<0.01.

of testis showed a significant (P<0.01) difference among all the groups. Pampiniform plexus thickness (PPT) was significantly different between CSO group and control / LSO group (P<0.01). Back fat thickness remained comparable among all groups. Although the values of control group were significantly higher in comparison with CSO and LSO groups, but the rate of growth of MT, CET and PPT was much better in dietary supplemented groups than in control group, whereas the BFT growth rate remained the same. It signifies that although there was no significant improvement in the testicular size yet the growth rate was much high in dietary supplemented groups. Stage of maturity had significantly affected the ultrasonographic parameters except CET. It implies that in CSO and LSO supplemented groups, the seminiferous tubules development along with the pampiniform plexus size had grown with a faster rate.

The report towards comparison of testicular ultrasonographic parameters of immature buck was not available in literature. However, ultrasonography revealed testicular parenchyma as homogenously greyish and moderately hypoechoic, while the mediastinum testes appeared as a white hyper-echoic thin line in the center. The scrotal wall appeared as a thick hyper-echoic semi-circular layer forming the periphery at caudo-dorsal portion surrounding the scrotal contents. Cauda epididymis appeared as a small round dark hypo-echoic structure on the caudo-ventral portion. However, the pampiniform plexus gave appearance of multiple hypo-echoic loops surrounded by hyper-echoic circular layer.

*Sperm attributes:* Colour of the semen varied from creamy white to yellowish in bucks fed un-supplemented and LSO supplemented diet, whereas in animals fed CSO supplemented diet, it was creamy white (Table 3). Ejaculate volume was significantly higher (P<0.05) in bucks fed CSO (105%) and LSO (22%) supplemented in comparison to that observed in bucks fed un-supplemented diet. Per cent

Table 3. Effect of dietary supplementation of omega-3 rich oils on semen attributes, lipid peroxidation and antioxidant enzymes in spermatozoa and testosterone in blood plasma of bucks

| Semen attribute | Control | LSO | Chia oil | P value |
|---|---|---|---|---|
| *Macroscopic attributes* | | | | |
| Colour | Creamy white-yellow | Creamy white-yellow | Creamy white | – |
| Volume (mL)** | 0.60[a] | 0.73[a] | 1.23[b] | 0.009 |
| *Microscopic attributes* | | | | |
| Mass Activity | +2 to +3 | +3 to +4 | +3 to +4 | – |
| Individual motility (%)*** | 56.67[a] | 70.56[b] | 85.00[c] | 0.000 |
| Abnormalities (%)* | 18.83[b] | 16.89[ab] | 10.83[a] | 0.020 |
| Viability (%)*** | 30.67[a] | 49.67[b] | 71.00[c] | 0.000 |
| Sperm concentration ($\times 10^6$/ml)*** | 242.50[a] | 752.56[b] | 1239.83[c] | 0.000 |
| Membrane Integrity (%)*** | 17.33[a] | 35.67[b] | 55.50[c] | 0.000 |
| Acrosome Integrity (%)*** | 26.17[a] | 48.78[b] | 66.50[c] | 0.000 |
| *Enzymes activity/$10^9$ spermatozoa* | | | | |
| Malondialdehyde (µmole)*** | 161.83[a] | 87.22[b] | 43.83[c] | 0.000 |
| Superoxide dismutase (IU/min)*** | 99.00[a] | 248.89[b] | 588.66[c] | 0.000 |
| Catalase (KU/min)*** | 91.67[a] | 187.78[a] | 516.33[b] | 0.000 |
| Glutathione reductase (IU/min)* | 1.90[a] | 5.54[ab] | 7.58[b] | 0.027 |
| *Testosterone concentration in blood plasma, ng/mL* | | | | |
| [#]15 days* | 4.60[a] | 8.80[b] | 6.00[ab] | 0.015 |
| 45 days | 4.20 | 4.60 | 7.40 | 0.055 |
| 75 days | 5.10 | 6.30 | 5.90 | 0.526 |
| Mean | 4.60 | 6.50 | 6.40 | 0.076 |

[#]Days after supplementation.

