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Effect of season, late embryonic mortality and progesterone production on pregnancy rates in pluriparous buffaloes (*Bubalus bubalis*) after artificial insemination with sexed semen

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ABSTRACT

The use of sexed semen technology in buffaloes is nowadays becoming more and more accepted by farmers, to overcome the burden of unwanted male calves with related costs and to more efficiently improve production and genetic gain. The aim of this study was to verify the coupling of some variables on the efficiency of pregnancy outcome after deposition of sexed semen through AI. Pluriparous buffaloes from two different farms (N = 152) were screened, selected, and subjected to Ovsynch protocol for AI using nonsexed and sexed semen from four tested bulls. AI was performed in two distinct periods of the year: September to October and January to February. Neither farms nor bulls had a significant effect on pregnancy rates pooled from the two periods. The process for sexing sperm cells did not affect pregnancy rates at 28 days after AI, for nonsexed and sexed semen, respectively 44/73 (60.2%) and 50/79 (63.2%), P = 0.70, and at 45 days after AI, for nonsexed and sexed semen, respectively 33/73 (45.2%) and 33/79 (49.3%), P = 0.60. Pregnancy rate at 28 days after AI during the transitional period of January to February was higher when compared with September to October, respectively 47/67 (70.1%) versus 47/85 (55.2%), P = 0.06. When the same pregnant animals were checked at Day 45 after AI, the difference disappeared between the two periods, because of a higher embryonic mortality, respectively 32/67 (47.7%) versus 40/85 (47.0%), P = 0.93. Hematic progesterone concentration at Day 10 after AI did not distinguish animals pregnant at Day 28 that would or would not maintain pregnancy until Day 45 (P = 0.21). On the contrary, when blood samples were taken at Day 20 after AI, the difference in progesterone concentration between pregnant animals that would maintain their pregnancy until Day 45 was significant for both pooled (P = 0.00) and nonsexed (P = 0.00) and sexed semen (P = 0.09). A similar trend was reported when blood samples were taken at Day 25, being highly significant for pooled, nonsexed, and sexed semen (P = 0.00). Hematic progesterone concentration between the two periods of the year was highly significant for pregnant animals at 28 days from AI when blood samples were taken at Day 20 after AI for pooled, nonsexed, and sexed semen, respectively P = 0.00, 0.00, and 0.06, and for pregnant animals at Day 45 for pooled, nonsexed, and sexed semen, respectively P = 0.00, 0.00, and 0.01. From these results, it can be stated that hematic progesterone concentration measurement since Day 20 after AI can be predictive of possible pregnancy

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maintenance until Day 45. Furthermore, the transitional period of January to February, although characterized by a higher pregnancy outcome when compared with September to October, suffers from a higher late embryonic mortality as evidenced by a significant different hematic progesterone concentration between the two periods at Day 20 after AI.

1. Introduction

Some studies and field trials have unequivocably shown that the use of sexed semen in the buffalo (*Bubalus bubalis*) is reliable and efficient for the establishment of pregnancies and consequent birth of live calves, similar to what is obtained with the use of nonsexed semen. In fact, the first pregnancies and birth of live buffalo calves were reported after the deep deposition of less number of sexed sperm cells at the uterotubal junction using a special catheter [1,2]. More recently, similar success with sexed semen has been reported in conjunction with *in vitro* embryo production procedures [3] and artificial insemination [4]. In the latter study, even lower concentration of sexed sperm cells were deposited either at the body of the uterus or at the very beginning of the uterine horn, ipsilateral to the ovary bearing the ovulatory follicle, using an ordinary catheter for AI. The successful application of reproductive technologies, and in particular of the sexed semen technology in association with artificial insemination, can be hampered by some conditions, some of which are physiologically intrinsic to the buffalo species and others are shared by cattle: (1) reproductive efficiency as related to season and photoperiod; (2) inadequate progesterone production by the gravidic corpus luteum, and (3) late embryonic mortality (LEM). At latitudes where the present study was conducted, buffaloes are reported as characterized by a different seasonal reproductive efficiency, being higher during the months of decreasing light hours [5]. Such differential seasonal output in terms of cyclicity, pregnancy rates, and calving is clearly evident when animals are left to naturally occurring mating with the exclusion of any human intervention, and when they are subjected to controlled breeding through the adoption of various reproductive technologies such as AI, hormonal stimulation, and *in vitro* embryo production procedures in association also with Ovum Pick Up [6]. In ruminants, the establishment and maintenance of pregnancy is dependent on progesterone production by the functional CL at the early stages of embryo development, and supported by additional progesterone production by the fetoplacental unit at later stages of pregnancy [7]. It has been reported in buffaloes that progesterone production follows a seasonal pattern in diverse geographic climatic conditions, both in the course of estrous cycles in extreme hot climate [8,9], and after parturition when monitoring ovarian function [10]. Seasonality in the buffalo species is also responsible for a differential progesterone production linked to maintenance or loss of pregnancy according to the period of the year [11]. The reasons for higher embryonic mortality in buffaloes during specific periods of the year are not fully understood. However, it seems that it might be, at least partially, because of a decrease in progesterone secretion by the CL during early pregnancy [12]. This study was conducted to outline possible relationships among some important variables, namely season and periods of the year, nonsexed versus sexed semen, and progesterone production on the establishment and maintenance of pregnancy in buffaloes after AI with nonsexed and sexed semen.

