

Dietary fish oil supplementation improved ovarian function, conceptus growth and certain reproductive variables in Marwari mares

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ABSTRACT

We investigated the effect of dietary fish oil supplementation on the development of the ovarian follicles, corpus luteum (CL), conceptus and certain reproductive events in Marwari mares, since it is reported to improve reproduction in cows. Accordingly, non-lactating mares (n = 20) were randomly assigned into two groups (10 per group) and fed either the control diet (CTR) or a diet enriched with fish oil (FOS) to supplement n-3 polyunsaturated fatty acids (PUFA) at the rate of 64 mg/kg body weight/day for 70 days or until 45 days post-ovulation in the mares that became pregnant. Estrus was detected using a teaser and insemination was performed using frozen thawed semen in the experimental mares. Development of the ovarian follicle, CL and conceptus were recorded using trans-rectal ultrasonography. Plasma concentrations of progesterone and estradiol were estimated by radioimmunoassay. In the FOS group, the diameter of the largest follicle from day 4 of estrus until ovulation, and the diameter of the CL on day 7 post-ovulation (D7_{po}) were greater (P<0.05). However, on day 15 post-ovulation (D15_{po}), the CL diameter increased significantly in the pregnant mares. Dietary fish oil significantly improved the development of the embryo as evidenced by an increase in the diameter of the embryonic vesicle on day 15 post-ovulation (D15_{po}) and the embryo proper on day 28 post ovulation (D28_{po}).

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Further, the mean plasma estradiol concentration was higher on the day of estrus onset ($P < 0.05$) and day 4 of estrus ($P < 0.01$) in the FOS group. Similarly, dietary fish oil significantly increased the plasma progesterone on D15_{po} in the pregnant mares ($P < 0.01$). Although the duration of estrus was shorter by 19 hours ($P < 0.05$), the length of the estrous cycle did not vary in the FOS group. A non-significant increase in the pregnancy rate was observed in the mares that received fish oil. It was concluded that dietary fish oil supplementation improved ovarian function and embryonic development in the Marwari mares.

Key words: embryo; fish oil; follicle; mare; n-3 PUFA

Introduction

Early embryonic death (EED), which occurs between day 15 and 39 of gestation, is a major cause of conception failure in mares and its incidence ranges from 13.28% (PAPA et al., 1998) to 46.2% (RASHEED et al., 2015). The cause of EED is imputed either to lack of maternal inhibition of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or increased secretion of $PGF_{2\alpha}$ due to endometrial inflammation (PENROD et al., 2013). Supplementation of n-3 polyunsaturated fatty acid (PUFA) inhibits the endometrial production of $PGF_{2\alpha}$ *in vivo* (MATTOS et al., 2004) or *in vitro* (MATTOS et al., 2003), and, thereby, favors the early development of an embryo in dairy cows (MATTOS et al., 2000), sow (WEBEL et al., 2004) and does (MAHLA et al., 2017).

n-3 PUFA influences follicular growth and ovulation (ROBINSON et al., 2002), increases progesterone production from the corpus luteum (CL) (LOPES et al., 2009) and suppresses the luteolytic pulses of $PGF_{2\alpha}$ during the window of maternal recognition of pregnancy (MATTOS et al., 2004). n-3 PUFA rich fish oil, which contains eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) inhibits endometrial $PGF_{2\alpha}$ secretion in cows (THATCHER et al., 1997), She buffaloes (MALIK et al., 2011) and does (MAHLA et al., 2017). It is believed that n-3 PUFA attenuates the synthesis of $PGF_{2\alpha}$ by altering the membrane phospholipid composition of cells, which may lead to competitive inhibition of the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), by changing the availability of substrates. Increased quantities of EPA and DHA decrease the arachidonic acid (AA) metabolism (WATHES et al., 2007), and reduce the activity of phospholipase A2 (PLA2) in the endometrium (WATERS et al., 2012). Further, displacement of AA by EPA or DHA in the membrane phospholipids can shift the production from series-2 PG to the less bioactive series-3 PG (MATTOS et al., 2003). Recently, dietary supplementation of n-3 PUFA in stallions has been investigated for its role in improving semen quality (RAVI et al., 2016; RODRIGUES et al., 2017). However, the influence of n-3 PUFA rich fish oil on reproductive functions in mares has not been previously studied. Therefore, the present study investigated the effect of dietary supplementation of fish oil on the ovarian function, conceptus development and pregnancy rate in Marwari mares.

