# Effect of different forms of rumen-protected fat from palm oil on body weight and sperm quality in Malin sheep

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## Abstract

The study purpose was to evaluate the effect of different forms of rumen-protected fat (RPF) that is high in fatty acid on body weight and sperm quality in male Malin sheep. Twenty male adult Malin sheep were randomly assigned to four treatment groups namely; CON: control, PF: 2% prilled fat, CS: 2% calcium salt fatty acid and CO: 2% canola oil respectively. No significant difference was observed in the body weight changes among the treatment group. Rams that received PF had the highest ($P<0.05$) semen volume while the animal received CO had the highest ($P<0.05$) sperm concentration than other treatment group. A significant increase ($P<0.05$) in the sperm membrane integrity in PF (44%) and CS (42%) than CON. Live sperm was also significantly higher in PF and CS group (79%) than control. No significant effect ($P>0.05$) was found in abnormal sperm percentage in animals with different dietary supplements with control. In conclusion, the result indicates that supplementation of rumen-protected fat from palm oil to adult male Malin sheep helped to improve the sperm plasma membrane integrity and live sperm.

**Keywords:** rumen-protected fat, · sheep, · sperm, · fatty acids · Malin sheep

## Introduction

Nutrient shortage can lead to poor reproduction performances thus decreasing animal productivity on the farm. Traditionally, concentrate or grain-based diets have been used as a solution to increase the energy density in the diet of the animals. However, there are limits on how much the concentrate and grain can be taken resulting in the high cost of production, lack of production and the risk of acidosis. Small ruminants like goats and sheep are also facing nutrients shortages in Asia because of unpredictable climatic patterns, declivity in land resources and a fall in feed resources (Ben Salem & Smith, 2008). As a result, there is energy deficiency in ruminant livestock and therefore this deficiency will affect the growth, production, and subsequent breeding performance (Devasena et al., 2007). In mature rams, changes in feed intake can cause changes in sperm production due to changes in the size of the seminiferous tubules and spermatogenesis efficiency (Martin et al., 2010). Underfeeding in adult rams in 65 days, can reduce spermatogenesis and more sperm DNA damage (Guan et al, 2014). Therefore, it is important to gather more information on whether feeding any supplement can overcome the problem.

Rumen-protected fat (RPF) has been used in the livestock industry to increase the energy

density of the animal feed to compensate for the energy deficiency in dairy animals but there is a lacks of knowledge on the effect of the RPF on the reproduction system in ram. Fat or also known as lipid have been used to increase the energy density of animal feeds. Lack of energy in ruminants can be due to low-quality roughages or reduce feed intake by the animals. Usage of fat supplementation has become a common practice to increase the energy density of the diet for ruminants. In Malaysia, palm oil defined product has been mostly used in livestock industry because they do not have the negative effect on rumen fermentation as unsaturated oils (Manso et al., 2009). Studies have shown that usage of lipid supplements can increase milk yield (Alstrup et al., 2015), body condition (Bhatt et al., 2013), conception rate in dairy cows (McNamara et al., 2003) and increase semen concentration in male animals (Fair et al., 2014). Protected fat can safely be used up to 7.5% without any adverse effect on dry matter intake and rumen fermentation (Sirohi et al., 2001) while another study showed the inclusion of protected fat with calcium can be used up to 10% (Ramana et al., 2003). Increasing the percentage of inclusion mean more energy can be given to the animal and using a locally produced product can save cost because the rumen-protected fat are from the byproduct of palm oil. This will be affordable to farmers compare to concentrate or grains and safer because it will not disturb the rumen microbes and metabolism. Therefore, the present study was conducted to determine the effect of supplementing different forms of rumen-protected fat from palm oil on the bodyweight changes and sperm quality in Malin sheep.

## Materials and Methods

*Animals*

The study was carried at the National Institute of Veterinary Biodiversity, Jerantut, Pahang. Twenty male Malin sheep with an average body weight of 36.6kg ± 5.57 kg and aged between 10-14 months old were used for the experiment. Before the experiment, the rams were fed with *Bracharia humidicola* grass and a commercial pellet. The rams were kept in an individual pen equipped with a feed and water trough in a raised floor housing. Before the study, attempts were made to train all rams for semen collection using an artificial vagina and teaser ewe. Each ram received concentrate at 0900 h and grass at 1100 h the rams received the grass respectively per animal requirement. The rumen-protected fat and canola oil were mixed with the concentrate before feeding. The rams have free access to water *ad libitum*.

