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In Vitro Sperm-Ovum Interaction Utilizing
Golden Hamster and Squirrel Monkey Spermatozoa
with Hamster Zona-Free Ova.

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Daniel Bernard Burke

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W. Richard Dukelow
Major professor

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IN VITRO SPERM-OVUM INTERACTION UTILIZING GOLDEN HAMSTER AND SQUIRREL MONKEY SPERMATOZOA WITH HAMSTER ZONA-FREE OVA

By

Daniel Bernard Burke

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ABSTRACT

IN VITRO SPERM-OVUM INTERACTION UTILIZING GOLDEN HAMSTER AND SQUIRREL MONKEY SPERMATOZOA WITH HAMSTER ZONA-FREE OVA

By

Daniel Bernard Burke

Studies were made on the interaction of golden hamster (*Mesocricetus auratus*) and squirrel monkey (*Saimiri sciureus*) sperm with hamster zona-free ova *in vitro*. Sperm obtained from both species was incubated separately in a 5% carbon dioxide in air atmosphere at 37°C mixed with one of a variety of medium and serum combinations. Ova from superovulated females were stripped of the zona pellucida by immersion in 0.1% trypsin. These zona-free ova were then added to the sperm culture and observed periodically. Several cultures provided sustained sperm motility. Sperm of both species bound zonal and zona-free ova; however, only zona-free ova were penetrated. Hamster sperm capacitation began at 1.5 to 2.0 hours and squirrel monkey within 8.0 hours. Seven cultures promoted hamster sperm penetration (60% of ova penetrated) and in two cultures there was evidence of squirrel monkey sperm penetration of ova.

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INTRODUCTION

The study of sperm and ovum interaction *in vitro* allows for detailed and controlled experiments not possible with *in vivo* situations. A system has been developed, utilizing an ovum enzymatically stripped of the zona pellucida, which has been reported to allow study of sperm characteristics of a variety of species. The vitelline membrane of the hamster ovum is believed to be devoid of a species-specific blocking mechanism. The zona pellucida provides this blockage in the untreated, zona-intact ovum. Once free of this protective cover, the zona-free ovum is subject to binding and penetration by *in vitro* capacitated sperm of the rat, mouse, guinea pig, boar, and human. Based upon these studies, it became apparent that this hamster zona-free ovum may be used as an easily obtainable biological assay for evaluation of the *in vitro* sperm capacitation time and male fertility of a variety of mammalian species.

Human sperm was the only primate species tested thus far in this system. Testing of squirrel monkey (*Saimiri sciureus*) sperm would provide a test of nonhuman primate sperm interaction with this ovum as well as provide greater insight into the characteristics of squirrel monkey sperm *in vitro*. Successful *in vitro* fertilization has been reported in this species, but sperm culture parameters were not extensively evaluated. While sperm capacitation has been revealed as an essential pre-penetration event in many mammalian

sperm, including one nonhuman primate (rhesus), it requires further elucidation in regard to the squirrel monkey.

Culture conditions were utilized in this study which differed from those previously reported in successful *in vitro* experiments. Attempts were made to devise an *in vitro* system which promoted hamster and squirrel monkey sperm penetration of the zona-free hamster ovum. Based upon this penetration, capacitation parameters of squirrel monkey could be evaluated. A visually noticeable change in hamster sperm motility, termed activation, accompanies capacitation in this species. Hamster sperm penetration of the zona-free ovum will be used as a test of the ability of this system to support homologous penetration.