Materials and methods

Experimental animals and diet. Non lactating cyclic and diestrus mares aged 4-11 years, 1-6 parity and weighing 350-410 kg were randomly allocated to either the control (CTR; n = 10) or the fish oil supplemented group (FOS; n = 10). Reproductive ultrasonography revealed that the ovary and uterus were normal. Mares in the CTR group were fed a standard diet that included 3 kg concentrate with 3% mineral mixture including salt, plus 9 kg green and dry fodder in a ratio of 3:1. Mares in the FOS group were supplemented with fish oil containing 15.28% eicosapentaenoic acid (EPA) and 10.41% docosahexaenoic acid (DHA) to supplement combined n-3 PUFA at the rate of 64 mg/kg BW/day in the standard diet for 70 days, or until 45 days post ovulation in pregnant mares. The energy density of the fish oil was 7 calories per gram. The mares were maintained under isomanagerial conditions and allowed drinking water *ad lib*. The experiment was approved by the Institute Animal Ethics Committee.

Estrus detection, monitoring ovarian follicle and CL growth. A stallion was paraded twice daily near to the mares' paddock to detect signs of behavioral estrus that was confirmed by trans-rectal ultrasonography, with commencement of experimental feeding. The day of estrus onset (D_0) was determined on the basis of the first observation of behavioral estrus along with the presence of an antral follicle (approximate 20-25 mm in diameter) on either ovary, and/or the presence of uterine edema. The number of small (6-10 mm), medium (10.1-25 mm) and large (>25 mm) sized follicles was counted on D_0 . The mean cross diameter of the developing follicle was measured on alternate days from D_0 to day 4 of estrus (D_4). Then the follicular diameter was measured at 12 h intervals until its disappearance. The day of ovulation (D_{ov}) was constructed when the pre-ovulatory follicle (POF) disappeared after the last observation. The mean cross diameters of the CL was measured on day 7 post-ovulation ($D7_{po}$) in all the experimental mares, and the diameter was measured on day 15 post-ovulation ($D15_{po}$) in the mares that became pregnant.

Artificial insemination. AI was performed near to ovulation following trans-rectal assessment of follicle consistency (soft) as well as ultrasonographic assessment of follicle size (≥ 36 mm) and shape (tear drop or guitar pick). Mares were inseminated with frozen thawed at 12 h intervals until ovulation or up to 6 h post ovulation. Thawed frozen semen of proven farm stallions, with approximately 300×10^6 progressively motile sperm per insemination was used. Insemination was done for three successive estrous cycles, if a mare failed to conceive on the preceding cycle.

Pregnancy diagnosis and monitoring the conceptus growth. Pregnancy was confirmed by the presence of an embryonic vesicle on $D15_{po}$ using trans-rectal ultrasonography. The diameter of the embryonic vesicle was measured at weekly intervals starting from $D15_{po}$ until day 42 post-ovulation. The length and width of the embryo proper were also recorded

on day 28 (D28_{po}), day 35 (D35_{po}) and day 42 (D42_{po}) post-ovulation. Embryonic loss was confirmed by the disappearance or collapse of the embryonic vesicle, and/or absence of heart beat. The day of loss was assigned to the interval in which embryo was present on the first day of increment and absent on the last day.

Assay of estradiol (E₂) and progesterone (P₄). Blood samples were collected from all the experimental mares until D7_{po}; however, the sample on D15_{po} was collected from the pregnant mares of both groups. The plasma was stored at -20 °C until assay. Estimation of E₂ and P₄ was done using radioimmunoassay (RIA) kits (Immunotech, France). Analytical sensitivity for P₄ was 0.05 ng/mL, and the coefficient of intra and inter-assay variation was 6.5 and 7.2%, respectively. Similarly, the sensitivity of E₂ was <6 pg/mL and the coefficient of intra and inter-assay variation was 12.1 and 11.2%, respectively.

