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Original article

Platelet-rich plasma uterine infusion and pregnancy rate in barren mares with chronic degenerative endometritis

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Abstract

The chronic degenerative endometritis (CDE) is recognised as directly related to age and infertility in the mare. In this study, the 14 days post ovulation (PO) pregnancy rate was assessed in 60 barren mares affected by CDE, submitted to platelet-rich-plasma intrauterine infusion (PRPI) 24 hours PO. Data showed a significant positive effect of PRPI on the chance to become pregnant in mares affected by Kenney I-II CDE. The overall 14 days PO pregnancy rate was 75%. Sixty-nine % of the overall pregnancies was achieved with the 1st PRPI, but an additional 31% was obtained with the 2nd PRPI at the subsequent heat, increasing significantly the number of pregnancies. Although the 1st PRPI lead to a higher pregnancy rate than the 2nd PRPI (52 vs 23%), the repetition of the treatment had a significant positive effect on the overall pregnancies. Mare's young age positively influenced the chance to become pregnant and was associated to endometritis Kenney I. Mares with endometritis Kenney I had better chance to become pregnant than those with endometritis Kenney II. The PRPI proved, therefore, to be beneficial in barren mares with CDE, and resulted in high overall pregnancy rate achievement in barren mares.

Key words: horse, barren mares, platelet-rich-plasma, pregnancy rate, chronic degenerative endometritis

Introduction

Differently to other species, such as bovine and swine, because of the high economic value of their progeny, valuable broodmares are bred until old age, implying decreasing fertility and pregnancy rates. However, different age cut-offs limits have been related to fertility problems, according to some authors starting from > 9 years, or starting when mares are > 15 years old.

The impaired fertility of the so-called "problem mare" is often the result of several causes. Among these

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causes, the advanced age of the mare (Buczkowska et al. 2014, Cuervo-Arango et al. 2019) and the status of the endometrium play a central role, sometimes with a synergic effect played by these two factors (Reghini et al. 2016).

Among the multiple factors affecting the probability for a mare to become pregnant and give birth to a foal, chronic degenerative endometritis (CDE) is recognised as directly related to age and infertility (Ricketts and Alonso 1991, Hoffmann et al. 2009). Chronic degenerative endometritis is reported to be an alteration of the uterine glands and the surrounding stroma, characterized by the re-arrangement of myofibroblasts, deposition of extra-cellular matrix, glandular modification and fibrosis (Walter et al. 2001, Hoffmann et al. 2003). Fibrosis of the endometrium is therefore a progressive condition, with a trend to worsen with increasing age (Woodward et al. 2012).

Endometrial biopsies score represents the gold standard for uterine conditions assessment (Ludwig et al. 2001, Schlafer 2007). According to the Kenney and Doig (1986) endometrial score, 4 grades were identified: I, with no endometrial fibrosis, IIa, with weak fibrosis, IIb with moderate fibrosis and III, with severe fibrosis.

Several treatments were proposed for the therapy of CDE in the mare, aimed to correct inflammation, anatomic defect, bacterial contamination and, recently, to modulate the immune response (Reghini et al. 2016), with different percentage of recovery and post-treatment pregnancy rate.

In the last decade, the use of platelet-rich-plasma (PRP) for orthopaedic surgery and for several non-genital soft tissue lesions resolution, was reported (Lopez et al. 2010, Ridermann et al. 2010). More recently, its use was also proposed for the treatment of CDE (Reghini et al. 2016), and persisting mating-induced endometritis (Segabinazzi et al. 2017) in the mare.

The PRP contains a mixture of growth factors, with positive actions on cells mitosis, chemotaxis, neovascular formation and inflammation (Gonshor 2002, Kevy et al. 2004, Mazzocca et al. 2013, Kim et al. 2014). Nonetheless, treatment with PRP was shown to act by increasing some growth factors in the injured tissues (El-Sharkawy et al. 2007).

Besides the use of PRP for the treatment of many non-genital diseases, the uterine infusion of PRP was found to be effective in the modulation of the excessive uterine inflammatory response to semen in mares affected by CDE (Reghini et al. 2016), and for increasing the successful of pregnancy in mares with persisting mating-induced endometritis (Segabinazzi et al. 2017).

