Effects of Japanese Kampo Medicines on Physiological Function of Frozen-Thawed Bovine Spermatozoa in In Vitro Fertilization

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Abstract: *The present series of experiments were conducted to examine the effect of Japanese Kampo Medicines (JKMs), namely Tokishakuyakusan (TJ23), Keishibukuryogan (TJ25), Shakuyakukansouto (TJ68) and Unkeito (TJ106) on the physiological function of frozen-thawed bovine spermatozoa in in vitro fertilization. In the first experiment (experiment 1), the most effective JKM was determined by adding JKMs to the BSA-free in vitro fertilization medium (m-Hepes-BO) and examining the developmental capacity of oocytes after in vitro fertilization. In the second experiment (experiment 2), a comparison of the effect of the most effective JKM on in vitro fertilization with that of BSA was conducted in the same manner as in experiment 1. The result of experiment 1 showed that Unkeito (TJ106) was the most effective JKM when added to BSA-free m-Hepes-BO medium, although very few blastocysts were obtained. Furthermore, recording the survivability of the frozen-thawed spermatozoa incubated in fertilization medium supplemented with TJ106 and/or BSA showed that the survivability of the spermatozoa was improved by TJ106. In experiment 2, when TJ106 was added to m-Hepes-BO medium together with BSA, the rates of morulae and blastocysts were better than in the case of TJ106 alone. Moreover, the morphological characteristics of oocytes after 6 h of insemination in fertilization medium with BSA and/or TJ106 as the supplements, also indicated that BSA was indispensable in in vitro fertilization of bovine oocytes, althought the physiological functions of the spermatozoa were somewhat improved by JKM in the present work.*

Key words: *In vitro fertilization, Bovine oocyte, Japanese Kampo Medicines, Morphological characteristics, TEM.*

Japanese Kampo Medicines (JKMs) have been shown to be used only empirically without any clarification of the mechanism of their efficacies. Recently, however, JKMs have been extensively used for women who were suffering from infertility and climacteric symptoms in clinical gynecology in Japan [1–4]. Many experiments have been carried out in order that the endocrine profiles of the effect of JKMs may be examined [5–7]. Laboratory animals have generally been used in experiments to evaluate the effects of JKMs in both *in vivo* [5, 6] and *in vitro* [7].

In our previous experiments, JKMs, which have been reported to be effective for the treatment of reproductive failure in both man and rats, have been demonstrated to have some favourable effects on *in vitro* maturation of bovine oocytes and the subsequent developmental capacity of the embryos [8]. Mammalian spermatozoa must undergo capacitation followed by acrosome reaction before they are capable of penetrating oocytes. A number of procedures have been developed to induce capacitation of bovine spermatozoa [9, 10]. It has been reported that various chemicals such as caffeine [11, 12], heparin [13, 14], and caffeine plus heparin [15] can induce capacitation of bovine spermatozoa *in vitro*. Proteinaceous macromolecules, especially bovine serum albumin (BSA) may play an important role in the process of capacitation and in the induction of acrosome reaction in bovine spermatozoa [16] and they have always been included in the culture media, although it is not understood how BSA supports sperm capacitation.

The growing interest in JKMs as potential new medical resources and the need to establish an *in vitro* culture system for bovine oocytes led us to conduct the present series of experiments to examine the effect of JKMs on the physiological function of frozen-thawed bovine spermatozoa in *in vitro* fertilization. Furthermore, the morphological characteristics of treated oocytes after insemination were also examined by transmission electron microscopy (TEM).

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Materials and Methods

The names of the JKMs used in the present study are: *Tokishakuyakusan* (TJ23), *Keishibukuryogan* (TJ25), *Shakuyakukansouto* (TJ68) and *Unkeito* (TJ106). These JKMs were chosen for use because of the results of previous experiments which were conducted to evaluate their effects on bovine oocyte maturation and embryonic development *in vitro*. The most effective dose of JKM was 100 μg/ml for the maturation of bovine oocytes [8].