Statistical analysis. Data generated were tested for normality by the Shapiro-Wilk test and transformed to square roots, natural logarithms or ranks when indicated. Assumption of homoscedasticity of the data was ascertained by Levene's test. Data were analyzed for the main effect of supplementation, S (CTR vs. FOS), day of sampling, D; and their interaction, SD using the General Linear Model Univariate of SPSS (Version 18) software package with the following model:

$$Y_{ijk} = \mu + S_i + D_j + (SD)_{ij} + \varepsilon_{ijk}$$

Where, Y_{ijk} = kth data value of jth level of factor D (days) and ith level of factor S (supplementation), μ = grand mean, D_i = ith level of factor D, D_j = jth level of factor D, (SD)_k = kth interaction, ε_{ijk} = residual error with respect to Y_{ijk}.

Pairwise comparisons of means within supplementation and between day differences were further examined by the least significant difference test. The relationship between different parameters was analyzed using Pearson's correlation coefficient. Data are presented as mean \pm SEM, unless otherwise stated.

Results

Follicular and luteal development. The mean number of small, medium and large size follicles on the day of estrus onset did not differ significantly (P>0.05) between the groups (Fig. 1). Supplementation of fish oil resulted in a significant increase in the diameter of the largest follicle (P<0.05) from day 4 of estrus until ovulation (Fig. 2). The mean diameter of CL (mm) on D7_{po} was larger in the FOS group as compared to CTR (31.01 \pm 0.8 vs 26.76 \pm 0.8; P<0.01). Similarly, the CL size was significantly greater (P<0.01) on D15_{po} in the FOS group than that of the controls (35.48 \pm 0.86 vs 23.73 \pm 1.0; P<0.01). However, there was no effect of day (P>0.05).

Embryonic development. The effect of day on the diameter of the embryonic vesicle was significant (P<0.001); however, the effect of fish oil supplementation and the

interaction between the treatment and day failed to achieve significance ($P>0.05$). In the FOS mares, the mean diameter of the embryonic vesicle increased significantly ($P<0.01$) on D15_{PO}, D22_{PO}, D28_{PO}, D35_{PO} and D42_{PO}. Similarly, in the CTR group, the diameter of embryonic vesicle increased significantly ($P<0.01$) on all the days except from D22_{PO} to D28_{PO} (Table 1). On D15_{PO}, the mares in the FOS group had significantly ($P<0.05$) larger embryonic vesicles than in the CTR.

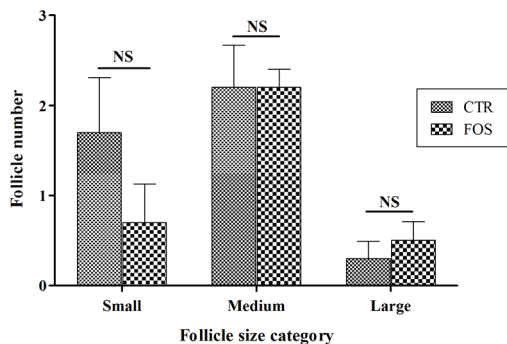


Fig. 1. Number of follicles at the onset of estrus in the control (CTR) and fish oil supplemented (FOS) Marwari mare. Each bar represents Mean \pm SEM (n = 10). NS = $P>0.05$

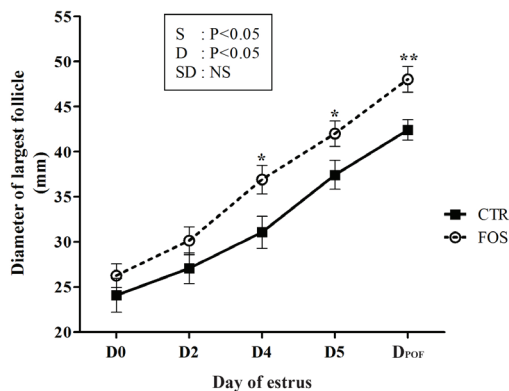


Fig. 2. Diameter of the largest follicle on different days of estrus in the control (CTR) and fish oil supplemented (FOS) Marwari mares. Each bar represents Mean \pm SEM (n = 10). S, D and SD represent treatment, day and their interaction effects. * $P<0.05$ and ** $P<0.01$

The mean length and width of the embryo proper were higher ($P<0.05$) on D28_{PO} in the FOS group than that in the CTR (Table 1). The length and width of the embryo proper

increased significantly ($P<0.01$) on $D28_{PO}$, $D35_{PO}$ and $D42_{PO}$ in mares of both groups. However, the interaction between supplementation and day was not significant ($P>0.05$).

