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Artikel Asli/*Original Article*

Development of Sperm Separation System Using Electrical Current for Bull

(Pembangunan Sistem Pemisahan Sperma Menggunakan Arus Elektrik bagi Lembu)

FARAH HANAN FATHIHAH JAFFAR, SITI FATIMAH IBRAHIM, MOHD ISWADI ISMAIL, CHEW FANG NANG, SYARIFAH FAEZAH SYED MOHAMAD, NUR HILWANI ISMAIL, NUROL AFIZA ABDUL WAHAB, BALKHIS BASHURI & KHAIRUL OSMAN

ABSTRACT

A novel electrophoretic separation system has been successfully applied for the preparation of human sperm prior to the execution of assisted reproductive techniques (ARTs). This new system is designed to overcome the generation of reactive oxygen species (ROS) through centrifugation in conventional sperm preparation. Since the previous study showed favorable outcomes in humans, this study intends to implement this new system for animal sperm preparation particularly in bull. Fresh semen from adult bulls were used. Optimization of the electrophoretic system for optimum bull sperm separation involved different strength of voltage and separation time. The voltages applied were 10V, 20V, 30V, 40V, 50V, and 60V. For each voltage applied, the system was operated for a duration of 12 min. An average of 10 µl fractionalized semen was taken out at the collection site at every 2-min interval. Every fractionated sperm was then evaluated for percentage of viability, motility, and DNA damage assessment. Result showed that electrophoresis at 20V and 6 min yielded more than 80% viable and more than 70% motile sperm population with the lowest DNA damage. In conclusion, the system was able to fractionate high quality bull sperm at 20V and 6 min.

Keywords: Bull Sperm; sperm DNA; electrophoresis separation; sperm motility; sperm viability

ABSTRAK

Sistem pemisahan secara elektroforesis telah berjaya diaplikasikan dalam penyediaan sperma manusia sebelum teknik bantuan kehamilan (ARTs) dilaksanakan. Sistem ini direka untuk mengatasi masalah penghasilan spesies oksigen reaktif (ROS) dari proses pengemparan semasa proses penyediaan konvensional. Kajian lepas menunjukkan hasil yang memberangsangkan pada semen manusia. Maka, kajian ini dilakukan untuk mengaplikasikan teknik yang sama terhadap penyediaan sperma haiwan khususnya sperma lembu. Semen segar dari lembu jantan dewasa telah digunakan. Penentuan voltan yang optimum untuk pemisahan sperma lembu dilakukan dengan menggunakan kekuatan voltan dan masa pemisahan yang berbeza. Voltan yang digunakan ialah 10V, 20V, 30V, 40V, 50V dan 60V. Masa yang digunakan bagi setiap voltan ialah 12 minit. Secara purata, sebanyak 10 µl sampel diambil dari tempat pengumpulan sampel pada setiap sela masa 2 minit. Bagi setiap sampel yang diasingkan, peratusan viabiliti, motiliti dan kerosakan DNA ditentukan. Hasil kajian menunjukkan pemisahan pada 20V dan minit ke-6 menghasilkan peratusan viabiliti melebihi 80%, peratusan motiliti melebihi 70% dan kerosakan DNA yang paling rendah. Kesimpulannya, sistem ini berupaya memisahkan sperma yang berkualiti pada 20V dan minit ke-6.

Kata kunci: Sperma lembu; DNA sperma; pemisahan elektroforesis; motiliti sperma; viabiliti sperma

INTRODUCTION

Sperm preparation is a mandatory step prior to any assisted reproductive techniques (ARTs). Sperm have to be separated from seminal plasma soon after semen collection to avoid increase in percentage of decapitated sperm (Mortimer 2000; Parks 2013). Sperm preparation also separate motile and viable sperm population from non-motile sperm cells, dead cells, cell debris, leukocytes and other contaminating microorganisms (Henkel & Schill 2003; Moreno et al. 2017).

The most commonly applied conventional sperm preparations are swim-up and density gradient centrifugation. Swim-up procedure isolates sperm population with high motility (Esteves et al. 2000; Beydola et al. 2013). On the other hand, density gradient centrifugations have the ability to isolate sperm population according to its density. As completely mature sperm cells have a higher cell density compared with sperm containing cytoplasmic droplet, density gradient centrifugation is therefore efficient in isolating sperm with normal morphology and motility (Malvezzi et al. 2014). Thus, utilization of either procedure will ensure that only sperm with the required quality and characteristics are used for ARTs.