Because the goal of horse breeding husbandry is to achieve the as highest as possible pregnancy rate also in valuable "problem mares", the aim of the present study was to verify the recovery of fertility, assessed by pregnancy rate in barren mares affected by CDE and treated with PRP uterine infusion (PRPI), taking in consideration the number of treatments, the age of the mare, the degree of endometritis and the interaction between factors on mares' outcome (pregnant/not pregnant).

Materials and Methods

The study was approved by the Ethical Committee "Comitato per il Benessere degli Animali dell'Università degli Studi di Bologna" Prot.n. 17710 -P-2017.

Animals

One hundred and four light horse barren mares were referred to the Veterinary Teaching Hospital belonging to the Veterinary Faculty, University of Teramo, Italy, during the breeding seasons 2017-2018. All the mares resulted not pregnant in the previous breeding season, and also after three attempts of insemination in the current breeding season, despite timely artificially insemination, with semen of proven fertility stallions.

Mares, housed in single boxes (night time) and in open paddocks (day time), were all in good body conditions (BCS 6-7/9) and were fed with 3 kg/day fodder and 10 kg/day good quality hay, with water and salt ad libitum.

Before entering the study, a breeding soundness examination was performed and only mares without external genital conformation defects, with negative uterine culture and negative cytology (<5% neutrophils) (Brook 1993), evidencing a noninflamed endometrium, were enrolled.

Therefore, a total of 60 mares affected by CDE, 3-21 years old, 400-650 kg body weight, were selected for the study.

According to age, mares were grouped in four classes: <5 (n=5), 5-10 (n=17), 11-15 (n=22), and >15 years old (n=16).

Endometrial biopsy and classification of mares according to endometritis Kenney score

At estrus, the 60 selected mares were submitted to endometrial biopsy from the cranial uterine body by a Jackson alligator jaw biopsy forceps (Jorgensen Laboratories, Loveland, USA CO), immediately placed in 10% neutral buffered formalin solution, and soon embedded for histology. Tissues sections of 5 µm thickness were obtained and stained with haematoxylin and

eosin, as reported by Rickets (1975). Endometrial biopsies were classified on the basis of the Kenney and Doig (1986) and Schoon et al. (2000) histopathologic and additional findings as follows:

- grade I: normal endometrium or mild and focal inflammatory infiltrates within the endometrium or mild and focal fibrosis surrounding the endometrial glands;
- grade IIa: mild-to-moderate inflammatory infiltrates within the endometrium or multifocal fibrosis (1-to-3 layers of fibroblasts surrounding the endometrial glands) or <2 glandular nests/5 mm linear field or mild lymphatic lacunae or partial atrophy of the endometrium during the late breeding season;
- grade IIb: moderate inflammatory infiltrates within the endometrium or moderate multifo -cal-to-diffuse fibrosis (> 4 layers of fibroblasts surrounding the endometrial glands) or 2-4 glandular nests/5 mm linear field or moderate lymphatic lacunae or barreness ≥ 2 years or combined presence of two IIa microscopic findings;
- grade III: severe inflammatory infiltrates within the endometrium or severe and diffuse peri-glandular fibrosis or ≥ 5 glandular nests/5 mm linear field or marked lymphatic lacunae or deep endometrial atrophy during the breeding season or combined presence of ≥ three IIa microscopic findings or ≥ combined presence of ≥ two IIb or III microscopic findings.

According to the Kenney and Doig (1986), histopathologic endometrial classification 23 mares were classified as having endometritis grade I and 37 as having endometritis grade II: 25 endometritis grade IIa, and 12 endometritis grade IIb.

Estrus monitoring and insemination

At estrus, the mares were submitted to daily ultrasound examination (Mindray, DP-2200VET, mounting a 7.5 MHz linear probe, Nanshan, Shenzhen 518057 P.R. China) of the genital tract. At the finding of a follicle with diameter ≥ 40 mm, coupled with ≥ grade 2 endometrial oedema, ovulation was induced by a single i.v. injection of 2500 IU hCG (Chorulon, MSD Animal Health srl, Milan, Italy). Ovulation occurrence was monitored by ultrasound examination every 12 hours, and evidenced by the disappearance of the dominant follicle. Twenty-four hours after hCG injection, mares were artificially inseminated with fresh semen collected from proven fertility stallions, and providing about 1 billion of total spermatozoa.