In vitro maturation (IVM) of oocytes

The ovaries were obtained from cows at a local slaughterhouse and were brought to the laboratory in warm (37°C) physiological saline (0.85% NaCl) within 2 h after collection. The cumulus-oocytes complexes (COCs) were collected from follicles 2 to 8 mm in diameter with a 20-gauge needle attached to a 5-ml syringe containing SFRE-2 solution [8] supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 μg/ml Gentamicin) and pooled in a watch glass. They were washed twice in Dulbecco's PBS and then three times in the maturation medium, which is SFRE-2 medium supplemented with 10% FBS, 1 μg/ml FSH, 1 μg/ml estradiol 17β and 50 μg/ml gentamicin. Ten to fifteen COCs were introduced into a 50 μl drop of the maturation medium in 35-mm petri dishes under mineral oil. The oocytes were then incubated for 21–23 h at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity.

In vitro fertilization (IVF)

Semen preparation was carried out according to the method of Ohboshi *et al.* [17]. Briefly, frozen bull semen was thawed in a water bath at 37°C and washed in basic fertilization medium, namely modified-Hepes-BO medium (m-Hepes-BO) described by Brackett and Oliphant [18]. The sperm suspension was diluted with the fertilization media containing 10 μg/ml heparin and 5.0 mM theophyline to obtain a final concentration of 10×10^6 sperm cells/ml. The oocytes after *in vitro* maturation culture were washed three times in the *in vitro* fertilization medium and introduced into a 100 μl droplet of the sperm suspension (15 to 20 COCs/drop) and incubated at 39°C for about 6 h under mineral oil in 5% CO_2 in air with maximum humidity.

In experiment 1, JKMs were added to the fertilization BSA-free medium at a concentration of 100 μg/ml and insemination was then carried out as described above. In experiment 2, the most effective JKM in experiment 1 and/or BSA were added to the fertilization medium, respectively.

In vitro culture (IVC)

The medium used for IVC was modified SOF (m-SOF) [19]. After 6 h of insemination, the presumptive zygotes were washed twice in mSOF supplemented with 1.0% calf serum (CS) and cumulus cells surrounding zygotes were partly removed before being transferred into drops of mSOF. At 48 h after the insemination, the medium was replaced with fresh mSOF supplemented with 5.0% CS and the cumulus cells surrounding the embryos were removed by repeated pipetting, while the cumulus cells attached to the surface of the culture dish were retained. The embryos were cultured for an additional 8 days and the medium was replaced with fresh medium every 48 h. The embryos which underwent cleavage on Day 2 were examined. The number of morulae on Day 6 and that of blastocysts on Day 8 were also recorded.

Survivability of frozen-thawed spermatozoa

The preparation of spermatozoa is described above. The fertilization media supplemented with BSA and/or JKM, respectively, were used to dilute the sperm suspension to a final concentration of 6×10^6/ml. Incubation of spermatozoa was carried out at 39°C in 5% CO_2 in air with maximum humidity and the survivabilities at 1, 4, 7 and 10 h of the incubation and finally the total survival time of spermatozoa in different media were recorded.

Preparation of oocytes for transmission electron microscope (TEM)

In the preliminary experiment, the most effective JKM for *in vitro* fertilization was determined to be *Unkeito* (TJ 106).

The TEM was conducted on oocytes after 6 h of insemination in different medium, namely m-Hepes-BO supplemented with BSA, TJ106 or TJ106 together with BSA, respectively. The method was described in our previous report [1]. Briefly, oocytes were fixed in a solution consisting of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at room temperature, and post-fixed for 30 m at 0°C in 1% osmium tetraoxide dissolved in 0.1 M cacodylate buffer. After routine dehydration, the oocytes were embedded in Beem capsules with epoxy resin. Thin sections were cut with glass knives and stained with 1% toluidine blue. Ultrathin sections cut with diamond knives were double-stained with both uranyl acetate and lead citrate, and examined with a JEM-200CX TEM.