As the technique requires centrifugation, excessive ROS production (Aitken and Clarkson 1988; Lampiao et al. 2010) and direct destructive mechanical forces

(Alvarez et al. 1993; Len et al. 2010) could damage the sperm. Therefore, the ability of conventional sperm preparation techniques to isolate good quality sperm is questionable. It is speculated that the above techniques are likely to produce sperm with unacceptable damage (Sakkas et al. 2000). This would be translated into an unfavorable ARTs outcome.

Several new techniques have been put forth to overcome the drawbacks of the conventional sperm preparation procedures. This include a novel sperm preparation procedure known as electrophoresis sperm separation which was successfully applied for separation of human sperm. This new technique is believed to effectively isolate sperm with less DNA damage within a very short time.

This study had implemented this new technique for bull sperm separation. Thus, the objective of the study was to identify the optimal separation voltage and time for electrophoresis required for bull sperm separation.

MATERIAL AND METHODS

SEMEN COLLECTION

A total of 6 fresh semen ejaculates from male adult Charolais bull were collected by using artificial vagina (AV) at National Biotechnology Institute of Veterinary (IBVK), Jerantut, Pahang, Malaysia. Each ejaculation had yielded around 4 to 8 ml of semen. The procedures were performed in accordance to Universiti Kebangsaan Malaysia animal ethics committee (FF-341-2010).

SPERM ELECTROPHORESIS SEPARATION SYSTEM

This present study had designed an electrophoretic separation system for bull sperm separation which had consisted of a 7.5 cm glass tube with 3 holes on the lateral surface. The hole located at the middle was the injection site and the remaining holes were the collection sites. Both ends were sealed off by using Isopore membrane filter with a pore size of 3 µm (Figure 1). This was to ensure that the sperm remained within the glass tube during separation. Later, the glass tube was dipped into a specially designed electrophoresis tank filled with Bioxcell® extender. This extender had acted as a buffer and supplied nutrient to the sperm. A constant volume of 20 ml buffer was used throughout the experiment. The buffer was allowed to fill into the glass tube slowly to ensure that no bubble was present in the tube. Following this, 30 µl of fresh semen was then injected into the injection site. Voltage was then immediately applied and time was recorded.

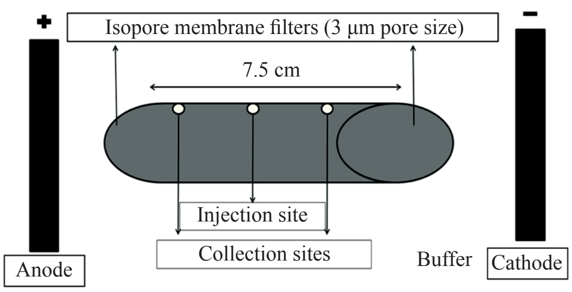

FIGURE 1. Schematic diagram of sperm electrophoretic separation system

OPTIMIZATION OF VOLTAGE AND TIME OF SEPARATION

Separations of fresh semen were conducted at different voltages - 10V, 20V, 30V, 40V, 50V and 60V. For each voltage, the system was operated for the duration of only 12 min. Three repetition were done for each voltage. An average of 10 µl fractionalized semen was taken out at the collection site every 2-min interval. The percentage of viability, motility and DNA damage for each sub-population were then calculated. Based on these parameters, the optimized voltage and duration were determined.

PERCENTAGE OF MOTILE AND VIABLE SPERM

Percentage of motile sperm for each sub-population was assessed using a Makler Chamber (10 µm deep; Sefi Medical Intrument Ltd., Haifa, Israel). Sperm were classified as A (fast progressive movement), B (slow progressive movement), C (non-progressive movement) and D (non-motile) (Kvist and Björndahl 2002). Percentage of motile sperm was counted as A+B/ total counts of sperm (A+B+C+D) x100. While the percentage of viable sperm were calculated as A+B+C/ total counts of sperm (A+B+C+D) x 100 (Ibrahim et. al, 2013).

