Studies on the Artificial Insemination in the Domestic Fowl

III. Sperm Concentration of Semen at Collection and Sperm-quality of the Semen

Hisayoshi NISHIYAMA, Kiyohiko OGAWA
and Yoshihiko NAKANISHI
(*Laboratory of Animal Reproduction*)

Introduction

Dense-semen is preferred for the use of artificial insemination because the quality of semen is considered to get better as the sperm concentration of semen increases. Consequently, Lake (1957[1], 1958[2]), and Taneja and Gowe (1961a[3], b[4]) obtained the semen containing no transparent fluid, Kamar (1958)[5] collected dense-semen without milking the copulatory organ and Nishiyama et al. (1967[6], 1968[7]) gathered the semen by sucking only the dense-semen-fraction, using a pipette.

However, it remains obscure whether the quality of sperm themselves contained in the dense-semen is superior to that of the sperm in the semen with lower sperm concentrations.

So, in this experiment, not only to clarify the necessity of collecting the dense-semen but to estimate the influence of the accessory reproductive fluid to sperm, three semen samples with different concentrations were separately collected, and the quality of sperm was compared among the three, by examining the life-span in vitro and by determining the fertilizing capacity after the artificial insemination.

Materials and Methods

1. Semen collection and assessment of semen quality:

For semen collection, highly fecund S. C. White Leghorn cockerels, ejaculating large quantities of semen were used. Semen was collected by Burrows and Quinn's method[8] with a few modifications[6]. Sperm concentration of semen varies during collection, usually it is the densest at the first ejaculation, decreasing with successive ones following the first. According to the density of ejaculates, dense-white (called dense-semen in this paper), milky-white (moderate-semen) and dilute-semen (watery-semen) were separately collected from a cockerel, using a semen collecting pipette[6]. Three semen samples with different sperm concentrations collected from several cockerels were pooled

separately and used for the experiments. Only the clean semen samples without any contamination of feces, urine or dirt, were used.

2. Determination of life-span of spermatozoa:

Two experiments were carried out. In the first experiment, each of the three semen samples with different concentrations was divided into two parts, one part being diluted 5 times with Ringer solution[9], and the other part with Lake's solution[10]. Six semen samples in total, each 0.5 *ml* in quantity, were stored in small test-tubes (8*mm* in diameter, 3 4 *cm* in length), and then the tubes were placed in a large test-tube (3.5 × 10*cm*), then it was soaked in the ice-water put in a vacum bottle. Motility was determined every day from the day of collection to the day on which all sperm in a sample ceased their motilities (Motility=0). Determinations were repeated 23 times, and comparisons were made, among three semen samples with different sperm concentrations, on the life-span, and on the decreasing curve of motility shown by the daily mean of the rates of motilty. Statistical analysis was performed by Snedecor's method[11].

In the second experiment, three semen samples with different sperm concentrations were also collected, each sample being divided into three parts (nine semen samples in total). The first part of the semen composed of three semen samples with different concentrations, 0. 1 *ml* in quantity, was centrifuged for 5 minutes at a speed of 3000 *r. p. m.*, to remove seminal plasma; and to the sperm clots were added 0.5 *ml* of phosphate buffer[12] and then stored in the same manner described above. The second part of the semen samples was prepared in the same manner as the first part of semen, and stored in the phosphate buffer containing 0.4 per cent glucose. The third part of the semen samples, 0.5 to 0.6 *ml* in quantity, was stored as orignal semen without centrifugation and dilution. Life-spans and rates of motility of sperm were determined 20 times, and comparisons were made as in the first experiment.

3. Fertility determination:

a) Determination of fertility of fresh semen:

Three semen samples, the dense-, moderate-, and watery-semen, were collected. Number of sperm of the semen-samples was determined as soon as possible, by optical-density-method,[7] these semen samples being diluted with Ringer solution or phosphate buffer up to 1 million sperm per cubic millimeter ; the rate of the dilution of each semen sample was determined, according to the concentratoin of the original semen; for example, assuming the concentration of sperm in the dense-, moderate-, and watery semen were 8, 3, and 1 millions per cubic millimeter, then the former two were diluted 8 and 3 times respectively, and the last was left untouched. S. C. White Leghorn pullets were inseminated 0.1 *ml* of the semen by Burrows and Ouinn's method.[8] Therefore, the number of sperm inseminated was 100 millions. When the sperm concentration of the watery-semen was less than one million per cubic millimeter, then pullets were inseminated more than 0.1 *ml* semen in order to insminate 100 million sperm; for example, supposing the sperm concentration of the watery-semen was 0.8 million, pullets were inseminated 0.125 *ml* of original semen without dilution.

