Development of a bovine sperm selection procedure for improvement of livestock fertility

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Improving the reproductive performance of livestock has wide-ranging significance that includes promotion of local industry, bioeconomy, and stabilization of food supply. Our research focused on sperm manipulation to improve the reproductive performance of cattle. Our experiments were based on previous studies on infertility treatment for humans by relying on the advantages of motile spermatozoa, i.e. spermatozoa that are capable of swimming against the flow of solutions, which is regarded as an attribute of healthy and physiologically functional spermatozoa. For the first time, we succeeded in collecting a number of spermatozoa that can be used for artificial insemination and obtained good conception results in a field trial. In addition, the field trial clarifies the advantageous relationship between sperm trajectory and conception.

Keywords : Chemical engineering, fluidics, livestock, breeding, spermatozoa

1 Introduction

The increase in world population is projected to continue for some time in the coming years, and with the changes in diet accompanying economic growth, in addition to quantitative food demand, changes in qualitative demand such as for animal-based protein are expected. Therefore, there is high technological demand for increased production in livestock industry. In addition, sustainable development of agriculture including livestock has wide-ranging significance, such as contribution to local food culture, and promotion of local and regional industrial recovery. Recently, social demands have incorporated new concepts such as bioeconomy, sustainable development goals (SDGs), and animal welfare, thus emphasizing the necessity of conducting R&D wherein various concepts from different disciplines are included. Particularly, bioeconomy is expected to become one of the main axes of technological innovation in the near future. For example, the Ministry of Economy, Trade and Industry has published Reference [1] "Toward a new bioeconomy society through bio x digital," and the market size is expected to reach 1.6 trillion dollars in 2030.

In Japan, livestock holds about 35 % of the agricultural production, and occupies the number one position among agricultural products leaving behind rice, vegetables, and fruits.[2] On the other hand, since it involves animal husbandry, constant work is necessary, and it is an archetypal example of industry in which "one cannot take time off."[3] It is also particularly hard hit by a shrinking agricultural workforce and aging population. Among livestock, "cattle" is the most influential in terms of market size and environmental impact. In this study, the focus is placed on the stage of "breeding" rather than "fattening" which is a stage where the cattle are raised to a large size. A cow gives birth only once a year even in ideal breeding situations, and unlike hogs, it produces only one calf per birth. Since the price per animal is high and since the animal is large, the cost of feed is high, and the success and failure of breeding greatly affects the farm business. Sperms collected from a bull that has excellent economic traits and reproductive ability are diluted, divided into 0.5 mL amounts, sealed and frozen in straw-like containers, and are distributed commercially. Breeding by artificial insemination (AI) is done by thawing the sperms and then introducing them into the cows' reproductive organs. That is, a natural mating program utilizing service live bulls does not occur in cattle breeding under the AI program. Currently, most cattle breeding (90 % or more) is done by AI. Although *in vitro* fertilization and transplantation of fertilized eggs have been put to practice as next-generation technologies, they have not diffused widely, since AI is simple and there is plenty of practical experience gained in such a method.[4] The rate of breeding success (conception rate) by AI in Japan is in a long-term downward trend, and currently it is 50–60 % for beef cattle and 40–50 % for dairy cattle.[5] To increase the reproductive capacity, studies from both the bull side (spermatozoa and sperms) and cow side (eggs and reproductive organs) are being conducted. Many involve selective breeding, clinical veterinary medicine from the cow side, and estrus monitoring using ICT in recent years, but the production method, for example, of frozen sperms has not really changed since the 1950s,[6] and

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not much research has been conducted on the bull side.

On the other hand, in the field of human infertility treatment, against a background of progress in analytical devices and embryo culturing technology, the importance of spermatozoa factors is becoming clear. For example, it is reported that healthy spermatozoa have significantly higher advantage in the rate of implantation, conception, and abortion.[7] The "healthiness" of spermatozoa here specifically means the integrity of elements and functions as reproductive cells such as "having little fragmentation of DNA." Moreover, it has been reported that spermatozoa with high motility[8] and those with good morphology[9] are relatively healthy and functional. It is also reported that high motility is advantageous in traveling through female reproductive organs and this is important in increasing the pregnancy rate.[10] It is also reported that about one-fourth of infertility in cattle actually involves extremely early miscarriages of which one may be even unaware of conception.[11] It is indicated that improvement of the health of spermatozoa is important, from the perspective of ensuring the healthiness of the embryo which does not stop developing after fertilization. That is, to improve the pregnancy rate of cattle and to increase productivity, thereby reducing workload in the Japanese cattle industry, we considered the prospect of improvement in the male factor and started R&D for improvement of semen quality by selecting high quality spermatozoa, a rather unexplored area.