DNA DAMAGE

Assessment of DNA damage was done using neutral Comet Assay. The protocol was optimized according to Boe-Hansen et al. (2005). The sample for every collection site was mixed with 0.5% low melting agar and then casted on 1% normal melting agar pre-treated frosted slide. These slides were then incubated in lysis buffer which consisted of 2.5 M NaCl, 100 mM EDTA, 10 mMTris Base, 1% Triton X-100 and 40 mM DTT. After 1 hour of incubation, the slides were then transferred into a 37ºC pre-warmed lysis buffer with an additional 500 µl of 10 µg/ml proteinase K. The incubation was then continued for another 24 hours. Subsequently, these slides were then washed 3 times with distilled water and immediately transferred to an electrophoresis tank. Electrophoresis buffer had consisted of 54 g/l Tris Base, 27.5 g/l boric acid and 0.5 M EDTA. The slides were then left for 20 min in the electrophoresis buffer before a voltage was applied for another 20 min at 25V (0.01 A). The slides were then neutralized using neutralizing buffer consisted of 40 mMtrisHCl, 2 mg/ml spermine and 50%

ethanol. Subsequently, the slides were stained with 10 µl of 50 µg/ml ethidium bromide. About 200 sperm cells were counted for each replicate of every sub-population and a total of 3 replicates were prepared for each of the sub-population.

STATISTICAL ANALYSIS

The differences for all parameters between groups were analyzed using SPSS software version 12.0.1. Analysis of variance (ANOVA) with Post-Hoc (Tukey) test was performed. Results were expressed as mean ± SEM. For DNA damage severity, Mann-Whitney U-Test was performed. Any results were considered significant when $p < 0.05$.

RESULTS

The optimum voltage and time of separation were based on the percentage of viable sperm, motile sperm and DNA damage status in each sub-population. Result for percentage of viable sperm showed that there were no significant differences between 10, 20, 30 and 40V for every minute of separation. All of the voltages applied were able to yield more than 70% of viable sperm. However, viability reduced significantly at 50V, 10 until maximum run time of 12 min ($F(5,12) = 38.331$, $p = 0.01$). At 60V, no viable sperm was found at 8 to 12 min ($F(5,11) = 38.925$, $p = 0.001$) (Table 1).

TABLE 1. Percentage of viable sperm for each minutes and voltage applied

Voltage/V	Separation time/minutes					
	2	4	6	8	10	12
10 (n = 6)	89.06 ± 3.58	93.31 ± 1.48	80.93 ± 7.57	85.56 ± 2.93	81.82 ± 2.22^{e,f}	74.88 ± 0.79^{e,f}
20 (n = 6)	80.76 ± 0.21	84.87 ± 4.22	81.11 ± 2.30	83.56 ± 5.43^{f}	75.69 ± 4.08^{e,f}	73.77 ± 4.62^{e,f}
30 (n = 6)	76.26 ± 4.88	79.66 ± 1.75	82.90 ± 3.38	77.97 ± 8.00^{f}	80.15 ± 5.81^{e,f}	77.91 ± 6.57^{e,f}
40 (n = 6)	84.42 ± 4.32	88.15 ± 2.11	87.43 ± 3.60	84.70 ± 2.22^{f}	87.26 ± 2.50^{e,f}	75.27 ± 8.18^{e,f}
50 (n = 6)	86.64 ± 3.58	87.00 ± 0.56	83.21 ± 7.43	68.64 ± 2.28^{f}	28.74 ± 11.86^{f}	8.97 ± 7.14
60 (n = 6)	87.99 ± 8.14	81.07 ± 7.18	93.08 ± 4.44	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values represent means ± standard error mean (SEM). ^{e,f}supercripts within the same column show significant difference with 50V and 60V respectively ($p < 0.05$).

Percentage of motile sperm showed similar trend as viability. There were no significant differences for 10, 20, 30 and 40V applied for every minute of separation. However, the percentage of motile sperm declined significantly at the application of 50V, 8 min ($F(5,12) = 38.331$, $p = 0.01$) until the end of run time. Interestingly, no motile sperm was found at the application of 60V, 8 min until the end of run time ($F(5,9) = 55.723$, $p = 0.001$) (Table 2).

