Possible Improvement of Spermatozoan Function and In Vitro Fertilization by Japanese Kampo Medicines (JKMs) in Mammals

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Abstract: *The present study was conducted to examine the effect of Japanese Kampo Medicines (JKMs) on mammalian spermatozoa and in vitro fertilization. Two kinds of experiments were carried out in this study to determine (1) the most effective mix ratios of JKMs which were determined by using PBS supplemented with JKMs, and (2) the most successful in vitro fertilization and subsequent embryonic development. In the first experiment, frozen-thawed bovine and goat spermatozoa were examined. Frozen thawed spermatozoa were kept for 3 h at 37°C in a 5% CO_2 incubator in media with added JKMs at different mix ratios and different concentrations. In the second experiment, in vitro fertilization (IVF) and consequent embryonic cleavage ratios were also determined. The present results show that all of the JKMs were effective at enhancing sperm function and in vitro fertilization, especially Syouhangekabukuryoto (JKMs21) and Hotyuekkito (JKMs41). In addition to this, all of the JKMs were confirmed to enhance spermatozoan function, in vitro fertilization and early embryonic development.*

Key words: *Mammals, Japanese Kampo Medicines (JKMs), Spermatozoa, Oocytes, IVF, Embryo*

The efficacy of JKMs on living things has been considered to be the result of various kinds of cooperative interactions [1]. On the other hand, digestive enzymes and intestinal bacilli also affect easily the function of JKMs [2]. It has been a difficult tasks to determine the effects of JKMs on *in vitro* culture systems. In addition, a lot of JKMs are more effective on functional disorders than on mental illnesses [3]. For example, most cases of male sterility are induced by declines in the ability of spermatogenesis, which is known as spontaneous spermatogenous disorder, and we have no effective cure for these diseases.

Recently, however, it has been reported that the administration of some JKMs to such patients, improvemed their spermatogenous ability [4, 5]. Furthermore, ferulic acid, an active constituent of various herbs, has been proved to be beneficial to sperm viability and motility in both fertile and infertile individuals. It is, therefore, possible that ferulic acid may also be used for the treatment of asthenozoospermic infertility [6].

Lately, Feng *et al.* [7] have reported that a kind of JKM, Unkeito, slightly enhanced the survivability of bovine spermatozoa, suggesting that some JKMs may induce actions effective for the treatment of reproductive failure in both men and rats. Their report has demonstrated that JKMs may have favorable effects on *in vitro* maturation of bovine oocytes and the subsequent developmental capacity of the embryos [8, 9]. The mechanisms by which some physiological functions are induced by JKMs have not yet been reported, however, with respect to this point, some experiments have been done, suggesting the possible efficacy of JKMs on germinal cells.

In the present study, therefore, the physiological effects of some JKMs on mammalian sperm function, in vitro fertilization and consequent development of early stage embryos were examined using bovine sperm cells and oocytes. This study was also designed to investigate the feasibility of applying some of the present results to the restoration of endangered wild animals via germinal cells.

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Materials and Methods

Experiment 1 Effect of the JKMs on physiological function of Chengdu native goat spermatozoa

Semen preparation: Goat semen, which had been obtained from Chengdu native goats, was cryopreserved in LN_2 in straws which are routinely used for bovine semen preservation. The preserved semen was thawed in a water bath at 37°C and washed once in 10 ml basic washing TALP solution, centrifugated at 300 × g for 10 min at room temperature (22–25°C). After removing the supernatant from the sperm suspension, 4 ml TALP were added and the resultant solution was cultured at 39°C with 5% CO_2 in an incubator for 30 min. Sperm suspension was washed again by the same centrifugation protocol. After again removing the supernatant of the sperm suspension, 1.0 ml TALP was added to prepare IVF.

Preparation of Mixed Chinese herb medicines: We mixed 8 kinds of Chinese herb medicines (Toutyuukasou, Syakuyaku, Keipi,Touki, Ougi and Kanzou), which are often used for various kinds of genital disorders by women in China. In the present study, three methods of mixing the medicines were examined and they were compounded based on the basic combination rate of JKMs (Mix-1, Mix-2 and Mix-3; Table). A large guantity of pH nentrol distilled water was added to each JKM for making an extract. The extract was filtered with suitable paper and stored at 4°C in a refrigerator as a stock solution for a week. Just before the experiment, the stock solutions were diluted 100 times with TALP in order to carry out the IVF and mixed with respective extracts of herbal materials.