Table 1. Effects of Japanese Kampo Medicines (JKMs) (100 μg/ml) on *in vitro* fertilization and development of *in vitro* matured bovine oocytes

Fertilization medium	No. of oocytes inseminated	No. of cleaved oocytes on Day 2	No. of morulae on Day 6 (%)	No. of blastocysts on Day 8 (%)
Control	78	28	2 (7.1)	1 (3.6)
TJ23	75	36	3 (8.3)	0 (0)
TJ25	78	32	2 (6.3)	1 (3.7)
TJ68	77	32	4 (12.5)	1 (3.1)
TJ106	79	40	7 (17.5)*	3 (7.5)

Experiments were repeated 3 times for each medium. Percentages in parentheses are per cleaved oocyte. *P<0.05 compared to Control.

Table 2. Effect of Unkeito (TJ106) and BSA on *in vitro* fertilization and development of *in vitro* matured bovine oocytes

Fertilization medium	No. of oocytes inseminated	No. of cleaved oocytes on Day 2	No. of morulae on Day 6 (%)	No. of blastocysts on Day 8 (%)
BSA	76	45	6 (11.1)	6 (11.1)
TJ106	75	41	5 (12.2)	3 (7.3)
BSA+TJ106	78	57	10 (17.5)	7 (12.3)

Experiments were repeated 3 times for each medium. Percentages in parentheses are per cleaved oocyte.

Statistical analysis

All data obtained in the experiment were subjected to statistical analysis by chi-square test [20]. Statements of significance were based on P<0.05 unless otherwise noted.

Results

Effect of the JKMs (100 μg/ml) added to BSA-free in vitro fertilization media on IVM-IVF oocytes (experiment 1)

The oocytes after the maturation culture were assigned randomly to sperm suspensions and the developmental capacity of the oocytes after *in vitro* fertilization in JKMs-added BSA-free media was examined. TJ106 had a tendency to improve the cleavage rate of the oocytes as well as the development to morula and blastocyst stages following *in vitro* fertilization though there were no significant (P<0.05) differences between the treatments (Table 1). Furthermore, without BSA supplementation, very few blastocysts were obtained in experiment 1.

Effect of Unkeito (TJ106) and BSA on in vitro fertilization of bovine oocytes (experiment 2)

After *in vitro* fertilization in BSA-, TJ106- and BSA-TJ106- containing media, comparison of the developmental capacity of oocytes was made among experimental groups.

As shown in Table 2, oocytes fertilized *in vitro* in media containing both BSA and TJ106 showed a higher rate of cleavage to morulae and blastocysts, when compared with those of BSA or TJ106 alone. Firstly, this finding showed that there was a kind of interaction between BSA and TJ106 which seemed to induce an increase in the proportion of oocytes developing to the morula and blastocyst stages. Secondly, such interaction between BSA and TJ106, however, did not seem to be enough to make the increase significant (P<0.05).

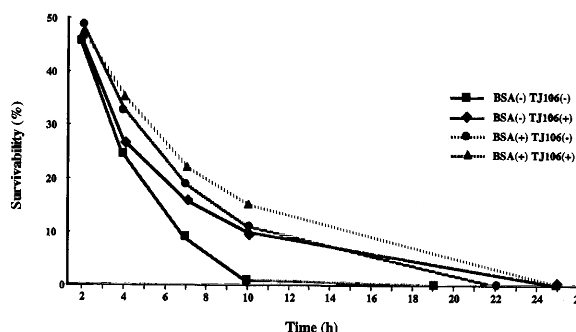

Fig. 1. Effect of Japanese Kampo Medicine on *in vitro* survivability of frozen-thawed bovine spermatozoa.

Effect of Unkeito (TJ106) on in vitro survivability of frozen-thawed bovine spermatozoa

Figure 1 indicates that frozen-thawed bovine spermatozoa incubated in TJ106-containing media showed