In order to elucidate the cause of declined in the percentage of viable and motile sperm, temperature of the buffer for each of the applied voltage were measured. Based on Figure 2, the noticed that temperature began to rise significantly during the application of 40V ($F(5,12) = 106.302$, $p = 0.01$) at 12 min run. The buffer temperature has also risen dramatically when 50V ($F(5,12) = 106.302$, $p < 0.05$) and 60V ($F(5,12) = 106.302$, $p = 0.01$) were applied.

The utilization of voltage 50V and 60V had been discontinued based on the unfavorable yields in percentage of viable sperm, motile sperm and buffer temperature elevation. Study was then only further conducted on sperm DNA damage using 10V, 20V, 30V and 40V. Result showed that sperm population separated at 10V ($z = -2.185$, $p < 0.05$), 30V ($z = -4.989$, $p = 0.001$) and 40 V ($z = -7.477$, $p = 0.001$) had higher DNA fragmentation compared to 20V.

TABLE 2. Percentage of motile sperm for each minutes and voltage applied

Voltage/V	Separation time/minutes					
	2	4	6	8	10	12
10 (n = 6)	86.63 ± 4.43	90.01 ± 3.62	69.87 ± 7.23	80.19 ± 2.70^{f}	76.65 ± 1.89^{e,f}	67.81 ± 1.70^{e,f}
20 (n = 6)	75.02 ± 0.83	80.51 ± 5.69	77.27 ± 2.45	79.92 ± 5.25^{f}	69.76 ± 3.21^{e,f}	68.14 ± 4.16^{e,f}
30 (n = 6)	67.38 ± 6.23	75.80 ± 2.55	77.30 ± 2.91	83.89 ± 3.53^{e,f}	76.46 ± 7.78^{e,f}	69.74 ± 9.26^{e,f}
40 (n = 6)	81.18 ± 4.32	86.11 ± 2.80	85.41 ± 3.58	82.91 ± 2.94^{e,f}	75.32 ± 7.74^{e,f}	76.73 ± 12.83^{e,f}
50 (n = 6)	81.99 ± 5.75	85.71 ± 0.60	79.80 ± 8.96	60.02 ± 6.45^{f}	7.68 ± 3.88	3.85 ± 2.22
60 (n = 6)	84.19 ± 8.01	79.00 ± 7.58	90.88 ± 5.65	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Values represent means ± standard error mean (SEM). ^{e,f}supercripts within the same column show significant difference with 50V and 60V respectively ($p < 0.05$).

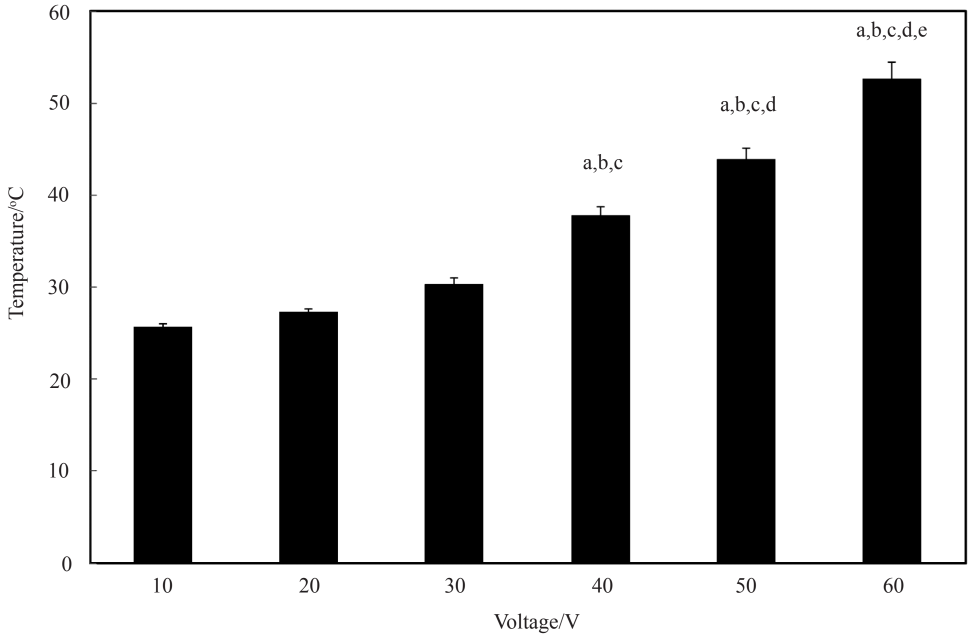

FIGURE 2. Temperature of the buffer for each voltage applied for 12 min electrophoresis duration. [a]significantly different compared to 10V; [b]significantly different compared to 20V; [c]significantly different compared to 30V; [d]significantly different compared to 40V; significantly different compared to 50V

