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Evaluation of Glucose-BSA as Extender of Chilled Canine Spermatozoa for Long-term Storage

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장기간 냉각 보존을 위한 개 정자 희석액으로서 Glucose-BSA의 유효성 평가

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SUMMARY

본 연구의 목적은 3주 동안 장기간 냉각상태로 보관된 개 정자의 기능적 특성을 알아보고자 두 종류의 정자 희석액, Glucose-BSA(G-BSA), Dimitropoulos-II(DIMI)가 정자의 기능적 특성에 미치는 영향을 알아보고자 한다. 개 정액을 수지법에 의해 채취하여 원심분리한 후 정장은 제거하였다. 정자를 G-BSA나 DIMI으로 희석하여 최종 농도를 100×10^6 sperm/ml로 하였다. 희석된 정자를 정자 운송 시스템을 이용하여 루지애나 주립 대학에서 뉴올리언즈 실험실까지 운송하였다. 정자 운송 시스템은 20℃에서 9℃까지 분당 0.08℃ 냉각율로 설정하였다. 희석된 정자는 4℃에서 3주간 보관하였다. 보관 1일부터, 매주 단위로 정자의 활력, 정자 원형질막 고유성(생존성), 정자 첨체의 고유성을 검사하였다. 또한 체외조건하에서의 정자 번식 능력을 평가하기 위해 zona binding assay를 실시하였다. 실험 결과 냉각 상태로 3주 동안 보관된 개 정자는 여전히 정자의 기능적 특성을 나타냈다. 냉각기간이 길어질수록 정자의 활력 및 생존성은 감소하였으며($P<0.01$), 첨체의 고유성은 냉각기간에 따라 감소하였으나 유의성은 없었다. 정자의 특성 중 활력이 다른 특성에 비해 냉각처리에 민감한 반응을 보였다. G-BSA 배지에 보관된 정자의 활력 및 생존성이 DIMI에 비해 높게 나타났으며, 1 주 동안 보관된 정자의 생존성의 비교시 유의성이 높게 나타났다($P<0.05$). 그러나 첨체의 고유성은 1일 동안 DIMI에 보관된 정자에서 높게 나타났다($P<0.05$). 3주 동안 냉각상태로 보관된 개 정자는 여전히 난자의 투명대에 결합하는 능력을 보였으며, G-BSA에 보관된 정자의 경우 난자에 부착되는 평균 정자 수가 보관일수에 따라 유의적으로 감소하였다($P<0.05$). 그러나 희석액 종류에 따른 난자에 부착되는 평균 정자 수의 유의적 차이는 없었다. 정자의 활력 및 생존성과 난자의 투명대에 결합하는 정자 수와의 상관관계를 본 결과, 활력 및 생존성이 높은 정자일수록 난자의 투명대에 많이 결합하여 정자의 잠재적 번식력이 증가함을 보였다. 결론적으로 G-BSA나 DIMI의 희석액으로 희석된 후 4℃에서 냉각 보관된 정자는 3주 동안 정자의 기능적 특성을 유지하였으며 냉각 보관 2주까지 좋은 성적의 정자 특성을 나타냈다. 개 정자 냉각 보존에 대안적인 희석액으로 G-BSA도 사용 가능한 것으로 사료된다.

(Key words: dog chilled spermatozoa, G-BSA, DIMI, zona binding assay)

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INTRODUCTION

Chilling and freezing are useful methods to conserve the dog semen for artificial insemination (AI). Chilling is relatively simple and inexpensive method to prepare dog semen (Verstegen *et al.*, 2005). It also comes with the generalized use of in-clinic progesterone assay, and good pregnancy rates and optimum litter size can be achieved (Pena *et al.*, 2006). Canine spermatozoa has been routinely frozen and stored in liquid nitrogen, yet freezing procedure makes intracellular ice in spermatozoa and give the damage to spermatozoa. Dog sperm acrosomes were more damaged after freezing than cold storage (Burgess *et al.*, 2001; Oettle, 1986).

