



## Influence of various dietary fat sources on freezing capacity of Moghani ram semen

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### Abstract

The aim of this study was to investigate the effects of dietary supplementation of protected fish oil (FO) and Persia fat<sup>®</sup> (PF) on the quality of Moghani ram semen. For this purpose, a total of 96 ejaculates were collected from 12 healthy mature Moghani rams, which were divided into three distinct groups (n = 4) and were assigned to one of three experimental diets. The first group (control) received a diet supplemented with palm oil (PO), while the second and third groups received encapsulated FO and PF, respectively. After primary evaluation, semen samples of each group were pooled to eliminate individual differences, and then evaluated for semen concentration and volume. Afterwards, the samples were diluted with a Tris-based extender and frozen with a standard protocol. After thawing, motion kinetics, viability, membrane functionality and abnormality were assessed. The results showed that the group that received FO had significantly higher viability (quadratic), progressive motility (PM) (%), average path velocity (VAP) ( $\mu\text{m/s}$ ), curvilinear velocity (VCL) ( $\mu\text{m/s}$ ) (linear), amplitude of lateral head displacement (ALH) ( $\mu\text{m}$ ) (quadratic) and sperm concentration (linear) than the others. Additionally, total motility (TM) (%) and straight-line velocity (VSL) ( $\mu\text{m/s}$ ) were significantly higher in the groups that received FO and PF compared with the control (quadratic). The results indicated that sperm abnormalities in the control group were significantly higher than the other groups. In conclusion, enrichment of the diet with FO or Persia fat could enhance ram sperm quality after freeze-thawing process.

**Keywords:** fish oil, frozen spermatozoa, ram, motion parameters

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### Introduction

Lipids are an integral part of cell membrane bilayers, which play a pivotal role in cell functions. Phospholipids, sterols and triglycerides are the best-known lipids in animal cell walls. Also, cell wall fatty acids (FA) and unsaturated/saturated fatty acid ratio are more effective. Animals cannot synthesize n-6 and n-3 fatty acids de novo owing to a lack of appropriate fatty acid desaturase enzymes and the need to obtain these fatty acids from their diet (Wathes *et al.*, 2007). In addition, dietary FA are known to affect the FA composition of sperm membranes in humans (Conquer *et al.*, 2000), bulls (Moallem *et al.*, 2015), stallions (Brinsko *et al.*, 2005; Rodrigues *et al.*, 2017), pigs (Cook & McMaster, 2002) and roosters (Cerolini *et al.*, 2006). In many mammalian species, the plasma membrane has more than 60% polyunsaturated fatty acids (PUFAs), although sperm membrane lipids vary between species (Saether *et al.*, 2007).

Mammalian sperm plasma is characterized by a high proportion of PUFAs, which play an important role in the fluidity and flexibility of the plasma membrane. On the other hand, plasma membrane functionality is a crucial factor in preserving sperm viability and fertilizing capacity. This physiological exclusivity may improve sperm resistance against stresses such as cold shock. A high concentration of docosahexaenoic acid (DHA) has been positively associated with sperm motility in human semen and sperm (Ollero *et al.*, 2001).

Lipids are used as fuel for Sertoli cells, and in membrane remodelling of developing germ cells (Rato *et al.*, 2014). For this purpose, fatty acids should accumulate in testicular cells through two processes: i) passive diffusion through the lipid bilayer and ii) protein-facilitated transport that is mediated by CD36 glycoprotein, which is widely expressed in Sertoli cells.

Mammalian testicular cells and spermatozoa contain high amounts of C20:4, C20:5, C22:5 and C22:6 FA (n-3 and n-6 PUFAs), derived from dietary linoleic (C18:2) and linolenic acids (C18:3), elongation and desaturation process (Rato *et al.*, 2014). Thus, decreasing PUFAs in sperm plasma membranes leads to decreases in motility and viability owing to cryoinjury in the freeze-thawing process (Tran *et al.*, 2016). Persia fat<sup>®</sup> (PF) is an Iranian product with the same concentration of n-3 and n-6 FA as supplementary fat. Many studies have demonstrated that dietary n-3 and n-6 fatty acids enhance the preservation of spermatozoa after cold shock and ice crystal formation in humans (Vireque *et al.*, 2016), bulls (Gholami *et al.*, 2010; Khoshvaght *et al.*, 2016), goats (Dolatpanah *et al.*, 2008), stallions (Rodrigues *et al.*, 2017) and roosters (Cerolini *et al.*, 2006). On the other hand, Karimi *et al.* (2017) demonstrated that supplementation of the diet of bulls with conjugated linoleic acid (CLA) did not affect fresh and frozen semen quality. To the best of the authors' knowledge, there is a dearth of information about the effects of dietary supplementation of n-3 and n-6 fatty acids on sperm motion kinematics in rams. Therefore, this study was conducted to investigate the protective effects of three lipid sources on the semen quality of cold-stressed Moghani rams

