American Journal of Animal and Veterinary Sciences

Original Research Paper

# Corpus Luteum Morphology and Function After Timed Artificial Insemination (TAI) in Nelore Heifers Supplemented with Sunflower Seed in their Diets

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*Article history*
Received: 01-03-2021
Revised: 27-05-2021
Accepted: 24-06-2021

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**Abstract:** The dietary Polyunsaturated Fatty Acids (PUFA) supplementation, before the Timed Artificial Insemination (TAI) protocol, may contribute to a reduction in embryonic mortality. In this context, we hypothesized that dietary supplementation with sunflower seed, before the TAI protocol, will promote an increase in the ovulatory Follicle Diameter (FD), with a consequent increase in the Corpus Luteum Diameter (CLD) and the plasma concentration of Progesterone (P4) in the subsequent diestrum. We evaluated the effect of sunflower seed supplementation, prior to TAI, on the ovulatory FD, CLD, lipid profile and plasma P4 concentration of both pregnant and non-pregnant Nelore heifers. Thirty heifers of 24-36 months old, kept on pasture with water and mineral salt *ad libitum*, were divided into two groups. The heifers received a 1.7 kg/day supplement containing 53% soybean meal and 47% corn (control group, $n$ = 15) or 40% soybean meal and 60% sunflower seed (sunflower seed group, $n$ = 15) for 56 days. All heifers received the TAI protocol 24 h after the final supplementation. At the time of TAI (D0), the ovaries of all animals were evaluated by ultrasound examinations to determine the FD. Every 48 h for 26 days, the CLD was measured using ultrasound. Blood samples were collected for P4 determinations (except on D8, D20 and D24) and total cholesterol, triglycerides, High-Density Lipoprotein cholesterol (HDL) and Low-density Lipoprotein Cholesterol (LDL), analyses were conducted on D0 and D21. There was no effect of sunflower seed supplementation on mean FD on TAI day, CLD and P4 concentration or lipid profile after TAI in pregnant or non-pregnant heifers. In conclusion, sunflower seed supplementation for 56 days preceding TAI did not change the FD, CLD, P4 concentration, or lipid profile in Nelore heifers.

**Keywords:** Sunflower Seed, Dominant Follicle, Progesterone, Cholesterol, Nelore Heifers

## Introduction

The influence of nutrition on reproductive performance is a topic that has generated interest in researchers, especially supplementation with Polyunsaturated Fatty Acids (PUFA). Animals do not synthesize some PUFA, such as linolenic and linoleic acid, due to the absence of specific enzymes for such synthesis (Santos *et al*., 2008). Among the PUFA-rich oilseeds, especially omega 6, sunflower seed (*Helianthus annuus L.*) is economically viable in ruminant nutrition. Such seed consists of 42% total lipids; of these, 68% are represented by linoleic acid (Staples *et al*., 1998).

The mechanisms involved in such reproductive enhancement appear to occur in the development of a larger ovulatory follicle (Mattos *et al*., 2002) and greater embryo development and quality (Fouladi-Nashta *et al*., 2007; Cerri *et al*., 2009), as well as decreased luteolytic signals during the maternal recognition of pregnancy (Mattos *et al*., 2000), which may contribute to a reduction in embryonic mortality.

In most studies, the addition of PUFA to the diet promoted an increase in plasma cholesterol and the cholesterol concentration in follicular fluid and luteal tissue events. These events determine an increase in the steroidogenesis synthesis capacity of the follicle and the corpus luteum (Ryan *et al*., 1992; Hawkins *et al*., 1995; Lammoglia *et al*., 1996; Staples *et al*., 1998; Cordeiro *et al*., 2015).

Cordeiro *et al*. (2015) supplementing the diet of *Bos indicus* females with sunflower seeds immediately following a progesterone/estradiol-based Timed Artificial Insemination (TAI) protocol. They found a significant increase in the pregnancy rate of TAI cows (66.7% versus 47.8%in the control) and the Fixed-Time Embryo Transfer (FTET) in heifers with in vitro-produced embryos (55, 7% versus 36.9% in the control).