Table 1. Embryonic vesicle diameter, length and width of embryo proper in the control (CTR) and fish oil supplemented (FOS) mares

Day of postovulation	Parameters					
	Diameter of Embryonic vesicle (mm)		Length of embryo proper (mm)		Width of embryo proper (mm)	
	Group		Group		Group	
	CTR	FOS	CTR	FOS	CTR	FOS
$D15_{PO}$	15.27 $\pm 1.25^{C,a}$	21.41 $\pm 0.71^{D,a}$				
$D22_{PO}$	26.99 $\pm 1.29^b$	27.20 $\pm 1.32^b$				
$D28_{PO}$	30.33 $\pm 0.54^b$	31.67 $\pm 0.8^c$	12.93 $\pm 1.08^{A,a}$	15.77 $\pm 0.88^{B,a}$	6.03 $\pm 0.34^{A,a}$	7.76 $\pm 0.29^{B,a}$
$D35_{PO}$	43.31 $\pm 1.41^c$	44.04 $\pm 0.8^d$	20.89 $\pm 1.56^b$	22.40 $\pm 1.16^b$	10.87 $\pm 1.04^b$	11.46 $\pm 0.33^b$
$D42_{PO}$	55.90 $\pm 1.8^d$	57.77 $\pm 1.4^c$	30.11 $\pm 1.68^c$	35.15 $\pm 2.10^c$	16.40 $\pm 0.92^c$	18.45 $\pm 1.16^c$

The values under each parameter bearing different superscripts within rows [A:B ($P<0.05$) and C:D ($P<0.01$)] and within column [a:b:c:d:e ($P<0.05$)] differ significantly in CTR and FOS group $P<0.05$.

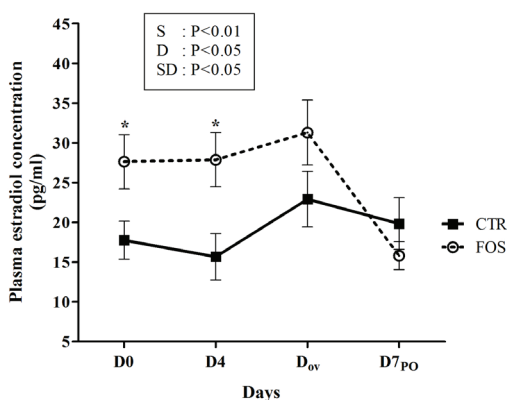


Fig. 3. Estradiol concentration (pg/mL) on different days of estrus (D_0 ; D_4 ; D_{ov}) and post-ovulation ($D7_{po}$) in the control (CTR) and fish oil supplemented (FOS) Marwari mares. Each bar represents Mean \pm SEM ($n = 10$). S, D and SD represent treatment, day and their interaction effects. $*P<0.05$ and $**P<0.01$

Plasma E_2 and P_4 concentration. The mean concentrations of plasma E_2 were significantly higher on D_0 ($P<0.05$) and D_4 ($P<0.01$) in the FOS group; however, they did not differ significantly between the groups on D_{ov} and $D7_{po}$ (Fig. 3). The mean plasma P_4 concentrations were significantly higher on $D15_{po}$ in the FOS group than the CTR ($P<0.01$). In contrast, they did not differ significantly ($P>0.05$) on D_0 , D_4 and $D7_{po}$ between the groups (Fig. 4).

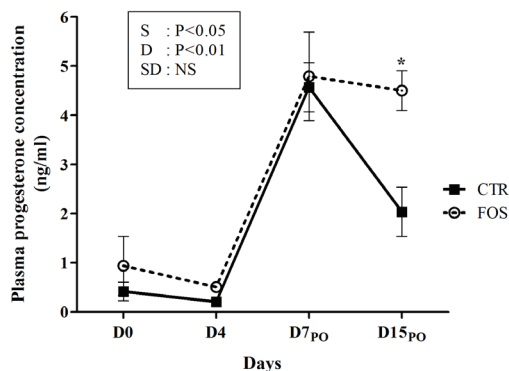


Fig. 4. Mean progesterone concentration (ng/mL) on different days of estrus (D_0 ; D_4) and post-ovulation ($D7_{po}$; $D15_{po}$) in the control (CTR) and fish oil supplemented (FOS) Marwari mares. Each bar represents Mean \pm SEM ($n = 10$). S, D and SD represent treatment, day and their interaction effects. * $P<0.05$ and ** $P<0.01$.