Several studies have determined that the range of 4～5℃ was the appropriate temperature to preserve sperm fertility (Ponglowhapan *et al.*, 2004; Rota *et al.*, 1995; Kumi-Diaka and Badtram, 1994; Bouchard *et al.*, 1990). In most studies, dog sperm characteristics observed for up to 4 days (Rota *et al.*, 1995; Kimi-Diaka, 1994). Properly chilled semen can maintain motility and membrane integrity at least 5～7 days (Pena *et al.*, 2006). There a few observation over a longer period (Ponglowhapan *et al.*, 2004; Iguer-ouada and Verstegen, 2001).

Most studies evaluated motility, membrane integrity or hypoosmotic swelling of chilled canine spermatozoa as the functional sperm characteristics (Rota *et al.*, 1995; Kumi-Diaka and Badtram, 1994; Bouchard *et al.*, 1990). In addition, zona pellucida binding assays provide an essential information of sperm potential fertility (Hermansson *et al.*, 2006; Strom Holst *et al.*, 2000).

Sugars provide the external energy source essential to maintain the motility of chilled canine spermatozoa (Ponglowhapan *et al.*, 2004). Dimitropoulos-II extender (DIMI) is a complex extender composed of glucose, fructose, salt, and egg yolk, whereas glucose-bovine serum albumin (G-BSA) consists of only glucose and bovine serum albumin.

The purpose of the present study was to determine functional characteristics of chilled dog spermatozoa as assessed by motility, membrane integrity, and acrosome integrity, and the potential fertility *in vitro* using zona binding assay during 3 weeks. In addition, we compared two extenders, G-BSA and DIMI to determine an appropriate extender for chilling dog semen.

MATERIALS AND METHODS

1. Experimental Animals

Three dogs (Walker hounds; ages ranging from 1 to 6 years) that have been proven to be fertile were used in this study. All males were housed at the School of Veterinary Medicine, Louisiana State University and routinely used for semen collection and AI program.

The dogs were caged individually and were provided water *ad libitum* and twice daily given Purina dry dog food formulated for laboratory canines. All males were allowed to exercise for 1～1.5 h daily.

2. Semen Collection, Dilution, and Transport

Ejaculates were collected by manual stimulation with no estrous bitch presented. The sperm volume, concentration, motility, and morphology of the ejaculates were evaluated. The sample was centrifuged at 200 g for 5 min, and the supernatant was discarded. The sperm pellet was diluted with one of the semen extenders described below to obtain a sperm concentration of 100×10^6 sperm/ml. Mean sperm motility and membrane integrity were 93% and 88%, respectively when semen was collected.

The spermatozoa were diluted in Glucose-Bovine Serum Albumin extender (G-BSA) (Yu, 2004) or Dimitropoulos-II extender (DIMI) (Yu *et al.*, 2002). G-BSA was composed of 0.33 M glucose supplemented with 3% (w/v) bovine serum albumin (G-BSA).

The diluted sample was then placed into a commercial semen transport system (Pinto *et al.*, 1999) that was constructed to cool from 20 to 9℃ at a rate of 0.08℃/min. The diluted sample was then transported overnight to the laboratory located in New Orleans from Louisiana State University (LSU). The interval between semen collection and studies was ~18 h. Extended semen was divided into four equal aliquots, placed in four cap closed tubes (1.5 ml) and stored at 4℃.

3. Sperm Motility

Sperm concentration was determined using a Neubauer haemocytometer. Progressive sperm percentage was assessed subjectively by microscopic examination at a magnification of 200 × using a scale of 0~4 described by Mortimer (1994).

4. Sperm Membrane Integrity (Viability) and Acrosome Integrity

The integrity of sperm plasma membranes was measured by the method of Garner and Johnson (1995) using the fluorescent double stain Fertilight® (Molecular Probes Inc., Eugene, OR). For each sample, two slides were examined, and appropriately 200 spermatozoa were counted for each slide. For each sample, the number of spermatozoa with green or red fluorescence was counted, and the percentage of membrane-intact spermatozoa (green fluorescence) was calculated.

Acrosome integrity was determined using a method from that described by Yu and Leibo (2002). For each sample, two slides were examined, and approximately 200 spermatozoa were counted for each slide. Percentage of spermatozoa with intact acrosome were calculated.