In commercial TAI programs in Brazil, a 50% pregnancy rate has been achieved. In nulliparous beef heifers, this rate tends to be smaller at approximately 39.6% (Sá Filho *et al*., 2009). In addition, the diameter of the dominant follicle during insemination influences the diameter of the corpus luteum, production of P4 and, consequently, the percentage of pregnant females after TAI (Sá Filho *et al*., 2010).

In this context, we hypothesized that dietary PUFA supplementation in the form of sunflower seed, before the TAI protocol, will promote an increase in the ovulatory Follicle Diameter (FD), with consequent increases in the Corpus Luteum Diameter (CLD) and the concentration of P4 in the subsequent diestrus. In addition, we measured the lipid profile to test whether the effect on P4 could be due to the increase in its precursor. Thus, we evaluate the luteal development, lipid profile and P4 concentration after TAI in both pregnant and non-pregnant Nelore heifers supplemented with sunflower seed for 56 days.

## Materials and Methods

### *Animals*

The experiment was conducted between November to January 2017/2018 in the University of Western São Paulo (UNOESTE) Experimental Farm, Brazil. The experimental procedures were approved by the Animal Use Ethics Commission of the UNOESTE (protocol number 2342).

Thirty Nelore heifers aged between 24 and 36 Months were used. A gynecological examination was performed using ultrasound and only females with healthy reproductive tracts were used. The females' mean weight ± Standard Error of the Mean (SEM) on the first day of supplementation was 403.8±20.9 kg for the untreated control group (CG, n = 15) and 398.1± 26.7 kg for the group supplemented with a blend of soybean meal and Sunflower Seed (SSG, n = 15). All heifers were evaluated and assigned a Body Condition Score (BCS) on a scale of 1 to 5 (Lowman *et al*., 1976). A single evaluator determined the BCS of heifers, showing a mean BCS ± SEM of 3.0±0.1 in heifers of both groups.

### *Nutritional Management and Treatments*

The heifers had free access to water, mineral salts and pasture (*Cynodonnlemfüensis*). At the start of grazing period, the forage dry matter was 36.1 kg/ha in the CG group and 35.5 kg/ha in the SSG group. The chemical composition of pasture and the supplement consumed by heifers in the CG and SSG groups are shown in Table 1.

Eight paddocks, each of 1.5 $m^2$ area and the heifers were maintained in each paddock for 3 days. The height of entry into the pasture was 32 cm and that of exit was 19 cm. After 3 days in a paddock, the heifers were transferred to another paddock. On D29 and D56, the heifers from both groups received 0.333 kg/day commercial concentrate (Ração Bovinos 18%; Premix Nutrição de Resultados, Presidente Prudente, SP, Brazil).

The heifers in the CG group ($n$ = 15) received 1.7 kg/day of supplement containing 53% soybean meal (44% crude protein, CP) and 47% corn and the heifers in the SSG group ($n$ = 15) received 1.7 kg/day of supplement containing 40% soybean meal (44% CP) and 60% sunflower seed supplement (Cordeiro *et al*., 2015). The supplements provided to both groups were balanced in energy and proteins. Both supplements had 72% total digestible nutrients and 24% CP, with (SSG) or without (CG) sunflower seeds.

**Table1:** Chemical composition and forage and supplement, given to the control (CG) and sunflower given Nelore heifers group (SSG)

| Food sample | DM (%) | MM (%) | CP (%) | ADF (%) | NDF (%) | EE (%) | TDN (%) |
|---|---|---|---|---|---|---|---|
| Forage CG group | 91.6 | 7.9 | 14.3 | 77.5 | 74.0 | 2.8 | 53.5 |
| Forage SSG group | 91.2 | 7.4 | 15.6 | 75.3 | 72.8 | 2.5 | 54.1 |
| CG group supplement | 87.5 | 5.0 | 30.9 | 21.4 | 7.0 | 5.7 | 83.6 |
| SSG group supplement | 90.5 | 5.5 | 31.4 | 46.7 | 38.9 | 24.4 | 87.1 |

DM, Dry Matter; MM, Mineral Matter; CP, Crude Protein; ADF, Acid Detergent Fiber; NDF, Neutral Detergent Fibre; EE, Ether Extract; TDN, Total Digestible Nutrients

The supplements were provided twice daily, early morning and late afternoon, for 56 consecutive days before the TAI. The supplementation period for sunflower seeds in this study was determined to establish the approximate period of development of a primary follicle until reaching the antral phases (Webb *et al*., 2004).