Eggs laid after insemination were collected and were incubated, to determine the

fertility, and the results were expressed as the rate of fertility during one week beginning from the 3rd day after insemination and as the duration of fertility beginning from the same day.

Culling of the lowered fertile pullets from experimental ones, artificial insemination and fertility determination were carrid out, with the same methods described in the previous paper[6].

b) Determination of fertility of the stored semen

The number of sperm was determined on the three semen-samples with different concentrations, and the samples were centrifuged to remove the seminal plasma. To the centrifuged sperm-clots was added phosphate buffer, in order to make the diluted semen have a concentration of one million sperm per cubic millimeter. The diluted semen-samples were stored at 0°C for a day, then the samples were centrifuged to remove the buffer and then, fresh phosphate buffer was added, to give the concentration of 3 million sperm per cubic millimeter. Pullets were inseminated 0.1 *ml* of the semen or 300 million sperm. The methods of semen collection, centrifugation, artificial insemination and comparison of ferility among three semen samples were the same as those described already.

Results of Experiment

1. Density of semen at collection and the life-span of spermatozoa in vitro

a) Experiment 1 : Life-span of sperm of the three sorts of semen with different concentrations, diluted with Ringer or Lake's solution (Fig. 1 and Table 1).

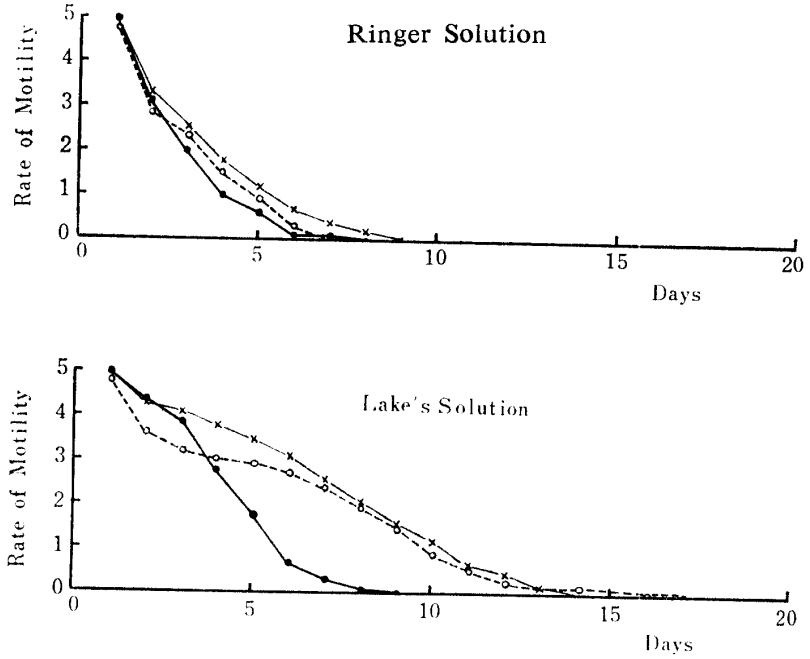

Fig. 1. Decreasing curves of the rate of sperm motility during storage.
•——• Dense-semen (6.24 million/mm^3)
×——× Moderate-semen (2.89 million/mm^3)
○·····○ Watery-semen (0.82 million/mm^3)

Table 1. Life span of sperm of the semen with different concentrations.

Kind of original semen	No. of samples	Sperm concentration of semen (millions/mm^3)	Life-span of semen (Days)	
			Ringer solution	Lake's solution
Dense semen	23	6.24±0.75†	4.3±1.3	5.5±1.2
Moderate semen	23	2.89±0.49†	5.6±1.6**	9.9±3.4**
Watery semen	23	0.82±0.29†	5.2±1.4*	10.0±2.9**

† The difference is significant at 1% level each other.
* Significant from dense-semen at 5% level.
** Significant from dense-semen at 1% level.