There are complex reasons for the long-term downward trend of the pregnancy rate. They include complex combinations of genetic degradation (that leads to inbreeding depression or inability to survive and reproduce) occurred as a result of inbreeding in pursuit of traits with economic value, as well as aging of personnel in charge of estrus monitoring which is dependent on tacit knowledge. It is apparent that various kinds of research are necessary in such areas. As the importance of the spermatozoa became known in recent years in the field of human infertility treatment, we decided to work on the improvement of frozen sperms on which hardly any studies have so far been done.

2 Design of research plan

Figure 1 summarizes the process of investigation from organization of social demand to building of specific research.

For the final goal which is transfer of technology, it was necessary not only to obtain highly motile spermatozoa but also to conduct field trials at farms, and this was an issue

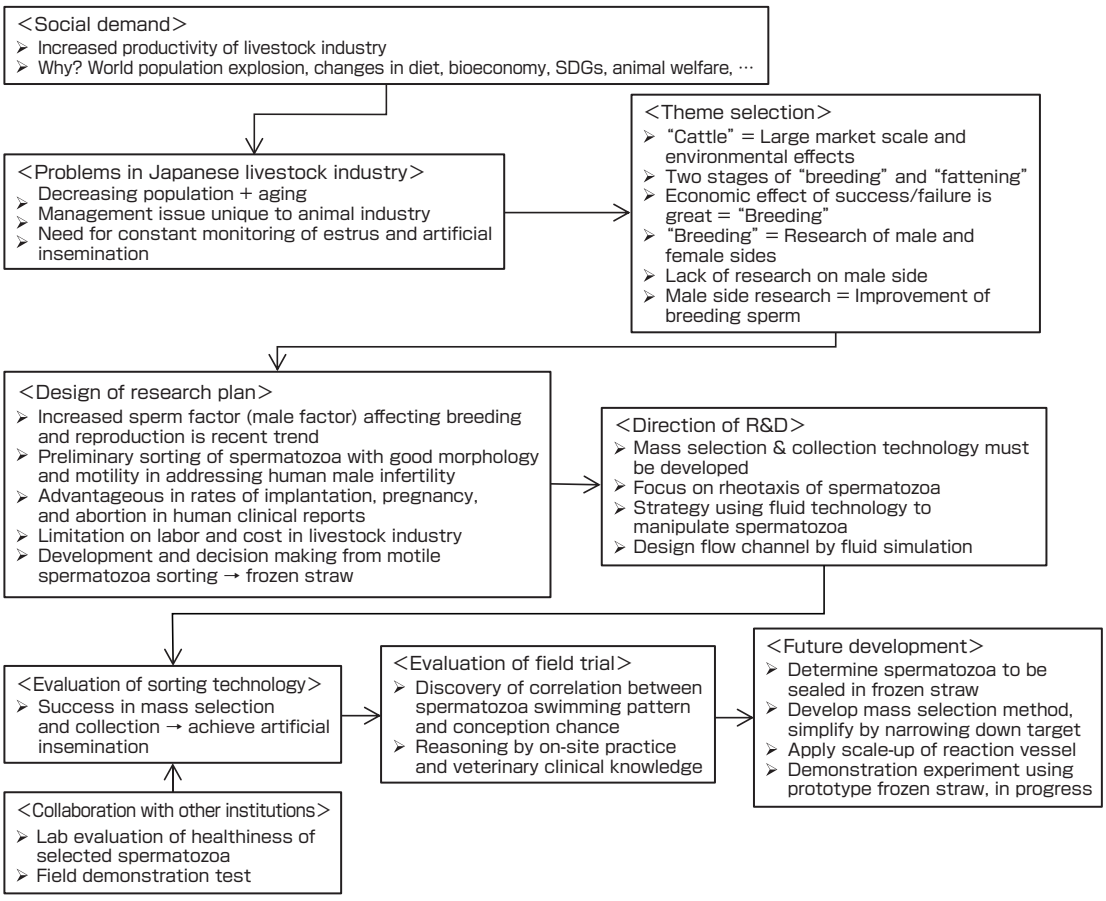

Fig. 1 Conceptual diagram of concept flow where research plan based on social demand is broken down into specific actions

