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Separation of Highly Motile Bovine Spermatozoa

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=국문초록=

고활력 우정자의 분리

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소의 번식효율을 증가시키기 위한 기초실험으로서 6%, 10% 및 20%의 bovine serum albumin을 사용한 선인들의 방법과 tyrode액을 사용하여 시도한 저자의 방법으로 소의 원정액, 희석정액 및 일반시판 냉동정액으로부터 고활력정자를 분리수집하여 정자의 각종 성상과 광학현미경적 형태를 비교관찰한 결과는 다음과 같다.

1. 원정액으로부터 bovine serum albumin을 사용하여 분리한 정자는 대조군에 비하여 운동성, 운동성 정자수, 정상정자율 및 전진운동성이 현저히 높았고 정자회수율은 6%일 때 가장 높았다.
2. 원정액으로부터 bovine serum albumin을 사용하여 고활력정자를 분리한 후 냉동한 정액의 정자운동성, 정상정자율 및 전진운동성은 대조군에 비하여 현저히 높았고, 이러한 현상은 bovine serum albumin의 농도가 20%일 때에 가장 현저하게 나타났다.
3. Bovine serum albumin을 사용하여 분리한 고활력정자의 냉동전 및 냉동후의 광학현미경적인 기형율은 대조군에 비하여 현저히 낮았다.
4. Bovine serum albumin을 사용하여 분리한 고활력정자는 전자현미경적으로 세포막의 확장 및 공포형성, acrosome의 확장과 density loss의 변형율이 대조군에 비하여 낮았다.
5. 일반시판 냉동정액으로부터의 고활력정자의 분리는 bovine serum albumin을 사용할 때는 어려웠으나, tyrode액을 사용한 이 실험에서는 가능하였다.
6. 원정액, 희석정액 및 냉동정액의 고활력정자회수율은 tyrode액을 이용하여 80분간 정치하였을때 현저히 높았다.
7. Tyrode액을 이용하여 원정액, 희석정액 및 냉동정액으로부터 분리된 고활력정자의 운동성, 전진운동성 및 정상정자율은 대조군에 비하여 현저히 높았다.
8. Tyrode액을 이용하여 원정액, 희석정액 및 냉동정액으로부터 분리한 고활력정자의 광학현미경적 기형율은 대조군에 비하여 현저히 낮았다.

INSTRUCTION

After Leewenhoek and Hamm first identified sperm under the microscope, the first scientific research in artificial insemination of animals was performed on dogs in 1780 by Spallanzani. Through a lot of studies after that time, artificial insemination in various kinds of domestic animal was used practically in Russia, Denmark, England and United States after 1930's (Reberts, 1971).

Semen which were used at that time were raw semen or extended semen. Egg yolk (Phillips and Lardy, 1940; Salisbury et al., 1941), skim milk (Thacker and Almquist, 1953; Almquist, 1954; Melrose, 1956), buffers (Van Demark and Sharma, 1957; Van Demark and Bartlett, 1957; Foote et al., 1958), antibiotics (Almquist, 1951; Orthey and Gilman, 1954; McEntee et al., 1954), enzymes (Foote and Dunn, 1962) added to extender for in-

crease of fertility rate in use of extended semen. But there was problem, namely possible duration of conception in extended semen could hardly prolong over 4-5 dayes (Almquist and Wickersham, 1962).

But, after Polge et al, reported tghat spermatozoa of animal could be survived at a temperature of-79c, problem of preservation was solved as frozen semen used practically in 1960's (Herman, 1965).

However, about one-half to two-thirds of the motile sperm will be rendered immotile by freezing and thawing (Morrow, 1980).

Thus, as Rutherford (1982) indicated, fertility of frozen semen is low because post-thaw motility is about 35%.

According to author's investigation, post-thaw motility often is below 35% in domestic marketed frozen semen.

We could consider following two methods which could increase fertility by improvement of frozen semen motility.

First method is to improve sperm motility by freezing and thawing of highly motile sperm selectively separated from raw semen.

Second method is to separate and pool highly motile sperm from post-thawed marketed frozen semen.

The hormonal treatment of males with HCG, testosterone and clomiphene, may result in an improvement of the sperm counts but not of the sperm motility (Broer and Dauber, 1978).

Ericsson and others (1973) firstly reported the method which could separated the highly motile sperm, and remove most seminal debris and many morphologically abnormal forms from human semen.

The efficacy of this method was confirmed by Evans et al. (1975). Ross et al. (1975), Glaub et al. (1976), Broer et al. (1977a), David et al. (1977), Jeulin (1980), Singer et al. (1980), Leslie and Quinlivan (1980), Quinlivan et al. (1982), Weeda and Cohen (1982). The method by Ericsson et al. (1973) is wellfounded in physiological swimming ability of sperm.

When human semen was layered on columns of bovine serum albumin, dead sperm and weakly-motile sperm couldn't pass bovine serum albumin of high viscosity.

But highly-motile sperm could pass bovine serum albumin with downward swimming (Glass and Ericsson, 1982).

The fact that highly-motile human sperm selectively could be separated by human serum albumin instead of bovine serum albumin, was reported (Dmowski et al., 1979; Binor et al., 1981; Beernink and Ericsson, 1981, 1982; Sherman and Dmowski, 1982).

In patient's semen such as asthenospermia (Broer et al., 1977b; Broer and Dauber, 1978), male factor infertility (Ericsson and Glass, 1977; Glass and Ericsson, 1978; Black et al., 1978) and oligospermia (Koper et al., 1979), and induction of pregnancy in a woman with seminal plasma allergy (Shapiro et al; 1981), highly-motile sperm separation usin human serum albumin successfully was tried.

Also, Faust et al. (1976) and Wall et al. (1980) selectively separated highly-motile govine sperm with bovine serum albumin, and Illyes et al. (1977) froze separated highly-motile sperm.

Illyes et al. (1980) and Rutherford (1982) improved fertility by artificial insemination with frozen, separated highly-motile sperm from bull, and White et al. (1982) reported that morphologically normal sperm selectively was separated.

Also, Goodeaux and Kreider (1979) reported that highly-motile sperm could be separated from stallion's semen with bovine serum albumin, so normal parturition rate was improved by artificial insemination with separated semen.

Dixon et al. (1980) reported that highly-motile sperm could be separated from boar's semen with bovine serum albumin, but significant difference was no in parturition rate by artificial insemination with separted semen.

Well et al. (1980) reported that highly-motile sperm could be separated from rabbit's semen with bovine serum albumin, thus fertility was improved by artificial insemination with separated semen.

Authors tried highly-motile sperm separation using bovine serum albumin in order to increase the

conception rate from the experimental frozen semen and the marketed frozen semen, and then it was possible to improve sperm motility after thawing by freezing of separated highly-motile sperm from liquid semen but it was impossible to separate highly-motile sperm from marketed frozen semen which already was frozen.

It was considered that many difficulties were in separation of highly-motile sperm from raw semen at the sight of demand and supply of semen in Korea.