Autologous platelet-rich-plasma preparation

Before estrus, the 60 mares were submitted to blood collection for autologous PRP preparation.

Up to 450 ml blood were collected within 25-30 min, by the jugular vein by using quadruple blood bag system (Compoflex; Fresenius Kabi, Bad Homburg, Germany), and frequently gently shacked to allow the action of the added anticoagulant. Within 2 hours from collection, blood, maintained at a constant 20-22°C temperature, was transferred to the Veterinary Blood Transfusion Unit of the Veterinary Teaching Hospital of Teramo.

After a first platelet count performed by an automatic laser analyser (ADVIA 120,38 Siemens Health Care srl, Milan, Italy), blood was submitted to centrifugation (1920 rpm for 10 min at 22°C), using a centrifuge for blood transfusion bags (Rotixa 50RS; Hettich Italia srl, Cimadolmo, TV, Italy). The first obtained PRP was transferred by a plasma extractor (Bag-Extractor BIO15, Vasini Strumenti srl, Ravenna, Italy) in a bag without anticoagulant, and centrifuged again at 3960 rpm for 6 min, to obtain a further platelets concentration. In this second PRP, a shaker (Orbital shaker SO1, Stuart® Shakers Cole Parmer instruments Company LTD, St Neots, UK) was used at 22°C for 16-18 hours to disaggregate the platelets. Meanwhile, the plasma obtained from the second centrifugation was stored at 4°C (Frigoemoteca 170 ECT-F TOUCH, Fiocchetti, Luzzara, RE, Italy). After the 16-18 hours a subsequent platelet count was performed and the final platelets concentration units set to contain 1-1.2 million platelets/µl. Those final PRP units were stored at -20°C (Superfreezer 130 ECO, Fiocchetti, Luzzara, RE, Italy) until their use for treatment.

PRPI and pregnancy assessment

Twenty-four hours after the detected ovulation, all the 60 mares were intrauterinely infused with an autologous PRP unit (18-22 g, providing 1-1.2 million platelets/µl, within a volume of 15 ml) by using an insemination catheter, and checked 24 hours later to assess for possible intrauterine fluid accumulation.

Pregnancy was firstly checked by ultrasonography at 14 days PO, and confirmed at 28 days, before the mares returned to the owners. Mares losing pregnancy between 14- and 28-days PO were recorded.

The non pregnant mares were submitted to a second PRPI at the subsequent oestrous cycle, with the same schedule. In all cases not more than 2 PRPI were performed.

By follow-up, the number of mares losing pregnancy beyond 28 days were recorded.

Table 1. Distribution of pregnancies (number and %) at 14 days PO achieved after the 1st and those achieved after the 2nd PRPI in mares.

	Pregnant 14 days PO	Not Pregnant 14 days PO	Total
1st PRPI	31/60 (51.7%)	29/60 (48.3%)	60/60 (100%)
2nd PRPI	14/29 (48.3%)	15/29 (51.7%)	29/29 (100%)

Table 2. Pregnancy rate at 14 days PO obtained with the 1st and the 2nd PRPI in the 60 mares, grouped according to the class of age.

	Age				Total
	<5 ys	5-10 ys	11-15 ys	>15 ys	
Pregnant at 1st PRPI	3/5 (60%)	9/17 (52.9%)	13/22 (59.1%)	6/16 (37.5%)	31/60 (51.7%)
Pregnant at 2nd PRPI	1/5 (20%)	7/17 (41.2%)	4/22 (18.2%)	2/16 (12.5%)	14/60 (23.3%)
Total pregnant	4/5 (80%) [a]	16/17 (94.1%)	17/22 (77.3%)	8/16 (50%) [b]	45/60 (75%)

[a,b] denote within row significant differences with $p<0.05$

Table 3. Pregnancy rate at 14 days PO obtained with the 1st and the 2nd PRPI in the 60 mares, grouped according to the endometritis Kenney score (I/II).