that could not be dealt with by AIST alone. Meaning, an interdisciplinary fusion was necessary, and considering that the sites at which research could be jointly conducted are dispersed in the regional farmland areas (due to the nature of the livestock industry), a regional collaborative effort was called for in order for the project to materialize. In general, the points that need attention in the interdisciplinary fusion (and thus, crossing knowledge boundaries) include differences in premises that were considered common knowledge, and lack of common understanding for expected outputs, and for heights of barriers that need to be overcome. These were also apparent in this research, and, for example, although good results were obtained in the field as a result of sorting highly motile spermatozoa, in order to achieve social implementation, there was a hurdle faced in the area of chemical engineering in terms of scaling-up of sperm sorting. The essence of sperm sorting could not be judged beforehand, faced with the obstacle presented by scale-up, and vice versa, for scale-up to be realized, the necessity of determining specific conditions of sperm sorting. Moreover, from the AIST side, we had no idea which work would incur a specific workload and in what level, and other institutions have seen the research as not realistic. Therefore, in conducting this research, we only set an outline regarding research policies and division of roles, and each institution was asked to come up with specific experimental methods. By assessing technical superiority based on the data obtained, we gradually clarified the topics that had to be tackled next. That is, there was no specific work plan prepared from the beginning. When conducting such research, testing opportunities are limited because trials involve large animals like cattle, and satisfactory trial plans cannot be drawn. Therefore, it was necessary to repeatedly scrutinize the data and plan the next trial.

In conducting improvement of semen by sperm selection for livestock breeding, R&D was planned under the assumption that unlike human male infertility, a large-scale facility, manpower, and cost could not be obtained. In human infertility treatment, conventional methods involved obtaining healthy spermatozoa by selecting spermatozoa with high motility. However, only a small quantity of spermatozoa can be collected by this method, and it was mainly used for micro-fertilization. On the other hand, cattle breeding is primarily done by artificial insemination, and it was necessary to increase the number of selected spermatozoa to several hundreds of thousand times which was normally obtained by conventional methods. We thought that this should be conducted mainly by engineering methods, and AIST became in charge. Since it would eventually become necessary to conduct field trials wherein work must be done by closely observing cattle, it should not involve complex work procedures. On the other hand, the institutions at which field trials were conducted evaluated the properties of spermatozoa every time prior to artificial insemination, and performed reproductive health checks of the cows. They also kept records of the time when estrous behavior was observed, time at which insemination was performed, sizes of the follicles, and estimated time of ovulation. Universities conducted cellular biological analysis of the spermatozoa, to evaluate the adequacy and efficiency of AIST's sperm sorting technology, and to investigate the cause-and-effect relationship with conception.

It was also agreed that the "final product will be frozen sperm straws" in organizing future concepts. This meant that the work of sealing livestock breeding spermatozoa into straw-shaped containers and providing the product in this standard format, must be routine work without incurring any additional burden to the cattle farmer. This means without the requirement of learning new techniques or installing new facilities. It was clarified and identified what type of spermatozoa should be sealed, and once the method to select and collect large amounts of such spermatozoa was developed, as the next step, a common understanding that the procedure to create frozen straws would be conducted was organized. The main role of AIST was to develop the process technology of developing an inexpensive and simple manufacturing facility that was highly compatible with the current sperm straw manufacturing process. However, in the structure of interdisciplinary fusion, expectation ran high, and it was necessary to constantly check the awareness on the height of the obstacle of each phase.

3 Selection of research elements by considering specific role of participating institutions

The general goal to improve livestock breeding through the improvement of breeding sperms involves three factors of research: to develop technology to select and collect large amounts of highly motile spermatozoa; to check the isolated spermatozoa for their healthiness as reproductive cells; and to conduct field trials by using the sorted spermatozoa in the breeding process at farms. AIST was mainly in charge of the development of technology to select spermatozoa with high motility. Analyses of selected and isolated spermatozoa were conducted mainly by the National Agriculture and Food Research Organization, Saga University, University of Toyama, and Toyama Prefectural Agricultural, Forestry and Fisheries Research Center. Field trials at farms were done by the National Livestock Breeding Center, Saga Prefectural Livestock Experiment Station, and Morinaga Rakunou Co., Ltd. AIST approached these institutions, shared the understanding that improvement of spermatozoa was important to increase livestock breeding, obtained agreement to select spermatozoa with high motility as a specific method, and asked cooperation considering work content and facilities of the institutions.

There are several conventional technologies and methods

for sorting highly motile spermatozoa that AIST was in charge. The simplest method is routine work of capturing highly motile spermatozoa with a pipette when they gather at the laminar interface. The swim-up method is a method in which sperms are centrifuged and allowed to settle, and then highly motile spermatozoa that swim upward are collected. This method is conducted widely, and there are tools used to perform this procedure easily and plainly. There are also methods that use Percoll, a solution that creates density gradients during centrifugation.[12] Such conventional methods can be distinguished from our concept, if considered from the perspective that the conventional methods are designed to remove spermatozoa that died or lost motility, rather than gathering the ones with high motility. Such pretreatments are commonly done in bovine *in vitro* fertilization and human infertility treatment.