### *TAI Protocol*

All heifers received a progestogen ear implant (Crestar, Norgestomet, MSD, Brazil) 24 h after the last supplement was provided. On the same day, they underwent ultrasound-guided follicular aspiration for ovum pick-up according to the technique described by Surjus *et al*. (2014). Our aim was to induce new follicular wave emergence. After 6 days, the auricular implant was removed and 500 µg of prostaglandin (Sincrocio, Sodium Cloprostenol, OuroFino, Brazil), 1 mg of estradiol cypionate (ECP, Zoetis, Brazil) and 300 IU of chorionic gonadotropin (Novormon, Zoetis, Brazil) were administered intramuscularly. Approximately 50 h after implant removal, TAI was performed (D0 = TAI day). Using doses of semen from a single Nelore bull and the inseminations were performed by a single inseminator.

### *Ultrasound Evaluations*

Approximately 50 h after implant removal, i.e., at the time of TAI (D0), the ovaries were scanned by ultrasound to determine the FD. Of the 30 heifers used, 26 females (86.7%) ovulated synchronously, of which 15 were a part of the CG group and 11 were a part of the SSG group. Mean weight ± SEM of females on the day of TAI was 422.2±20.5 kg in the CG group and 415.7±24.0 kg in the SSG group. The heifers were evaluated by ultrasound every 48 h from the day of TAI (D0) until 26 days. Both ovaries were scanned with a real time B-mode ultrasound scanner, 7.5 MHz transrectal transducer and the location of the CL was recorded for retrospective analyses of each animal. In the evaluations, the FD and the CLD were estimated using the average measurements (height and width). The CLD was evaluated on D2 and every other day until D26. The total CL volume was calculated using the formula $V = 4/3 \times \pi \times R^3$ using the radius (R) obtained with the formula $R = (L/2 + W/2)/2$ (Carvalho *et al*., 2015). Pregnancy diagnosis by ultrasound was achieved at day 30 post TAI. All ultrasonic examinations were performed by a single technician using a Honda machine (model HS-2000 VET) equipped with a 7.5 MHz transrectal transducer.

### *Blood Collection, Radioimmunoassay and Lipid Measurements*

Immediately after each CL ultrasound measurement was performed, blood samples were collected except on days D8, D20 and D24. Blood was collected either by coccygeal or jugular venipuncture using a 21 G needle and placed in a 10 mL-tube containing heparin. The blood samples were cooled for a maximum of 2 h and centrifuged at 2500 rpm for 15 min. Plasma samples were removed and stored in a freezer at -20°C for later progesterone determinations using Radioimmunoassay (RIA). For the measurement of progesterone concentration, a RIA kit (MP Biomedicals, USA) was used with a sensitivity of 0.01 ng/mL. The intra-and inter-assay coefficients of variability of the high and low controls were 16.17 and 0.29 and 11.31 and 14.68%, respectively.

In addition, blood samples were collected for lipid fractions determinations viacoccygeal venipuncture on D0 (at the time of TAI) and D22 into a 10mL tube containing EDTA (Ethylene diamine tetra acetic acid). Plasma was removed after centrifugation at 2,900 rpm for 15 min and stored at -20°C. Plasma lipid fractions were measured with an enzymatic method in an automated analyzer (RX Daytona, Randox Laboratories, UK).

### *Statistical Analyses*

The normality of the data was tested using the Cramer von Mises test and Levene's test was used to test for homogeneity of variance. Statistical analyses were performed in a completely randomized design in the two groups (GC and SSG) and the reproductive parameters (ovulatory FD, CLD and plasma P4 concentration) were evaluated in the different reproductive conditions (pregnant and non-pregnant) in each experimental group. The lipid profile was evaluated in each experimental group (GC and SSD). Student t test was performed for parametric data and Mann Whitney for nonparametric data, using a significance level of 5% ($P<0.05$). Analyses were performed using the software SAS (SAS University Edition; SAS Institute Inc., Cary, NC).