	Kenney I	Kenney II	Total
Pregnant at 1st PRPI	15/23 (65.2%)	16/37 (43.2%)	31/60 (51.7%)
Pregnant at 2nd PRPI	5/23 (21.7%)	9/37 (24.3%)	14/60 (23.3%)
Total pregnant	20/23 (87%)[a]	25/37 (67.6%)[b]	45/60 (75%)

[a,b] denote within row significant differences with $p<0.05$

Statistical analysis

A statistical analysis was performed to assess the possible effect of PRPI, mares' age, and the presence of endometritis on the chance to become pregnant using the ANOVA for repeated measures test. The possible effect played by each age class, each endometritis Kenney score (I/II), and each PRPI on mares' outcome (pregnant/not pregnant), was firstly assessed by post hoc ANOVA. Moreover, the possible effect of each mares' age class and each endometritis Kenney score (I/II), on the need for the repetition of PRPI was tested by post hoc ANOVA. At last, the possible effect played by the interaction of mares' age, endometritis Kenney score and number of PRPI on mares' outcome, was also assessed by ANOVA for repeated measures. To allow a suitable statistical evaluation, Kenney IIa and Kenney IIb were merged in a single class Kenney II.

Results

None of the treated mares developed intrauterine fluid accumulation 24 hours after the 1st or the 2nd PRPI.

Pregnancy and foaling rate

The overall 14 days PO pregnancy rate (number of pregnant/number of inseminated mares) was 45/60 (75%), while overall pregnancy losses (total losses from 14 days PO to term) account for 5/45 (11.1%), and foaling rate (number of foaling/number of inseminated mares) was 40/60 (66.7%).

Pregnancy losses occurred in 2 mares within 28 days PO, while in 3 mares beyond the 5th month of pregnancy. Four of the 5 mares losing pregnancies, were endometritis Kenney II scored.

Effect of PRP uterine infusion numbers

Out of 45/60 14 days PO pregnancies, 31/60 (52%) were achieved at the 1st PRPI, while 14/60 (23%) mares conceived at the 2nd PRPI.

The distribution of pregnancies at 14 days PO achieved at the 1st and those achieved at the 2nd PRPI, is reported in Table 1.

The ANOVA for repeated measures showed a significant effect of PRPI ($p<0.05$) on the chance to become pregnant for the 60 barren mares affected by CDE. No significant differences were found between the 1st and the 2nd PRPI on pregnancy achievement.

However, 31/45 (69%) were achieved with only

Table 4. Distribution (number and %) of the 60 mares submitted to only the 1st PRPI, and those submitted also to a 2nd PRPI, grouped according to endometritis Kenney score and age classification.

	Kenney I				Kenney II			
	<5 ys	5-10 ys	11-15 ys	>15 ys	<5 ys	5-10 ys	11-15 ys	>15 ys
1st PRPI	3/5 (60%)	5/17 (29%)	6/22 (27%)	1/16 (6%)	0	4/17 (23%)	7/22 (32%)	5/16 (31%)
2nd PRPI	2/5 (40%)	2/17 (12%)	2/22 (9%)	2/16 (12%)	0	6/17 (35%)	7/22 (32%)	8/16 (50%)

the 1st PRPI, but additional 14/45 (31%) pregnancies were obtained thanks to a 2nd PRPI at the subsequent heat, increasing the total number of pregnancies. The statistical analysis showed that the repetition of PRPI had a significant ($p<0.001$) positive effect on the overall pregnancy success.

Effect of age

Pregnancy rate at 14 days PO obtained with the 1st and the 2nd PRPI in the mares, grouped according to the class of age, is reported in Table 2.

The ANOVA for repeated measures showed a significant effect of mares' age class ($p<0.05$), on the chance to become pregnant in the 60 barren mares affected by CDE and treated with PRPI.

The post hoc ANOVA showed that mares 5-10 years old had significant ($p<0.05$) higher chance to became pregnant than mares >15 years old.

Mares' age also related to endometritis ($p<0.01$). Young age was associated to endometritis Kenney I, with significant ($p<0.01$) differences between mares <5 years old than all the other three age classes. By contrary, mare's age was not associated to the need of a 2nd PRPI.

Effect of endometritis Kenney score

In mares with endometritis Kenney I, pregnancy was achieved with the 1st PRPI in a higher number of mares than in those with endometritis Kenney II.

Pregnancy rate at 14 days PO obtained with only the 1st and obtained with the 2nd PRPI in the 60 mares, grouped according to the endometritis Kenney score (I/II), is reported in Table 3.