On the other hand, the number and quality of spermatozoa gathered by such conventional methods could not be maintained at the required levels simultaneously. If qualitative homogeneity was to be maintained from the perspective of healthiness, the number of spermatozoa necessary was in units of several hundreds or thousands. On the other hand, it is difficult to maintain quality when a high number of spermatozoa is desired. For AI application which is the main approach to cattle reproduction, a large number approximately approaching several to tens of millions spermatozoa is necessary. With the conventional methods, it is difficult to gather large amounts of highly qualitatively healthy spermatozoa, and it is unknown how many spermatozoa of such high quality is necessary to conduct a successful AI.

Based on previous studies, a microfluidic approach is the most appropriate platform to ensure the quality of the selected spermatozoa. The relationship of the size of spermatozoa (several ten micrometer unit) and the size of microchannels (several hundred micrometer unit) is suitable for effective spermatozoa sorting, apart from the sieve method. Various devices have been developed, and several reports present investigation on the qualitative property of the sorted spermatozoa.[13] However, as the size of the microchannel relative to the size of spermatozoa to maintain swimming against the flow has a limit, the number of spermatozoa that could be selected at a specific time is also limited, thus, challenging the microfluidic approach in terms of surpassing such limits. Looking at the property of spermatozoa motility, the phenomenon of "rheotaxis" is well-known. Rheotaxis is described as the property of "swimming against the flow." The phenomenon itself has been known for quite some time, but recently, detailed reports have been provided on the physics of the actual behavior.[14] This phenomenon was considered for the possibility of guiding the navigation of spermatozoa with the flow, and this led to the technological idea of "having the spermatozoa gather by themselves." This initially seemed to be a strange idea for researchers who have been involved in conventional livestock breeding.

On the other hand, in the field trials conducted by institutions other than AIST, the experiments could not be similarly planned as the organized condition in a laboratory, and therefore, technologies such as machine learning were applied for data organization. Details will be explained later. While AI is conducted based on the observation of estrous behavior, at actual sites, it can be missed depending on the scale and form of the feeding facility or the number of available personnel. There were two feeding forms at the institutions involved in this study: one in which cattle were kept in individual sections of a barn; and another in which cattle moved from pasture to barn at regular intervals. In the latter case, it was difficult to observe estrous behavior or conduct AI while cattle were in the pasture. When the cattle were kept in a barn, stickers could be placed on their backs to record estrous behavior or frequent monitoring could be done after observing follicles by echo. These ensured the detection of estrous behavior, and it was also possible to check ovulation by echo AI.

4 R&D of each element

To realize the basic research structure for using the flow to gather large amounts of highly motile spermatozoa and to guide the spermatozoa to self-gather for collection, we investigated specific methods to be used.[15][16]

To "guide the spermatozoa" by "flow" or to link the gap between two elements of fluid manipulation technology and spermatozoa motility, investigation was done using fluid simulation technology. Here, the parameters set included the property of sperm movement (the rate is several tens to hundred micrometers per second) or the size of the microchannel of 100 μm that could be realistically fabricated.

The flow in the microchannel was a slow "laminar flow," and the central part of the channel had a faster flow rate than alongside the wall. It was calculated that the number of spermatozoa that could be transported with just one microchannel was far from enough. That is, unless the difference between the parts where the flow was fast and where the flow was slow could be minimized as much as possible, the separation efficiency of spermatozoa would not increase. The solution to this issue was simple; a partition board was placed in the channel. At the same time, the size of spermatozoa and the size of the wall that could be realistically fabricated were set as prerequisites of simulation. For example, if the walls were made too thin to increase the number of spermatozoa, the partition would not stand on its own, and might break when it was removed from the mold. However, the number of spermatozoa that could be selected

was still insufficient.

Pressed by the necessity to gather spermatozoa more aggressively, we decided to set a layered crescent-shaped channel as shown in Fig. 2. Spermatozoa are sucked in and gathered at the edge of the crescent regardless of motility, and then transported to an area near the entrance of the microchannel. Of the selected spermatozoa, those with high motility will swim up as they sense the flow in the microchannel, while those without motility will be pushed along with the flow. Although the point of using a crescent-shaped flow channel as a structure that allows continuous separation may seem to be a breakthrough, anyone with knowledge of mechanical engineering that looks at the viscous force of fluids will more likely consider that given a flow suddenly entering a wide space, a flow that pulls in the surrounding fluid is formed. A flow that repeats such separation and a flow channel that realizes this action were designed by fluid simulation, and the overall design of the device was determined considering the ease of use at farms and the actual amount of sperms that would be treated. Following the overall design, a device was fabricated by a mold cutting process and transferred to silicon rubber. The sorting device for highly motile spermatozoa fabricated by this technology is simple, as shown in the schematic diagram of Fig. 2 and the photograph of Fig. 3. Fluids are delivered by the height difference of the liquid surface of the three fluid reservoirs of the device, and no exterior mechanism such as pumps is required.