So authors intensely felt necessity of research which could separate highly-motile sperm from marketed frozen semen.

Authors thought that it might be possible to separate selectively highly-motile sperm from common frozen semen when physiological characteristic of sperm (Sorensen, 1979) which show universal movement were used. Thus, authors report that new theory was obtained as a result of selectively separation experiment using Tyrode's solution.

Fertilization rate could be improved, and abortion rate could be decreased in the cows inseminated with isolated highly motile semen by Tyrode method, as compared with the cows inseminated with non-isolated marketed frozen semen of which motility was 20-40%, meanwhile, human inseminated with sperm isolated by Tyrode method got successfully conception (Chung, 1984).

Sperm analysis in couples with more than 2 daughthers showed significantly lower in sperm motility compared with sperm motility in couples with 2 or more sons, it may suggested one of factor that influenced to be female offspring is the sperm motility rate of that ejaculate sperm. Since Tyrodes method, 6% and 10% albumin method are the method which collect highly motile sperm, How does the sex ratio when thus highly motile sperm is used for insmination?

Do the Y bearing sperm go more rapidly when the highly motile sperm are inseninated, while reading the fine of ovulation?

Secondary sex ratio of cows inseminated artificially and mated naturally were observed in the field, and male ratio of group inseminated artificially was highly than that of group mated naturally.

After all, insemination is that sperm is put into uterine cavity. Even if our data is small, it is assumed that sex ratio will be male predominant if cows inseminated with isolated sperm by Tyrode's solution.

Cows inseminated with marketed frozen semen showed also male prevalent sex ratio is the offspring. Therefore, it is also interesting how the sex ratio is in the cows inseminated with sperm separated by Tyrode's solution.

METERIALS AND METHODS

Meterials

1. Sperm

Sperm were used with raw semen which collected with artificial vagina method from five sexually mature F_1 of Korean native-Charolais bull, and with frozen semen of Korean native bull and Holstein bull.

Semen was classified into 3 kinds of type, namely raw semen, extended semen and frozen semen.

Producing method of extended semena and frozen semen are as follows. Extended semen was made with following method. Semen cooled down until the semen reaches a temperature of 5°C in about 4 hours after 0.5ml of raw semen was diluted with 9.5ml of egg yolk-citrate extender.

Frozen semen was made with following method. Semen cooled down until the sperm reaches a temperature of 5°C in about 4 hours after adding the first extender to the raw semen. And then equal volume of the second extender was added to the first either in 4 equal amounts at 20 minutes intervals. So the number of sperm per 0.5ml straw was maded about 2.5×10^7. Then the temperature was lowered as rapidly as possible to −79°C.

This required about 10 minutes. And it was stored with frozen semen by dipping in liquid nitrogen. Before using the frozen semen was thawed by placing in warm water of 30-35°C for 15-20 seconds.

Human semen samples were obtained from 122 adult males. The ejaculates were collected by

masturbation following a minimum of 48 hours of sexual abstinence.

2. Bovine serum albumin (called BSA hereafter)

25% BSA (Sigma Co.) was diluted with Tyrode's solution to make 6%, 10% and 20% BSA. And then it was used.

3. Tyrode's solution

Tyrode's solution which was used in this experiment followed wade (1977) and the prescription was as follows.

Namely 8g NaCl, 0.2g KCl, 0.1g $MgCl_2$ $6H_2O$, 0.05g NaH_2PO_4, 1g Glucose, 1g $NaHCO_3$ and 0.2g $CaCl_2$ were diluted per 1000ml distilled water. So it was adjusted to pH 7.4.

METHODS

1. Sperm isolation using boving serum albumin

1) Faust's method using 6% bovine serum albumin

1.5ml raw semen was diluted with Tyrode's solution at the ratio of 1:1. It was centrifuged at 3000 rpm for 3 minutes. Supernatant was poured off and the pellet was diluted with Tyrode's solution to contain 100×10^6 sperm per ml.

Four tubes (13×125mm) were placed in the stand vertically. 6ml of 6% bovine serum albumin was layered gently into each column. 2ml of sperm-Tyrode (100×6^6 sperm/ml) was layered gently into each column to avoid mix with bovine serum albumin. And it was allowed to remain in each column for one hour. Upper layer from each column and lower layer from each column was pooled respectively. And sperm characteristics were observed.

2) Ericsson's method using 10% bovine serum albumin

1.5ml raw semen was diluted with Tyrode's solution at the ratio of 1:1. It was centrifuged at 3000 rpm for 3 minutes. Supernatants was poured off and the pellet was diluted with Tyrode's solution to contain 20×10^6 sperm per ml. 80 tubes (6×75mm) were placed in the stand vertically. 1ml of 10% bovine serum albumin was layered gently into each column. 0.5 ml of sperm-Tyrode (20×10^6/ml) was layered gently into each column to avoid mix with bovine serum albumin. And it was allowed to remain in each column for one hour. Upper layer from each column and lower layer from each column was pooled respectively. And sperm characteristics were observed.

3) Ericsson's method using 20% boving serum albumin

1.5ml raw semen was diluted with Tyrode's solution at the ratio of 1:1. It was centrifuged at 3000 rpm for 3 minutes. Supernatant was poured off and the Tyrode's solution to contain 80×10^6 sperm per ml. 20 tubes (6×75mm) were placed in the stand vertically. 1ml of 10% bovine serum albumin was layered gently into each column. 0.5ml of sperm-Tyrode (80×10^6/ml) was layered gently into each column to avoid mix with bovine serum albumin. And it was allowed to remain in each column for 1 hour.

After one hour, upper layer from each column was removed. And lower layer from each column was pooled respectively.

It was centrifuged at 3000 rpm for 3 minutes. Supernatant was poured off and the pellet was diluted with Tyrode's solution to contain 60×10^6 sperm per ml.

Each tube which contains 0.5ml of 20% bovine serum albumin and 1ml of 12% bovine serum albumin was placed in the stand vertically.

0.5ml of sperm-Tyrode (60×10^6/ml) was layered gently into each column. And it was allowed to remain in each column for one hour. After upper layer from each column was removed, it was allowed again to remain in each column for 30 minutes. Upper layer from each column was removed and lower layer from each column was pooled respectively. And sperm characteristics were observed.

Control sperm of this experiment was the sperm which didn't used bovine serum albumin.

2. Characteristics of sperm separated by Tyrode's solution at various time intervals

Raw semen, extended semen and frozen semen were diluted with Tyrode's solution at the ratio of 1:1. It was centrifuged at 3000 rpm for 3 minutes.

Supernatant was poured off and tubes were inclined to 45° angle. 2ml of Tyrode's solution was layered gently into each column. After it was allowed to remain in each column for 20, 40, 60, 80, 100, and 120 minutes respectively, recovery rate of sperm, motility and progressive motility were observed at various time intervals.