## Results

The pregnancy rate was 40% (6/15) in the CG and 45.6% (5/11) in the SSG. The ovulatory follicle diameters on the day of the TAI showed no significant effect of the treatment on the FD in non-pregnant heifers (9.5 ± 0.5 and 9.9 ± 0.9 mm in CG and SSG, respectively; $P = 0.7364$) or pregnant heifers (9.0±0.7 and 9.5±1.0 mm, in CG and SSG, respectively; $P = 0.7143$). There were no significant effects due to the treatment on diameter or volume of the CL either in pregnant or non-pregnant heifers (Fig. 1). The maximum CL diameter (mm) in non-pregnant heifers was found on D12 (19.3±1.2) and D14 (17.9±0.8) in CG and SSG, respectively; In pregnant heifers the maximum CL diameter was (19.3±1.2) on D12 and (17.9±0.8) D14, in CG and SSG, respectively.

Table 2 shows the P4 concentrations obtained. There were no treatment effects on P4 concentrations on any tested days. The maximum mean P4 concentration in non-pregnant heifers was found on D12 amounting to 6.6 and

6.3 ng/mL in CG and SSG, respectively. However, these figures on D26 in pregnant heifers were 8.2 and 8.3 ng/mL in CG and SSG, respectively.

As shown in Table 3, there was no treatment effect on blood lipid fractions (e.g., triglycerides, HDL, LDL and total cholesterol) on D0 and D21.

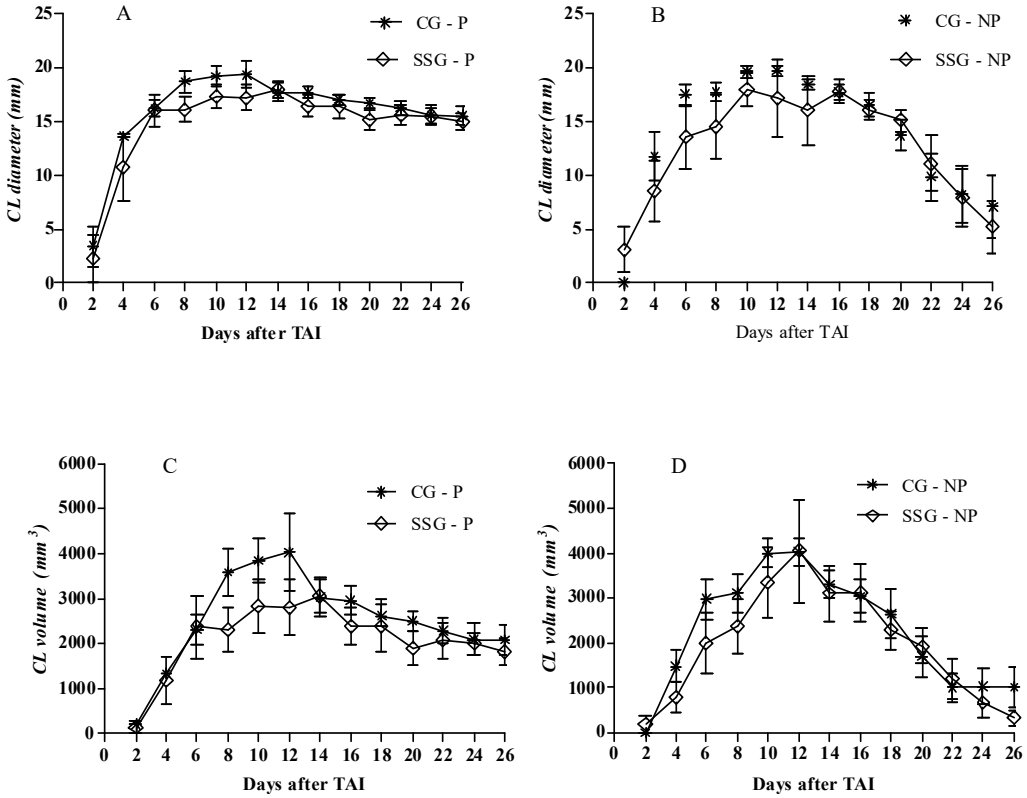


**Fig. 1:** Mean (± SEM) corpus luteum diameter (CL; mm-fig. A and B) and volume ($mm^3$ -fig. C and D) from Day 2 (D2) to 26 (D26) after timed AI in control Nelore heifers (Control Group, CG) or supplemented with Sunflower Seed (SSG) Pregnant (P) or Not Pregnant (NP)