Examination of spermatozoa motility: Sperm suspension (TALP: semen = 1:1) was prepared and 2.0 μl of semen sample was introduced into a 100 μl droplet of three kinds of mixed solution, and incubated at 37°C with 5% CO_2 in air under maximum humidity for 5 h (final concentration of sperm cells: 1.5×10^5). Sperm motility was determined under a phase-contrast microscope at 400 × magnification.

Experiment 2: Effect of JKMs on the function of bovine spermatozoa

Semen preparation: Commercially purchased frozen bovine semen was thawed in a water bath at 37°C and washed twice with PBS by 300 × g centrifugation procedures. The supernatant of the sperm suspension was removed and then 1.0 ml of PBS was added. The final concentration of sperm cells was about 4.0×10^7/ml. In the present study, it was found that initial sperm motility was very high, so this freshly thawed semen could not be used for the experiment for comparison with the semen samples from wild animals [7]. In the present study, therefore, the sperm suspension was warmed in water at 46°C for 10 min to obtain a sort of weakened spermatozoa with slightly decreased motility which might be comparable with spermatozoa from endangered wild animals such as Giant Pandas. In the case of freshly thawed semen, the administration of JKMs has not shown any effect on spermatozoa activity and motility.

Table 1. Mixing ratios of Japanese Kampo Medicines (JKMs)

Herb	Mix 1	Mix 2	Mix 3
Toutyukaso	—	3	3
Ninjin	—	1	—
Taizo	2	3	2
Keipi	2	1	2
Syakuyaku	3	1	2
Ougi	2	—	2
Touki	2	—	2
Kanzo	1	—	1

Japanese Kampo Medicines (JKMs) used in this study: The JKMs used in this experiment were Syouhangekabukuryoto (JKMs21), Hotyuekkito (JKMs41), Ougikentyuto (JKMs98), Ninjineiyouto (JKMs108) which were provided by Tsumura & Co and Wakunaga Pharmaceutical CO., Ltd. of Japan. These materials were also chosen because of their sufficient efficacy for human infertility. These medicines were granules and weighed 100 μg/ml aq. All of these JKMs and Mix-3 (Table 1) were boiled for 1.5 h with distilled water, extracted, and filtered. The extracts were diluted with PBS at 10 times and stored at 4°C in a refrigerator for a week.

Examination of spermatozoan activity

(1) Classifications of sperm activity

Two μl of sperm suspension was added to a 100 μl droplets of JKMs (JKMs21, JKMs41, JKMs108) and Mix-3, and incubated at 37°C under 5% CO_2 in air with maximum humidity for 6 h. Sperm activity was measured under a phase-contrast microscope at × 400 magnification, and sperm activity was classified into five categories as follow: 5, vigorous advancing motion; 4, advancing motion; 3, vigorous swinging motion; 2, slightly swinging motion; and 1, no motion.

(2) TMMR (transmembrane migration ratio) determination

The TMMR is the method of determining sperm mo-

tility using the transmembrane migration ratio [1]. Two microliters of sperm suspension were added to 10 μl droplets of JKMs (JKMs: 21, JKMs: 41, JKMs: 98, JKMs: 108) and Mix-3, incubated at 37°C with 5% CO_2 in air under maximum humidity for 1 h or 3 h. An aliquot (90 μl) of semen sample was introduced into a glass pipe covered with a 5 μm membrane filter, and cultured for 1 h under the same conditions as mentioned above. The glass pipe with attached membrane filter was then removed and the number of sperm cells in the dish was counted with a haemocytometer. The number of sperm cells that moved across the membrane into the lower chamber was calculated. The results obtained were the transmembrane migration ratio (TMMR). In these experiments, we made equipment to determine TMMR based on the method of Hong.

In vitro maturation (IVM) of oocytes

The bovine ovaries were obtained from cows at a local slaughterhouse and were returned to the laboratory in saline at 35–37°C within 3 h of collection. Oocytes were aspirated from follicles of 2–7 mm in diameter with a 21-gauge needle attached to a 20 ml syringe. The collected cumulus-oocytes complexes (COCs) were washed three times with PBS and maturation medium. Only oocytes surrounded by compact cumulus layers were used in this study. Around 20 COCs were cultured in a 100 ml drop of maturation medium under mineral oil at 39°C with 5% CO_2 in air under a high humidity atmosphere for 22 h. The maturation medium, TCM199, included HEPES and was supplemented with 10% FCS, 1 mg/ml 17β-estradiol and 20 IU hCG. The total time taken from aspiration of oocytes to culture was about 1 h.