5. Oocyte Collection and Preparation for the Zona-binding Assay

Dog ovaries were obtained from bitches undergoing ovariohysterectomy at local veterinary clinics. Ovaries were transported to the laboratory in plastic bags containing sterile 0.9% saline solution at ambient temperature (15~25℃). To recover ovaries. ovaries were rinsed with 0.9% saline, placed into a Petri dish containing Dulbecco's PBS (D-PBS), and the tissue was minced into small pieces with a scalpel to release cumulus-oocyte complexes. Then, those oocytes were transferred into 3 ml of 3% sodium citrate buffer in a 5 ml test tube and shaken vigorously to remove cumulus cells. The oocytes were washed with D-PBS containing 0.4% bovine serum albumin, and placed into 0.25 ml straws; the straws were healed, placed into a freezer at −20℃ overnight, and then plunged into liquid nitrogen for storage. For zona-binding assay, straws were thawed for 1min at 37℃, the oocytes washed with canine capacitation medium (CCM, Mahi and Yanagimachi, 1976) and incubated for 10~20 min in a humidified atmosphere of 5% CO_2 in air at 37℃.

6. Zona Binding Assay

Binding of spermatozoa to the zona pellucida of oocytes was assessed by a method from that described by Yu and Leibo (2002). Six oocytes were placed into 100 ul droplets of CCM containing 5×10^6 spermatozoa/ml under mineral oil for 1 min. The oocytes were washed to remove loosely bound sperm by pipetting them vigorously in a fresh CCM, and then incubated for an additional 1 h in a droplet of fresh CCM under oil at 38.5℃ in an atmosphere of 5% CO_2 in air. The oocytes and spermatozoa were stained with Fertilight®. Spermatozoa that were bound to zona and that exhibited green or red fluorescence were counted.

7. Experimental Design

Dog spermatozoa extended with DIMI or G-BSA were stored for 3 weeks at 4℃. Sperm motility, plasma membrane integrity, acrosome integrity, and zona binding assay were assessed on day 1 and every week.

8. Statistical Analysis

The mean percentages of motility, membrane integrity, and acrosome integrity were compared by one way ANOVA with Turky-Kramer multiple comparisons test. Sperm characteristics between G-BSA and DIMI were compared by a paired *t*-Test. The analysis was performed using GraphPad InStat, GraphPad Software, San Diego, CA, USA. Differences were considered significant when $P<0.05$.

RESULTS

The sperm characteristics of dog spermatozoa extended with DIMI or G-BSA and stored at 4℃ during 3 weeks are shown in Fig. 1.

Long-term chilled dog spermatozoa still retained sperm functional characteristics, although motility and membrane integrity were significantly decreased with storage time ($P<0.01$). Acrosome integrity appeared to decrease with storage time but there was not significant difference. About 50∼70% of spermatozoa had intact membrane integrity at weeks 2. Sperm motility was more sensitive to storage time, in that motility decreased abruptly from ∼70 to 10 ∼20%. Acrosome integrity of spermatozoa had stored at 4℃ during 3 weeks was more than 80% regardless of extenders.

G-BSA extender preserved better sperm motility, membrane integrity, and acrosome integrity than DIMI except day 1. However, membrane integrity on only week 1 showed significant difference and acrosome integrities was significantly different on day 1 ($P<0.05$).

Overall sperm characteristics redrawn according to extender are shown in Fig. 2. The mean percentage of sperm motility was the lowest among sperm characteristics regardless of storage time. There were similar appearances in both extenders. Sperm motility was the most sensitive to cooling. Sperm acrosome integrity showed the highest percentages regardless of storage time in both extenders and appeared to be the most resistant characteristic to cooling.