2. Materials and methods

2.1. Animals

The study was conducted during the breeding season, characterized by decreasing light hours (September to October), and the transition period (January to February), characterized by increased light hours. All the pluriparous buffaloes (N = 152), in good general and reproductive health, completed 140 ± 5.3 days after parturition and confirmed to be cycling (having ovulatory follicles/functional CLs) by two ultrasound examinations at an interval of 10 days. They were included in the study at two different farms in the south of Italy located at latitude 40.5 to 41.5 N and longitude 13.5 to 15.5.

2.2. Synchronization of ovulation

Buffaloes were synchronized for ovulation by implementation of a conventional Ovsynch protocol [13] by GnRH administration of 12 μg buserelin acetate (Receptal, Intervet, Milan, Italy) im on Day 0, followed by 0.524 mg of synthetic prostaglandin (Cloprostenol, Estrumate, Schering-Plough Animal Health, Milan, Italy) on Day 7 and an additional 12 μg buserelin acetate on Day 9. Artificial insemination was performed 16 to 20 hours after the second GnRH administration.

2.3. Semen and AI

Two different collaborating companies (Cogent Breeding Ltd, UK, and Centro Tori Chiacchierini, Perugia, Italy), were responsible for semen collection, processing, sexing, freezing, and distribution. Unsexed and sexed semen from the same ejaculate of each of four buffalo bulls was used for AI. Sperm sorting was performed according to the Beltsville Sperm Sorting Technology [14], starting with dilution of semen up to 80×10^6 spermatozoa per mL with modified Tyrode's albumin lactate pyruvate extender. Then, 50 μL of 5 mg/mL Bisbenzimide (Hoechst 33342; Sigma, St. Louis, MO, USA) and 27 μL food dye FD#40 (Warner Jenkinson Company Inc., St. Louis, MO, USA) were added to samples and incubated at 35.5 °C for 30 minutes, and filtered through a 30 μm filter (Partec, GmbH). Samples were then sorted at a rate of 5500 cells per second and sorting pressure of 40 psi into 50 mL conical plastic tubes (BD Biosciences) prefilled

with 3 mL egg yolk extender. After sorting, samples were centrifuged at 800 × *g* for 20 minutes. The supernatant was discarded and the pellet resuspended with a TRIS freezing extender. Viability characteristics of sexed semen from the four bulls, after sorting of the two cell populations, were as follows: (1) purity ranged from 92% to 97% and mean ± SEM 94 ± 0.7; (2) progressive linear motility ranged from 30% to 60% and mean ± SEM 48.7 ± 4.4; (3) membrane integrity test (propidium iodide test) ranged from 59% to 69% and mean ± SEM 62.7 ± 2.1; and (4) osmotic resistance test ranged from 43% to 56% and mean ± SEM 48.1 ± 2.3. A total content of 2 million live sorted sperm cells were packaged into 0.25 mL straws and frozen according to the Cogent Breeding Ltd, UK proprietary technology. Reanalysis of sorting purity was performed from frozen-thawed samples, as described above at an event rate of 100 cells per second and purity was analyzed by curve fitting statistics. Nonsexed semen from the same bulls was used at a concentration of 20 million per dose, packaged similarly into 0.25 mL straws. Both nonsexed and sexed semen was deposited at the very beginning of the uterine horn ipsilateral to the ovary bearing the ovulatory follicle. Only animals with a follicle ≥10 mm and a tonic uterus with or without vaginal mucous discharge were considered to be in heat with impeding ovulation and subjected to AI. Nonsexed and sexed semen were used in 73 and 79 buffaloes, respectively. Pregnancy rates were assessed by ultrasound at Day 28 and confirmed at Day 45.