**Table 2:** Mean (± SEM) plasma progesterone concentration (ng/mL), during D2 to D26 after TAI in Nelore heifers not supplemented (CG, n = 15) or supplemented with Sunflower Seed (SSG, n = 11)

| Days after TAI (D0 = TAI day) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| NP | D2 | D4 | D6 | D10 | D12 | D14 | D16 | D18 | D22 | D26 |
| CG (n=9) | 0.58±0.12 | 2.28±0.50 | 3.82±0.37 | 5.76±0.67 | 6.58±0.67 | 5.75±0.64 | 4.44±1.07 | 2.79±1.26 | 0.54±0.17 | 2.63±0.65 |
| SSG (n=6) | 1.07±0.36 | 2.17±0.58 | 3.24±0.99 | 5.10±1.14 | 6.31±1.22 | 6.12±0.63 | 4.86±0.89 | 4.03±1.34 | 2.42±1.39 | 0.82±0.44 |
| *P* value | 0.1557 | 0.8869 | 0.5384 | 0.6036 | 0.8344 | 0.7045 | 0.7888 | 0.5236 | 0.7389* | 0.0803 |
| P | | | | | | | | | | |
| CG (n = 6) | 1.44±0.28 | 3.24±0.76 | 4.87±0.99 | 7.93±1.38 | 7.83±1.55 | 7.28±0.98 | 7,48±1.06 | 7.90±0.89 | 7.60±1.11 | 8.18±1.20 |
| SSG (n = 5) | 0.73±0.15 | 2.31±0.51 | 3.58±0.42 | 5.40±0.64 | 5.35±0.71 | 6.96±0.73 | 6.48±0.56 | 6.86±0.73 | 6.77±0.72 | 8.32±0.70 |
| *P* value | 0.0528 | 0.3573 | 0.2909 | 0.1556 | 0.2082 | 0.8078 | 0.4576 | 0.3990 | 0.5658 | 0.9266 |

Statistical analysis was performed using T-Student test and *Mann-Whitney ($p<0.05$); NP = Non pregnant; P = Pregnant

**Table 3:** Mean (± SEM) plasma concentrations (mg/dL) of triglycerides, HDL, LDL and total cholesterol in beef heifers in CG (control group, *n* = 15) and SSG (sunflower seed group, n = 11) feed supplemented for 56 days

| | Day 0 | | | Day 21 | | |
|---|---|---|---|---|---|---|
| | CG | SSG | p value | CG | SSG | p value |
| Triglycerides | 14.5±1.2 | 11.7±1 | 0.8252 | 16.6±0.9 | 16.9±0.5 | 0.7756 |
| HDL | 42.03±2.7 | 44.7±3.4 | 0.5434 | 38.5±2 | 38±2.5 | 0.8613 |
| LDL | 41.4±4.6 | 42.9±4.8 | 0.090 | 38.3±2.4 | 35.9±3.3 | 0.5619 |
| Cholesterol | 86.3±3.3 | 89.9±3.7 | 0.4799 | 80.1±2.5 | 77.5±2.2 | 0.4692 |

## Discussion

Contrary to the initial hypothesis, sunflower seed supplementation for 56 days before the TAI protocol did not show significant effects on the FD, CLD, or P4 concentrations at the time of the TAI. In addition, the supplementation with sunflower seed did not alter the lipid profile (cholesterol, triglycerides, HDL and LDL) in the present study.

Pregnancy rate did not vary among groups (40% *vs*. 45.5% in the CG *vs*. SSG). Although this experiment was not designed to evaluate pregnancy rates due to its small sample size, pregnancy in the SSG was 5.5% higher than that of the CG. Contrariwise, other studies have produced interesting results. Cordeiro *et al*. (2015), working with Nelore females, observed increase in pregnancy rates after TAI and FTET (fixed-timed embryo transfer). Positive results were obtained by Lopes *et al*. (2009) after supplementing Nelore females with protected fat (Megalac-E). The crucial difference among the studies is time of supplementation. In the present study, supplementation was started 56 days before TAI. The pregnancy rate was increased when supplementation with sunflower seed was from the day of TAI until 21 or 28 days later (Cordeiro *at al*., 2015; Lopes *et al*., 2015).