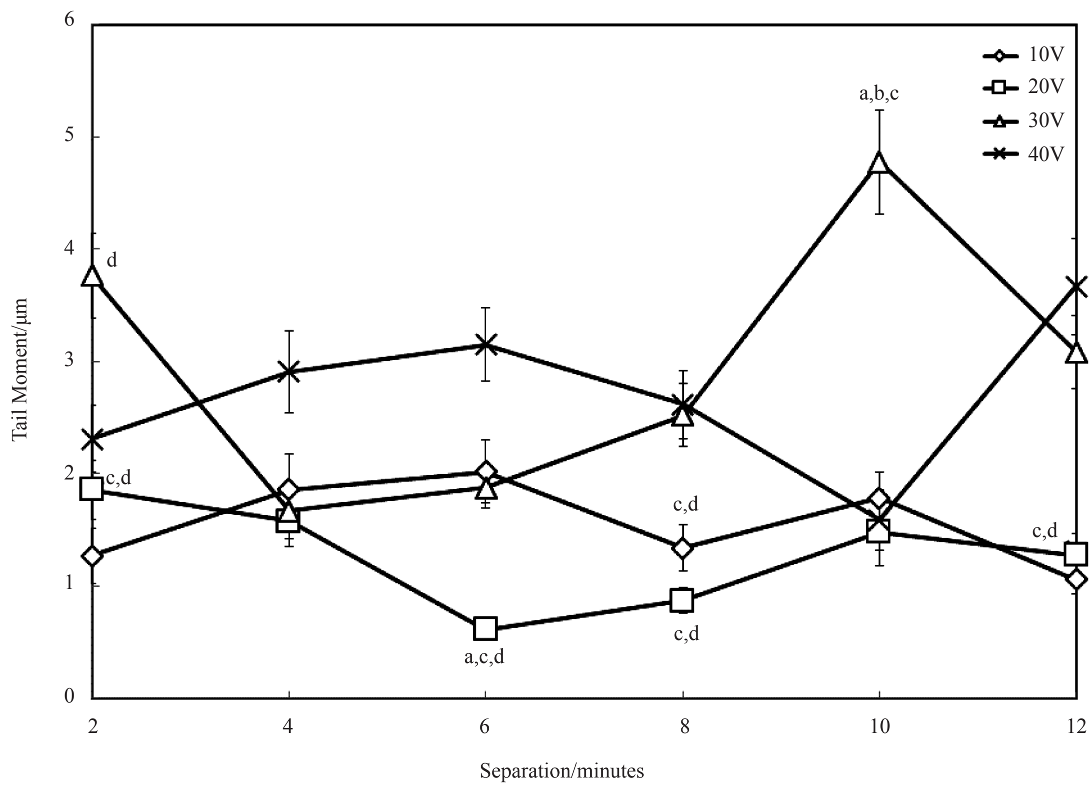

FIGURE 3. Tail moment of sub-population isolated at 10 to 40V for every 2 min of separation. [a]significantly different compared to 10V; [b]significantly different compared to 20V; [c]significantly different compared to 30V; [d]significantly different compared to 40V; [e]significantly different compared to 50V; fsignificantly different compared to 60V

The sperm DNA damage at the application of 20V and 6 min separation time demonstrated a significantly lower DNA damage compared to 2 min (z = −4.678, p = 0.001), 4 min (z = −3.084, p = 0.01), 10 min (z = −2.760, p = 0.01) and 12 min (z = −4.614, p = 0.001) (Figure 3) run time.

DISCUSSION

This current study had successfully developed and implement a new sperm separation system known as sperm electrophoresis separation system for animal model. The system was also to circumvent the various problems associated with swim-up and density gradient centrifugation. During this study, optimization of voltage and duration of separation was done based on the percentage of viable sperm, percentage of motile sperm and the severity of the DNA damage.