## Material and Methods

Eosin Y (CAS no. 15086-94-9), nigrosin (CAS no. 101357-32-8), Tris (CAS no. 77-86-1), citric acid (CAS no. 77-92-9), fructose (CAS no. 57-48-7) and sodium citrate (CAS no. 6132-54-3) were obtained from Sigma & Merck (St. Louis, MO, USA).

Semen samples were collected from 12 healthy mature Moghani rams (3 - 5 years old, weight 60 - 70 kg). Rams were housed at the Moghani Sheep Husbandry and Breeding Centre in Ardabil, Jaafar Abad, Iran (39°38'49"N 48°02'23"E), and were maintained under uniform management conditions. Before the beginning of the experiment, rams were classified according to their subjective sperm motility (good versus moderate to poor quality). Afterwards, they were divided into three equal groups (n = 4), which were randomized according to their sperm motility. Each group included one poor semen quality ram (60% ≤ TM ≤ 70%), one moderate (70% ≤ TM ≤ 80%) and two good (TM ≤ 80%) semen quality rams. They received one of the experimental isoenergetic and isonitrogenic diets, which were enriched with fat sources (3% dry matter intake) (Table 1). The first group (control) received a diet supplemented with palm oil (PO), while the second and third groups received encapsulated fish oil (FO) (n-3 source) and PF (Kimia Danesh Alvand Co, Qom, Iran) (Table 2). The rams were fed the experimental diets from two weeks before sample collection until eight weeks throughout it. A total of 96 ejaculates were collected weekly from 12 rams (eight weeks) (each collection time was regarded as an experimental repeat) during the non-breeding season using an artificial vagina (IMV Technologies, L'Aigle, Basse-Normandie, France). Rams were stimulated for ejaculation by a ewe that received a dose of oestradiol (0.5 mL) one day prior to collection to show oestrus behaviour. Immediately after collection, the ejaculates were immersed in a warm water bath at 37 °C until assessment in the laboratory (utmost 30 min). The ejaculates with a volume 0.75 - 2 mL, sperm concentration ≥ 2.5 × 10<sup>9</sup> sperm/mL, TM ≥ 60%, and sperm abnormalities < 10% were used for cryopreservation. To eliminate individual differences in each group, semen samples (n = 4) were pooled, evaluated for semen concentration and volume, and then processed for freezing process (Seifi-Jamadi *et al.*, 2017).

The Tris-based extender used in this study comprised 253.42 mM Tris, 85.36 mM citric acid, 70 mM fructose, and 500 IU/mL penicillin (Shafa Farmed Co, Iran) and 20% (v/v) egg yolk. The osmolarity and pH of this base extender were set at 325 - 350 mOsm/Kg and 6.9 - 7.1, respectively. For each group, the pooled ejaculates were diluted with the basic extenders. After dilution, the final sperm concentration was 200 × 10<sup>6</sup> spermatozoa/mL. The diluted semen samples were loaded into 0.25 mL straws (IMV Technologies, France) sealed with polyvinyl alcohol, and equilibrated at 5 °C for 150 min. After equilibration, the straws were frozen in liquid nitrogen (LN<sub>2</sub>) vapours resting 4 cm above the liquid nitrogen for 12 min (Najafi *et al.*, 2013). Afterwards, the straws were plunged into liquid nitrogen (LN<sub>2</sub>) for storage. For post-thaw evaluation, the frozen straws were thawed individually at 37 °C for 30 seconds in a water bath. Sperm evaluation was performed immediately after thawing (Uysal & Bucak, 2007).