2.4. Progesterone measurement

The cyclic ovarian status of the buffaloes and luteal function were evaluated by measuring progesterone (P4) concentrations by RIA in blood samples collected on Days 10, 20, and 25 after AI [15,16]. Blood P4 concentrations greater than 1.5 ng/mL were considered to be indicative of a functional CL [17]. The minimum detectable amount of progesterone was 2.1 ± 0.08 pg. Intra- and interassay coefficients of variation were 6.2% and 11.8%, respectively.

2.5. Statistical analysis

Results are expressed as mean and SEM or as frequencies and percentage. Differences among percentages were assessed by the chi-square test or, when appropriate, by Fisher exact test. Progesterone concentration is expressed as ng/mL. Continuous variables were compared using the *t* test for unpaired data or by Mann–Whitney test. All statistical analyses were performed using STATA software version 11.2 (STATA Corporation, College Station, TX, USA).

3. Results

3.1. Farm and bull effect

Farm management and environment did not affect pregnancy rate at 28 Days post AI when semen was considered as a whole (P = 0.64) and split into nonsexed (P = 0.70) and sexed semen (P = 0.30). The same trend was observed at confirmation of pregnancy at Day 45 for semen (P = 0.26) split into nonsexed (P = 0.97) and sexed semen (P = 0.11). Similarly, bulls did not affect pregnancy outcome at 28 Days post AI, when results from the use of nonsexed and sexed semen were pooled together (P = 0.31), and when they were singularly evaluated as nonsexed semen (P = 0.57) and sexed semen (P = 0.56). A similar trend was reported at confirmation of pregnancy at 45 Days for pooled semen (P = 0.74), nonsexed semen (P = 0.54), and sexed semen (P = 0.93).

3.2. Semen and period of the year on pregnancy rates and LEM

When pooling data from both periods of the year considered in this study, nonsexed and sexed semen gave similar rates of pregnancy at 28 Days post AI (P = 0.70) and a similar trend was reported at 45 Days post AI (P = 0.60). No differences in LEM were reported between nonsexed and sexed semen (P = 0.78). When considering singularly the transitional and the breeding seasons, a higher pregnancy rate at 28 Days post AI in the former as opposed to the latter was reported when results from nonsexed and sexed semen were pooled together (P = 0.06). Such difference disappeared when only nonsexed semen was evaluated (P = 0.40), but was more evident with the use of only sexed semen, respectively (P = 0.07). A significantly higher incidence (P = 0.05) of LEM reported for pooled semen during the transitional period, obviates any difference in pregnancy rates at 45 Days post AI between two periods of the year. This is confirmed for pooled semen (P = 0.93), and nonsexed semen (P = 0.48) and sexed semen (P = 0.43). Collective data are presented in Table 1.

3.3. Birth of calves and sex rate

From the 20 pregnancies derived from AI during the breeding season with nonsexed semen, an abortion at 7 months of gestation was reported and the birth at term of 10 male and nine female live calves. On the contrary, no abortions were reported within the 20 pregnancies obtained with sexed semen, resulting in the birth of two male and 18 female live calves. All pregnancies obtained during the transitional period led to the birth of live calves. The 13 pregnancies derived from the use of nonsexed semen resulted in the birth of 13 male and seven female calves, and all 19 pregnancies obtained with sexed semen resulted in the birth of female calves.