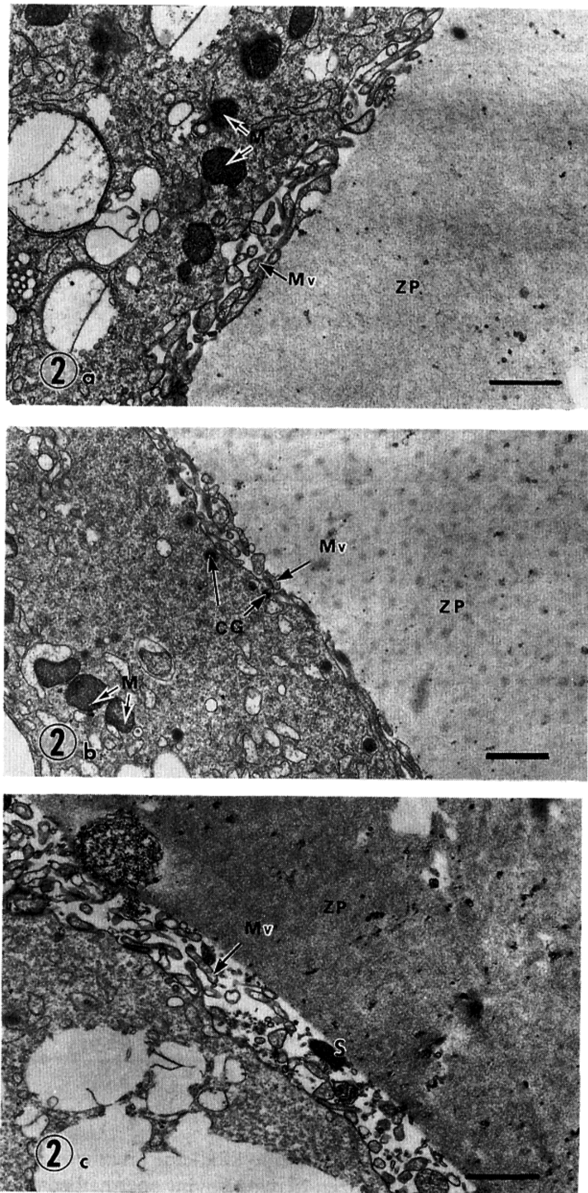

Fig. 2. Distribution of cortical granules (CGs) in inseminated bovine oocytes. a) oocyte from BSA-containing medium; b) oocyte from TJ106-containing medium; c) oocyte from BSA-TJ106-containing medium. Abbreviations: CC, cumulus cell; CG, cortical granule; M, mitochondria; Mv, microvilli; O, oolemma; ZP, zona pellucida. Scale bars: 1 μm.