The number of sperm of the original ones in the dense-, moderate-, and watery-semen was 6.24, 2.89, and 0.82 millions per cubic millimeter on an average, the difference being highly significant each other.

Against expectation, the life-span of sperm was the shortest in the dense-semen; in case of the storage with Ringer solution, the life-spans of the dense-, moderate-, and watery-semen were 4.3, 5.6 and 5.2 days, respectively, and the differences between the dense- and moderate-semen and the dense- and watery-semen were significant at 1 per cent and 5 per cent level, the difference between the moderate- and watery-semen being not significant. In the Lake's solution, the life-span of sperm was longer than that in Ringer solution, showing significant difference between them by analysis of variance. The life-spans of sperm in three semen samples with different concentrations, however, showed the same tendency as in the Ringer solution; the shortest in the dense-semen (5.5 days), and about the same in the moderate- (9.9 days) and watery-semen (10.0 days). The difference between the dense- and moderate-, or the dense- and watery-semen was significant at 1 per cent level. The rate of sperm-motility in the dense-semen also declined more rapidly than the other two samples with lower sperm concentration (Fig. 1).

From the above experiment, it became clear that the life-span of sperm of the dense-semen was shorter than that of the moderate- or watery-semen. This fact was more clearly demonstrated, in the experiment 2 in which the original undiluted semen was stored in the same method as in the experiment 1.

b) Experiment 2: Life-spans of sperm of the three sorts of original semen with different concentrations and the effect of removal of seminal plasma (Fig. 2 and Table 2).

The number of sperm of the original ones in the dense-, moderate-, and watery-semen was 4.84, 2.78 and 1.27 millions per cubic millimeter, the difference being highly significant each other. When the original undiluted semen was stored, the longest life-span was observed in the watery-semen, followed by the moderate-semen and the shortest in the dense-semen, the difference between them being highly significant each other at 1 per cent level. The rate of sperm motility declined sharply in the dense-semen, gradually, in the moderate-semen, and slowly, in the watery-semen.

On the other hand, when semen was centrifuged to remove the effect of seminal plasma and was stored in phosphate buffer, the difference of life-span of sperm between