We did not observe an effect of supplementation on the follicular diameter on the day of TAI, which varied from 9.0 to 9.9 mm on average. Studies with *Bos taurus* females show that animals whose diets were supplemented with fat have larger follicles (Lammoglia *et al*., 1996; Staples *et al*., 1998; Robinson *et al*., 2002). Attempts to elucidate the mechanism of action of PUFAs have shown an increase in the diameter of the ovulatory follicle (Bilby *et al*., 2006) and in the subsequent CL P4 production (Vasconcelos *et al*., 2001). In addition, the FD at the time of insemination influences the ovulation rate and, consequently, the percentage of pregnant females after TAI (Vasconcelos *et al*., 2001; Pfeifer *et al*., 2012). The ovulatory follicle diameter at the time of TAI was significantly higher (10.7 vs. 8.5 mm) in pregnant Nelore cows (da Silveira *et al*., 2011).

Sunflower seed supplementation did not have a significant effect on P4 concentrations. These results are in agreement with others who did not observe an increase in serum P4 concentrations when calcium salts of PUFA were added to the diets of dairy (Reis *et al*., 2012; Moriel *et al*., 2014) and beef cows (Lopes *et al*., 2011).

We observed maximum diameters of the CL of 19.7 and 18.9 mm, respectively in the GC and SSG. Nelore heifers with a CL diameter of >13mm were considered to have a functional CL (Andrade *et al*., 2019) and Holstein cows with a CL diameter of >23mm (Bicalho *et al*., 2008).

Considering that there was no significant difference in the mean FD between the groups studied on the day of the TAI or the lipid profile, this may have determined the non-significant effect in CL measurements and, consequently, P4 concentrations.

Another possible mechanism associated with improved conception may be related to embryos' need for essential fatty acids (Haggarty *et al*., 2006). Supplementing the diets of females with essential fatty acids may lead to improved embryonic quality and development (Thangavelu *et al*., 2007; Cerri *et al*., 2009).

Therefore, supplementation before TAI, with the aim of affecting the oocyte, the dominant ovulatory follicle and consequently the CL, did not result in the hypothesized effect. On the other hand, positive results were observed in the literature when supplementation started at the time of AI and continued until after the pregnancy was confirmed. Thus, the positive effects of PUFAs on pregnancy rates may be due to direct changes in the embryo and uterine microenvironment or during maternal recognition of pregnancy, rather than changes in the FD, CLD, or P4 production.

In addition, the positive results in the reproductive sphere using polyunsaturated fatty acids are highly variable between studies, as they depend on the type of fatty acid used, time of use, reproductive status (heifers vs. cows) and breed of females studied.

## Conclusion

It can be concluded that supplementation with sunflower seed to the Nelore heifer's diets for 56 days before TAI did not alter the FD, CLD, plasma concentrations of total cholesterol, triglycerides, HDL and LDL or P4 production.

## Acknowledgements

The authors are grateful to the CNPq (Project 480783/2013-6) and FAPESP (Project 2016/02805-4) for their financial assistance in this project under the responsibility of Profa. Dra. Claudia Maria Bertan Membrive, UNESP, Brazil.

## Author's Contributions

**Gabriel Molinari de Mattos:** Responsible for the development of the entire experimental part, data collection, study writing, manuscript preparation, literature search and final revision of the study.

**Beatriz de Moraes Ropelli and Angélica Leão Baltazar:** Responsible for the development of the entire experimental part and data collection.

**Claudia Maria Bertan Membrive:** Responsible for experimental design, study writing, manuscript preparation, data analysis and interpretation of data, literature search and final revision of the study.

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**Guilherme de Paula Nogueira:** Responsible for Progesterone dosages by the RIA, interpretation of data and final approval of the version the manuscript.

**Luiz Fernando Coelho da Cunha Filho:** Responsible for development of nutritional management of animals and scientific review of the article's content.

**Caliê Castilho:** Project coordinator, responsible for experimental design, study writing, manuscript preparation, data analysis and interpretation of data, literature search and final revision of the study.

## Ethics

This article is original and contains unpublished material. The corresponding author confirms that no ethical issues involved.

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