3. Sperm isolation using Tyrode's solution

Experimental method was same as method 2 which is preparatory experiment, but stationary time was 80 minutes in this experiment because recovery rate of sperm was the highest at 80 minutes. 1.5ml of upper layer was collected gently with pasteur pipette and it was named Tyrode layer. Remaining part in each column was named lower layer. And sperm characteristics were observed. Control sperm of this experiment was the sperm which didn't used Tyrode's solution.

4. Freezing experiment of sperm separated by bovine serum albumin Each experimental group separated by bovine serum albumin was frozen and thawed with the above-mentioned method. Prefreeze and post-thaw sperm characteristics were observed, as compared with control sperm.

1) Observation of sperm characteristics

Observation of total sperm count, motile sperm count and motility followed Sorensen method (1979), and progressive motility followed Glaub method (1976).

2) Observation of sperm morphology

Light-microscopic morphology was examined under 1000X microscope using hematoxylin-eosin staining (Schoenfeld et al., 1981).

Normal sperm, microcephalic sperm, macrocephalic sperm, absent sperm head, amorphous sperm, double tailed sperm, tail abnormality and immature sperm were differentiated from each other, standard of differentiation were as follows.

microcephalic sperm: sperm which is below 4u in head's length and is below 2u in head's wideht.

macrocephalic sperm: sperm which is above 12u in head's length and os above 6u in head's length and is above 6u head's width.

Absent sperm head: sperm which has no head.

Amorphous sperm: sperm which has abnormal figure in head's morphology.

Double tailed sperm: soerm which has two tails.

Tail abnormality: sperm which has coiled, bent or short tail.

Immature spem: sperm with a cytoplasmic droplet.

Electron microscopic morphology was examined after each specimen was processed as Luft (1961).

Collected specimen was fixed with 3% glutaraldehyde.

It was washed twice with phosphate buffer (pH 7.4).

Upper layer was removed after it was centrifuged at 2000 rpm. The pellet was embedded in 2% agar, postfixed for 2 hours at 4°C in osmium tetraoxide.

The pellet was dehydrated in ethanol and propylene oxide, embedded in Epon solution, incubated at 60°C for 3 days.

Thin sections were stained lead citrate and uranyl acetate and viewed with an electron microscope (H-500, Hitachi). Intact, dilation and vesiculation of cell membrane, intact, dilation and density loss of acrosome were observed.

5. Statistical analysis

The significance of differences between two experimental group was dealt with t-test and the significance of differences between three experiment or more was performed by F-test.

RESULTS

Selective separation of highly motile sperm from bovine semen was tried by different methods using 6%, 10% and 20% bovine serum albumin and Tyrode's solution respectively. The separated sperm were examined for motility, motile sperm count, percent of normal sperm, progressive motility, percent of sperm recovery and morphological characteristics. The results from each method were compared with each other.

Table 1. Light Microscopic Characteristics of Marketed Frozen Semen Passed Through 6% Bovine Serum Albumin (n=10; mean±S.D.)

		6% BSA	Control
Total sperm count ($\times 10_6$)		5.1±1.77	24.5±0.97
Motility (%)		17.8±4.29	22.5±4.72
Motile sperm count ($\times 10_6$)		0.9±0.44	5.4±1.06
Normal sperm (%)		94.6±1.43	88.6±2.17
Progressive motility		2.1±0.31	1.9±0.33
Sperm recovery (%)	Total	21.2±7.26	
	Motile	16.9±6.27	

BSA: Bovine serum albumin
Progressive motility: Glaub (1976)

Table 2. Light Microscopic Characteristics of Raw Bovine Semen Passed Through Bovine Serum Albumin (n=10; mean ±S.D.)

		6% BSA	10% BSA	20% BSA	Control
Total sperm count ($\times 10_6$)		252.8 ±29.10	211.0±37.13	60.8 ±14.76	800.0**
Motility (%)		83.6 ± 8.28	86.5± 7.58	95.2**± 3.82	48.0 ± 8.56
Motile sperm count ($\times 10_6$)		211.3**±43.20	182.7±45.26	57.6 ±15.57	384.0 ±68.51
Normal sperm (%)		95.5 ± 1.62	95.1± 2.42	96.4* ± 1.71	85.6 ± 5.68
Progressive motility		3.7 ± 0.21	3.7± 0.16	3.8**± 0.17	2.7 ± 0.23
Sperm recovery (%)	Total	31.6**± 3.64	26.4± 4.64	7.6 ± 1.90	
	Motile	55.0**± 3.29	47.8± 6.30	15.0 ± 2.49	

BSA: Bovine serum albumin **: P<0.01 *: P<0.05 Progressive motility: Glaub (1976)

1. Sperm separation using bovine serum albumin

1) Observation of sperm separated by bovine serum albumin

Frozen semen: The sperm separated from frozen semen using bovine serum albumin was observed. The result was summerized in Table 1. Motility of separated sperm and control sperm was 17.8±4.29 and 22.5±4.72 respectively. So motility of separated sperm was lower than that of control sperm. Normal sperm (%) of separated sperm and control sperm was 94.6% and 88.6% respectively. Therefore it had no meaning to separate highly motile sperm from marketed frozen semen using bovine serum albumin.

Raw semen: The highly motile sperm separated from raw semen using 6%, 10% or 20% bovine serum albumin was observed, and the result was summerized in Table 2.

Total sperm count: Sperm separated from 800×10^6 sperm were 252.8±29.10 (×10^6), 211.0±37.13 (×10^6) and 60.8±14.76 (×10^6) in 6%, 10% and 20% BSA group. Therefore sperm separated by 6% bovine serum albumin showed the highest total sperm count in separation experiment group (P<0.01).

Sperm motility: Sperm separated by 6%, 10% and 20% bovine serum albumin, and control sperm were 83.6±8.28, 86.5±7.58, 95.2±3.82 and 48.0± 8.56%. Motility of three experiment group was higher in all than that of control group. Sperm separated by 20% bovine serum albumin showed the highest value among separation experiment groups.

Motile sperm count: Sperm separated by 6%, 10% and 20% bovine serum albumin, and control sperm were 211.3±43.2(X10^6), 182.7±45.26

($X10^6$), $57.6 \pm 15.57V(X10^6)$ and 384.0 ± 68.51 ($X10^6$) respectively. Motile sperm count of three experiment group was smaller than that of control group ($P<0.01$). Sperm separated by 6% bovine serum albumin showed the highest number among separation experiment groups.

Percent of normal sperm: Sperm separated by 6%, 10% and 20% bovine serum albumin, and control sperm were 95.5 ± 1.62, 95.1 ± 2.42, 96.4 ± 1.71 and 85.6 ± 5.68% respectively. Values of experiment groups were higher in all than that of control sperm ($P<0.01$). There was no large difference among experiment groups.

Progressive motility: Sperm separated by 6%, 10%, and 20% bovine serum albumin, and control sperm were 3.7 ± 0.21, 3.7 ± 0.16, 3.8 ± 0.17 and 2.7 ± 0.23 respectively. Progressive motility of three experiment groups was higher than that of control group ($P<0.01$). There was no large difference among experiment groups.