*Experimental design*

The animals were randomly assigned into four groups namely i) control group with a basal diet, consisted of *Brachiaria humidicola* grass and commercial sheep pellet (CON); ii) PF, a basal diet supplemented with prilled fat at 2% per day of the total diet; iii): CS, a basal diet supplemented with calcium salt at 2% per day of the total diet; and iv) CO, a basal diet with 2% canola oil per day of the total diet. The experimented diet was formulated to be isocaloric and isonitrogenous for all groups according to (ICAR-NIANP, 2013) (Table 1). The period of the experiment lasts for 12 weeks. An adaptation period of two weeks has been given to all rams.

Table 1. Feed ingredients and composition of diets in control, prilled fat (PF), calcium salt fatty acids (CS) and canola oil (CO).

| Ingredients | Feed Formulation | | | |
|---|---|---|---|---|
| | CON | PF | CA | CO |
| Bracharia grass | 66 | 76 | 75 | 77 |
| Commercial pellet | 34 | 22 | 23 | 21 |
| Rumen-protected fat A (without calcium) | -- | 2 | -- | -- |
| Rumen-protected fat B (with calcium) | -- | -- | 2 | -- |
| Canola oil | -- | -- | -- | 2 |
| Total | 100 | 100 | 100 | 100 |
| Calculated analysis | | | | |
| ME (kcal/kg) | 2217 | 2211 | 2214 | 2210 |
| Crude protein (%) | 12.2 | 11.3 | 11.4 | 11.3 |

*Body weight, semen collection, semen volume and semen processing*

The body weight of the animals were taken and recorded once every two weeks. Based on the daily feed offered and refused records, the daily DM feed intake was calculated by dividing the total feed consumed by the animals (based on DM concentration) with the total number of experimental days. Semen collection was conducted in the morning once every two weeks for the entire experiment period using an artificial vagina. Conical graduated tubes were used to determine the semen volume ejaculated by the animals (Fair et al., 2014; Jafaroghli et al., 2014; Samadian et al., 2010) and transported to the laboratory within 10-15 min and placed in a water bath at 37˚C water bath until analysis for sperm concentration, live sperm, abnormal sperm and plasma membrane integrity.

*Determination of sperm concentration, morphology, and live sperm.*

A spectrophotometer (Minitube, SDM1 V1.7, Tiefenbach, Germany) was used to determine the sperm concentration of the ram. Sperm concentration evaluation was done by pipetted a 30µl semen sample into a cuvette and placed in the spectrophotometer. Examination of live sperm and abnormal sperm was done by mixing semen samples with eosin nigrosine stain. A small drop of semen sample was put onto a glass slide together with a larger drop of eosin-nigrosin into the semen sample and allowed to mix for one minute. A thin smear was made by smooth drawing using the edge of another slide and then the smear was air dry (Tran et al., 2016). Three smears were made for each sample. Approximately 200 sperms per slide were counted from each smear using a phase-contrast microscope under x400 magnification. Live and abnormal sperm were calculated and presented in percentage.

*Determination of sperm plasma membrane integrity*

Sperm plasma membrane integrity was examined by using the Hypo-osmotic swelling test which was described by Correa and Zavos (1994). The test was performed to evaluate the changes in functional plasma membrane integrity. About 1 ml of hypo-osmotic solution (fructose 13.51g, trisodium

citrate 7.35 g into distilled water 1000 ml; osmolarity of 150 mOsm/kg) was mixed with 0.1 ml of semen and incubated at $37^0$C for one hour (Tran et al., 2016). A drop of the mixed solution was put onto a glass slide and covered with a coverslip. Sperm tail curling which was an indication of swelling of the tail due to influx of water was recorded. A total of 200 spermatozoa were counted in different fields at 400x magnification under a phase-contrast microscope and expressed in percentage.