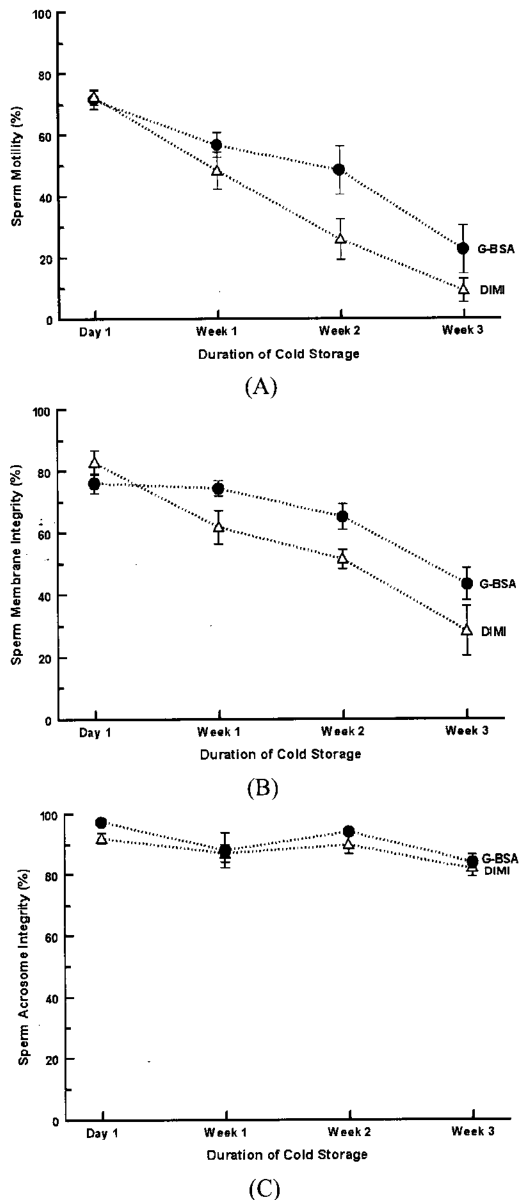
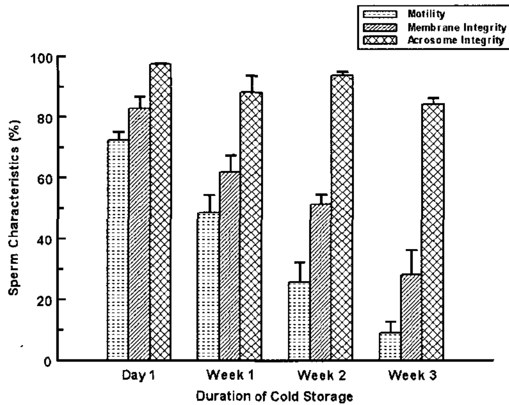
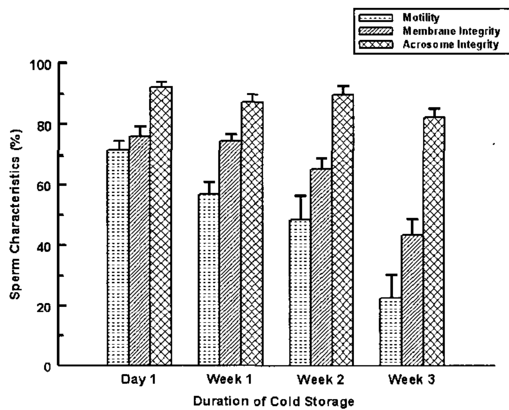


Fig. 1. Sperm characteristics of spermatozoa extended in G-BSA or DIMI and stored during 3 weeks at 4℃ are shown. (A) The mean±SEM percentage of motility is shown. (B) The mean± SEM percentage of membrane integrity is shown. (C) The mean±SEM percentage of acrosome integrity is shown.

The result of zona-binding assay of chilled dog



(A)



(B)

Fig. 2. Overall characteristics of spermatozoa according to extender are shown. (A) Characteristics of spermatozoa extended and stored in DIMI at 4℃ are shown. (B) Characteristics of spermatozoa extended and stored in G-BSA at 4℃ are shown. Mean±SEM for percentage values of motility, membrane integrity and acrosome integrity are shown.

spermatozoa with storage time is shown in Fig. 3. Spermatozoa had been preserved in DIMI or G-BSA for 3 weeks retained their capabilities to bind to canine oocyte zonae. However, mean numbers of spermatozoa bound to zona decreased with storage time in G-BSA ($P<0.05$). There was no significant difference between G-BSA and DIMI but spermatozoa stored in G-BSA had more capability to bind to zonae than those stored in DIMI until week 2.