Sperm motility parameters were analysed using a Casa system (VideoTest<sup>®</sup>, Sperm 3.1, Russia) equipped with a phase contrast microscope (Labomed<sup>®</sup> Lx400, USA). These variables were recorded for TM (%), PM (%), VSL (µm/s), VCL (µm/s), VAP (µm/s), LIN (%), amplitude of lateral head displacement (C) (µm), straightness (STR) (%), and beat/cross frequency (BCF) (Hz).

Viability was assessed through eosin-nigrosin staining (Björndahl *et al.*, 2003) The sperm suspension smears were prepared by mixing 5 µL semen sample with 5 µL eosin-nigrosin stain on a warm slide and then smeared. The viability was assessed by counting 200 cells under phase-contrast microscopy at 400x. Sperm

with white (unstained), light pink heads or a red appearance restricted to the neck region (leaky neck) were considered alive, while those with stained or partial stained heads were regarded as dead (WHO, 2010).

**Table 1** Ingredients and composition of the experimental diets that were used as treatments

Feed Ingredient	Experimental diets (%)		
	Fish oil	Persia fat	Palm oil
Alfalfa hay	27.47	27.47	27.47
Corn silage	24.50	24.50	24.50
Straw	10.50	10.50	10.50
Barley	20.17	20.13	20.13
Wheat bran	12.50	12.54	12.54
CaCO <sub>3</sub>	1.00	1.00	1.00
Premix (vitamin) <sup>a</sup>	0.50	0.50	0.50
Salt	0.36	0.36	0.36
Fish oil	3.00	-	-
Persia fat	-	3.00	-
Palm oil	-	-	3.00

<sup>a</sup> Each kg of the vitamin–mineral premix contained: vitamin A: 50 000 IU; vitamin D<sub>3</sub>: 10 000 IU; vitamin E: 0.1 g; calcium: 196 g; phosphorus: 96 g; sodium: 71 g; magnesium: 19 g; iron 3 g; copper: 0.3 g; manganese: 2 g; zinc: 3 g; cobalt: 0.1 g; iodine: 0.1 g; selenium: 0.001 g

**Table 2** Fatty acid composition of the rams experimental diets

Fatty acids (g/100 g FA)	Experimental diet		
	Fish oil	Persia fat	Palm oil
14:0	3.67	2.86	2.23
16:0	26.17	22.23	45.04
16:1	6.74	3.03	1.01
18:0	11.01	10.43	12.35
18:1	18.04	21.12	15.06
18:2 (n-6)	9.34	19.03	11.67
18:3 (n-3)	6.87	8.52	0.81
20:0	0.51	0.48	0.52
20:4 (n-6)	0.42	0.39	0.26
20:5 (n-3)	1.54	0.32	0.15
22:5 (n-6)	0.31	0.71	0.42
22:6 (n-3)	4.36	1.43	--
Unknown FA	11.02	9.45	10.48
n-3/n-6 ratio	1.27	0.51	0.08

The hypo-osmotic swelling test (HOST) was used to assess the functional functionality (MF) of the sperm membrane. The HOST was performed by incubating 30 µL semen with 300 µL of a 100 mOsm hypo-osmotic solution (0.9 g fructose and 0.49 g sodium citrate dissolved in 100 mL distilled water) at 37 °C for 30 min (Rahmatzadeh *et al.*, 2017). After incubation, 10 µL of the mixture was placed on a glass slide and covered with a cover slip to evaluate under a phase-contrast microscope (×400). A total of 200 spermatozoa

were counted in at least five microscopic fields. Then the percentage of spermatozoa with swollen and curled tails was recorded. Swelling is characterized by a coiled tail, indicating that the plasma membrane is intact (Seifi-Jamadi *et al.*, 2016a).

The sperm abnormalities were observed by adding three drops of each semen sample to a tube containing 1 mL Hancock solution 62.5 mL formalin 37%, 150 mL sodium saline, 1.8 g/100 mL double distilled water, 150 mL buffer solution (including Na<sub>2</sub>HPO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub> in distilled water) (Schäfer & Holzmann, 2000). One drop of this mixture was placed on a microscope slide and covered with a cover slip. The percentages of total sperm abnormalities (acrosome and caput abnormalities, detached heads, abnormal mid-pieces and tail defects) were determined by counting a total of 200 spermatozoa under phase contrast microscopy at 1000x (Seifi-Jamadi *et al.*, 2016b).