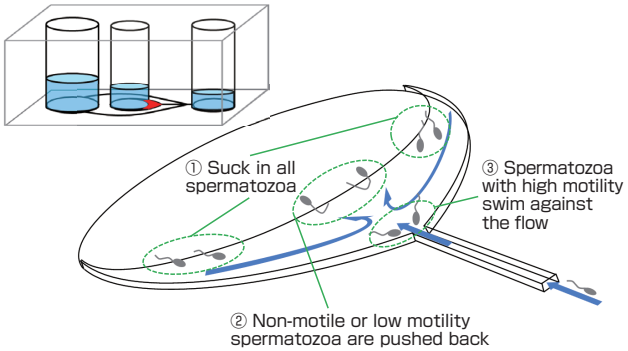

Fig. 2 Overall conceptual diagram of device for motile spermatozoa sorting, positioning of crescent-shaped layered flow channel (red part), and concept of flow of solution and motion of spermatozoa in crescent layered channel

Fig. 3 Photograph of device for sorting motile spermatozoa

The actual selection of highly motile spermatozoa was conducted using this device, and it was confirmed that, for example, about 1 million to 10 million highly motile spermatozoa could be obtained in a sorting process of about 30 minutes, using one typical straw of frozen sperms (number of contained spermatozoa 30–60 million, 0.5 mL fluid). The actual number of spermatozoa obtained is dependent on the concentration and quality of the spermatozoa in the original sperm. In the case of cattle, this number of spermatozoa fulfills the number of spermatozoa required for AI.

In addition, by adjusting the rate of fluid flow, it is possible to sort according to the manner of movement of spermatozoa, such as “straight swimming” or “zigzag swimming” (Fig. 4), and not merely whether they are motile. Spermatozoa do not possess fertilization ability from the beginning even if they become mature sperm cells. It is known that there is capacitation, or changes into fertilizable form through various biochemical reactions and increased motility as they travel through the female reproductive organ, and that the swimming forms change accordingly. That is, selecting spermatozoa based on the characteristic motility provides a means of selection of spermatozoa at a specific stage of change or a “capacitation event,” and this was the first time that we realized a method to study the characteristics of spermatozoa that is advantageous to AI.

5 Evaluation of results

The correlation between highly motile spermatozoa and qualitative healthiness had been known, but in this study, evaluation was done for the spermatozoa that were sorted using the developed technology. When the DNA

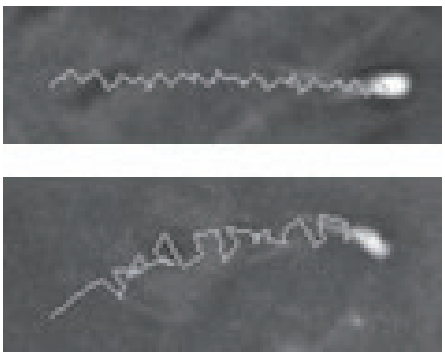

Fig. 4 Motion of spermatozoa is not uniform, and some may swim straight while others may swim zigzag. The swimming pattern is one of the indices that represent the condition of spermatozoa.

fragmentation rate of the selected motile spermatozoa was investigated, it was 7 % before treatment and 0.4 % after treatment, demonstrating and confirming a remarkable improvement in terms of DNA. Low DNA fragmentation, in other words, means that the integrity of DNA carried by the spermatozoa is high. Considering the fact that the DNA fragmentation rate of commercial breeding sperms is around 5 %,[17] the value obtained is sufficient. The sperm after treatment has high mitochondrial activity, and it was also found that the high activity was maintained over a long time. For example, the mitochondrial activity of untreated sperms decreased to about 20 %, but the treated sperms had about 60 % activity six hours after treatment.