Total sperm recovery: Sperm separated by 10% and 20% bovine serum albumin were 31.6 ± 3.64, 26.4 ± 4.64 and 7.6 ± 1.90% respectively. Total sperm recovery of experiment group using 6% bovine serum albumin was significantly higher than that of other groups ($P<0.01$).

Motile sperm recovrey: Sperm separated by 6%, 10% and 20% bovine serum albumin were 55.0 ± 3.29, 47.8 ± 6.30 and 15.0 ± 2.49% respectively. Motile sperm recovery of experiment group using 6% bovine serum albumin was significantly higher than that of other groups ($P<0.01$).

2) Freezing experiment of sperm separated by bovine serum albumin Sperm separated from raw semen using 6%, 10% and 20% bovine serum albumin was frozen and thawed, and observed as shown Table 3.

Sperm motility: They were 57.5 ± 8.78, 60.5 ± 11.1, 68.2 ± 8.48 and 12.8 ± 6.12% respectively when sperm separated by 6%, 10% and 20% bovine serum albumin was frozen and thawed. Motility of three experiment group was higher in all than that of control group. Sperm separated by 20% bovine serum albumin showed the highest value among separation experiment groups ($P<0.01$).

Percent of normal sperm: Sperm separated by 6%, 10% and 20% bovine serum albumin, and control sperm were 94.4 ± 2.37, 94.2 ± 2.39, 95.8 ± 2.10 and 84.1 ± 4.16% respectively. Values of experiment groups were higher in all than that of control group ($P<0.01$). There was no large difference among experiment groups.

Progressive motility: Sperm separated by 6%, 10% and 20% bovine serum albumin, and control sperm were 3.1 ± 0.27, 3.0 ± 0.17, 3.2 ± 0.25 and 2.2 ± 0.21 respectively. Progressive motility of three experiment groups was higher in all than that of control group ($P<0.01$). There was no large difference among experiment groups.

3) Comparison of characteristics of prefreeze and post-thaw sperm separated by bovine serum albumin

Comparative result of characteristics of prefreeze and post-thaw sperm separated by 6%, 10% and 20% bovine serum albumin was summerized in Table 4.

Sperm motility: Post-thaw reduction rate of sperm separated by 6%, 10% and 20% bovine serum albumin were 31.6 ± 4.46, 30.3 ± 9.48 and 28.5 ± 6.89% respectively, and that of control group was 73.6 ± 10.16%. Post-thaw reduction rate of sperm separated by bovine serum albumin was

Table 3. Light Microscopic Characteristics Bovine Semen Frozen and Thawed Following Passing Through Bovine Serum Albumin

(n=10; mean ±S.D.)

	6% BSA	10% BSA	20% BSA	Control
Motility (%)	57.5±8.78	60.5±11.10	68.2**±8.48	12.8±6.12
Normal sperm (%)	94.4±2.37	94.2± 2.39	95.8**±2.10	84.1±4.16
Progressive motility	3.1±0.27	3.0± 0.17	3.2**±0.25	2.2±0.21

BSA: Bovine serum albumin **: P<0.01 Progressive motility: Glaub (1976)

Table 4. Post-thaw Reduction Rate of Light Microscopic Quality of the Sperm Passed through Bovine Serum Albumin

	6% BSA			10% BSA			20% BSA			Control		
	Prefreeze	Post-thaw	Reduction rate (%)	Prefreeze	Post-thaw	Reduction rate (%)	Prefreeze	Post-thaw	Reduction rate (%)	Prefreeze	Post-thaw	Reduction rate (%)
Motility (%)	83.6 ±8.28	57.5 ±8.78	31.6 ±4.46	86.5 ±7.58	60.5 ±11.10	30.3 ±9.48	95.2 ±3.82	68.2 ±8.48	28.5 ±6.89	48.0 ±8.56	12.8 ±6.12	73.6 ±10.16
Motile sperm count ($\times 10_6$)	211.3 ±43.20	147.3 ±37.90	31.5 ±4.50	182.7 ±45.26	127.8 ±42.30	30.3 ±9.50	57.6 ±15.57	41.5 ±14.38	28.5 ±6.86	384.0 ±68.51	102.4 ±49.00	74.6 ±8.26
Normal sperm (%)	95.5 ±1.62	94.4 ±2.37	1.2 ±0.92	95.1 ±2.42	94.2 ±2.39	0.9 ±0.90	96.4 ±1.71	95.8 ±2.10	0.6 ±0.88	85.6 ±5.68	84.1 ±4.16	1.7 ±1.09
Progressive motility	3.7 ±0.21	3.1 ±0.27	16.3 ±3.17	3.7 ±0.16	3.0 ±0.17	18.3 ±3.21	3.8 ±0.17	3.2 ±0.25	16.9 ±4.98	2.7 ±0.23	2.2 ±0.21	17.3 ±6.21

BSA: Bovine serum albumin Progressive motility: Glaub (1976) Reduction rate $= \frac{\text{Prefreeze sperm quality} - \text{Postthaw sperm quality}}{\text{Prefreeze sperm quality}} \times 100$

significantly lower than that of control group.

Motile sperm count: Post-thaw reduction rate of sperm separated by 6%, 10% and 20% bovine serum albumin were 31.5±4.50, 30.3±9.50 and 28.5±6.86% respectively, and that of control group was 74.6±8.26%. Post-thaw reduction rate of sperm separated by bovine serum albumin was significantly lower than that of control group.

Percent of normal sperm: Post-thaw reduction rate of sperm separated by 6%, 10% and 20% bovine serum albumin were 1.2±0.92, 0.9±0.90 and 0.6±0.88% respectively, and that of control group was 0.6±0.88%. Post-thaw reduction rate of perm separated by bovine serum albumin was lower than that of control sperm, but there was no large difference.

Progressive motility: Post-thaw reduction rate of sperm separated by 6%, 10% and 20% bovine serum albumin were 16.3±3.17, 18.3±3.21 and 16.9±4.98% respectively, and that of control group was 16.9±4.98%. Post-thaw reduction rate of sperm separated by 6% and 20% bovine serum albumin was lower than that of control group, and reduction rate in 20% bovine serum albumin showed the lowest value.

4) Microscopic morphology (Table 5)

Microscopic abnormality in the sperm separated by 6%, 10% and 20% bovine serum albumin, and in control sperm were 4.5±1.62, 4.9±2.08, 3.6±1.35 and 14.6±5.68% respectively. Percent of abnormality in experiment groups was significantly lower than that of control group ($P<0.01$). Post-thaw microscopic abnormality in the sperm separated by 6%, 10% and 20% bovine serum albumin, and in control sperm were 5.6±1.44, 5.8±2.10, 4.2±1.03 and 15.9±4.16% respectively. So percent of abnormality in experiment groups was significantly lower than that of control group ($P<0.01$).

5) Electron microscopic morphology (Table 6)

Cell membrane: In the sperm separated by 6% bovine serum albumin, intact sperm (Fig. 12) was 88±4.1%, and sperm with dilatation (Fig. 13) and sperm with vesiculation were 4±1.3 and 8±2.9% respectively. In the sperm of control group, intact sperm was 81±2.4%, and sperm with dilatation and

sperm with vesiculation were 6±1.6 and 13±2.5% respectively. So sperm in experiment groups was lower than that of control group in the ultrastructural deformity of cell membrane.

