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Studies on the Improvement of Performance and Reproductive Efficiency in Farm Animals
Ⅳ. Assessment of Fertilizing Ability of Korean Native Bull by *In Vitro* Fertilization with Bovine Follicular Oocytes

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家畜의 改良 및 繁殖效率 增進에 關한 硏究
Ⅳ. 牛 卵胞卵과의 體外受精에 의한 韓牛 種牡牛의 受精能力 評價에 關한 硏究

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적 요

본 硏究는 種牡牛의 選拔方法으로 卵胞卵을 利用하여 實驗室內에서 精子의 受精能力을 直接 檢定하여 評價코자 시도되었다. 즉 본 실험은 後代檢定중에 있는 韓牛 後補種牡牛 15頭의 凍結融解精子의 受精能力을 評價하기 위하여 精液을 高張液(HIS)에 처리한 후 DM에서 6시간 그리고 소 卵胞液이 20% 첨가된 DM에서 4시간 前培養하여 受精能을 獲得시켜 精子의 活力과 尖體反應率을 조사하였고 前培養된 精子의 體內(토끼 난관) 또는 體外受精能力을 調査하기 위하여 FCS 15%, 발정암소혈청(CSS) 10%가 첨가된 mKRB에서 體外成熟된 韓牛卵胞卵과 受精시켜 受精能力을 評價하였으며 人工受精에 의한 個體別 受胎率과도 比較 檢討한 바 다음과 같은 결론을 얻었다.

1. 韓牛 卵胞卵의 體外成熟率은 BSA 添加區에서 43.8%, FCS 15% 添加區에서 67.4%, CSS10% 添加區에서 69.9%이었다.
2. 토끼 卵管에서 體外受精率은 BSA 參加區 50.0%, FCS 15% 添加區 41.2% 및 CSS 10% 添加區 35.0%이었다.
3. 後補種牡牛 15頭의 精液을 HIS-DM으로 處理후 6시간 前培養하였을 때 精子의 活力指數는 9~32%였고 尖體反應率은 19~44% 이었으며 20% 卵胞液을 添加하여 4시간 前培養하였을 때 精子의 活力指數는 9~13%이었고 尖體反應率은 20~43%로 個體間에 차이가 있었다.
4. 體外受精率은 6.6~85.7%였으며, 발정암소혈청(CSS) 10%가 첨가된 mKRB에서 成熟시킨 卵胞卵이 FCS 15% 첨가된 mKRB에서 成熟시킨 卵胞卵보다 다소 높았으나, 精子受精能獲

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得方法間에는 차이가 없었다.

5. 體外受精率에 있어서 前培養後 精子活力指數와는 負의 相關이 있었으며, 尖體反應率과는 낮은 正의 相關을 나타냈다.
6. 種牡牛의 受胎率은 體外受精率, 精子活力指數 및 尖體反應率과 낮은 正의 相關關係를 나타냈다.
7. 種牡牛의 個體間의 受胎率 優劣順位에서는 受精率順位와의 사이에 더욱 낮은 負의 相關關係를 보였다.
8. 이상의 연구결과에 비록 後代檢定중의 제한된 자료로 인하여 種牡牛 受胎率과 體外受精率간에 有意的인 相關關係는 없었으나, 連結 韓牛 受精率 評價에 대한 實驗室內의 檢定可能性을 찾을 수 있었다.

INTRODUCTION

A remarkable genetic progress of productive efficiency in cattle has been brought about by widespread use of highly selected, genetically superior AI sires. Recently, genetic engineering technique which combined genetic materials of these superior sires and embryo transfer in large farm animals is one of the exciting research fields. To apply this technique in livestock industry, there are many problems to be precedently developed in essential biotechnology such as semen quality test, IVF, embryo culture, genetic manipulation, embryo transfer, etc. Semen quality tests in AI industry have not still proven as good absolute indicators of semen fertility, because most tests were poorly correlated with fertility (Graham *et al.*, 1980). On the other hand, the rates of bovine *in vitro* fertilization(IVF) have been reported to vary among individual bulls used for IVF(Brackett *et al.*, 1982a; Lambert *et al.*, 1984; Iritani, 1987) and among the procedures of sperm treatment for *in vitro* capacitation (Fukui *et al.*,1983a). If the laboratory new test that would accurately predict the semen fertility in AI industry and biotechnology, more effective screening of sires will be possible, Such a possibility for predicting the bull fertility in laboratory has been reported form IVF system using zona-free hamster eggs (Brackett *et al.*,1982b; Calcote, 1980) ;Bousquet and Brachet, 1981,1982 ; Baird *et al.*, 1984; Chung *et al.*, 1986; Graham and Foote, 1985, 1987a,b) and bovine follicular oocytes (Iritani, 1987). In these two methods, establishment and application of a satisfactory oocyte maturation system will dramatically bring about the great growth in the fields of genetic manipulation as well as sperm physiology in cattle which are very difficult to obtain pronuclear eggs economically. The purpose of this study was to investigate the differences *in vitro* sperm capacitation rate and their *in vitro* fertilizing abilities with matured bovine follicular oocytes among frozen semen of individual young bull, and the relationship between the above IVF rates and AI fertility of frozen semen.