The data were analysed using the GLM procedure of SAS software (SAS, 2002). The dataset was checked for normal distribution using the univariate procedure and Shapiro-Wilk test and normalized through arcsine square root transformation. The results were expressed as *Ismeans* ± SE. The Duncan test was applied to determine the significant differences. Linear and quadratic effects of dietary fat sources (treatment as fixed effect) were tested, using orthogonal polynomials and the level of significance was set at  $P \leq 0.05$ .

## Results

The effects of FO and PF on the post-thaw sperm kinetics of the semen of Moghani rams are presented in Table 3. The results showed that PM, VAP, VCL (linear effect,  $P = 0.01$ ), and ALH (quadratic effect,  $P = 0.002$ ) in the group that received FO were higher than the control. Also, TM and VSL were higher in the groups that received FO and PF compared with the control (quadratic effect,  $P = 0.008$ , and  $P = 0.001$ , respectively). No difference was noted in STR and LIN among the groups ( $P \geq 0.05$ ).

**Table 3** Post-thaw characteristics of semen of Moghani rams after freeze-thawing (*Ismeans* ± SE)

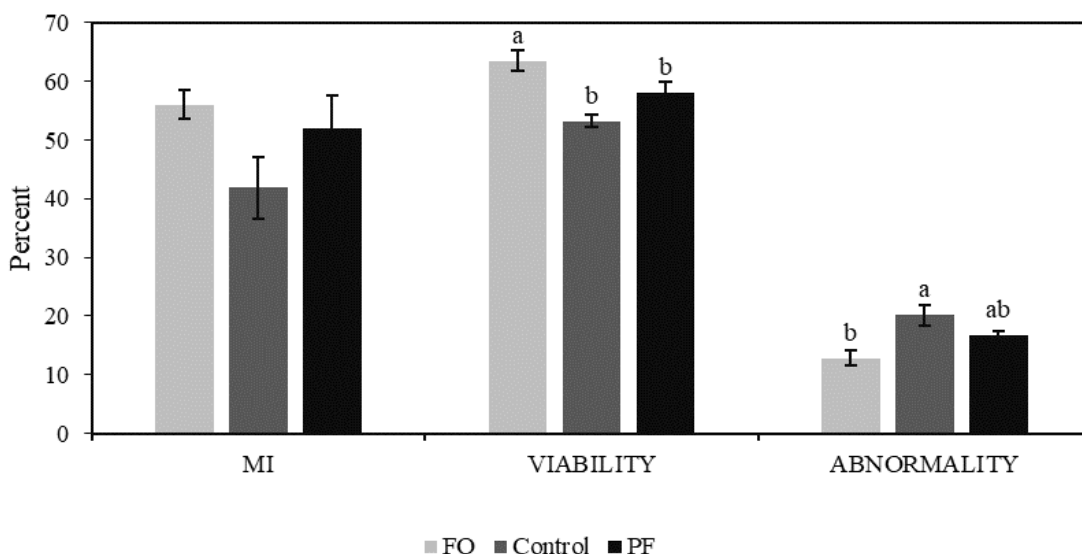
Parameters	Treatments			P-value	
	FO	Control	PF	Linear	Quadratic
TM (%)	58.75 <sup>a</sup> ± 1.69	43.10 <sup>b</sup> ± 2.43	51.93 <sup>a</sup> ± 1.72	NS	0.008
PM (%)	38.25 <sup>a</sup> ± 1.61	24.25 <sup>c</sup> ± 1.34	31.28 <sup>b</sup> ± 0.87	0.01	0.001
VAP(μm/s)	31.63 <sup>a</sup> ± 0.54	21.05 <sup>c</sup> ± 0.87	29.55 <sup>b</sup> ± 0.39	0.01	0.001
VSL(μm/s)	23.33 <sup>a</sup> ± 0.82	16.13 <sup>b</sup> ± 1.28	21.90 <sup>a</sup> ± 0.49	NS	0.001
VCL(μm/s)	67.53 <sup>a</sup> ± 1.58	47.73 <sup>c</sup> ± 1.49	55.08 <sup>b</sup> ± 0.61	0.01	0.001
STR (%)	70.58 ± 3.08	76.30 ± 3.75	74.20 ± 0.61	NS	NS
LIN (%)	30.05 ± 1.34	32.63 ± 2.40	29.75 ± 1.71	NS	NS
ALH (μm)	1.57 <sup>a</sup> ± 0.01	1.23 <sup>b</sup> ± 0.04	1.48 <sup>a</sup> ± 0.04	NS	0.002