Acrosome: In the sperm separated by 6% bovine serum albumin, intact sperm (Fig. 12) was 86±2.60%, and sperm with dilation (Fig. 15) and sperm with density loss (Fig. 16) were 9±3.0 and 5±1.6% respectively. In the sperm of control group, intact sperm was 75±2.4%, and sperm with dilation and sperm with density loss were 16±2.3 and 9±2.1% respectively. So sperm in experiment groups was lower than that of control group in the ultrastructural deformity of acrosome.

2. Characteristics of sperm separated by Tyrode's solution at various time intervals

Tyrode's solution was added to the sperm pellet

Table 5. Light Microscopic Abnormalities of Prefreeze and Post-thaw Sperm Passed through Bovine Serum Albumin (n=10; mean±S.D.)

	Prefreeze				Post-thaw			
	6% BSA (%)	10% BSA (%)	20% BSA (%)	Control (%)	6% BSA (%)	10% BSA (%)	20% BSA (%)	Control (%)
Small head	0.3±0.09	0.4±0.12	0.3±0.11	1.1±0.37	0.3±0.11	0.4±0.13	0.3±0.11	1.1±0.27
Large head	0.1±0.02	0.1±0.03	0.1±0.03	0.3±0.14	0.1±0.03	0.1±0.03	0.1±0.02	0.3±0.12
Absent head	0.8±0.37	0.6±0.21	0.3±0.15	2.8±1.03	1.2±0.35	0.9±0.29	0.4±0.16	3.4±1.02
Amorphous	0.1±0.03	0.1±0.03	0	0.2±0.06	0.1±0.02	0.1±0.02	0	0.2±0.05
Double tail	0.1±0.01	0.1±0.02	0	0.2±0.04	0.1±0.01	0.1±0.01	0	0.2±0.04
Tail abnormality	2.2±0.88	2.1±0.77	1.7±0.75	7.3±2.49	2.4±0.75	2.4±0.71	2.1±0.53	8.1±2.13
Immature sperm	0.9±0.41	1.5±0.36	1.2±0.32	2.5±0.77	1.4±0.62	1.8±0.60	1.3±0.04	2.6±1.12
Total abnormalities	4.5±1.62	4.9±2.08	3.6±1.35	14.6±5.68**	5.6±1.44	5.8±2.10	4.2±1.03	15.9±4.16**

BSA: Bovine serum albumin **: P<0.01

Table 6. Electron Microscopic Abnormalities of the Sperm Treated with 6% Bovine Serum Albumin (n=6; mean±S.D.)

	Acrosome			Cellmembrane		
	Intact (%)	Dilatation (%)	Vesiculation (%)	Intact (%)	Dilatation (%)	Density loss (%)
6% BSA	88*±4.1	4±1.3	8 ±2.9	86**±2.6	9 ±3.0	5 ±1.6
Control	81 ±2.4	6±1.6	13**±2.5	75 ±2.4	16*±2.3	9*±2.1

BSA: Bovine serum albumin **: P<0.01 *: P<0.05

Table 7. Per Cent of Motility of Sperm Separated by Tyrode's Solution at Various Time Intervals (n=10; mean±S.D.)

	20 min (%)	40 min (%)	60 min (%)	80 min (%)	100 min (%)	120 min (%)	Control (%)
Raw semen (KC)	16.2±2.20	27.5±2.46	57.4±6.70	89.8**±3.68	60.2±3.19	52.3±4.50	36.2±3.39
Extended semen (KC)	20.1±4.23	25.8±2.49	51.3±3.95	85.1**±2.92	58.6±4.84	47.3±5.00	28.3±3.74
Frozen semen (K)	23.6±3.44	32.4±4.70	43.7±4.06	83.7**±3.77	65.1±3.48	48.3±2.50	23.5±3.50
Frozen semen (H)	28.3±2.58	35.1±3.63	53.5±3.41	86.8**±2.66	67.8±2.20	42.9±3.73	27.3±2.75

KC: F_1 of Korean native-Charolais bull K: Korean native bull H: Holstein Friesian bull **: P<0.01

Table 8. Progressive Motility of Sperm Separated by Tyrode's Solution at Various Time Intervals

(n=10; mean±S.D.)

	20 min (%)	40 min (%)	60 min (%)	80 min (%)	100 min (%)	120 min (%)	Control (%)
Raw semen (KC)	2.4±0.23	2.7±0.23	3.1±0.19	3.8**±0.11	3.5±0.16	3.2±0.14	2.4±0.27
Extended semen (KC)	2.3±0.13	2.8±0.16	3.0±0.16	3.7**±0.20	3.4±0.18	3.0±0.15	2.2±0.12
Frozen semen (K)	2.2±0.12	3.0±0.19	3.2±0.19	3.8**±0.11	3.3±0.11	2.9±0.18	2.1±0.10
Frozen semen (H)	2.5±0.13	2.9±0.25	3.3±0.20	3.7**±0.18	3.2±0.18	3.1±0.19	2.2±0.16

Progressive motility: Glaub (1976) KC: F_1 of Korean native-Charolais bull K: Korean native bull
H: Holstein Friesian bull **: P<0.01

Table 9. Motile Sperm Recovery Rate of Sperm Separated by Tyrode's Solution at Various Time Intervals

(n=10; mean±S.D.)

Semen	20 min (%)	40 min (%)	60 min (%)	80 min (%)	100 min (%)	120 min (%)
Raw semen (KC)	5.1±1.20	23.6±3.37	37.2±2.15	76.3**±2.54	63.4±2.50	54.1±2.28
Extended semen (KC)	8.9±3.51	30.1±3.60	46.8±2.53	69.3**±4.00	59.2±2.30	57.4±2.80
Frozen semen (K)	2.6±0.20	13.8±1.99	26.3±3.30	64.2**±3.19	57.3±3.13	49.2±3.20
Frozen semen (H)	7.2±2.10	20.7±2.71	27.3±2.79	66.1**±2.77	54.5±2.42	46.8±3.68

F_1 of Korean native-Charolais bull K: Korean native bull H: Holstein Friesian bull **: P<0.01

Table 10. Motility of Sperm of Various Specimens Separated by Tyrode's Solution

(n=12; mean±S.D.)

	Tyrode layer (%)	Lower layer (%)	Control (%)
Raw semen (KC)	87.9**±6.36	7.6±2.04	34.4± 3.78
Extended semen (KC)	82.1**±8.59	4.3±2.12	26.9± 6.52
Frozen semen (K)	84.5**±4.38	5.6±0.67	22.1± 8.41
Frozen semen (H)	87.5**±5.89	6.1±2.45	28.2±10.12

KC: F_1 of Korean native-Charolais bull K: Korean native bull H: Holstein Friesian bull **: P<0.01

which was maded from semen by centrifuge. Thereafter highly motile sperm was collected and observed according to stationary time of 20, 40, 60, 80, 100 and 120 minutes. The result was summerized in Table 7, 8, and 9. Sperm motility, progressive motility and motile sperm recovery showed the highest value in the stationary time of 80 minutes (P<0.01). In stationary time between 20 and 80 minutes, the more time was passed, the higher value was shoed. After that time, the more time was passed, the lower value was showed.