Similar changes in viability and motility of sperm had also been reported by Aitken et al. (2011). The authors demonstrated that there was impairment in sperm motility when sperm were subjected through an electrophoretic separation at 12V for 10 min. When the electrophoresis duration was extended to 30 min, there were no motile sperm and only a small amount of sperm was still viable. The authors also performed oxidative stress test to confirm the mechanisms on how such impairment occurred. However, the extension of electrophoresis duration did not give significant effect on ROS production (Aitken et al. 2011). Thus, the impairment of the fractionation at high voltage might be due to increase in buffer temperature.

In order to validate this postulation, this study measured the buffer temperature at every electrophoresis run. The data demonstrated that there was an increased in buffer temperature as the voltage increase above 40V. Consequently, the percentage of viable sperm and percentage of motile sperm at 50V and 60V had decline tremendously at 8 min of separation. The mechanism by which the sperm motility and viability were affected by the rise of the separation buffer temperature still remains unclear.

However, it is believed that the increase in temperature had caused deterioration of several important pathways that regulates sperm motility. Two main pathways that could have been affected are (1) cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway (Lefievre et al. 2002; Lachance et al. 2014) and (2) Ca^{2+} signaling pathway (Ho et al. 2002; Chung et al. 2014; Chung et al. 2017). Both pathways are connected by an enzyme known as soluble adenylyl cyclase (sAC) (Carlson et al. 2007). Since enzymes are very sensitive to temperature changes (Daniel et al. 1996), thus elevation of temperature at high voltage might had caused denaturation of the afore mentioned enzymes. This in turn would cause sperm to lose their motility. However, the involvement of this pathway remains a postulation until further research is done in the future.

Based on severe impairment of sperm viability and motility in the present study, the application of 50V and 60V for sperm electrophoresis had been discontinued. Further determination of DNA damage was only done on populations separated at 10, 20, 30 and 40V. The results clearly demonstrate that the application of 30V and higher had caused greater DNA damage in the sperm population. On the other hand, high DNA damage recorded on the application of 10V might be due to the insufficient strength of the applied voltage to separate the injected sperm into extensive and minimal damaged DNA sperm groups.

The probable cause of the high DNA fragmentation observed in the sperm population separated at 30V and 40 V is might be due to the presence of high electrical charges which had lead to irreparable damage to the membrane (Rajasekaran et al. 1994). High electrical current applied in this study had probably affected the acrosome of the sperm. This would have probably caused a dramatic reduction of acrosome membrane potential. Enzymes contained within the acrosome will then leak out and bind to the other part of sperm membrane which eventually had caused severe membrane damage (Guha 1988). These damages had probably lead to exposure of sperm nucleus to the external environment and eventually caused extensive DNA damage.

Since the percentage of viable and motile sperm data were not conclusive, this study therefore relies on the significant different of sperm DNA damage. Based on the findings, this study concludes that bull sperm were best separated at 20V and 6 min by using this system.

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Farah Hanan Fathihah Jaffar
Siti Fatimah Ibrahim
Mohd Iswadi Ismail
Department of Physiology
Faculty of Medicine
Universiti Kebangsaan Malaysia
56000 Jalan Yaacob Latif
Bandar Tun Razak, Kuala Lumpur
Malaysia

Chew Fang Nang
Khairul Osman
Balkhis Bashuri
Centre for Applied Health Sciences
Faculty of Health Science
Universiti Kebangsaan Malaysia
50300 Jalan Raja Muda Abdul Aziz, Kuala Lumpur
Malaysia

Syarifah Faezah Syed Mohamad
Faculty of Applied Sciences
Universiti Teknologi Mara
26400 Jengka, Pahang
Malaysia

Nur Hilwani Ismail
Faculty of Applied Sciences
Universiti Teknologi Mara
40450 Shah Alam, Selangor
Malaysia

Nurol Afiza Abdul Wahab
National Veterinar Biotechnology Institute
27000 Jerantut, Pahang
Malaysia

Corresponding author: Khairul Osman
E-mail: khairos@ukm.edu.my

Tel: +603-9289 7607
Fax: +603-2693 8717

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