*Determination of sperm kinematic*

About 10µl of semen sample was diluted with 90µl of bio-excel extender (IMV Technologies, L'Aigle, Normandy, France). A drop of 5µl of the mixture was dropped onto a glass slide and cover with a cover glass. The slide was later placed onto a heated stage and observed under the microscope. The sperm kinematic was measured using computer-assisted sperm analysis (CASA, CEROS, Hamilton Thorne Biolab). The motility variable measured included motility percentage (MS, %), progressive motility percentage (PS, %), velocity curvilinear (VCL, um/s), velocity straight line (VSL, um/s), velocity average path (VAP, um/s) and straightness (STR). Motile sperm or slow cells were classified for sperm with VAP greater than 30 µm/s and VSL greater than 15 µm/s were considered motile and sperm with VAP more than 50µm/s and STR (VSL/VAP x 100) greater than 70% will be classified as progressive.

*Statistical analysis*

All statistical were analysed using Statistical Analysis Software (SAS, Version 9.4). Data were subjected to analysis using two-way ANOVA for interaction between time and dietary treatment. General Linear Model (GLM) was used to analyse the data and the mean between treatments was compared by Duncan's Multiple Range Test (DMRT). All statistical analyses that have $P<0.05$ was the significant value.

## Results

*Growth performance and feed intake*

The growth performance and feed intake for all treatments in response to dietary fat supplements are shown in Table 2. There was no significant effect ($P>0.05$) in the bodyweight changes for initial and final body weight among treatments. Closer inspection of the table showed, there was a tendency that rams received calcium salt of fatty acid have a higher body weight gain and average daily gain. There was a significantly different ($P<0.05$) on dry matter intake in the canola and prilled group relative to the CON and CS group. However, no significant differences ($P>0.05$) between PF and CO group in dry matter intake. Dry matter intake for the CS group was not significantly different ($P>0.05$) compare to CON but was significantly lower ($P<0.05$) compare to rams fed with PF and CO.

*Semen characteristics*

There was a significant effect of different dietary fat for semen ejaculation volume, sperm concentration, plasma membrane integrity, and live sperm (Table 3). No significant differences were found in abnormal sperm among treatments. The ejaculated semen volume was significantly higher ($P<0.05$) in the PF group and CON group compare to CS and CO group. Nonetheless, no significance was found between PF and CON groups in semen volume ejaculated. Similar results were also observed in CS and CO groups. There was a significant effect ($P<0.05$) of sperm concentration in response to the dietary treatment.

Table 2. Effect of different dietary treatments on body weight and dry matter intake of rams means±SE)

| Parameters | CON | PF | CS | CO |
|---|---|---|---|---|
| Initial BW (kg) | 35.7±2.98 | 37.1±2.25 | 36.6±2.95 | 36.8±2.86 |
| Final BW (kg) | 38.2±2.17 | 39.3±2.32 | 38.1±1.90 | 39.1±2.53 |
| Gain BW (kg) | 2.5±0.81 | 2.3±0.26 | 2.8±0.72 | 2.4±0.5 |
| ADG (g) | 32.5±10.47 | 30.3±3.35 | 36.8±9.34 | 30.8±6.44 |
| DMI (g/day) | 958.4±19.27[b] | 1055.4±8.33[a] | 973.7±26.60[b] | 1063.0±15.53[a] |

[a,b] Means bearing different superscripts in a row differ significantly (P<0.05)
Note:
CON, control group with a basal diet, consisted of *Brachiaria humidicola* grass and commercial sheep pellet;
PF, a basal diet supplemented with prilled fat at 2% per day of the total diet;
CS, a basal diet supplemented with calcium salt at 2% per day of the total diet;
CO, a basal diet with 2% canola oil per day of the total diet.

Table 3. Effect of different dietary treatments on the parameters of sperm quality (mean±SE)

| Parameters | CON | PF | CS | CO |
|---|---|---|---|---|
| Ejaculated semen volume (ml) | 0.75±0.04[a] | 0.79±0.03[a] | 0.65±0.03[b] | 0.62±0.03[b] |
| Sperm concentration ($10^6$/ml) | 1950.2±13.87[a] | 1894.7±10.21[b] | 1903.3±13.24[b] | 1954.9±10.32[a] |
| Sperm plasma membrane integrity (%) | 35.7±1.61[a] | 44.7±1.55[b] | 42.2±1.46[b] | 41.9±1.35[b] |
| Live sperm (%) | 75.3±1.25[a] | 79.0±0.97[b] | 79.2±0.98[b] | 76.6±1.07[ab] |
| Abnormal sperm (%) | 16.3±0.62 | 15.6±0.53 | 15.5±0.58 | 17.1±0.71 |

[a,b] Means bearing different superscripts in a row differ significantly (P<0.05)
Note:
CON, control group with a basal diet, consisted of *Brachiaria humidicola* grass and commercial sheep pellet;
PF, a basal diet supplemented with prilled fat at 2% per day of the total diet;
CS, a basal diet supplemented with calcium salt at 2% per day of the total diet;
CO, a basal diet with 2% canola oil per day of the total diet.