The ANOVA for repeated measures showed a significant ($p<0.05$) effect of endometritis on the chance to become pregnant in the 60 barren mares affected by CDE and treated with PRPI.

Mares with endometritis Kenney I have higher ($p<0.05$) chance to become pregnant than those with endometritis Kenney II.

The endometritis Kenney score (I/II) was not associated to the need of a 2nd PRPI.

Effect of the interaction between mares' age, endometritis Kenney score and number of PRPI on pregnancy achievement

The distribution of the 60 mares submitted to only the 1st and those submitted also to a 2nd PRPI, grouped according to endometritis Kenney score and age classification, is reported in Table 4.

The statistical analysis failed to detect any possible effect of the interaction between mare's age, number of PRP uterine infusions and endometritis Kenney score (I or II) on the chance to became pregnant in the 60 barren mares affected by CDE.

Discussion

Problem mares with fertility impairment represent an issue in horse husbandry, where the purpose is to achieve the as high as possible pregnancy rate in valuable broodmares, independently from age and reproductive conditions.

In the present study, only 37% of the mares were ≤10 years old, and a noticeable 27% were >15 years old, so most of the studied mares were aged or old, according to the >9 years cut-off age suggested by Allen et al. (2007). In this study, 58% barren mares were affected by CDE, demonstrating, once more, the role of CDE in impairing reproduction efficiency in "problem mares", especially with advancing age. The CDE was, in fact, largely reported to be related to fertility problems, and increasing age (Ricketts and Alonso 1991, Lehmann et al. 2011, Mambelli et al. 2013, Buczkowska et al. 2014, Rebordao et al. 2014, Cuervo-Arango et al. 2019).

According to Kenney and Doig classification (1986), beside 38% of mares classified with endometritis Kenney I, referring to a normal or with minimal alterations endometrium, 62% mares were classified with endometritis Kenney II, grouping endometritis of different degree of severity from mild to moderate, while no Kenney III endometritis was found. Therefore, the results from the present study highlights the role of PRPI in mares with normal or minimally altered endometrium and with mild-to-moderate endometritis.

The distribution of mares according to endometritis Kenney score was different from data by Lehmann et al. (2011) and Pascoe (1995), that reported a value of 84.3-93% of Kenney II scored mares.

Data from the present study showed that in barren mares affected by endometritis Kenney I-II CDE, treated with PRPI, an overall 14 days PO pregnancy rate of 75%, was obtained summing the pregnancies achieved after 1st (52%) and at the 2nd (23%) PRPI. Foaling rate was 67%, and the overall pregnancy losses accounted for about 11%, with 3/5 cases with pregnancy loss occurring beyond the 5th month of pregnancy, confirming the higher risk for mares affected by CDE to lose pregnancy late in gestation (Kenney 1978), possibly due to the reduced efficiency of fetal membranes in mares with CDE (Scoggin 2015). The 11% pregnancy loss rate is in agreement with the 11.7% reported by Morris and Allen (2002) for barren mares.

The obtained 75% pregnancy rate at 14 days PO is a bit higher than the 58-69% reported in mares affected by persisting breeding-induced endometritis and treated with PRP intrauterine infusion (Segabinazzi et al. 2017). In contrast to that study, in which a comparison was done with a control group (pregnancy rate 31%), the main limitation of the present study is the lack of an untreated control group, that could have corroborated the obtained results. However, all the selected mares were "problem mares" not pregnant in the previous breeding season and also in the current breeding season after three attempts of insemination. Therefore, although it cannot be ruled out, it is unlikely that a similar percentage of mares could have conceived without any treatments. Sharma et al. (2011) reported a Day 16 pregnancy rate of 45.9% in Thoroughbred mares submitted to different type of uterine treatment, foaling rate of 35.8% and overall pregnancy losses of 21.9%, all parameters lower than those observed in the present study. According to Pascoe (1995), uterine autologous plasma plus antibiotic treatment in barren mares tended to be effective in increasing pregnancy rate in comparison to the untreated (75 vs 66%) and improved pregnancy and foaling rate in barren to values similar to those of normal maiden mares.