3. Sperm separation using Tyrode's solution

1) Sperm motility

Highly motile sperm was separated from various semen using Tyrode's solution, and the motility of separated sperm was summerized in Table 10.

In raw semen, extended semen and two kinds of frozen semen, sperm motility of Tyrode layer were 87.9±6.36, 82.1±8.59, 84.5±4.38 and 87.5±5.89% respectively, and sperm motility of lower layer were 7.6±2.04, 4.3±2.12, 5.6±0.67 and 6.1±2.45% respectively.

Motility of sperm collected in Tyrode layer was significantly higher than that of sperm collected in lower layer, in all kinds of semen (P<0.01).

Also motility of sperm collected in Tyrode layer was significantly higher than that of control group,

and sperm motility of lower layer was significantly lower than that of control group.

2) Motile sperm count

Sperm was separated from various semen using Tyrode's solution, and motile sperm count of separated sperm was summerized in Table 11. In raw semen, extended semen and two kinds of frozen semen, motile sperm count ($\times 10_6$) of Tyrode layer were 116.3 ± 8.97, 85.5 ± 5.76, 3.2 ± 0.65 and 4.9 ± 0.72 respectively, and motile sperm count ($\times 10_6$) of lower layer were 24.2 ± 3.51, 15.9 ± 3.86, 1.08 ± 0.36 and 1.3 ± 0.36 respectively.

So motile sperm was significantly selectively collected in Tyrode layer ($P<0.01$).

Table 11. Number of Motile Sperm in Various Specimens Separated by Tyrode's Solution

(n=12; mean±S.D.)

	Tyrode layer ($\times 10_6$)	Lower layer ($\times 10_6$)
Raw semen (KC)	116.3**±8.97	24.2±3.51
Extended semen (KC)	85.5**±5.76	15.9±3.86
Frozen semen (K)	3.2**±0.65	1.1±0.36
Frozen semen (H)	4.9**±0.72	1.3±0.36

KC: F_1 of Korean native-Charolais bull
K: Korean native bull
H: Holstein Friesian bull
**: $P<0.01$

3) Percent of normal sperm

Sperm was separated from various semen using Tyrode's solution, and percent of normal sperm of separated sperm was summerized in Table 12. In raw semen, extended semen and two kinds of frozen semen, percent of normal sperm in Tyrode layer were 95.3 ± 3.20, 95.2 ± 1.46, 95.5 ± 1.41 and $95.5 \pm 2.32\%$ respectively. So percent of normal sperm of Tyrode layer was significantly higher than that of lower layer or control group ($P<0.05$, $P<0.01$).

4) Progressive motility

Sperm was separated from various semen using Tyrode's solution, and progressive motility of separated sperm was summerized in Table 13.

Table 12. Normal Sperm of Various Specimens Separated by Tyrode's Solution

(n=12; mean±S.D.)

Normal sperm / Specimen	Tyrode layer (%)	Lower layer (%)	Control (%)
Raw semen (KC)	95.3**±3.20	73.7±5.28	85.3±4.92
Extended semen (KC)	95.2**±1.46	76.5±2.43	85.5±2.00
Frozen semen (K)	95.5**±1.41	79.9±3.11	88.3±3.06
Frozen semen (H)	95.5**±2.32	77.5±2.76	88.4±2.63

KC: F_1 of Korean native-Charolais bull K: Korean native bull H: Holstein Friesian bull **: $P<0.01$ *: $P<0.05$

Table 13. Progressive Sperm Motility of Various Specimens Separated by Tyrode's Solution

(n=12; mean±S.D.)

	Tyrode layer	Lower layer	Control
Raw semen (KC)	3.9**±0.10	1.3±0.22	2.3±0.34
Extended semen (KC)	3.8**±0.15	1.3±0.23	2.1±0.33
Frozen semen (K)	3.7**±0.22	1.1±0.13	2.0±0.42
Frozen semen (H)	3.7**±0.27	1.3±0.19	2.2±0.40

Progressive motility: Glaub (1976) KC: F_1 of Korean native-Charolais bull K: Korean native bull
H: Holstein Friesian bull **: $P<0.01$

Table 14. Recovery Rate of Motile Sperm of Various Specimens Separated by Tyrode's Solution

(n=12; mean±S.D.)

Specimen \ Recovery rate	Tyrode layer (%)	Lower layer (%)
Raw semen (KC)	75.0**±9.78	15.6±3.37
Extended semen (KC)	67.1**±5.79	12.5±3.40
Frozen semen (K)	62.9**±7.88	21.2±4.15
Frozen semen (H)	65.0**±9.66	17.3±4.86

KC: F_1 of Korean native-Charolais bull K: Korean native bull H: Holstein Friesian bull **: P<0.01

Table 15. Morphological Abnormalities of the Sperm Separated by Tyrode's Solution

	Raw Semen (KC)			Extended Semen (KC)			Frozen Semen (K)			Frozen Semen (H)		
	Tyrode layer (%)	Lower layer (%)	Control (%)	Tyrode layer (%)	Lower layer (%)	Control (%)	Tyrode layer (%)	Lower layer (%)	Control (%)	Tyrode layer (%)	Lower layer (%)	Control (%)
Small head	0.8 ±0.31	1.3 ±0.27	0.9 ±0.32	0.3 ±0.05	0.9 ±0.27	0.8 ±1.01	0.3 ±0.11	1.2 ±0.35	0.8 ±0.27	0.2 ±0.04	1.3 ±0.67	0.5 ±0.15
Large head	0.1 ±0.2	0.5 ±0.20	0.3 ±0.11	0	0.5 ±0.13	0.2 ±0.13	0.1 ±0.03	0.3 ±0.11	0.2 ±0.03	0.1 ±0.02	0.4 ±0.13	0.3 ±0.09
Absent head	0.4 ±0.5	6.0 ±1.90	2.5 ±0.72	0.4 ±0.11	5.7 ±1.32	2.3 ±0.91	0.4 ±0.09	2.8 ±1.07	1.2 ±0.28	0.4 ±0.11	3.3 ±1.02	1.3 ±0.40
Amorphous	0.2 ±0.3	0.4 ±0.11	0.3 ±0.09	0.2 ±0.1	0.3 ±0.9	0.3 ±0.6	0.1 ±0.02	0.3 ±0.14	0.2 ±0.04	0.1 ±0.03	0.3 ±0.09	0.1 ±0.03
Double tail	0	0.2 ±0.03	0.1 ±0.01	0.1 ±0.01	0.2 ±0.01	0.2 ±0.04	0	0.2 ±0.05	0.1 ±0.01	0.1 ±0.01	0.2 ±0.03	0.2 ±0.04
Tail abnormality	2.0 ±0.66	15.2 ±4.70	8.3 ±3.20	2.6 ±0.08	13.0 ±1.78	8.2 ±1.32	2.8 ±1.08	12.7 ±2.07	7.1 ±2.07	2.5 ±1.03	14.3 ±2.32	7.2 ±2.17
Immature sperm	1.2 ±0.34	2.7 ±0.93	2.3 ±0.67	1.2 ±0.04	2.9 ±1.02	2.5 ±0.89	0.8 ±0.02	2.6 ±1.03	2.1 ±0.98	1.1 ±0.41	2.7 ±1.75	2.0 ±0.68
Total abnormalities	4.7 ±3.20	26.3 ±5.28**	14.7 ±4.92	4.8 ±1.46	23.5 ±2.43**	14.5 ±2.00	4.5 ±1.41	20.1 ±3.11**	11.7 ±3.06	4.5 ±2.32	22.5 ±2.76**	11.6 ±2.63