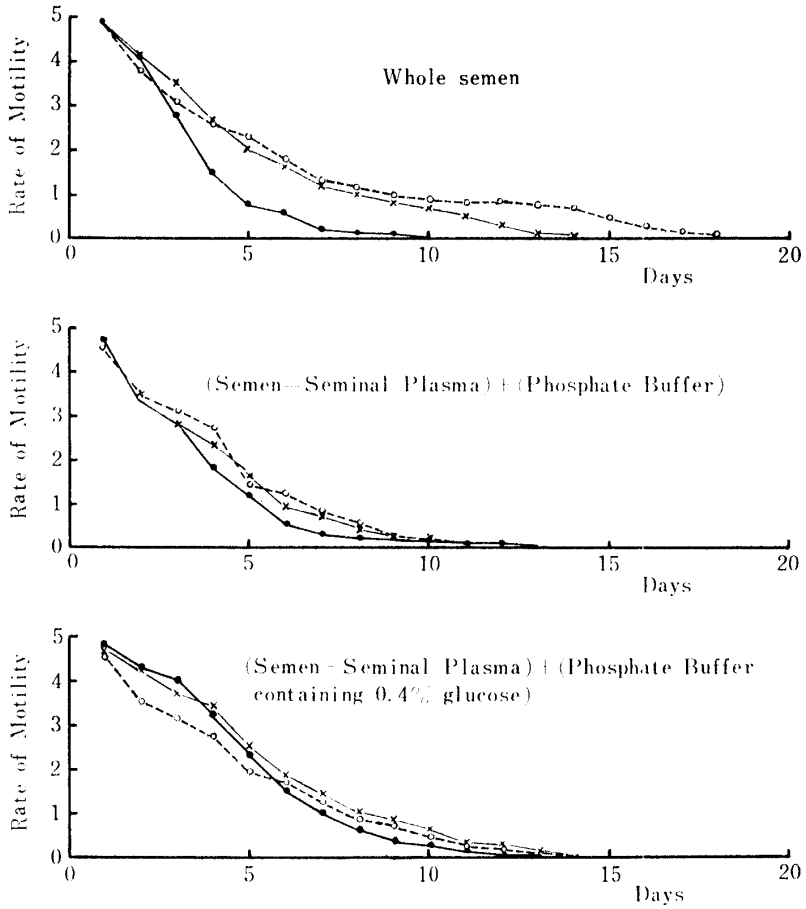

Fig. 2. Decreasing curves of the rate of sperm motility

·——· Dense semen (4.84 million/mm^3)
×——× Moderate semen (2.78 million/mm^3)
○·····○ Watery semen (1.27 million/mm^3)

Table 2. Life-spans of sperm of original semen with different concentrations and effects of removal of seminal plasma.

Kind of original semen	No. of samples	Sperm concentration of semen (millions/mm^3)	Life-span of semen (Days)		
			Original semen	(Semen-seminal plasma)+(phosphate buffer)	(Semen-seminal plasma)+(phosphate buffer containing 0.4% glucose)
Dense semen	20	4.84±0.76†	5.5±1.5†	6.0±2.1	8.6±1.6
Moderate semen	20	2.78±0.41†	10.2±2.4†	7.3±1.8*	10.1±1.5**
Watery semen	20	1.27±0.28†	14.6±2.5†	7.4±1.6*	8.9±2.3

† The difference is significant at 1% level each other.
* Significant from dense-semen at 5% level.
** Significant from dense-semen at 1% level.

the three semen samples with different concentrations became considerably small, although the life-span of sperm of the dense-semen was still shorter than the other two, moderate- or watery-semen (Table 2). The decreasing curves of the rate of sperm motility were also similar each other, because the motility of centrifuged sperm of the moderate- and watery-semen decreased earlier than that of original semen (Fig. 2).

From this result, it may be concluded that the longer survival of sperm of the moderate- or watery-semen is due, for the most part, to the favorable effect of seminal plasma, and also due, though only imperceptible, to the poor quality of sperm in the dense-semen.

Since the accessory reproductive fluid of cockerels contains glucose, centrifuged sperm were stored in phophate buffer containing 0.4 per cent glucose, and the life-spans of sperm were compared among the three semen samples with different concentrations. Differences of the life-spans of sperm among the three semen samples showed about the same tendency as those of the sperm stored in phoshpate buffer without glucose, and the life-span of sperm of the dense semen was also shorter than that of the moderate-semen. The decreasing curves of sperm motility showed also, similar tendency. On the other hand, comparing the life-span between the two sorts of diluent with- and without-glucose, the life-span of the former was longer than that of the latter, and the rate of sperm motility also decreased more slowly in the former.

2. Density of semen at collection and the fertilizing capacity of spermatozoa

a) Fertilizing capacity of fresh semen:

Two experiments were carried out; In the first one, the semen was diluted with Ringer solution, and in the second one, with phosphate buffer.

In the first experiment, the sperm concentrations of the dense-, moderate, and watery-semen were 7.28, 3.72 and 0.98 millions per cubic millimeter, the difference being highly significant (Table 3). Three groups of pullets showng no significant difference in laying intensities were inseminated 100 million sperm. Rates of fertility of the dense-, moderate- and watery-semen-groups were 70.9, 78.9 and 79.3 per cent respectively and the duration of fertility was 10.2, 12.4 and 10.7 days on an average, there were no significant differences between them in both fertility expressions.

In the second experiment, the concentrations of original semen were 5.01, 2.76 and 1.12 millions per cubic millimeter, the difference was also highly significant, and the laying intensities of pullets of the three groups were almost the same, showing no significant difference. The result was the same as at the first experiment, there being no significant differences among fertilizing capacities of three original semen samples with different concentrations; rates of fertility in the dense-, moderate- and watery-semen groups were 81.6, 79.6 and 69.0 per cent and the durations of fertility were 7.3, 7.7 and 6.9 days on the average respectively, and there were no significant differences among them in both (Table 3).