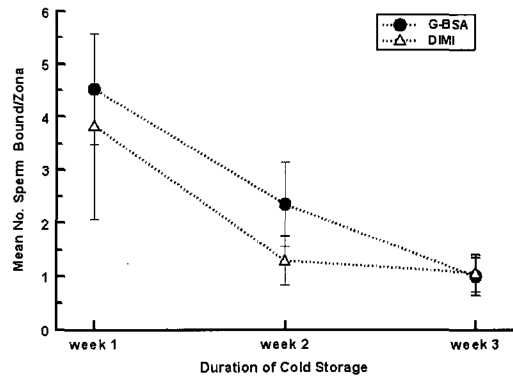


Fig. 3. The mean numbers of spermatozoa bound to oocytes zonae with storage time are shown. Mean±SEM for number of spermatozoa bound to zona is shown.

The motility and membrane integrity of spermatozoa were plotted as a function of the mean number of spermatozoa bound to zonae to determine the correlation between sperm characteristics and the mean number of spermatozoa bound to zonae (Fig. 4). As sperm motility and membrane integrity increased, the mean number of spermatozoa bound to oocyte zona increased regardless of extenders.

DISCUSSION

The possibility of conserving motile spermatozoa in cold condition has been reported (Ponglowhapan *et al.*, 2004; Rota *et al.*, 1995; Kumi-Diaka and Badtram, 1994). The long-term storage of dog spermatozoa extended up to 20 days (Verstegen *et al.*, 2005). In the present study, dog spermatozoa had stored at 4℃ for 3 weeks retained sperm characteristics. However, the overall sperm characteristics decreased with storage time (Fig. 1). Sperm motility appeared to much more sensitive to the cool storage condition, whereas sperm acrosome integrity was the most resistant among sperm characteristics (Fig. 2). There were studies showing a similar tendency to our findings (Ponglowhapan *et al.*, 2004; Yu *et al.*, 2002). Sperm motility is an indicator for the

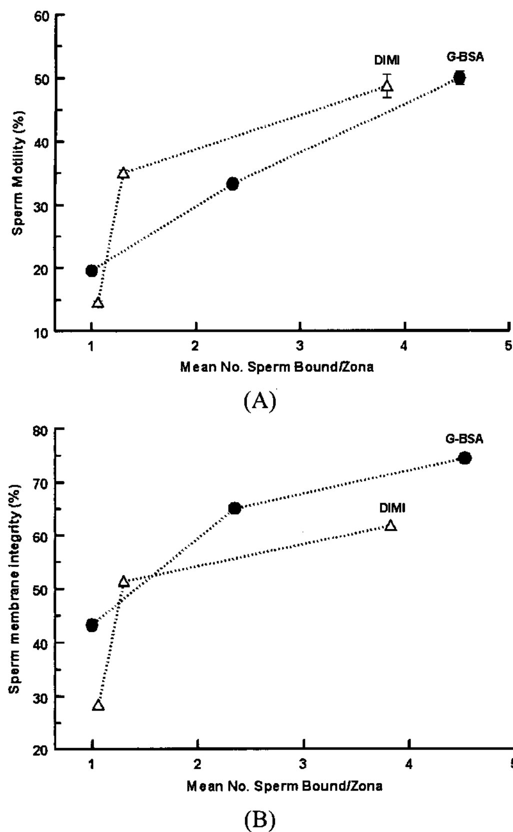


Fig. 4. The motility and membrane integrity of spermatozoa are plotted as a function of the mean number of spermatozoa bound to zonae. (A) Correlation between sperm motility and mean number spermatozoa bound to zonae is shown. (B) Correlation between sperm membrane integrity and mean number of spermatozoa bound to zonae is shown. Mean±SEM for number of spermatozoa bound to zona is shown.

viability of spermatozoa (Ponglowhapan *et al.*, 2004). This study also demonstrates that sperm motility is the most critical characteristic of spermatozoa chilled and preserved.

The percentages of membrane integrity and motility of spermatozoa stored in G-BSA was higher than those stored in DIMI except day 1. DIMI is mainly composed of fructose and glucose and is supplemented with other components. G-BSA is a simple extender composed of glucose and BSA. This result considers that glucose could be used as single energy substrate for sperm cold storage. Ponglowapan *et al.* (2004) indicated that dog sperm used glucose in preference to fructose. G-BSA might be used as an alternative extender for the long-term cold storage in clinical application of semen preservation.