KC: F_1 of Korean native-Charolais bull K: Korean native bull H: Holstein Friesian bull **: P<0.01

In raw semen, extended semen and two kinds of frozen semen, progressive motility of Tyrode layer were 3.9+0.10, 3.8+0.15 and 3.7+0.27 respectively. So progressive motility of Tyrode layer was significantly higher than that of lower layer or control group (P<0.01).

5) Motile sperm recovery

Sperm was separated from various semen using Tyrode's solution, and motile sperm recovery of separated sperm was summerized in Table 14. In raw semen, extended semen and two kinds of frozen semen, motile sperm recovery of Tyrode layer were 75.0+9.78, 67.1+5.79, 62.9+7.88 and 65.0+9.66% respectively. So motile sperm recovery of Tyrode layer was significantly higher than that of lower layer (P<0.01).

6) Morphological abnormality

Sperm separated by Tyrode's solution was observed for the examination of morphology, and distribution of various kinds of abnormality was summerized in Table 15.

In raw semen, extended semen and two kinds of frozen semen, percent of sperm abnormality in Tyrode layer were 4.7+3.20, 4.8+1.46, 4.5+1.41 and 4.5+2.32% respectively.

So percent of sperm abnormality of Tyrode layer

was significantly lower than that of lower layer or control group ($P<0.01$).

DISCUSSION

Lipshultz (1982) reported that it is the quality of the sperm rather than the quantity that is important in defining male fertility potential, and a complete semen analysis contists of five basic determinants: volume, density, motility, qualitative sperm movement (forward progression), and morphologic characteristics.

Rutherford (1982) reported that post-thaw motility of isolated sperm using serum albumin was 65% compared to 35% for normal sperm, cows inseminated with isolated semen showed 8% more birth rates than cows inseminated with non-isolated semen.

In this experiment, separation of highly motile sperm was tried by passing of raw semen through 6%, 10% and 20% bovine serum albumin. So motility, motile sperm count, percent of normal sperm and progressive motility of each treatment group showed significantly highly value in all than those of the control group that was not passed bovine serum albumin. But total sperm count was significantly lower than that of the control group (Table 2).

Glass and Ericsson (1982) stated that highly motile and morphologically normal sperm would pass easily through bovine serum albumin. The results in this experiment were similar to their results.

Motility, progressive motility and percent of normal sperm showed the higher value. But only motile sperm count and sperm recovery rate showed the highest value in 6% bovine serum albumin. Ericsson et al. (1973) also reported that sperm separated by 6%, 10% and 20% bovine serum albumin from human raw semen, showed the highest motility in 20%, and showed the highest sperm recovery rate in 6%.

But report which used 20% bovine serum albumin for the separation of highly motile bovine sperm, was not seen.

The results of present experiment, were similar to the Ericsson's results obtained from human raw semen.

According to the above-mentioned results, it was possible to separate highly motile sperm from bovine raw semen using bovine serum albumin, and best sperm in quality could be obtained selectively when 20% bovine serum albumin was used, but sperm recovery rate was low, and sperm characteristics in 10% bovine serum albumin was not different largely from those in 6% bovine serum albumin, but recovery rate was lower than that of 6% bovine serum albumin. In 6% bovine serum albumin, sperm recovery rate showed the highest value, and sperm characteristics were similar to the result of 10% bovine serum albumin, so it was considered that the use of 6% bovine serum albumin is profitable.

But to know the possibility of highly motile sperm separation from marketed frozen semen using bovine serum albumin, 6% bovine serum albumin was used firstly, therefore total sperm count, motility and motile sperm count in separated sperm were remarkably lower than those of control group not treated with bovine serum albumin (Table 1).

The results of this present study have shown that the value of highly motile sperm separation using bovine serum albumin was low for the marketed frozen semen.

It was considered that the reason why the value of highly motile sperm separation from marketed frozen semen was low, was that frozen semen after thawing couldn't pass easily bovine serum albumin layer with viscosity on account of post-thaw low sperm motility. Our conception rate in field with marketed frozen semen was also very low, it could be same reason which explained as above.

Rutherford (1982) reported that highly motile sperm was separated by bovine serum albumin, and that fertilization rate was increased by use of frozen and thawed separated sperm for artificial insemination.

Therefore in the present study, sperm selectively separated by 6%, 10% and 20% bovine serum albumin before freezing was frozen, thawed and observed as a means for the increase of post-thaw

sperm motility of frozen semen.

So motility, percent of normal sperm and progressive motility of experimental group showed significantly high value, as compared with control group namely that common sperm was frozen, thawed and observed (Table 3).

In this experiment, highly motile sperm separated by bovine serum albumin from raw semen was frozen, thawed and observed, so post-thaw sperm motility was reduced as compared with that of prefreezing, but reduction rate was significantly low as compared with control group. Then the higher density of bovine serum albumin which used was, the lower thus reduction rate was (Table 4). This result suggests the possibility that highly motile sperm separated by bovine serum albumin may have resisting power against temperature shock as compared with common sperm. Microscopic abnormality rate of highly motile sperm separated by 6%, 10% and 20% bovine serum albumin were 4.5, 4.9 and 3.6% respectively for prefreezing, and were 5.6, 5.8 and 4.2% respectively for post-freezing, and were significantly low in all, as compared with control group showed 14.6% of prefreezing and 15.9% of post-thawing (Table 5). Thus results were similar to result of observation by White et al. (1982).

Electron-microscopic deformity rate of the highly motile sperm separated with 6% bivine serum albumin were low as 4, 8, 9 and 5% respectively in the dilation and vesiculation of cell membrane, in dilation and density loss of acrosome than that of control sperm as 6, 13, 16 and 9% respectively in those of control sperm. Thus experimental results were similar to the result of Sherman et al. (1982) that highly motile sperm selectively separated from human semen showed decreased deformity of cell membrane as decreased from 18% to 11%, and decreased deformity of acrosome as decreased from 27% to 13%.