ALH: amplitude of lateral head displacement; BCF: beat cross frequency; FO: fish oil; LIN: linearity; PF: Persia fat; PM: progressive motility; STR: straightness; TM: total motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight line velocity

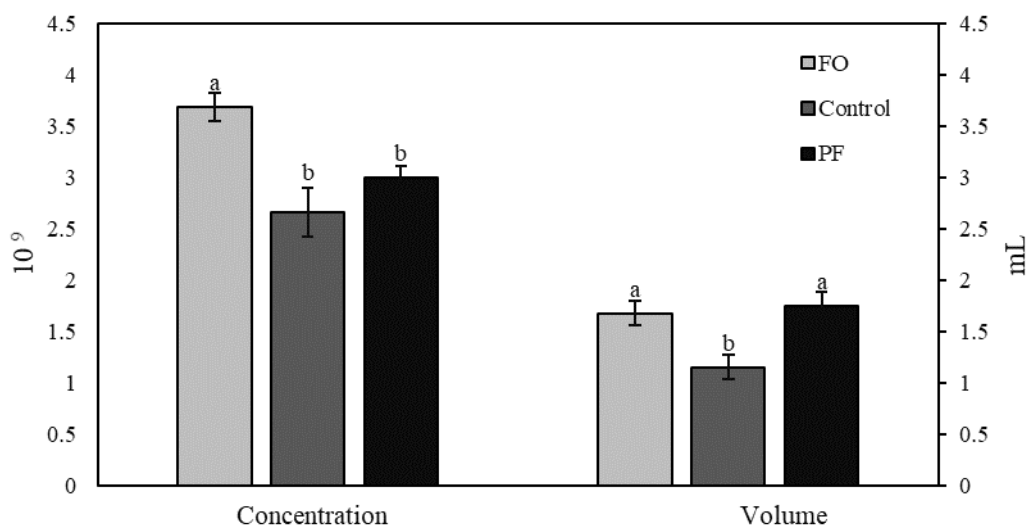
Different superscripts within the same line shows significant differences ( $P < 0.05$ )

According to the results shown in Figure 1, viability was higher in the FO group compared with the other groups (quadratic effect,  $P = 0.01$ ). Although the MF of the FO group was increased, there was no difference in MI among the groups ( $P \geq 0.05$ ). Also, the sperm abnormality after the freeze-thawing process was higher in the control group compared with other groups, but there was no difference in the number of sperm abnormalities between the control and the PF group ( $P \geq 0.05$ ). However, the difference between control and FO group was statistically significant (quadratic effect,  $P = 0.03$ ).

The results of the current study showed that the group that was treated with FO had a higher sperm concentration in comparison with other groups (linear effect,  $P = 0.04$ ). However, there was no difference in the semen volume of rams when the FO and PF groups were compared ( $P \geq 0.05$ ). But the difference between the FO and PF groups and the control was statistically significant (quadratic effect,  $P = 0.01$ ) (Figure 2).



**Figure 1** Effects of fish oil (FO) and Persia fat (PF) on viability, membrane functionality and abnormality of semen of Moghani rams after freeze-thawing process (Ismeans ± SE). Different superscripts in each parameter show significant differences ( $P < 0.05$ )



**Figure 2** Effects of fish oil (FO) and Persia fat (PF) on volume and sperm concentration of semen of Moghani rams (Ismeans ± SE). Different superscripts shows significant differences ( $P < 0.05$ )

**Discussion**

The aim of the current study was to investigate the effects of supplementing the diets of Moghani rams with three differently composed lipids on the quality of ram frozen-thawed semen. According to the literature, vertebrates cannot synthesize the n-3 and n-6 series of PUFA and these elements must be obtained through a balanced diet (Cook & McMaster, 2002). On the other hand, lipid composition of the diet could alter fatty acid composition of the semen and plasma membrane of spermatozoa (Blesbois *et al.*, 1997). PUFAs enhance frozen (Gürler *et al.*, 2015) or fresh (Karimi *et al.*, 2017) sperm quality by improving the fluidity of the plasma membrane lipids of the sperm head, body and tail. Increasing fluidity is necessary for sperm motility, plasma membrane integrity and functionality.