The 2nd PRPI provided a further 23% 14 days PO pregnancy rate in mares resulting not pregnant after the 1st PRPI, leading to a valuable overall pregnancy rate of 75%. The statistical analysis, indeed, showed a significant positive effect ($p<0.001$) of the 2nd PRPI on the overall 14 days PO pregnancy achievement, evidencing that, when the 1st PRPI is not followed by pregnancy achievement, a mare can be successfully submitted to a second PRPI at the subsequent heat, increasing the overall fertility recovery in barren mares affected by mild to moderate CDE (Perkin 2010).

As stated above, pregnancy success in the mare is related to many factors, such as age and endometrial conditions. Advancing age of the mare increases also the incidence of uterine treatments (Allen et al. 2007, Sharma et al. 2011) and to CDE (Ricketts and Alonso 1991). For these reasons, in the present study, the possible effect played by mares' age on pregnancy achievement, on the degree of CDE, and on the need for the 2nd PRPI, was also considered. The results confirmed the association between pregnancy achievement and young age in mares, with 5-10 years old mares showing ($p<0.05$) better chance to become pregnant in comparison to mares >15 years old. This finding was in agreement with the reported higher pregnancy rate in treated mares at ages of 3-7 (52.7%) and 8-12 (49.6%) years than ≥18 years old mares (29%) (Sharma et al. 2011). Moreover, the statistical analysis showed that mares <5 years old were associated ($p<0.01$) to Kenney I endometritis score, in comparison to all the other three age classes, confirming that in mares endometrial condition worsen with increasing age (Ricketts and Alonso 1991, Woodward et al. 2012, Buczkowska et al. 2014). However, age of mares was not associated to the need for a 2nd PRPI.

The results of the present study showed an association ($p<0.05$) between endometritis and the chance to become pregnant in the 60 barren mares affected by CDE and treated with PRPI.

On the basis of endometritis Kenney score, the overall 14 days after ovulation pregnancy rate was 87% for mares with endometritis Kenney I, and 67% for mares with endometritis Kenney II. Mares with endometritis Kenney I have higher ($p<0.05$) chance to become pregnant than those with endometritis Kenney II.

Although endometritis Kenney I score refers to normal endometrium with minimal alterations, in which the positive effect of the treatment should be considered cautiously, the 67% pregnancy rate in mares with endometritis Kenney II seems to suggest a positive association between PRPI and pregnancy achievement. The absence of mares affected by severe endometritis (Kenney III score) prevents the evaluation of the PRPI for the recovery of fertility in mares with severe CDE, representing a limit of the study.

The overall 67% foaling rate observed is in agreement with the expected 10-90% foaling rate in mares with endometritis Kenney I/II reported by Buczkowska et al. (2014).

According to the number of PRP uterine infusions, 14 days PO pregnancy rate was achieved at the 1st PRPI in 65% of mares with endometritis Kenney I, and

in 43% mares with endometritis Kenney II. A further 14 days PO pregnancy rate was achieved thanks to the 2nd PRPI in 22% of mares with endometritis Kenney I, and in 24% of mares with endometritis Kenney II.

The endometritis Kenney score (I/II) was not associated with the need of a 2nd PRPI.

At last, no effects of the interaction among mare's age, number of PRP uterine infusions and endometritis score Kenney (I or II) on the chance to became pregnant in the 60 barren mares affected by CDE were found. Although, on one hand this result could be affected by the number of studied mares, on the other hand it could suggest that one or repeated PRPI, the mares' age and endometritis Kenney score play significant independent roles on pregnancy achievement in barren mares affected by CDE Kenney I-II endometritis.

In conclusion, although it is expected that mares with Kenney I endometritis score have better chance to become pregnant in comparison to those with Kenney II, that young mares conceive easier than older ones, and that uterine treatments could improve the chance of becoming pregnant, data from the present study showed a significant effect of PRPI on the chance to become pregnant in barren mares affected by Kenney I-II CDE. Although the 1st PRPI lead to a higher pregnancy rate than the 2nd PRPI (52 vs 23%), the repetition of the treatment had a significant positive effect on the overall pregnancy achievement. Mare's young age positively influenced the chance to become pregnant and was associated to endometritis Kenney I. Mares with endometritis Kenney I had better chance to become pregnant than those with endometritis Kenney II.

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