3.4. Days open on pregnancy rates and LEM

Days from parturition to AI did not influence rate of pregnant versus nonpregnant buffaloes at Day 28 when nonsexed and sexed semen results were pooled, respectively 138.0 ± 6.6 versus 143.3 ± 9.0 (P = 0.63). A similar trend was reported when only nonsexed semen was considered, respectively 139.9 ± 9.0 versus 150.5 ± 13.2 (P = 0.49) and only sexed semen, respectively 136.3 ± 9.8 versus 136.0 ± 12.3 (P = 0.98). An absence of effect was also reported at confirmation of pregnancy at 45 Days for pooled semen, respectively 143.5 ± 7.5 versus 136.9 ± 7.6 (P = 0.54), and for nonsexed semen, respectively 143.5 ± 10.2 versus 144.6 ± 10.9 (P = 0.94), and for sexed semen, respectively 143.4 ± 10.9 versus 129.2 ± 10.7 (P = 0.35). The same interval

Table 1
Effect of breeding season (BS) versus transitional period (TP) and late embryonic mortality (LEM) on pregnancy rate (PR) after AI with nonsexed (NS), sexed (S), and pooled semen (PS) in buffalo.

Pregnancy/ Embryo Mortality	BS			TP			Pooled Seasons		
	NS	S	PS	NS	S	PS	NS	S	PS
PR (28 Days)	23/41 (56.1%)	24/44 (54.5%)	47/85 (55.2%)	21/32 (65.6%)	26/35 (74.2%)	47/67 (70.1%)	44/73 (60.2%)	50/79 (63.2%)	94/152 (61.8%)
PR (45 Days)	20/41 (48.7%)	20/44 (45.4%)	40/85 (47.0%)	13/32 (40.6%)	19/35 (54.2%)	32/67 (47.7%)	33/73 (45.2%)	39/79 (49.3%)	72/152 (47.3%)
LEM	3* (13.0%)	4** (16.6%)	7*** (14.8%)	8* (38.0%)	7** (26.9%)	15*** (31.9%)	11 (25%)	11 (22%)	22 (23.4%)

Within a row: * $P = 0.05$; ** $P = 0.38$; and *** $P = 0.05$.

from parturition to AI among pregnant buffaloes, did not influence the rate of LEM when nonsexed and sexed semen were considered (no LEM 143.5 ± 7.5 vs. LEM 120.0 ± 14.1; $P = 0.13$). Similarly, results from the use of only nonsexed semen highlighted a similar trend (no LEM 143.5 ± 10.2 vs. LEM 128.9 ± 19.1; $P = 0.48$) and sexed semen (no LEM 143.4 ± 10.9 vs. LEM 111.2 ± 21.4; $P = 0.17$).

3.5. Late embryonic mortality: progesterone on pregnancy and periods of the year

Progesterone values (ng/mL) at Day 10 after AI among all pregnant buffaloes could not help in discriminating animals that would terminate their pregnancy as opposed to animals that would carry their pregnancy until Day 45. Although the same trend was observed when only pregnancy from nonsexed semen was considered, with sexed semen progesterone concentration was found significantly lower in animals terminating their pregnancy as opposed to those maintaining up to 45 Days from AI. On the contrary and more evidently, when progesterone values were taken from all pregnant animals and considered at Day 20 after AI, a clear significant difference was reported. This difference was also evident when results from pooled semen were split into nonsexed and sexed semen. Progesterone values considered at Day 25 from AI confirmed the same significant difference between the two classes of animals, when considering pooled semen and singularly taken, nonsexed and sexed semen (Table 2).

Progesterone values at Day 10 after AI did not differ between buffaloes reported pregnant at Day 28 in the two different periods of the year considered in this study, September to October and January to February, for pooled semen and for nonsexed and sexed semen. The difference in progesterone levels were found, on the contrary, different in the two different periods examined among pregnant buffaloes when values were taken at Day 20 from AI for pooled semen and for nonsexed semen, and for sexed semen. Such difference in progesterone value was reported again nonsignificant when blood samples were taken at Day 25 from AI for pooled semen, nonsexed, and sexed semen (Table 3).

Animals that maintained pregnancy at Day 45 after AI followed the same trend as for pregnant animals at Day 28. In fact, only for progesterone values from blood samples taken at Day 20 from AI, a significant difference was reported between periods for all categories of semen considered (Table 4).