Reproductive event. The duration of estrus (day) in the mares of the FOS group (7.45 ± 0.2 ; range 6-9) was significantly shorter ($P<0.05$) than that of the CTR (8.26 ± 0.2 ; range 6-10). However, the length of the estrous cycle (day) did not vary significantly between FOS (25.87 ± 0.58) and CTR mares (24.93 ± 0.67). Mares in the FOS group achieved numerically more conceptions (10/10) as compared to the CTR group (7/10). The number of AI per conception was 1.2 in the FOS group, whereas it was 1.86 in the CTR group. The CTR mares needed approximately 1.5 times more AI per conception than the FOS mares in spite of the almost equal number of AI per cycle. In addition, no embryonic loss was recorded in the FOS group while one resorption was observed in a CTR mare on $D35_{po}$.

Discussion

The objective of the current study was to investigate the effect of dietary fish oil, a rich source of n-3 PUFA, on the development of ovarian follicles and CL, plasma estradiol and progesterone, embryonic development and other reproductive events in Marwari mares. The lack of significant difference regarding the number of small, medium

and large sized follicles is in contrast to the report regarding cows (ROBINSON et al., 2002; HERAVI-MOUSSAVI et al., 2008). The significant increase in the diameter of largest follicle is in accordance with earlier reports. The mean diameter of the ovulatory follicle was higher when dairy cows were fed diets high in n-3 (HERAVI-MOUSSAVI et al., 2008). It is likely that feeding PUFA increased the size of ovulatory follicle due to stimulation of LH by improved energy status (MATTOS et al., 2000), although isocaloric studies have shown this to be independent of energy content of the diet (ROBINSON et al., 2002). STAPLES and THATCHER (2005) summarized the studies on the effect of supplemental fat on the diameter of the dominant follicle in dairy cows and concluded that there was a 23% increase (3.2 mm larger) in the size of the dominant follicle when dairy cows were supplemented with lipid containing the protected PUFAs in the ration. Dietary lipid are known to enhance secretion of insulin in cattle (THOMAS and WILLIAMS, 1996), which favourably affects the follicular dynamics through its cognate receptors (PORETSKY and KALIN, 1987) or indirectly through increasing the sensitivity of the follicle to follicle stimulating hormone (SIMPSON et al., 1994). A significant increase in the plasma E_2 concentration during the period of estrus (Fig. 2) is concurrent with the reports in the cow (ROBINSON et al., 2002). A significant increase in the growth rate of the largest follicle (Fig. 1) supports the finding of increased plasma E_2 in the Marwari mare.

The size of POF is essential in determining the overall size of the CL. The ovulation of larger follicles may result in the formation of larger corpora lutea with increased production of P_4 to improve the conception (FUNSTON, 2004). A positive correlation between the size of CL and plasma P_4 concentration on D15_{po} in the pregnant mares of FOS group supports the above notion. This could be due to the inhibition of $PGF_{2\alpha}$ by the fish oil during the window of maternal recognition of pregnancy. It is reported that dietary n-3 PUFA upregulates the endometrial expression of peroxisome proliferator activated receptors to decrease the PGF production (MacLAREN et al., 2006); decreases plasma P_4 clearance (GALBREATH et al., 2008) and stimulates the release of P_4 (STEWART et al., 1995) through increased sensitivity to insulin (THOMAS and WILLIAMS, 1996). Increased diameter of CL in FOS mares was consistent with the reports in the dairy cow (PETIT et al., 2002). As the FOS and CTR groups are not isocaloric, it is difficult to partition the specific effects of n-3 PUFA and energy mediated effects of fish oil.