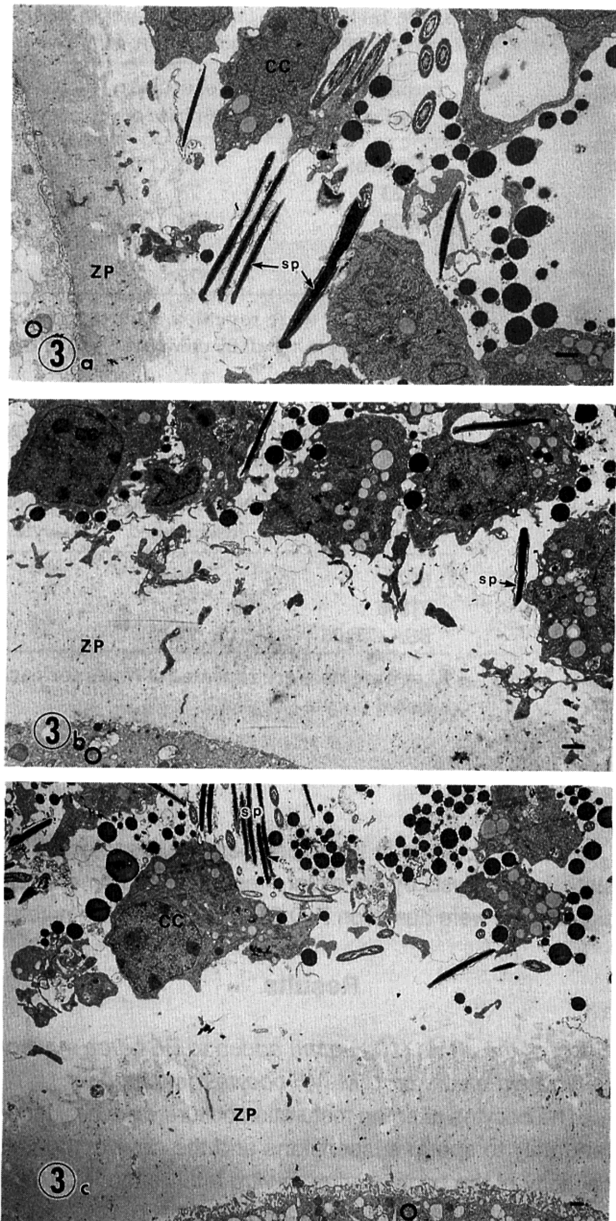

Fig. 3. Ultrastructural features of bovine oocytes inseminated in the medium supplemented with JKM and/or BSA with special reference to the distribution of spermatozoa. a) oocyte from BSA-containing medium; b) oocyte from TJ106-containing medium; c) oocyte from BSA-TJ106-containing medium. Note. Increase in the number of spermatozoa in the BSA (3a) and BSA+TJ106 (3c) groups compared with the TJ106 one (3b). Abbreviations and scale bars are the same as in Fig. 2.

higher survivability than those in the media without TJ106 supplementation.

Transmission electron microscopical observation of oocytes after insemination

Ultrastructural features of bovine oocytes inseminated in the media supplemented with BSA, TJ106 or BSA plus TJ106 are shown in Figs. 2 and 3.

Firstly, in regard to the distribution of cortical gran-

ules (CGs) in oocytes after different treatments (Figs. 2a, 2b and 2c), no CGs were observed in oocytes fertilized *in vitro* in BSA-containing media, indicating the completion of fertilization (Figs. 2a, 2c), but in those from the medium with only TJ106 supplementation, a few CG still remained (Fig. 2b).

Furthermore, a considerable difference in the distribution of spermatozoa around zona pellucida was observed between oocytes treated with BSA (Figs. 3a, 3c) and those treated with TJ106 alone (Fig. 3b), and a considerably larger number of spermatozoa were found in BSA- (Fig. 3a) and TJ106+BSA-containing (Fig. 3c) groups than in the group supplemented with TJ106 alone (Fig. 3b).

Discussion

The present series of experiments were conducted to examine the effect of JKMs on the physiological function of frozen-thawed bovine spermatozoa in *in vitro* fertilization, and the results showed that TJ106 considerably improved the survivability of bovine spermatozoa. Furthermore, TJ106 slightly promoted the rate of cleavage of oocytes fertilized *in vitro* (Table 1). But a comparison between TJ106 and BSA showed that when TJ106 was added to the medium in combination with BSA, the highest rates of cleavage to the stage of morulae and blastocysts after *in vitro* fertilization were obtained. This finding was in contrast to the results of our previous experiment revealing that TJ25 had a counteractive effect on the action of CS [1]. The reasons, however, still remain unknown.

The TEM morphological observation showed that the oocytes inseminated in media containing BSA, namely BSA-containing and BSA-TJ106-containing media showed much more distribution of spermatozoa near the zona pellucida than in the medium supplemented with TJ106 alone (Fig. 3). In the previous experiments, CGs were observed only in oocytes from TJ106-containing medium. This suggested that among the oocytes from TJ106-containing medium, there were still some unfertilized oocytes, while no unfertilized ones were observed in the oocytes subjected to other treatments. This ultrastructral evidence provided an explanation of the low cleavage rate for the oocytes from the TJ106-added medium shown in Table 2. It may be concluded, therefore, that BSA might be necessary to carry out *in vitro* fertilization efficiently, and this is consistent with the results of Bryd [16].

TJ106 also slightly improved the survivability of bovine spermatozoa (Fig. 1). Without the addition of BSA, however, *in vitro* fertilization of bovine oocytes is not necessarily improved despite TJ106 supplementation. It is generally accepted that the survivability of spermatozoa is not necessarily related to the fertilizability, which is in good accordance with the results of the present study [1].

Since the effective chemical components of JKMs remain unknown, further research needs to be carried out to explain the mechanism of action of TJ106 on the physiological function of bovine spermatozoa during *in vitro* fertilization.

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