Since the technology that we developed was for livestock breeding, it was necessary to conduct AI at farms and investigate the performance. The aforementioned devices were distributed to a number of farms, and when cows were observed to be in estrus, the thawed sperms were treated using the device, and selected sperms were used in AI. The determination of pregnancy or non-pregnancy was conducted 40–50 days later, and correlation of the test results on the motility of the selected sperms and pregnancy test results was established. One frozen straw containing cattle breeding sperms used in ordinary artificial insemination contains about 20 million to 30 million spermatozoa. In this test, AI was performed with about one million selected highly motile spermatozoa. Upon comparison, the pregnancy rate of cows using this remarkably lower number of selected spermatozoa was the same as the pregnancy rate of the past few years of the subject area (test farm). The progress of pregnancy and the calves born were normal.

Since field trials were done at a number of farms, there were differences on how work was handled at different farms, and the environmental conditions could not be kept completely uniform; a reasonable reality of field trials. Under such situation in which conditions could not be kept uniform, we attempted excavating new findings, and various statistical methods and machine learning were used for data analysis. As a result, we found that there was a correlation among the results of pregnancy and infertility, the motility of spermatozoa used in artificial insemination, and the timing of artificial insemination. Specifically, the pregnancy rate was better in spermatozoa that swam zigzag compared to the ones that swam straight. This tendency was more apparent when timing of AI was later from the discovery of estrus.

As mentioned earlier, spermatozoa do not possess fertilization ability from the beginning, but change to a state in which they can fertilize after undergoing various biochemical reactions and increased motility in the female reproductive organ, and the swimming form changes during this process. While a commercial spermatozoa motility analyzer can measure the number of vibrations of the head of the spermatozoa and travel speed such as linear and curve velocity (spermatozoa may travel arc-like while waving their heads, and there are several ways of representing the speed according to the interpretation of the track), there was no index for representing the "swimming form." Therefore, we originally defined SMI index as the index to represent the "swimming form," and conducted an evaluation. The SMI index was defined as the value in which the product of the linear velocity and the number of head vibrations was divided by the curve speed. The larger SMI indexes indicate linear swimming, while the smaller indexes show zigzag forward motion.

Figure 5 shows the results of pregnancy and infertility after AI. The time from the discovery of estrous behavior in cows to performing AI is shown in the horizontal axis, while the vertical axis shows the SMI index of the spermatozoa used in AI. The pregnancy and non-pregnancy plots are indicated by different colors. These results demonstrate that the pregnancy rate is higher for zigzag-swimming spermatozoa for the time span of 8 to 24 hours that is generally used for AI. For AIs conducted earlier than the above timing, the same or at the least not less pregnancy rate was observed in the straight-swimming spermatozoa. Such results suggest an existing relationship between the time required for the spermatozoa to travel in the female reproductive organ and the timing of ovulation. Moreover, these results clarify the property of spermatozoa, and identify the sperm population types that should be supplied and contained in the livestock breeding straw. In addition, we were able to discern and classify spermatozoa according to the swimming form or motility pattern. On the other hand, the plots representing non-pregnancy (infertility) show that SMI remains almost constant in a horizontal line against time, suggesting infertility to be ascribed to other factors such as the physical conditions of the cows.

6 Future development

To achieve technological implementation and realize the benefit of this work in terms of increasing reproduction, the following development has been considered while taking into account both the obtained scientific findings and the actual practice on site.

For AI of cattle, the "AM-PM method" had become the standard, in which insemination is done in the afternoon of the day when one finds the cow in estrus in the morning, and on the morning of the following day when estrus is observed in the afternoon. By this method, the interval between ovulation and AI is roughly controlled. Understanding and analysis of Fig. 5 will be facilitated better with the AM-PM method in mind. For the time frame starting from estrus discovery to AI, as shown in the horizontal axis, rather than the short time, the long time frame matches the timing of AI based on the AM-PM rule. Looking at the distribution of

pregnancy and fertility in the long time frame, the zigzag-swimming spermatozoa lead to higher pregnancy. With the AI purpose in mind, it is concluded that incorporating higher percentage of zigzag -swimming spermatozoa in the sealed frozen straws will yield more efficient reproduction in cows. This is based on the concept of fine-tuning the timing of the encounter of the ova and spermatozoa in the female reproductive organ.

Currently, these selected "spermatozoa appropriate for AI" are manufactured as frozen straws, and their efficiency in increasing conception rate is being conducted at farms. To produce frozen straws, it is necessary to scale up the selected and isolated highly motile spermatozoa while considering the manufacturing facility for mass production. This requires a review of the cryopreservation process.