Fish oil supplementation significantly increased the diameter of embryonic vesicle as well as the embryo proper (Table 1). The establishment of pregnancy in mare requires the embryo to develop in an appropriate way and show adequate intrauterine mobility pattern to inhibit the production of $PGF_{2\alpha}$, thereby allowing the persistence of CL (BOERBOOM et al., 2004). Our result is supported by earlier report in which the number of blastomeres were increased in the superovulated cows that were fed a diet rich in n-3 α -linolenic acid (ALA) as compared to those that consumed n-6 linolenic acid (LA) or saturated

stearic acid (THANGAVELU et al., 2007). While embryo recovery rate was not altered when cows were fed a ruminally protected long chain n-3 from fish oil, the number of degenerated embryos was reduced following dietary inclusion of n-3 PUFA (CHILDS et al., 2008). McEVOY et al. (2012) suggested that at less than 3% inclusion in the diet of ewes, there may be beneficial effects of fish oil fatty acids on embryo production *in vivo* and of the corresponding ewe serum during *in vitro* culture.

A decreased number of AI per conception and improved pregnancy rate in the FOS group is consistent with the reports in the cattle (HERAVI-MOUSSAVI et al., 2008) and buffalo (NAZIR et al., 2013) that were fed n-3 PUFA rich diet. Results from a number of studies indicate that n-3 PUFA may be associated with reduced embryo mortality (THANGAVELU et al., 2007; ELIS et al., 2016).

In conclusion, the results indicate that the supplementation of the diet with EPA and DHA rich fish oil improved the ovarian function by increasing the diameter of preovulatory follicle, CL, plasma progesterone as well as enhanced the embryonic development in the Marwari mare.

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SAŽETAK

Na temelju prethodnih izvješća o poboljšanju reprodukcije krava, kod kobila pasmine marvari istražili smo učinak ribljeg ulja kao prehrambenog dodatka na razvoj folikula jajnika, žutog tijela, koncepcije i određenih reproduksijskih pokazatelja. U skladu s ciljem, 20 kobila koje nisu bile u laktaciji je metodom slučajnog izbora podijeljeno u dvije skupine s po 10 kobila. Za razliku od kontrolne skupine, kobile u eksperimentalnoj skupini hranjene su obrokom obogaćenim ribljim uljem u obliku dodatka koji je sadržavao n-3 polinezasićene masne kiseline (PUFA), u dnevnoj količini od 64 mg na jedan kg tjelesne mase. Eksperiment je trajao 70 dana, odnosno za kobile koje su ostale gravidne do 45 dana nakon ovulacije. Estrus je praćen teaserom, a osjemenjivanje je provedeno zamrznutim sjemenom. Razvoj folikula jajnika i žutih tijela te gravidnosti praćeni su transrektalnim ultrazvukom. Koncentracija progesterona i estradiola u plazmi procijenjena je radioimunološkom metodom. U eksperimentalnoj skupini utvrđen je veći ($P<0,05$) promjer najvećeg folikula od 4. dana estrusa do ovulacije i veći promjer žutog tijela 7. dan nakon ovulacije. No, 15. dan nakon ovulacije promjer žutih tijela bio je signifikantno povećan kod gravidnih kobila. Obrok obogaćen ribljim uljem signifikantno je poboljšao razvoj embrija, što se očitalo povećanjem promjera embrionalne vezikule 15. dan nakon ovulacije i povećanjem embrija 28. dan nakon ovulacije. Nadalje, u eksperimentalnoj skupini kobila srednja koncentracija estradiola u plazmi bila je veća na dan početka estrusa ($P<0,05$) i 4. dan estrusa ($P<0,01$). Slično tome, obrok obogaćen ribljim uljem kod gravidnih je kobila signifikantno ($P<0,01$) povećao progesteron u plazmi 15. dan nakon ovulacije. U kobila eksperimentalne skupine je, uz varijacije estrusnog ciklusa, utvrđeno i za 19 sati kraće trajanje estrusa ($P<0,05$). Povećana stopa gravidnosti kod kobila dohranjivanih ribljim uljem nije bila signifikantna. Zaključeno je da je hranidba s dodatkom ribljeg ulja kod kobila pasmine marvari poboljšala funkciju jajnika i razvoj embrija.

Ključne riječi: embrij; riblje ulje; folikul; kobila; n-3 PUFA