Thus fact suggests the possibility that morphological deformation of sperm wasn't caused when the sperm passed bovine serum albumin, and that passage through bovine serum albumin was hard for the abnormal sperm.

In brief, it was difficult to separated the highly motile sperm with bovine serum albumin from common semen already frozen, but highly motile sperm separated from raw semen using bovine serum albumin showed superior sperm characteristics and low morphological abnormality rate, as compared with control group not using BSA. So this evidence suggests the possibility that highly motile sperm separation using bovine serum albumin may increase the fertilization rate.

According to the present condition at demand and supply of sperm, there are many difficulties in the highly motile sperm separation from raw semen using bovine serum albumin.

Therefore, it is considered that highly motile sperm separation from common frozen semen already maded and marketed, is needed strongly at reality.

Authors supposed that highly motile sperm could be separated because highly motile sperm would swim upward with preference and be suspended in buffer solution, when the precipitated sperm by centrifuging were stationed, after harmless buffer solution used to sperm experiment was mixed to semen.

Therefore, separation of highly motile sperm, was tried with Tyrode's solution used frequently in sperm experiment (Diasio and Grass, 1971; Bavister, 1975; Back et al., 1976; Goodall and Roberts, 1976; Ericsson, 1977; Dmowski et al., 1979) and embryo transfer (Danial, 1971).

In this experiment, motility, progressive motility and motile sperm recovery of raw semen, extended semen and two kinds of frozen semen, were compared respectively at the stationary time of 20, 40, 60, 80, 100 and 120 minutes in order to decide stationary time in the Tyrode's solution at highly motile sperm separation using Tyrode's solution.

So sperm motility of each group were 89.8, 85.1, 83.7 and 86.8% respectively at 80 minutes, and showed significantly high value as compared with those of another time intervals, and showed significantly high value as compared with 36.2, 28.3, 23.5 and 27.3% in control group not treated with Tyrode's solution (Table 7).

Also, progressive motility of each treatment group at 80 minutes were 3.8, 3.7, 3.8 and 3.7

respectively, and showed significantly high value as compared with those of another time intervals, and showed significantly high value as compared with 2.4, 2.2, 2.1 and 2.2 in control group not treated with Tyrode's solution (Table 8).

And motile sperm recovery of each treatment group at 80 minutes were 76.3, 69.3, 64.2 and 66.1% respectively, and showed significantly high value as compared with those of another time intervals (Table 9).

So the best results were showed when sperm stationary time in Tyrode's solution was 80 minutes, therefore stationary time in present experiment was fixed on 80 minutes.

It was considered that sperm motility was maintained during certain time in Tyrode's solution, because fertility rate was increased by artificial insemination with 0.25 ml of sperm-Tyrode, after human sperm separated by human serum albumin was pelleted and diluted with Tyrode's solution (Chung, 1984).

Highly motile sperm separation using Tyrode's solution was possible not only from raw semen and extended semen but also from marketed frozen semen, unlike when bovine serum albumin was used. Also highly motile sperm separated by Tyrode's solution showed significantly high value in motility, motile sperm count, progressive motility and percent of normal sperm, as compared with control group, and showed remarkably low value in morphological abnormality rate. And motile sperm recovery in each kind of semen showed high value in Tyrode layer. Namely, in motility, motile sperm count, percent of normal sperm and progressive motility of sperm separated by Tyrode's solution at 80 minutes interval from raw semen, extended semen and two kinds of frozen semen, sperm motility in Tyrode layer were 87.9, 82.1, 84.5 and 87.5% respectively (Table 10), motile sperm count ($\times 10_6$) in Tyrode layer were 116.3, 85.5, 3.2 and 4.9 respectively (Table 11), percent of normal sperm in Tyrode layer were 95.3, 95.2, 95.5 and 95.5% respectively (Table 12), and progressive motility in Tyrode layer were 3.9, 3.8, 3.7 and 3.7% respectively (Table 13). So sperm characteristics in Tyrode layer showed significantly high value in all, as compared with those of lower layer and control group.

Therefore, it was considered that thus result proved hypothesis established in this experiment, namely highly motile sperm should be separated because motile sperm swim first upward into Tyrode's solution from the sperm pellet made by centrifuging. Motile sperm recovery in Tyrode layer at 80 minutes were 75.0, 67.1, 62.9 and 65.0% respectively for raw semen, extended semen and two kinds of frozen semen, and showed significantly high value as compared with those of lower layer (Table 14).

It was considered that thus result proved that highly motile sperm was selectively separated with Tyrode's solution not only from raw semen and extended semen but also from frozen semen. In raw semen, extended semen and two kinds of frozen semen treated with Tyrode's solution, total abnormality in raw semen was 4.7%, and showed significantly low value as compared with 14.7% of control group and 26.3% of lower layer, and total abnormality in extended semen was 4.8%, and showed significantly low value as compared with 14.5% of control group and 23.5% of lower layer, and total abnormalities in two kinds of frozen semen were 4.5 and 4.5% respectively, and showed significantly low value as compared with 11.7 and 11.6% of control group and 20.1 and 22.5% of lower layer. So, total abnormalities of Tyrode layer were significantly low all in each treatment group (Table 15).

It was considered that abnormal sperm might hardly swim into Tyrode's solution because of it's weak motility or lack of motility.

The reason why sperm without motility was found occasionally in Tyrode's layer, was that dead sperm sticked to motile sperm might come out simultaneously when motile sperm swim upward into Tyrode's solution from the pellet.

Therefore, it was proved that highly motile sperm could be separated with Tyrode's solution, also the effect of exclusion of abnormal sperm could be obtained when Tyrode's solution was used.

Predecessor's report using Tyrode's solution was not seen yet, so present experiment couldn't be compared.

According to above-mentioned result, sperm separated by Tyrode's solution showed significantly high value in motility, motile sperm count, progressive motility and percent of normal sperm, and showed low value in abnormalities.

Thus result were better than those of sperm separated by bovine serum albumin in present experiment.

Especially, it was impossible to separate the highlY motile sperm from frozen semen with bovine serum albumin, but it was possible also from common marketed frozen semen with Tyrode's solution.

Therefore, it was considered that highly motile sperm could be separated by Tyrode's solution in order to increase fertility rate and normal parturition rate, and this method could be used practically.

Shettles (1970) claimed that change of pH with an acid douche (2 tablespoons of white vinegar to 1 quart of water) could improve female offspring. It is caused by motility.

There is male prevalent technic by sperm isolation, but male offspring could be increased also by artificial insemination. Ericsson et al. (1973) devised use of 20% serum albumin as male prevalent method, but consumption of energy might be made up in the sperm because sperm already had done an amount of movement or metabolism on account of process which requires 6 hours.

On the other hand, Tyrode method required only 80 minutes for the separation, so it was considered that Tyrode method may be favourable for the conception rate.

At natural mating, there are barriers for the sperm to the time of fertilization, like microorganism in vagina, cervix, cervical mucus, uterine cavity and oviduct. But barriers almost were vanished at artificial insemination, so Y-sperm which had faster motility would reach the egg first.

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