The sperm concentration of the CO group shown the highest mean and significantly (P<0.05) higher compare to PF and casa groups. Yet, there was no significant difference (P>0.05) in sperm concentration between CO and CON groups. Also, no significant effect was observed between PF and casa group in sperm concentration.

Plasma sperm integrity membrane shown rams feed with PF, CS and CO had significant difference (P<0.05) than CON diet. However, there was no significant difference in sperm

plasma membrane integrity among PF, CS and CO. Live sperm percent was significantly different (P<0.05) as recorded in PF, CS and CO group compared to CON. However, no significant difference (P>0.05) in live sperm among rams in the dietary fat supplement was noted in the present study. The live sperm result was also inconsistent with the sperm plasma integrity result which showed the same data pattern. Abnormal sperm percentage appeared to be unaffected by different dietary treatments as there were no significant differences observed among the group.

The results of sperm kinematic analysis in response to dietary treatment are set out in Table 4. The velocity straight line (VSL) parameter in sperm kinematic showed significantly different (P<0.05). The highest mean was observed in rams fed with casa than CON group yet VSL of CS group was not significantly different compare to PF and CO group. Other sperm kinematic analysis, rapid motility, medium motility, slow motility, static, VAP, VCL, ALH, BCF, STR, LIN, elongation and area of sperm head were not affected with dietary fat supplement (P>0.05).

Table 4. Effect of dietary treatments on the parameters of sperm kinematic and morphometric (mean±SE)

| Parameters | CON | PF | CS | CO |
|---|---|---|---|---|
| Motile | 84.55±1.08 | 84.29±0.89 | 84.60±0.99 | 82.97±0.70 |
| Progressive | 62.93±1.08 | 62.73±1.00 | 63.65±1.14 | 62.68±0.84 |
| Rapid | 78.85±1.31 | 78.61±1.14 | 78.67±1.33 | 77.36±0.97 |
| Medium | 5.52±0.47 | 5.41±0.42 | 5.64±0.53 | 5.76±0.49 |
| Slow | 6.13±0.48 | 6.11±0.36 | 6.24±0.45 | 6.00±0.40 |
| Static | 9.43±0.77 | 9.76±0.68 | 9.24±0.88 | 10.97±0.62 |
| VAP(μm/s) | 110.44±2.86 | 116.43±2.76 | 118.29±3.00 | 114.58±2.47 |
| VSL(μm/s) | 93.04±2.47[b] | 98.16±2.44[ab] | 100.79±2.79[a] | 97.43±2.28 [ab] |
| VCL(μm/s) | 160.07±4.15 | 169.82±3.86 | 167.48±4.26 | 165.10±4.21 |
| ALH(μm) | 5.87±0.11 | 6.02±0.10 | 5.87±0.09 | 6.21±0.27 |
| BCF(Hz) | 21.04±0.70 | 22.15±0.74 | 22.37±0.57 | 21.90±0.69 |
| STR(%) | 82.61±0.42 | 82.76±0.41 | 83.15±0.54 | 83.08±0.43 |
| LIN(%) | 58.80±0.62 | 58.40±0.57 | 60.25±0.68 | 58.79±0.58 |
| Elongation((μm) | 50.20±0.31 | 50.73±0.24 | 50.28±0.25 | 50.74±0.27 |
| Area($\mu m^2$) | 5.23±0.04 | 5.29±0.05 | 5.25±0.04 | 5.21±0.04 |

[a,b] Means bearing different superscripts in a column differ significantly (P<0.05)

Note:

CON, control group with a basal diet, consisted of *Brachiaria humidicola* grass and commercial sheep pellet;
PF, a basal diet supplemented with prilled fat at 2% per day of the total diet;
CS, a basal diet supplemented with calsium salt at 2% per day of the total diet;
CO, a basal diet with 2% canola oil per day of the total diet.