From the above experiment, it was concluded that sperm themselves contained in the dense-, moderate- and watery-semen were the same in the fertilizing capacity; and regardless of sperm concentration of original semen, about the same fertiliy would be obtained when pullets were inseminated the same number of sperm.

Table 3. Fertilizing capacity of sperm of the fresh semen with different concentrations.

Experiment / Item \ Kind of Original Semen	Experiment I			Experiment II		
	Dense semen	Moderate semen	Watery semen	Dense semen	Moderate semen	Watery semen
Concentration of sperm (millions/mm^3)	7.28±0.27	3.72±0.65	0.98±0.12	5.01±0.54	2.76±0.36	1.12±0.34
Diluent	Ringer Solution			Phosphate Buffer		
Dilution rate	7.28	3.72	No*	5.01	2.76	1.12*
Volume of the diluted semen inseminated (*ml*)	0.1	0.1	0.106	0.1	0.1	0.112
Number of sperm inseminated (millions)	100	100	100	100	100	100
Number of pullets inseminated	10	12	14	34	43	44
Laying intensity (%)	61.1	66.7	62.1	63.8	67.1	64.6
Rate of fertility (%)	70.9	78.9	79.3	81.6	79.6	69.0
Duration of fertility (days)	10.2	12.4	10.7	7.3	7.7	6.9

* When sperm concentration was higher than 1 million/mm^3, the semen was diluted with buffer and when sperm concentration was less than 1 million/mm^3, semen was not diluted and pullet was inseminated original semen.

b) Fertilizing capacity of the stored semen:

To clarify whether there may be any difference in the ability to maintain the fertilizing capacity among sperm of the three semen samples with different sperm concentrations, centrifuged sperm of the three samples were stored in phosphate buffer for a day, and then three groups of pullets were inseminated 300 million sperm per pullet.

The concentrations of sperm of the original semen were 5.13, 2.90 and 1.30 millions per cubic millimeter in the dense-, moderate-, and watery-semen, the difference being highly significant. The laying intensities of pullets were almost the same.

The rate of fertility was slightly higher in the moderate-semen-group (78.9 %) than in the other two groups (65.2 and 64.9 %), but the difference was not significant. The duration of fertility was significantly longer (at 95 % confidence) in the moderate-semen-group (7.5 days) than in the other two groups, the dense-semen- and watery-semen-groups being almost the same, 5.7 and 5.6 days, respectively (Table 4). Judging from the non-existence of significant difference in the rate of fertility and from the existence of signiffcant difference in the duration of fertility, the superiority of sperm of the moderate-semen in the abilities to maintain the fertilizing capacity, if it ever exists, may be slight. So that, it may be concluded that the sperm of the three sorts of semen with different concentrations were about the same in their abilities to maintain the fertilizing capacity.

Table 4. Fertilizing capacity of sperm of the stored semen with different concentrations.

Item \ Kind of Original Semen	Dense semen	Moderate semen	Watery semen
Concentration of sperm (millions/mm^3)	5.13±0.82	2.90±0.36	1.30±0.35
Volume of the diluted semen inseminated (*ml*)	0.1	0.1	0.1
Number of sperm inseminated (millions)	300	300	300
Number of pullets inseminated	24	27	25
Laying intensity (%)	53.0	51.3	50.3
Rate of fertility (%)	65.2	78.9	64.9
Duration of fertility (days)	5.7	7.5	5.6