Number of spermatozoa bound to zonae decreased with storage time (Fig. 3). One day chilled semen had better zona binding capacity than had been chilled for 2 days prior to zona binding (Hermansson *et al.*, 2006). Strom Holst *et al.* (2000) found that the number of spermatozoa bound was decreased on day 4 when it was compared to that on day 1. These were in agreement with our result, although above results did not assay zona binding capacity of spermatozoa for the long chilling period.

The zona binding capacity of dog spermatozoa diluted in a tris-citric acid-fructose and stored at 4℃ reduced after 4 days and the mean number of spermatozoa bound to zona pellucida *in vitro* was reduced from 4.8 on day 1 to 0.9 on day 4 (Strom Holst *et al.* 2000). In the present study, mean spermatozoa bound to oocyte zonae was about 1 on week 3 regardless of extenders and more than 2 spermatozoa preserved in G-BSA bound to zona. Further studies are required to attempt AI for sperm survival *in vivo* or perform intracytoplasmic sperm injection using small number of spermatozoa.

The motility of spermatozoa stored at 4℃ was plotted as a function of the mean number of spermatozoa bound to zonae (Fig. 4A). We found that these two characteristics appear to be correlated, although there was difference between two extenders. As the mean percentage of sperm motility was higher, the mean number of spermatozoa bound to zonae increased. The sperm membrane integrity plotted as a function of the mean number of spermatozoa bound to zonae showed a similar tendency to Fig. 4A (Fig. 4B). Sperm membrane integrity also correlated with the mean number of spermatozoa

bound to zonae. These results confirmed that decrease of motility and membrane integrity caused decrease of sperm capability to bind to zonae and motility and membrane integrity are critical assays to estimate sperm fertility *in vitro*.

In conclusion, the present study demonstrates that chilled dog spermatozoa can be conserved up to 3 weeks and preserved with good sperm characteristics for at least 2 weeks. Chilled dog spermatozoa retained their ability to bind to zona pellucida even after 3 weeks. G-BSA might be also used as an alternative extender instead of an usual extender like tris-egg yolk for dog sperm chilling and storage.

CONCLUSION

The purpose of this study was to determine functional characteristics of canine chilled spermatozoa for 3 weeks and compared two extenders; Glucose-BSA (G-BSA) and Dimitropoulos-Ⅱ extender (DIMI).

Dog semen was collected by digital manipulation and centrifuged, and seminal plasma was removed. Spermatozoa were diluted with G-BSA or DIMI and sperm concentration was adjusted to 100×10^6 sperm/ml. The diluted sample was then placed into a commercial semen transport system that is constructed to cool from 20 to 9℃ at a rate of 0.08 ℃/min. The diluted sample was then transported overnight to the laboratory located in New Orleans from Louisiana State University (LSU) and stored at cold room (4℃) for 3 weeks. Sperm motility, plasma membrane integrity, and acrosome integrity were evaluated on day 1 and every week. Zona binding assay was also performed to find whether chilled spermatozoa could have an potential fertility to bind to dog oocytes.

Dog spermatozoa stored at 4℃ still retained sperm functional characteristics although they preserved for 3 weeks. The overall sperm characteristics were significantly decreased with storage time ($P<0.01$), whereas there was not significant decrease in acrosome integrity. Sperm motility was the most sensitive to cold storage. Membrane integrity and motility of spermatozoa stored in G-BSA were higher than those stored in DIMI. However, there was a significant difference between two extenders on only week 1. Acrosome integrity between two extenders was significantly different on only day 1. Spermatozoa preserved in DIMI or G-BSA for 3 weeks still showed their capabilities to bind to canine oocyte zona. Mean numbers bound to zona significantly decreased with storage time in G-BSA ($P<0.05$). There was no significant difference between DIMI and G-BSA. In plotting sperm membrane integrity and motility as a function of the mean number of spermatozoa bound to zonae, there was correlation between these sperm characteristics and the mean number of spermatozoa bound to zonae. The higher the mean percentage of sperm motility and membrane integrity was, the more the number of spermatozoa bound to zonae increased.

In conclusion, chilled dog spermatozoa can be conserved up to 3 weeks and preserved with good sperm characteristics for at least 2 weeks. G-BSA may be also used as an alternative extender for dog sperm chilling and storage.

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