4. Discussion

This study confirms the feasibility and applicability of the sexed semen technology in the buffalo farm management for reproductive control. It highlights once more the absence of difference between the use of sexed as opposed to nonsexed semen, and a similarity of results when confronting semen derived from different bulls, and farms. In cattle, several studies have shown a reduction in conception rate when sexed spermatozoa are used for AI, highlighting the low dose and the sorting process as the main causes responsible for such a decline, more relevant in cows than heifers [18,19]. On the contrary, in the first large trials conducted in buffalo heifers [4] and in pluriparous animals (present study), conception rates have not been greatly and significantly affected by the use of a reduced number of sexed spermatozoa. Such similarity can be accounted for by a rigorous selection of bulls and a highly validated semen processing technology [4]. Furthermore, there is evidence that the sexing process improves the DNA integrity of sexed semen samples [20], by eliminating male and female spermatozoa characterized by compromised DNA through the flow cytometry sorting procedure [21]. The evidence that a significantly reduced number of sexed sperm cells can give a conception rate similar to the use of conventional semen at full dosage, opens also the possibility to produce and commercialize doses of conventional semen containing a much lower number of sperm cells. A condition

Table 2
Difference in blood progesterone concentration (ng/mL) between pregnant animals that maintained or terminated pregnancy after AI with nonsexed and sexed semen.

Semen	Day 10 post AI	Day 20 post AI	Day 25 post AI
Nonsexed semen	2.4 ± 0.1 vs. 2.7 ± 0.4	3.4 ± 0.3 vs. 1.0 ± 0.2	3.8 ± 0.3 vs. 1.0 ± 0.1
P	0.40	0.00	0.00
Sexed semen	2.5 ± 0.1 vs. 1.7 ± 0.2	2.9 ± 0.1 vs. 2.2 ± 0.4	3.2 ± 0.2 vs. 1.6 ± 0.5
P	0.02	0.09	0.00
Pooled semen	2.4 ± 0.0 vs. 2.2 ± 0.2	3.1 ± 0.1 vs. 1.7 ± 0.2	3.5 ± 0.2 vs. 1.3 ± 0.3
P	0.21	0.00	0.00

Table 3
Difference in blood progesterone concentration (ng/mL) at different intervals among pregnant animals at Day 28 after AI with nonsexed and sexed semen, during the breeding season versus the transitional period.

Semen	D10[a]	D20[a]	D25[a]
Nonsexed semen	2.4 ± 0.1 vs. 2.6 ± 0.2	3.8 ± 0.4 vs. 1.8 ± 0.2	3.6 ± 0.4 vs. 2.5 ± 0.5
P	0.46	0.00	0.21
Sexed semen	2.5 ± 0.1 vs. 2.1 ± 0.1	3.0 ± 0.2 vs. 2.4 ± 0.2	2.8 ± 0.2 vs. 2.9 ± 0.4
P	0.14	0.06	0.91
Pooled semen	2.4 ± 0.1 vs. 2.3 ± 0.1	3.4 ± 0.2 vs. 2.1 ± 0.1	3.2 ± 0.2 vs. 2.7 ± 0.3
P	0.47	0.00	0.32

[a] Blood samples taken at Day (D) 10, 20, and 25 after AI for animals pregnant at 28 days from AI.