Discussion

It has been generally accepted that the quality of semen of fowls is the best one in the dense-semen, and this idea probably depends on several reasons or opinions. A major reason is based on Lake's opinion[1] that the transparent fluid or the accessory reproductive fluid (he called blood plasma transudate) of the fowl is not a normal constituent of semen, and more dilute-semen contains more of the fluid excerting ill effect on sperm. This Lake's opinion, however, was denied by Nishiyama and Fujishima (1961)[13], who demonstrated that the transparent fluid was a normal constituent of fowl's semen and was ejected in the natural copulation. Second reason may be a higher initial motility of sperm in the dense- and moderate-semen. Under microscopical observation, the motility of these semen samplesappears to be very vigorous, the rate of motility appearing very higher than that of the dilute- or watery-semen. As described by Rice et al. (1957)[14], the appearance of these semen is that of millions of sperm in vigorous motion with fierce eddies and currents and constant undulating movement. It is difficult to distinguish the behavior of individual sperm and even dead sperm appear to move because of the motion set up by the live sperm. So that, the appearance of these semen differs greatly from that of the diluted semen containing the sperm of the same motility; in the diluted semen, there is little or no movement of the liquid itself and the motility of sperm appears very lower than that of dense-semen. Therefore, the motility of sperm of dilute-semen should be determined on the basis of the type and percentage of motile sperm. In this experiment, initial motility was the highest in the dense-semen, followed by the moderate-semen and the lowest in the watery-semen, the difference, however, being little and not significant (Fig. 1 and 2).

The third reason may be that when dense-semen is diluted at the same time as dilute-semen, and then pullets are inseminated the same volume of the diluted semen, a higher fertility might be obtained out of the dense-semen. Such a higher fertility, however, may be considered to be due to largely the greater number of sperm inseminated.

From these considerations, such an idea that semen is the most superior in the dense-

semen and that only the dense-semen should be collected for the use of artificial insemination might require re-examination.

In this experiment, the life-span of sperm of the moderate-and watery-semen were longer than that of the dense-semen. Considering the cause of this fact, the senescence (aging) of sperm in the dense-semen and favorable effects of seminal plasma in the moderate- and watery-semen may be assumed. The senescence of sperm, however, seems to be not probable, because the semen used in this experiment was collected from cockerels at intervals of one to three days. In spite of this expectation, comparing with sperm of the moderate-semen, poor quality of sperm was assumed in the dense-semen by the experiment as to life-span of sperm, the effect of seminal plasma of which had been removed beforehand (Table 2). However, the differences of the life-span and of decreasing rate of motility among three sorts of semen with different concentrations were not so large, and we were led to assume that poor quality of sperm in the dense-semen might be imperceptible. The other presumed factor for longer life-span of sperm in the moderate-and watery-semen, in other words, the favorable effect of seminal plasma, seems to be probable; because the major difference among three sorts of semen is the concentration of sperm, in other words, the difference in the quantity of the accessory reproductive fluid: for example, if we suppose that the concentration of sperm in the dense-semen is 5 millions per cubic millimeter, 3 millions in the moderate-semen and 1 million in the watery-semen, the rates of the accessory reproductive fluid added to the semen ejaculated from the vas deferens are estimated to be about 60 %, 270 % and 800 % respectively, because the sperm concentration of the semen in the vas deferens is about 8 millions per cubic millimeter[1), 13), 15)]. Seminal plasma of the ejaculated semen is, of course, composed of seminal plasma of the semen ejected from the vas deferens and the accessory reproductive fluid ejected from the lymph-fold,[13), 15), 16)] but it is impossible to assume favorable effect of the former in the moderate- and watery-semen because the rate of the former in the seminal plasma of the dilute-semen ought to be lesser than that of the dense-one, and the former can not be considered to be exerting ill-effect to the life-span of sperm in the dense-semen. Therefore, the favorable effect of seminal plasma to the life-span of sperm may be attributed to that of the accessory reproductive fluid rather than to that of seminal plasma of the semen ejected from the vas deferens. Favorable effects of seminal plasma or accessory reproductive fluid on the life-span of sperm were shown more clearly in case of the storage of undiluted semen, because, in this case, seminal plasma had not been diluted with phosphate buffer; accordingly accessory reproductive fluid contained in them presumably might be assumed to have exerted its favorable effect to the sperm directly.

The accessory redroductive fluid contains glucose-quantity comparable to the level of blood serum[13)], the level reported by several authors being 230- 300 *mg.* per 100 *ml.*[17)] And it was also reported that glucose present in cock semen was utilized by sperm,[18), 19)] and that the life-span of sperm was able to be lengthened by adding it into diluents.[20)] In this experiment, as well, the life-span and the rate of motility of sperm during storage were better in the buffer containing glucose than those in the buffer without it. So that, one of the favorable factors of the accessory reproductive fluid might be presumed to be glucose contained in it.