METERIALS AND METHODS

1. Semen and oocytes

This study was performed with frozen semen of 15 Korean native young bulls having their AI records and follicular immature oocytes of slaughtered Korean native cows.

2. Collection and *in vitro* maturation of follicular oocytes

Ovaries were obtained from slaughterhouse and transported to the laboratory in 0.9% saline(37℃) containing antibiotics within 4 hr after slaughter. The ovaries were washed twice with saline to remove adhering blood.

Oocytes were collected from follicles of 2~5mm in diameter by aspiration with a 5ml syringe and 20-guage needle according to the procedure of Ball *et al.*(1982, 1984). Only oocytes with a compact cumulus and normal cytoplasm were used after washing twice with maturtion medium. The medium used for oocyte maturation was a modified Krebs-Ringer-Bicarbonate solutio (mKRB) (Shea *et al.*, 1976; Iritani *et al.*, 1984) containing 15%(v/v) fetal calf serum (FCS) or 10%(v/v) cow serum(CSS) at standing estrus instead of BSA. All media were filtered with a 0.2 μm Millipore filter. About 10 oocytes were introduced into 2.5ml maturation medium in plastic culture dish that previously covered with paraffin oil and equlibrated under the same condition with oocytes maturation, and oocytes were cultured for 28hr in a CO_2 incubator(5% CO_2 in air at 38℃, 100% humidity).

3. Sperm treatment

Frozen-straw semen were thawed for 30 sec at 38℃ and evaluated for sperm concentration and sperm motility index(SMI). Sperm were treated and capacitated *in vitro* by two methods:(1) for 6 hr in DM after HIS(Brackett and Oliphant, 1975) and (2) for 4 hr in DM plus 20%(v/v) bovine follicular fluid(BFF) (Breuer and Wells, 1977) and capacitated *in vivo* for 4~6 hr in rabbit uterus by the procedure of Fukui *et al.* (1983b). The *in vitro* capacitated sperm were evaluated for their acrosomal characteristic, acrosome loss, by staining method of Lenz *et al.* (1983).

4. Fertilization *in vivo* and *in vitro*

For *in vivo* fertilization, 5~7 matured oocytes were surgical transferred into an oviduct of rabbit that artificially inseminated with frozen-thawed bull semen 4~6 hr before oocyte transfer. The transferred oocytes were recovered from oviduct after 28 hr. For IVF, sperm suspension adjusted to be $1\sim2\times10^6$ live sperm/ml was introduced into 2.5ml medium of a petri culture dish containing 5~7 oocytes and gametes were coincubated for 36 hr in a CO_2 incubator. Oocytes were fixed with acetic alcohol (1:3) and stained with 1% aceto-orcein. The formation of pronuclei and cleavage as a criterium for fertilization were examined before and after staining by phase-contrast microscopy.

RESULTS AND DISCUSSION

1. Oocyte maturation *in vitro*

As shown in Table 1, maturation rates in mKRB plus CSS or FCS were higher than that of control(mKRB plus BSA) and cleavage of these matured oocytes were also higher, although their IVF rates were slightly lower than that of control. IVF rate of control was similar to that of these result showed trends similar to those of Sanbuissho and Threlfall (1985).