In vitro fertilization (IVF)

A half of a 0.5 ml straw of frozen semen obtained from the Holstein breed was thawed in a water bath at 37°C. Spermatozoa were washed twice in PBS by centrifugation at 300 × g for a period of 5 min each. The supernatant was removed and diluted with PBS to obtain a final concentration of 4.4×10^7 sperm cells/ml. After warming in water at 46°C for 10 min, about 2 μl of sperm suspension was added to 100 μl droplets of JKMs and Mix-3 media. The oocytes after *in vitro* maturation culture were washed three times in PBS and introduced into the spermatozoa-JKMs mixture droplet, and cultured at 39°C with 5% CO_2 in air under high humidity atmosphere for 6 h. After insemination, the oocytes were washed three times with TCM199 including HEPES and supplemented with 10% FBS, and 1 mg/ml 17β-estradiol, which was maturation medium without hCG.

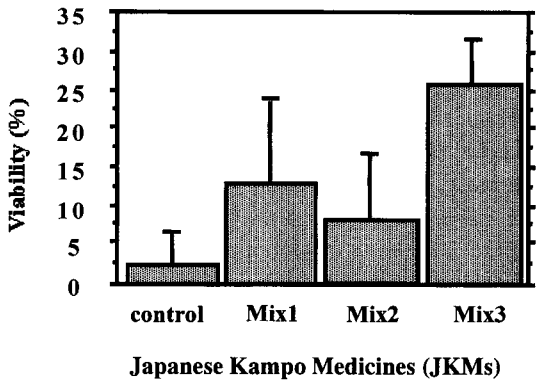

Fig. 1. Effect of Japanese Kampo Medicines (JKMs) on Chengdu goat sperm viability. Graph shows rate of viability after 5 h incubation at 37°C under 5% CO_2 in air with maximum humidity. Mean ± SD.

Then the oocytes were transferred into 100 μl drop under the same conditions for an additional 24 h culture *in vitro*. Following the in vitro culture, the oocytes were denuded by 0.1% hyaluronidase in PBS, fixed in 25% acetic alcohol for 48–72 h at room temperature (22–25°C), stained with 1% orcein in 45% acetic acid, and examined for fertility and cleavage under a phase-contrast microscope at × 400 magnification.

Results

Effect of JKMs Mixtures on in vitro preservation of frozen-thawed Chengdu goat spermatozoa

The result obtained by the addition of Mix-1, Mix-2 and Mix-3 to the Chengdu goat spermatozoa *in vitro* indicated higher viability among the treatment groups than in the control group (Fig. 1). The mixtures of JKMs improved sperm motility (data not shown). Most spermatozoa in the droplets of Mix-1 and Mix-3 showed vigorous progressing motion. Goat sperms showed 32% abnormality in this experiment.

Effect of JKMs on in vitro preservation of frozen-thawed bovine spermatozoa

(1) Classification of sperm motility

Figure 2 indicates that frozen-thawed bovine spermatozoa incubated in PBS medium with several kinds of JKMs included (JKMs-21 or JKMs-41 or JKMs-108), showed significantly ($p<0.05$) higher sperm activity than those in PBS without JKMs supplementation.