The damage from the freezing and thawing process could decrease sperm plasma membrane functionality (Celeghini *et al.*, 2008; Seifi-Jamadi *et al.*, 2017). Thus, enrichment of the mammalian diet with

unsaturated fatty acids would cause the sperm plasma membrane to better resist damage arising from the formation of ice crystals during the freezing process (Khoshvaght *et al.*, 2016). Most mammalian species, such as bulls, rams and primates, have sperm with a high content of DHA. It follows that the loss of sperm plasma membrane PUFAs leads to a decrease in survival and fertilizing ability (Speake *et al.*, 2003).

The results of this study showed that the groups that received FO (as a n-3 fatty acid source) and PF (source of both n-3 and n-6 fatty acids) improved total and progressive motility, viability, VSL, VCL and VAP of ram semen compared with the control group. Additionally, the n-3 group showed a significant increase in PM, VAP and VCL compared with the PF group. These results are similar to those of studies in boars (Rooke *et al.*, 2001) and turkeys (Zaniboni & Cerolini, 2009), but differ from those in stallions (Brinsko *et al.*, 2005). The n-3 fatty acid family consists of three members, namely  $\alpha$ -linoleic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The n-6 family consists of linoleic acid (LA) and arachidonic acid (AA) (Safarinejad *et al.*, 2010). n-3 FAs are important in sperm membrane and acrosome integrity compared with the n-6 family. Moallem *et al.* (2015) demonstrated that sperm tail and head DHA content are the most important factors in enhancing the motility because of increasing the fluidity. Additionally, most DHA content is located in the sperm tail, which plays an important role in flagella function and sperm motion (Connor *et al.*, 1998). In this regard, Ansari *et al.* (2012) suggested that the positive effect of n-3 on sperm quality is related to the conversion of n-3 fatty acids to DHA. According to Cerolini *et al.* (2006), the DHA content of the sperm decreased significantly after the freeze–thawing process, but Blesbois *et al.* (1997) suggested that this decrease is because of DHA's reduction of reactive oxygen species (ROS). N-3 FAs are considered potentially important antioxidants (Hodge *et al.*, 2006). Therefore, DHA could reduce toxic ROS that arise because of the sperm freeze-thawing process (Blesbois *et al.*, 1997).

Aksoy *et al.* (2006) reported that asthenozoospermic, oligozoospermic and oligoasthenozoospermic human beings had lower DHA levels than normozoospermic (Aksoy *et al.*, 2006). The current results are in agreement with studies in ruminants (Masoudi *et al.*, 2016; Tran *et al.*, 2016) and humans (Vireque *et al.*, 2016; Ferramosca *et al.*, 2017). However, other researchers reported different results (Mourvaki *et al.*, 2010; Karimi *et al.*, 2017). Khoshvaght *et al.* (2016) concluded that feeding FO could improve the quality of fresh and frozen thawed semen by altering the sperm membrane fatty acid composition. Based on the current results, feeding the rams with the addition of FO and PF could increase the seminal volume compared with that of the control, but it did not have significant effects on sperm concentration. Karimi *et al.* (2017) showed that CLA could not increase bull semen volume and spermatozoa concentration. A probable reason or mechanism for seminal volume increasing with PUFAs is the synthesis and secretion of seminal plasma (Rooke *et al.*, 2001; Khoshvaght *et al.*, 2016). However, in some researches, this effect was not observed (Cerolini *et al.*, 2006; Dolatpanah *et al.*, 2008).

## Conclusion

Based on the results the authors concluded that diet composition of FA could alter the quality of ram semen after the freeze-thawing process. Also, the results emphasized that enrichment of the diet of the rams with FO and PF could enhance sperm quality after the freeze-thawing process.

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## Authors' Contributions

NH-E design the study, analysed the data and wrote the manuscript. FM performed the experiment and wrote the manuscript. VV performed experiment and wrote the manuscript. BN and AS-J wrote the manuscript.

## Conflict of Interest Declaration

The authors declare no competing financial interests.

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