2. Sperm capacitation and motility among bulls

Table 2 contains data for individual bulls showing SMI and %AR after 6 hr incubation in DM. Their SMI and %AR ranged from 9 to 32 and from 19 to 44%, respectively, which were small variations. Sperm incubated for 4 hr in DM plus BFF showed %AR similar to those of DM, but their SMI were slightly lower than those of DM. To compare the effect of BFF ad-

Table 1. Effects of media on follicular oocyte maturation *in vitro* and fertilization *in vivo*

Medium	No. of oocytes					
	Matrued	Trans-ferred	Recovered[1]	Fertilized		
				Pronuclei	Cleaved	Total(%)
mKRB+	21/48	21	8	4	–	4/8
BSA(4mg/ml)	(43.8)		(38.1)			(50.0)
mKRB+	31/46	30	17	5	2	7/17
FCS(15%,v/v)	(67.4)		(56.7)			(41.2)
mKRB+	32/46	30	20	5	2	7/20
CSS(10%,v/v)	(69.6)		(66.7)			(35.0)

1) Proprtions of oocytes from oviduct 28 hr after transfer.

Table 2. Sperm motility index and percent acrosome reaction of sperm incubated *in vitro* in DM after HIS or DM plus bovine follicular fluid

Bull code	HIS,DM(6 hr)		DM+20%BFF(4 hr)	
	SM[1]	%AR[2]	SMI	%AR
A	18.3	31.0	12.8	31.0
B	18.3	33.5	–	–
C	12.5	34.0	–	–
D	14.7	44.0	13.1	39.5
E	24.2	25.5	–	–
F	14.9	37.5	9.7	42.5
G	31.7	27.5	–	–
H	8.9	27.5	8.7	28.0
I	26.4	25.5	–	–
J	12.5	33.0	–	–
K	28.3	23.5	10.8	19.5
L	24.2	19.0	–	–
M	14.2	38.5	–	–
N	20.2	38.0	10.7	31.5
O	17.8	32.5	–	–

1) Initial SMI before incubation ranged from 75 to 85.

2) Percent AR was expressed as proportion of acrosome "loss" sperm.

dition to capacitation medium, sperm from only 2 bulls per group(low : bulls K,N;middle:F. H and high:A,D according to IVF rates with mKRB plus CSS matured oocytes) had been selected. Small variations of SMI between bulls were different from those of Brackett and Oliphant(1975) in rabbit. Percent AR which appeared similar proportions with that of DM were also different from result of Breuer and Wells (1977), in which BFF were able to promote capacitation and acrosome reaction.

3. Fertilization *in vitro* among bulls

IVF rates of sperm capacitated *in vitro* in DM and DM plus BFF are shown in Table 3. IVF rates with mKRB plus FCS-and mKRB plus

Table 3. Fertilization *in vitro* of matured follicular oocytes with sperm capacitated in DM after HIS or DM plus 20% bovine follicular fluid

Bull code	mKRB + 15%FCS1			mKRB + 10%FCS					
	HIS DM2			HIS, DM			DM + 20%BFF		
	Exam.	Fert.	%	Exam.	Fert.	%	Exam.	Fert.	%
A	15	10	66.7	14	12	85.7	19	9	47.3
B	15	5	33.3	12	10	83.3	–	–	–
C	17	4	23.5	14	10	71.4	–	–	–
D	15	7	46.7	16	10	62.5	17	10	58.8
E	17	4	23.5	20	12	60.0	–	–	–
F	15	5	33.3	15	9	60.0	20	8	40.0
G	24	5	20.0	17	10	60.0	–	–	–
H	18	9	50.0	16	9	58.8	20	6	30.0
I	18	4	22.2	15	8	56.3	–	–	–
J	15	5	33.3	17	8	53.3	–	–	–
K	22	4	18.2	11	5	47.1	20	4	20.0
L	16	2	12.5	20	9	45.0	–	–	–
M	15	6	40.0	12	5	41.7	–	–	–
N	15	1	6.6	14	5	35.7	21	5	23.8
O	25	6	24.0	12	2	16.7	–	–	–

1) Oocyte maturation 2) Sperm capacitaion 3) No. of oocytes examined and fertilized

CSS-matured oocytes ranged form 7 to 67% and from 36 to 86%, respectively, which showed broad ranges among individual bulls and slightly higher rates in the latter sperm. DM plus BFF-capacitated sperm classified into 3 groups also showed ranges similar to those of DM-capacitated sperm. This great variations between individuals have been reproted (Brackett *et al.*, 1982a; Lambert *et al.*, 1984); Sirard *et al.*, 1984, 1985 and Iritani, 1987). The results that IVF rate of sperm was not different between DM and BFF treatment, but was different between DM and BFF treatment, but was different between DM and BFF treatment, but was different between maturation media were similar trends to that of Sanbissho and Threlfall (1985) than Fukui *et al.* (1983a,b).