Setting aside the favorable effect of the accessory reproductive fluid, it was revealed

from the experiment of life-span of centrifuged sperm that the sperm themselves contained in the dense-, moderate- and watery-semen were almost the same in their qualities.

Fertilizing capacities of sperm in the dense-, moderate- and watery-semen were also about the same in both experiments carried out, using fresh and stored semen, although in the experiment using stored semen, rather better fertility was obtained with the moderate-semen than with the dense-semen, and this result might have some connection with longer life-span of sperm in the moderate-semen, when the centrifuged sperm were stored in phoshate buffer.

Therefore, it may be concluded that there are no great differences in the qualities of sperm themsleves, regardless of the sperm concentration of the original semen.

Considering these results of this experiment, it is hard to accept the idea that the dense-semen is the best in its quality. And it is concluded that more dilute-semen, at least, the moderate-semen, can be used for artificial insemination. Of course, we have no intention to refuse the dense-semen: on the contrary, we recommend the dense-semen for the use of artificial insemination, because the semen is able to dilute at a higher rate. It means that the moderate-semen which is usually ejected after the ejection of the dense-semen is able to use effectively for artificial insemination, and that it might be unnecessary to pay special considerations for the techniques or cares to collect the dense-semen. The use of the watery-semen may not be practical, because the semen must be used, without dilution, as much as 0.1 *ml* of original semen for one pullet to obtain a favorable fertility.

The semen used in this experiment is clean one and is collected from fecund cockerels, so that the results of this experiment should not be applied to the semen which was diluted by contamination of feces, urine or other alien substance or fluid and also should not be applied to the dilute-semen obtained from unfecund cockerels. These sorts of semen should not be used for artificial insemination.

Summary

The present investigation re-examined the generally acepted idea that the quality of dense-semen was the best one for the use of artificial insemination.

Three sorts of semen with different concentrations, the dense-, moderate- and watery-semen, were collected separately from a fecund cockerel, and each semen obtained from several cockerels was pooled separately for examination. Qualities of sperm of the three sorts of semen were compared by determining the life-span of sperm in vitro, and by determining the fertility of pullets inseminated the same number of sperm.

The results obtained were as follows:

1. The life-span of sperm of the original semen was the longest in the watery-semen, then in the moderate-semen, and the shortest in the dense-semen, the difference among them being highly significant each other. The rate of sperm motility declined more sharply in case of the dense-semen, gradually in the moderate-semen and slowly in the watery-semen.

2. The life-span of sperm in Ringer or Lake's solution was also the shortest in the

dense-semen and almost the same in the other two sorts of semen. Comparing the life-span within two diluents, sperm in Lake's solution showed significantly longer survival than that in Ringer solution. The rate of sperm motility also showed the same results as that of life-span; it decreased more sharply in the dense-semen, and more slowly in the moderate- and watery-semen.

3. When sperm were centrifuged to remove the seminal plasma and were stored in phosphate buffer, the differnce of life-span of sperm among the three sorts of semen became considerably small, although the life-span of sperm of the dense-semen was still shorter than that in the other two, moderate- and watery-semen. The decreasing curve of rate of motility was also similar each other. Life-spans of the sperm stored in the phosphate buffer containing 0.4 per cent glucose were longer than that in the buffer-without glucose. Accordingly, the longer survival of sperm of the moderate- and watery-semen was considered to be due, for the most part, to the favorable effect of the accessory reproductive fluid, and, even though only imperceptible, to the poor quality of sperm in the dense-semen. And one of the favorable factors of the fluid was estimated to be the glucose contained in it.

4. Provided that pullets were inseminated the same number of spem, fertilizing capacities of sperm in the dense-, moderate- and watery-semen were about the same in both experiments, using fresh- and stored-semen.

From these results, it is hard to accept the idea that the quality of the dense-semen is the best one for the use of artificial insemination, and it is concluded that the semen of lower concentrations, at least the moderate-semen, can be used effectively for artificial insemination and that there might be no necessity to pay the special considerations for techniques or cares for the collection of dense-semen.

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