Scaling up of the selection of highly motile spermatozoa is investigated based on the concept of the microfluidic device. By narrowing the target to zigzag-swimming spermatozoa, we are working to enable easy operation during on-site implementation, including simplifying the setting conditions of actual sperm selection maneuvers. On the other hand, for the cryopreservation process, the method of adding a cryoprotectant to the spermatozoa solution and spermatozoa concentration must be simultaneously investigated. Egg yolk and glycerol are used as cryoprotectants. The technology for sorting highly motile spermatozoa involves the selection of traveling spermatozoa, and the presence of granular substances like egg yolk in amounts beyond the limit tolerable by a specific number of spermatozoa must be avoided, as such substances may damage the spermatozoa and consequently inhibit forward motility and sperm function. Cryopreservation takes the course in which temperature is gradually decreased to 4 ºC and then rapid freezing is done, and the components of the cryopreservation solution needed to protect the spermatozoa at each stage are fixed. Therefore, each component of the cryoprotectant solution must be considered and investigation must be performed on what specific component must be added at which particular stage of cryopreservation.

Field trials will be done by creating frozen straws under a combination of various conditions, checking the motion of spermatozoa after thawing, and after achieving the most appropriate frozen straws verifying its efficiency by investigating the pregnancy rate by performing AI at farms. This is a time-consuming trial but we are making progress with the cooperation of farmers, and in the near future, we expect to release a new livestock breeding sperm product.

7 Summary

This research was aimed to increase the livestock reproductivity, particularly cattle by improving the frozen semen used in AI thru healthy and functional sperm selection. The specific procedures mainly depends on reports and literature on human infertility treatment. Table 1 presents the reports in the field of human infertility treatment, things expected to be applicable to livestock breeding by analogy, and the findings obtained in this research. In addition, our study developed the technology to select highly motile spermatozoa by several millions or more in number, and the relationship between the swimming form and pregnancy rate

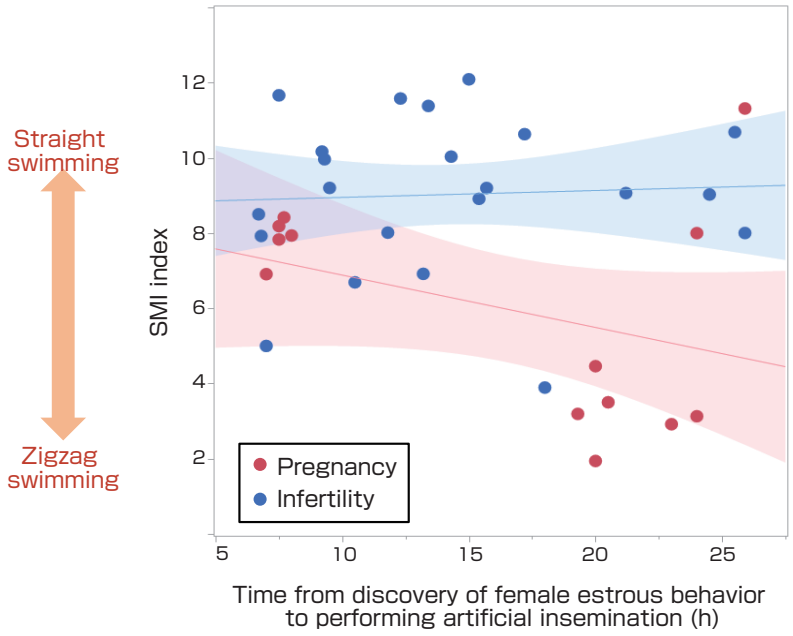

Fig. 5 Relationship between swimming pattern of spermatozoa and artificial insemination rate. Shaded area represents 95 % confidence interval 95 % for pregnancy/infertility.

Table 1. Reports in the field of human infertility treatment and the effectiveness of sorting technology for highly motile spermatozoa in cattle breeding

Perspective	Findings in human infertility treatment	What is expected in livestock breeding	What became clear from the study
Reason for improving sperm factor (rather than female factor)	Significance of sperm factor is becoming evident	Effect of improvement in breeding sperm exceeds expectation	Obtained almost same conception rate as conventional method by artificial insemination with about one-tenth the number of spermatozoa, by conducting motile spermatozoa selection
Qualitative healthiness of spermatozoa as a gamete	Sperm with good morphology and motility has good quality such as low DNA fragmentation	Strategy of selecting highly motile spermatozoa to improve livestock breeding is effective	Spermatozoa collected by this original sorting technology had greatly reduced DNA fragmentation (7 % → 0.4 %)
Clinical performance	High implantation rate, high conception rate, and low miscarriage rate	Increased conception rate and reduced embryonic death (very early miscarriage)	Conception rate was maintained even with less number of spermatozoa
Direction of improvement in breeding sperm	(Not applicable)	Clarify direction of improvement of livestock breeding sperm Possibility of achieving frozen straw containing spermatozoa capable of increasing conception	Relationship between swimming pattern of spermatozoa and conception rate in artificial insemination
Actualization of mass selection by ripple effect	(Not applicable)	Increase efficiency of breeding by in vitro fertilization and fertilized egg implantation	Succeeded in artificial insemination by establishing mass collection technology by selecting motile sperm

was clarified from the trial results using this technology. We believe on the potential of these findings to be extended to human infertility treatment.