4. Ranking comparison of IVF among bulls

As shown in Table 4, bulls were listed in desending order of IVF rates of DM-capacitated sperm. This order especially coincided more for sperm of bulls with low IVF rates, but such a tendency did not appeared in AI fertility due to the limited data.

5. Relationships of sperm capacitaion, IVF and semen fertility

Results in Tables 2 to 4 were conclusively summarized as correlation coefficients in Table 5. IVF rate showed negative correlation with SMI, but positive correlation with %AR. Also, correlation between SMI and %AR was negative. Bull fertility showed lower posiitive correlations with the other measures. Rank correlation was positive between IVF rates and negative between IVF rate and fertility. The rest except 2 correlation coefficients showed no significant correlation. Hall(1981) reported that % motile human sperm was not correlated with sperm penetration into hamster eggs. Graham and Foote(1987) showed lower correlation be-

Table 4. Ranking comparison among individual bulls in IVF rate and semen fertility

Bull code	IVF rate			Semen fertility of first AI (50days-NR)			
	Sperm capacitation						
	HIS, DM		DM+20%BFF	No. of cows			
	Oocyte maturation(mKRB)			Insemi-	Concei-	%	Rank
	+10%CSS	+15%FCS	+10%CSS	nated	ved		
A	1	1	2	10	2	20.0	15
B	2	6	−	11	6	54.5	4
C	3	9	1	9	4	44.4	8
D	4	3	−	12	6	50.0	6
E	5	10	−	15	5	33.3	11
F	6	7	3	10	3	30.0	14
G	7	12	−	8	4	50.0	7
H	8	2	4	16	7	43.8	9
I	9	11	−	31	18	58.1	3
J	10	5	−	11	6	54.5	5
K	11	13	6	5	4	80.0	1
L	12	14	−	15	6	40.0	10
M	13	4	−	9	3	33.3	12
N	14	15	5	13	4	30.8	13
O	15	8	−	10	6	60.0	2

Table 5. Correlations between IVF rate and different measures

Variable	Correlation coefficients[1)]					
	1	2	3	4	5	6
1. IVF rate,mKRB+FCS	1.0	0.87 (0.44)	−0.52		0.27	0.30 (−0.08)
2. IVF rate,mKRB+CSS		1.0		−0.39	−0.41	0.01 (−0.19)
3. SMI related to variable 1			1.0	0.30	−	0.22
4. SMI related to variable 2				1.0	1.0	0.20
5. AR related to variable 1						0.15
6. Fertility of AI						1.0

1) n=pairs= 15: $r>0.51$, $p<0.05$. Values in parentheses are the rank correlation coefficient.

tween sperm motility and bull fertility and negative correlation between %AR and fertility. Ax *et al.* (1985) reported high-fertility bulls showed higher AR as compared with lower-fertility bulls.

Barackett *et al.*(1982b) suggested inverse relationship between IVF of bull sperm with zona-free hamster ova and fertility.

SUMMARY

Frozen-thawed sperm from 15 Korean native young bulls being progency test were evaluated their *in vivo*(rabbit oviduct) and *in vitro* fer-

tilizing ability with bovine follicular oocytes which matured in mKRB plus 15%(v/v) FCS or plus 10%(v/v) cow serum at standing estrus(CSS). Rates of *in vivo* and *in vitro* fertilization(IVF) ranged from 35 to 50% and from 6.6 to 85.7%, respectively and IVF rates showed a great variation among individual bulls. Follicular oocytes matured in mKRB plus CSS showed slightly more higher IVF rates than those matured in mKRB plus FCS. However, no difference had between sperm capacitation methods. IVF rates had negative correlation with sperm motility index(SMI) after incubation and low positive correlation with percent acrosome reaction(AR). Semen fertility(50 days-NR) showed low positive correlations with IVF rates, SMI and percent AR, respectively. But the fertility of individual bulls had lower negative rank correlatio with their IVF rates. It was concluded that although IVF rates among individual yong bulls did not show a significant correlation with bull's AI fertility due to the limited data, these findings could provide a possibility of semen quality test to estimate the fertility of frozen bull semen.

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