(2) TMMR determination

In the case of in vitro culture for 1 h, frozen-thawed

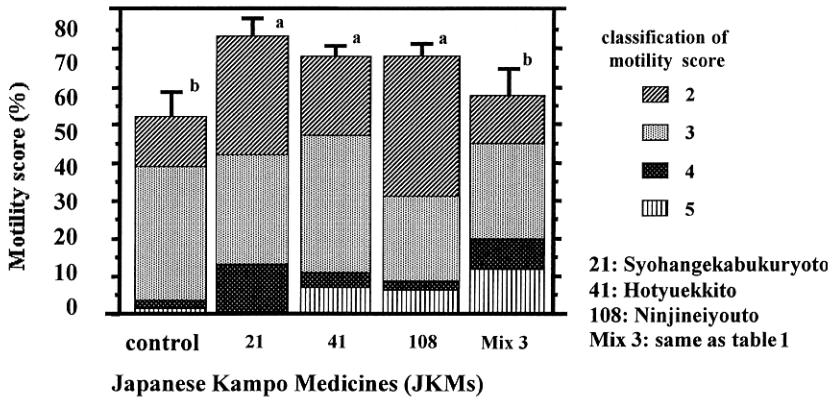

Fig. 2. Effect of JKMs on bovine spermatozoa motility with 5 classifications. Spermatozoa activity was classified into five categories as follow: 5 (straight line), vigorous advancing motion; 4 (white dot), advancing motion; 3 (empty square), vigorous swinging motion; 2 (slash), slightly swinging motion; 1 (not shown); no motion. a>b=p<0.05.

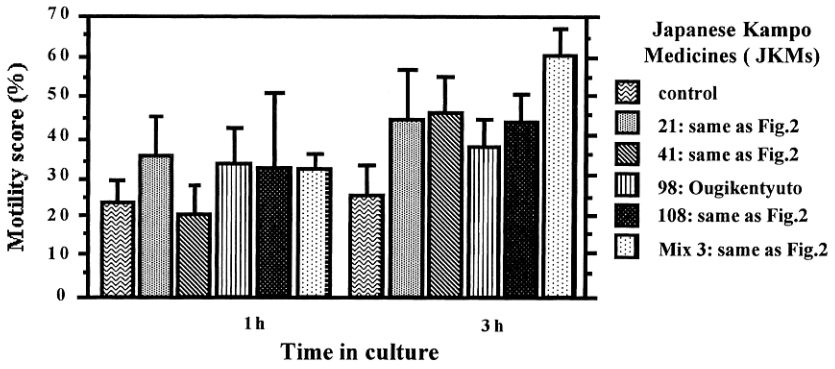

Fig. 3. Effect of JKMs on bovine spermatozoa motility measured by the trans membrane migration ratio (TMMR). Bovine spermatozoa motility after 1 h and 3 h incubation at 37°C under 5% CO_2 in air with maximum humidity. JKMs are 21 (empty square), Syohangekabukuryoto; 41 (slash), Hotyuekkito; 98 (straight line), Ougikentyuto; 108 (white dot), Ninjinyoeito; Mix3 (black dot) and Control is shown as a wary line. Mean ± SE.

bovine spermatozoa incubated in the experimental media, except for JKMs-41, had a tendency to improve sperm activity. After 3 h of culture, differences of sperm activity between control and trials became more apparent and all of the treated groups showed higher sperm activity (Fig. 2).

Effect of the JKMs on IVM-IVF of oocytes

Only the PBS with added JKMs enabled frozen thawed spermatozoa to fertilize bovine oocytes *in vitro*. Especially, JKMs-21 and JKMs-41 had a tendency to improve the fertilizability of bovine spermatozoa, though no significant (P>0.05) differences were found between the treatments (Fig. 3). Regarding *in vitro* fertilization, as shown in Fig. 4, the oocytes fertilized in PBS containing JKMs showed a higher rate of cleavage, when compared with that of PBS alone, though the effects of JKMs were not enough to induce significant enhancement.

Discussion

The results obtained by the TMMR method indicated that the membrane transmigration ratio (activity) of frozen-thawed bovine spermatozoa was improved when spermatozoa had been exposed to JKMs for a certain period. The co-culture of spermatozoa with JKMs at

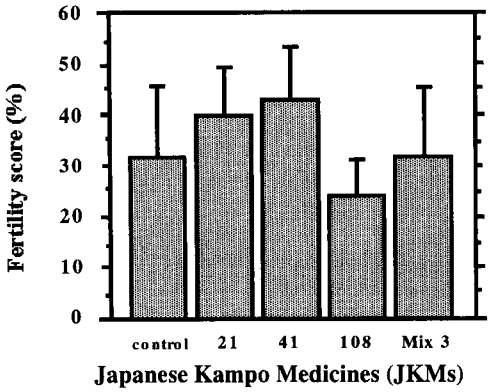

Fig. 4. Effect of Japanese Kampo Medicine (JKMs) on bovine in vitro fertilization. Graph shows fertilization rate as assessed under a phase-contrast microscope at × 400 magnification. Mean ± SE. JKMs 21, 41, 108 and Mix3 are the same as Fig. 2.