that, on the contrary, typically limits the reproductive efficiency in the buffalo species is the period of the year considered. Buffaloes are in fact short-day breeders and therefore tend to perform better, during natural mating than while implementing reproductive technologies, in the time of the year of decreasing light hours [22]. The two periods taken into account in this study, can be seen as one in which the highest reproductive efficiency is usually reported (September to October), and the other (January to February) to be considered transitional into the season of the year of increasing daylight hours, and therefore suboptimal with regard to reproductive performance [23]. In this study, a higher pregnancy rate has been reported among buffaloes during the transitional period at Day 28 post AI, but leveling between the two periods when pregnancy check was re-evaluated at Day 45 because of a higher embryonic mortality. Mortality of the conceptus at the early stages of development has been studied in domestic large ruminants, and it appears that in buffaloes, unlike cattle, the greatest incidence occurs between 25 and 45 days after mating or AI. This timeframe of embryo mortality accounts for most loss and is termed late, opposed to an earlier window of occurrence between 15 and 24 days [24]. In buffaloes, seasonality has already been reported to be responsible for a differential LEM, significantly higher with increasing daylight length when compared with the opposing period of the year characterized by decreasing light hours. It has been reported that progesterone concentrations follow a progressive decline from 10 days after AI in pregnant animals at Day 25 that will not maintain pregnancy at Day 45 [22]. In buffaloes, as for many other mammalian species, this is a sensitive period encompassing the transitory attachment phase up to the completion of the embryo–uterine attachment [25]. In this regard, the mucine transmembrane glycoprotein (MUC-1) plays an identified important role when available in the uterine environment and when progesterone concentration is low: in fact, its presence and the abundance of MUC-1 endometrial receptors are keys in preventing the embryo attachment to the endometrium. On the contrary, when progesterone concentration is higher and adequate for pregnancy maintenance, it results in the blockage of the MUC-1 endometrial receptors and by negative feedback to the halt in MUC-1 synthesis. This process leads to the attachment of the embryo to the epithelial lining of the endometrium through the concomitant action of some adhesive molecules [26,27]. In this study, a significantly higher progesterone production was reported at Day 20 and 25 post AI in pregnant animals that would maintain their pregnancy as opposed to animals that would be found not pregnant up to Day 45 post AI. Together with the causative action determined by the low progesterone concentration, the termination of pregnancy found at Day 45 can be also speculatively attributed to a reduced embryonic growth [28] and consequently to a smaller area for caruncles attachment as already shown in sheep [29–31]. Based on these findings, concentration of hematic progesterone since Day 20 after AI can be predictive of possible maintenance of pregnancy up to Day 45 or on the contrary of its failure and termination. The two periods of the year investigated in this study showed a differential response to LEM. It has already been reported by Vecchio et al. [32], a close link between increasing light hours length, reduced progesterone production, and increased LEM. This study confirms a close connection among these elements considered, and highlights a significant difference in progesterone production in pregnant buffaloes at Day 20 post AI between the two periods of the year considered, being higher in months characterized by a high ratio of dark to light hours. This result is confirmed by a previously reported significant decrease of hematic progesterone during the seasonal transitional period together with a reduced CL size, by Campanile et al. [33]. In addition, in domestic large ruminants, blood flow and vascularization have

Table 4
Difference in blood progesterone concentration in (ng/mL) at different intervals among pregnant animals at Day 45 after AI with nonsexed and sexed semen during the breeding versus the transitional period.

Semen	D10[a]	D20[a]	D25[a]
Nonsexed semen	2.5 ± 0.1 vs. 2.3 ± 0.1	4.1 ± 0.4 vs. 2.2 ± 0.3	4.0 ± 0.4 vs. 3.0 ± 0.5
P	0.56	0.00	0.26
Sexed semen	2.6 ± 0.1 vs. 2.3 ± 0.2	3.2 ± 0.2 vs. 2.4 ± 0.2	3.1 ± 0.2 vs. 3.3 ± 0.4
P	0.31	0.01	0.71
Pooled semen	2.5 ± 0.1 vs. 2.3 ± 0.1	3.7 ± 0.2 vs. 2.3 ± 0.1	3.6 ± 0.2 vs. 3.1 ± 0.3
P	0.24	0.00	0.43

[a] Blood samples taken at Day (D) 10, 20, and 25 after AI for animals pregnant at 45 days from AI.

been strongly linked to progesterone synthesis and secretion in cattle [34], and in buffaloes [35] throughout the estrous cycle. The link between CL function, angiogenesis, and progesterone production in buffaloes can be inferred by the change of vascular endothelial growth factor expression within the CL itself in the course of the estrous cycle similar to that already described in cattle [11,36]. As a cascade mechanism, insufficient P4 production has been associated with an impaired capacity of the developing embryo to produce bovine trophoblastic protein-1, also called interferon-tau at the needed amount to prevent luteolysis from 16 days after AI or natural mating [37]. Such protein ensures maternal recognition of pregnancy through avoidance of CL regression by either inhibiting oxytocin receptors development on the endometrium [38], or by activating a prostaglandin inhibitor [39].

4.1. Conclusions

The results from this study, of a differential progesterone production by the gravidic CL after AI with sexed and nonsexed semen between two periods of the year considered and inductive of a different LEM, associated with previously reported similar evidence strongly support the notion of a causative effect of season on the reproductive efficiency in buffaloes.

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