These are the points that make this research original.

- We used fluidics to guide highly motile spermatozoa. Selection of appropriate number of spermatozoa was achieved using this technology, and for the first time in the world has succeeded in artificial insemination by pretreatment of spermatozoa and without incurring damage to this gamete.
- The characteristic of spermatozoa appropriate for conception was identified by their swimming form. We achieved a method for isolating a homogenous group of healthy and functional spermatozoa based on their swimming form.

Currently, research is being conducted targeting artificial insemination that dominates most of the breeding practice, but we hope to apply the findings obtained to *in vitro* fertilization in the future.

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Authors

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Discussions with Reviewers

1 Overall

Comment (YUMOTO Noboru, National Cerebral and Cardiovascular Center)

This is an interesting paper in which technology was developed to sort motile spermatozoa from frozen cattle sperms using fluidics, and collecting a sufficient number of spermatozoa that can be used directly for artificial insemination. It also demonstrated in actual cattle breeding that the technology was advantageous for conception. I evaluate the paper as appropriate for publication in *Synthesiology*.

Comment (IKEGAMI Keiichi, AIST)

Using engineering methods, the authors developed sorting technology of spermatozoa that may contribute greatly to increased productivity of livestock, through collaboration with bioscience university labs and institutions that engage in field trials at farms. In this R&D, a premise was set from the planning stage that the developed technology must be applicable at sites of livestock production, where conditions such as individual animals and environments cannot be controlled. Synthetic R&D was conducted, and the case study is very thought-provoking for the readers of *Synthesiology*.

2 Building of collaborative framework

Comment (IKEGAMI Keiichi)

It will be great if you could provide explanation from the perspective of regional collaboration, not just interdisciplinary collaboration.

Comment (YUMOTO Noboru)

I believe there were many hardships in building the collaboration, but how did you overcome them? Building of a collaborative framework is an important point in the scenario of Synthesiology, so can you please add descriptions as much as you can?

Answer (YAMASHITA Kenichi)

The most important point you are asking, I think, is "why these members?" I added descriptions to Chapter 3 "Selection of the research element considering work of participating institutions." The flow of collaboration building was that AIST made the approach and got the cooperation of those that agreed to the theme of research and had facilities where actual experiments could be conducted. Rather than in the building of collaboration, the hardship was in how to process the trial results since trial conditions could not be unified among the institutions, and there was an accumulation of trial results at each institution in its area of specialty. The solution to this issue is explained in the final

paragraph of Chapter 3.

3 Comparison between research for human infertility and cattle breeding

Comment (YUMOTO Noboru)

I think you should create a table that lays out in an easy to understand manner what is known, not known, and what was found in the authors' research, concerning in vitro fertilization in human infertility treatment and artificial insemination for cattle breeding.

Answer (YAMASHITA Kenichi)

We added a summary which is presented in Table 1, and likewise added descriptions in Chapter 7 "Summary." Our research has accomplished what was not known or could not be accomplished in human infertility treatment, and therefore, we addressed the possibility of feedback to human infertility treatment in the text.

4 Relationship between spermatozoa motility and pregnancy rate

Comment (IKEGAMI Keiichi)

Can you explain so the non-experts can understand, how the number of non-motile spermatozoa sealed in the same straw affects the pregnancy rate, assuming that there are a million motile spermatozoa? I understand that spermatozoa with damaged DNA can have adverse effects, but I think you should provide a clearer explanation. For example, does inclusion of some poor spermatozoa make no difference because non-motile spermatozoa have low probability of reaching the egg? Those are the points that are unclear to those who have no expertise in the field.

Answer (YAMASHITA Kenichi)

There are a number of phases in which spermatozoa travel inside the female reproductive organ, arrive in the proximity of an egg, and cooperate together as a group to form a pathway to the egg. Then, fertilization occurs, and the process progresses to cell division and development. It has been reported earlier that active oxygen generated by dead spermatozoa may be harmful, and there have been similar recent reports. Dead spermatozoa (however, non-motile spermatozoa are not necessarily dead) may have adverse effects, and the transfer of damaged DNA may also have ill effects, but I think it is difficult to clearly and quantitatively state what factors affected how much at which stage.