change in individual motility, viability, sperm concentration, membrane integrity and acrosome integrity was observed to be significantly higher (P<0.05) in bucks fed CSO as compared to LSO control bucks. The percentage of spermatozoa with abnormal morphology was significantly (P<0.05) less in bucks fed CSO (42%) and LSO supplemented (10%) than that observed in control group. Increase in ejaculate volume is also reported in goats (Dolatpanah *et al.* 2008) and rams (Baiomy and Mottelib 2009) after feeding omega-3 and omega-6 rich feedstuffs. Habibi *et al.* (2015) recorded a 65% increase in semen volume in ovine after supplementation of their diets with fish oil. On the contrary, Dolatpanah *et al.* (2008), failed to achieve significant difference in semen volume following PUFA supplementation in goat. Similarly, fish oil fed for 9 weeks did not affect the total semen volume in the ram (Fair *et al*. 2014). Increase in percentage of viable spermatozoa due to dietary supplementation of LSO and CSO seed oil during the present study is also in line with the observations of feeding fish oil (Dolatpanah *et al.* 2008), LSO (Mourvaki *et al.* 2010), omega 3 fatty acids (Gholami *et al.* 2011) in goat and Holstein bulls, respectively. On the contrary, dietary supplementation of omega-3 PUFA or DHA-enriched nutraceutical and unsaturated fatty acids didn't improve sperm viability and count in rams (Fair *et al*. 2014) and buffalo (Tran *et al.* 2016). Increase in membrane integrity in semen of LSO and CSO fed bucks is comparable to observations in Nili Ravi bulls (Shah *et al.* 2016). Similar to our observations on acrosome integrity, an improvement in acrosomal integrity as a result of fish oil supplementation was also reported in fresh and frozen-thawed ram spermatozoa (Habibi *et al.* 2015).

The concentration of MDA (µmole/$10^9$ sperm), activity of SOD (IU/$10^9$ spermatozoa/min), GRE (IU/$10^9$ spermatozoa/min) and CAT (KU/$10^9$ spermatozoa/min) in spermatozoa were significantly (P<0.05) higher in semen of bucks fed CSO supplemented diet in comparison to control or LSO fed group.

*Plasma testosterone profile:* Plasma testosterone levels in bucks fed LSO and CSO diet had non-significantly higher (P>0.05) concentrations (Table 3) than that observed in control bucks, however, after 15 days of supplementation, the concentration of testosterone was significantly higher in LSO fed group in comparison to control but comparable to CSO supplemented bucks. Many reports suggest that PUFA supplementation improve steroidogenesis *in vivo* through steroidogenic acute regulatory protein expression (Hughes *et al*. 2011) and altering the function of transcription factors (Wathes *et al.* 2007). Therefore, fatty acid composition of both oils may have improved steroidogenesis and ultimately higher level of testosterone in CSO and LSO supplemented groups.

*Characterization of sperm proteins by SDS-PAGE:* SDS-PAGE of sperm extracts of bucks resulted in resolution of 22 bands ranging from 7 kDa to 366 kDa. The proteins of 70, 26 and 22 kDa were detected only in two bucks fed LSO supplemented diet. Quantity of 366, 333, 100, 90 kDa proteins was significantly (P<0.05) high in bucks fed LSO compared to those fed CSO supplemented and control diet. Proteins of 66 and 11 kDa were significantly more (P<0.05) in bucks fed CSO supplemented than those fed LSO supplemented and un-supplemented diet. Amount of two proteins of 39 and 38 kDa was significantly (P<0.05) higher in bucks fed CSO and LSO supplemented diet as compared to bucks fed control diet. Intensity of 7 kDa protein was significantly (P<0.05) higher in bucks fed CSO supplemented and control diet in comparison to those fed LSO supplemented diet. Quantity of 135, 68, 45, 40, 35, 31, 28, 25, 20 and 17 kDa proteins was not significantly different among the three groups. Electrophoretic profile of sperm proteins revealed the presence of twenty proteins in sperm extracts of goat bucks during the present study. Deori *et al.* (2016) also indicated 20 different protein bands with molecular weight ranging from 10 kDa to 75 kDa in Assam hill goat buck spermatozoa. Among the 20 proteins, only 6 proteins bands were consistently present in all 8 bucks. Protein bands with molecular weight 10, 14, 22, 28, 55, 57 and 60 kDa showed significant positive correlation with certain parameters of the fresh semen, while 47 kDa protein band presented negative correlation with fresh semen characteristics in AHG bucks. A variation in proteins was also noticeable in our study, which may be due to individual variation rather than effect of LSO or CSO supplementation. In a study, the replacement of soybean meal by castor cake in the diet of bucks revealed alteration in the expression of certain seminal plasma and sperm membrane proteins in ricin supplemented bucks (Oliveira *et al.* 2015).

From present study it may be concluded that CSO supplementation in the diets may be helpful in improving the process of spermatogenesis, sperm output, quality and ultimately fertility of bucks.

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