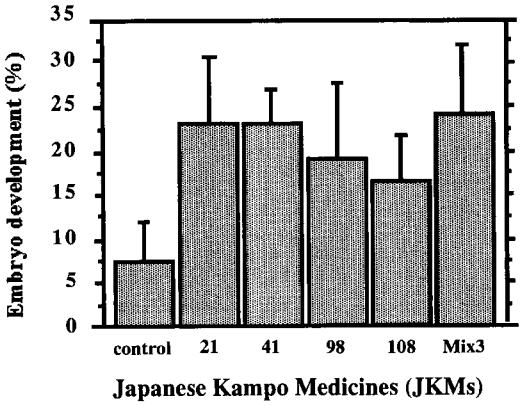

Fig. 5. Effect of JKMs on bovine embryonic development. Graph shows cleavage rate as observed by a phase-contrast microscope at × 400 magnification. Mean ± SE. JKMs 21, 41, 98, 108 and Mix3 are the same as Fig. 3.

39°C with 5% CO_2 in air under a high humidity atmosphere for 3 h before insemination was shown to improve *in vitro* fertilizability. In addition, JKMs-21 and JKMs-41 also slightly promoted the rate of fertilization. All JKMs and Mix-3 were effective at improving sperm activity and at promoting the cleavage ratio of bovine embryos. Hitherto, herbal medicines such as JKMs-41 and JKMs-108 have been reported to cause a kind of active acrosomal reaction for human spermatozoa [10], and these JKMs also enhanced sperm concentration and sperm activity. In addition to this, other kinds of JKMs such as Yamidiougan and Gosyajinkigan had favorable effects on the function of human spermatozoa. While JKMs-108 and Gosyajinkigan increased spermatozoan activity via increase of cAMP, this effect was temporary. With regards to the physiological function of JKMs, it has been reported that the effects of JKMs-41 and JKMs-108 disappeared 3–4 months after administration in humans. Unkeitou has also been reported to have a tendency to stimulate the cleavage rate of oocytes as well as the embryonic development to morula and blastocyst stages following in vitro fertilization, though there were no significant ($p>0.05$) differences between the treatments [7]. Based on these reports and the present results, it is likely that some of the JKMs had favorable effects on the motility and activity of bovine spermatozoa and on the in vitro fertilization and subsequent cleavage rates under a certain physiological condition. The special effect of JKMs was found on sperm activity (Fig. 1), leading to the highest motility for Mix-3. Mix-3 also had a beneficial effect on the cleavage rate of fertilized oocytes (Fig. 4). However, the results of sperm motility for Mix-3 showed the lowest rate, with no significant ($p<0.05$) differences between the treatments, while another trial had significant ($P<0.05$) differences. Therefore, the relationship between improvements of embryonic viability and fertilizability (or cleavage rate) was difficult to explain in detail in the present study. These differences might arise from the difficulty of preparing bovine oocytes with same developmental stage. Another possibility is that the high temperature (46°C) treatment of the semen was related to the physiological changes of spermatozoa.

In the case of human beings, one of the functional characteristics of JKMs was thougt to be dependent upon the dosage for each person. In the present experiments, no significant ($P>0.05$) differences were observed between the various JKMs. Another point of view is that the JKMs are composed of many kinds of ingredients which are quite different from those of western medicines. Therefore, the efficacy of JKMs may be induced by suitable combination of herbal medicines. The embryonic developmental rates of in vitro fertilized bovine oocytes in PBS-JKMs medium was lower than the case of IVF in BO medium, suggesting that it will be necessary to study some of the practical usages of JKMs.

Recently, it has been reported that some herbal medicines worked as scavengers for oxidized products in living things. The JKMs may have the ability to remove free radicals in living cells, therefore, they might have maintained higher bovine sperm motility in the present

experiments. The present results suggest that JKMs have a favorable effect on preservation of bovine spermatozoa, in vitro fertilization and embryonic development. In the present experiments, some of the JKMs improved sperm motility, the rates of fertilization and embryonic development. Based on this study, JKMs may provide a possible alternative to expensive chemicals such as bovine serum or other high priced materials, especially in Asian countries.

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