## Discussion

*Growth performance and feed intake*

In this study, two different sources of the fat supplement were used. The first source was from palm oil which is high in palmitic acid (C16:0) and oleic acid (C18:1) but low in PUFA. The second source of fat supplement use in the study were canola oil that was high in oleic acid (C18:1) and PUFA (Giakoumis, 2018). The usage of different rumen-protected fat from palm oil showed no significance in growth performance against CON ($P>0.05$). However, there was a tendency that rams received CS have higher body weight gain and average daily gain. Bodyweight gain and ADG tend to be higher in CS group in the present study and it may be due to feeding calcium salt of palm oil fatty acids can increased energy intake of diet (Jenkins & Palmquist, 1984) as it is protected from hydrolysis and biohydrogenation in the rumen and causing no harmful effect on rumen fermentation. Rumen-protected fat such as calcium fatty acids is a significant source of energy owing to its high fatty acids contents (84% fatty acids), for that reason it has been used to supply the energy demand in ruminants (Abd El-Hamid et al., 2016). The dietary fat could be safely used up to 7.5% without any adverse effect on dry matter intake and rumen fermentation (Sirohi et al., 2001).

Another possible explanation for this is that calcium salt of fatty acids was more protected or inert compare to prilled fat. Gulati et al. (1997) studied showed that supplement of calcium salt of fatty acid 21% more inert than prilled fat thus, higher fatty acids reach the small intestine for absorption. This is further support by Sirohi et al. (2001) that calcium salt of vegetable oils does not affect rumen fermentation indicated by unvarying microbial protein synthesis.

Feeding canola oil and prilled fat have higher dry matter intake in the present study compared to control. It is in contrast with Manso et al. (2009) that reported no significant difference was observed in dry matter intake between lipid supplements high in saturated fat or unsaturated fat. This may be due to different amounts of lipid added as he is using a higher percentage of hydrogenated palm oil at 4% was used in their study compared to our present study which uses only 2% of lipid supplement. Previous studies by Bhatt et al., (2011) demonstrated that usage of unprotected fat or conventional oil has a negative effect on voluntary intake and fibre digestibility at a high level of supplementation. This is in line with Sutton et al. (1983) studied that showed natural oil impairs digestibility by 40%. A study on meta-analysis literature by Patra (2014) indicated that low concentrations of fat in the diet do not cause an adverse effect in rumen fermentation but at high concentration may reduce the fibre digestibility. In this case, supplementing canola oil at a low level did not influence the dry matter intake of the animals involved in this study. These results further support the observation of Doreau and Chilliard (1997) that adding fat to ruminant not only depend on the nature or type of fat but also the amount of fat added in the feed. Studies on the impact of rumen-protected fat on feed intake have produced varied results. Lower dry matter intake in CS group was different with the data reported by Bhatt et al. (2013) that feed intake increased with calcium salt fatty acids supplementation. However, the present study is inconsistent with Ganjkhanlou et al. (2009) which has found a similar outcome of a reduction in dry matter intake by the animal. It suggests that by adding calcium salt of fatty acid in the diet, it may still have depressed the rumen fibre digestion resulting in decreased rumen fermentation, thus decreased dry matter intake. While Allen (2000) suggested that

increased long-chain fatty acid intake and increased supply to the small intestine may decreased dry matter intake. It has been proposed that the digestive processes in the hindgut compensated for the possible reduction in digestion in the rumen resulting in a limited effect on whole tract digestion (Sutton et al., 1983).

*Sperm quality*

Many thoughts have been given to supplement animals with a diet high in PUFA because semen from domesticated animals comprise a high level of PUFA (Brinsko et al., 2005). PUFA are synthesis from a shorter chain of linolenic and linoleic acid through a process of desaturation and elongation. Linolenic and linoleic acids alone cannot be synthesised and must be consumed from the diet (Lands, 1992). However, a saturated fatty acid is also important especially providing the energy that would support the reproduction activities of the animals. A study by Matoba et al. (2008) has noted the importance of energy requirement in males. Testis specific morphogenetic changes in germ cell differentiation suggest that male gonads required higher energy demand than ovaries and this discrete metabolic characteristic, emphasis on the activity of mitochondria might also contribute to the sex determination of sperm (Ramalho-santos and Amaral 2013).

Amaral et al. (2013) have identified that usage of energy from beta-oxidation of fatty acid have a greater proportion in metabolism and energy production in sperm. It is interesting to note that the study found sperm that incubated with fatty acid oxidation inhibitor showed a significant decrease in sperm motility proving the importance of energy in sperm fertility and the role of fatty acids in energy production in sperm.

In the present study, there was a significant effect of the different dietary fat supplements on semen ejaculation volume, sperm concentration, plasma membrane integrity and live sperm. Feeding prilled fat rich in saturated fat significantly increased the semen volume ejaculated than others group which disagrees with Tran et al. (2016) whose found that no significance was observed in ejaculated semen volume in animals fed with prilled fat. This can be due to Tran et al. (2016) fed unequal concentrations of lipid supplement between prilled fat (4%) and a calcium salt of fatty acid (4.67%) to the animals for comparison. Nevertheless, the finding in this study was consistent with data obtained by Fair et al. (2014) who found that animals given saturated fatty acid had a strong tendency to ejaculate a higher volume of semen than animals fed with a diet high in PUFA.

There was a significant difference in sperm concentration in response to dietary treatment. The sperm concentration of animals that received canola oil shown the highest mean than animals that received prilled fat and calcium salt fatty acids in the present study. The same result was reported by Fair et al. (2014) that feeding a diet high in n-3 PUFA significantly yield higher value of sperm concentration than animal received saturated palmitic acid. This may be due to a diet high in PUFA effectively increase serum PUFA (Conquer et al., 2000) which in the end will increase sperm production.

Live sperm is significantly higher in prilled fat and calcium salt fatty acids group than in control, yet there was no significant difference with live sperm in canola oil. This outcome is contrary to that of Tran et al. (2016) who found feeding a diet high in PUFA have significant higher live sperm and it was consistent with support evidence from previous observation by Khoshvaght et al. (2016) that dietary supplement of PUFA could improve sperm quality through alteration of the fatty acid profile of sperm lipid. However, PUFA is intensely susceptible to oxidative damage generated by reactive oxygen

species(Aitken & Baker, 2006) that can be associated with sperm disfunction. Another possible explanation for these results differences may due to the usage of different concentrations and sources of fatty acid used in the present experiment.

Results showed a significant increase in the sperm membrane integrity in CS and PF group compare to the CON group but not significantly different to the CO group. Sperm membrane integrity is a test in hypo-osmotic solution which sign that the sperm membrane was biochemically active and transport of water across the membrane occur normally (Nur et al. 2005, Correa and Zavos 1994). Nonetheless, this finding was not consistent with Tran et al. (2016) who reported usage of calcium salt high in PUFA to have better improvement than ram fed with a diet low in PUFA. The result in this studied may suggest that saturated fat play a major role in sperm membrane. Higher plasma integrity and live sperm for prilled fat and calcium salt fatty acids group may due to higher dietary fat levels that increase the concentration of cholesterol (Swecker et al., 1987). Cholesterol is an important element of all animal cell membranes, and significantly affect membrane fluidity, permeability, curvature and membrane protein interaction (Elustondo et al., 2017). The cholesterol content of different subcellular fractions contain approximately 40-fold higher level than endoplasmic reticulum and mitochondria (Horvath & Daum, 2013). These results reflect those of Fair et al. (2014) who found that animals that received prilled fat showed a tendency to have higher cholesterol in plasma compared to animals that received a diet high in PUFA.

Sperm kinetic result showed a significant increase in velocity straight line (VSL) for animals fed with calcium salt from palm oil. This might be due to the influence of long-chain fatty acids for sperms metabolism and energy production (Amaral et al., 2013) as calcium soaps of long-chain fatty acids are more inert than prilled fat (Gulati et al., 1997). High supplementation of PUFA has been proven to have no effect on sperm motility (Conquer et al., 2000).

## Conclusion

In conclusion, supplementation of rumen-protected fat from palm oil to adult male Malin sheep does not show a better growth performance compared to control. However, feeding rumen-protected fat showed an increase in the sperm plasma membrane integrity and live sperm compared to the control diet. The results of this study may also have indicated that prilled fat and calcium salt fatty acids supplementation that is high in saturated and monosaturated fatty acids influence by increasing the sperm plasma membrane integrity and liveability.

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## Conflict of interest statement

There is no conflict of interest among authors regarding the publication of this article.

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