LITERATURE REVIEW

The early work on mammalian *in vitro* fertilization laid the foundation upon which Yanagimachi (1972) based his work on cross-species fertilization with guinea pig spermatozoa and zona pellucida-free hamster ova. Success in fertilization in the rabbit was reported as early as 1954 (Dauzier, Thibault and Winterberger, 1954; Moricard, 1954). Schenk (1878) and Long (1912) worked with guinea pigs and rats and noted second polar body extrusion in both species, as well as cleavage in the guinea pig with *in vitro* fertilization. However, it has been emphasized by some workers (Thibault, 1949; Chang, 1957; Austin, 1961) that formation of a second polar body and cleavage may occur by parthenogenic activation. Although rare, these events can occur spontaneously with unfertilized ova while in culture. Not until Yanagimachi and Chang (1964) reported the first successful *in vitro* fertilization of the golden hamster (*Mesocricetus auratus*) was rodent fertilization *in vitro* conclusively proven. Ova were considered fertilized only when enlarged sperm heads or male pronuclei were found in the vitellus. While sperm tails in and around the vitellus were also considered a good criterion for fertilization, female pronuclei alone and the presence of sperm in the perivitelline space were not considered as definitive proof of fertilization. The same evidence of fertilization holds for zona pellucida-free hamster ova inseminated with capacitated

hamster sperm. The importance of this zona-free ovum fertilization system is that it appears a wide variety of capacitated mammalian sperm are able to penetrate the ovum. This characteristic of the hamster zona-free ovum allows studies of sperm capacitation and male fertility in an *in vitro* biological system.

Hamster Maturation

The golden hamster has a life span of between 1 and 3 years. Generally these animals are considered senescent when 12 to 15 months of age in the female, and 2 years in the male. Greenwald and Peppler (1968) reported that the earliest spontaneous ovulations occur at 29 days of age. Bond (1945) found that litters caged from birth typically mated on or about the 42nd day. Therefore, by 42 days the male is capable of producing fertile spermatozoa and of ejaculation. Lubicz-Nawrocki (1976) studied the relationship between age and weight as it relates to sexual maturity. Male hamsters 6 weeks of age weighing 110 g had cauda epididymal sperm with high fertility, while those weighing 80 to 90 g had a lower fertility rate. By 7 weeks, even those animals weighing 80 to 90 g had maximum fertility. It is apparent that both size and age play a role in the sexual maturation of the golden hamster.

Ovum Structure and Maturation

By 8 days of age the germ cells have developed to dictyate oocytes in the hamster. The critical period for follicular development is between 21 and 28 days of age. Antral follicles are seen first at 26 days. The dictyate stage, arrived at after the 15 μ oogonia completes the first meiotic division, is characterized by the presence of a germinal vesicle. Luteinizing hormone (LH) prompts

the resumption of ovum maturation. At the time of ovulation most ova are at second metaphase (Iwamatsu and Yanagimachi, 1975). When dictyate oocytes are recovered from the follicles and put into the proper culture conditions, meiotic division resumes. This ability to mature in an *in vitro* environment seems to be a property of many mammalian ova. Iwamatsu and Yanagimachi (1975) reported that the 23 day old hamster has oocytes capable of maturation *in vitro*. Tables 1 and 2 show the stage of ovum maturation in relation to natural ovulation time, or following an ovulatory dose of human chorionic gonadotropin (HCG). From these tables, and confirmed by Austin (1956a) and Barros and Yanagimachi (1972), one can see that 75 to 80% of the ova are spontaneously activated by 18 hours after ovulation. According to Yanagimachi and Chang (1961), the presence of 2 pronuclei in the spontaneously activated ovum indicates that either the second polar body did not form or that the pronucleus broke down into smaller pronuclei. It is due to this spontaneous activation that the presence of 2 polar bodies, pronuclei and cleavage, are not in themselves absolute criteria for fertilization. They state that this spontaneous activation of ova begins at the time of loss of fertilizability of the ova, about 12 hours after ovulation.

At the time of ovulation, the mature ovum is surrounded by a dense mass of cells (the cumulus oophorus and the corona radiata). The zona pellucida, an acellular glycoprotein structure, constitutes the next innermost layer and immediately surrounds the ovum (Wolf *et al.*, 1976). The small perivitelline space separates the zona pellucida from the vitelline membrane. The entire surface of the ovum, except the area from which the second polar body is destined

Table 1. Time and sequence of oocyte maturation in adult female hamsters before ovulation[a]

Time (hrs)	Percent of Oocytes[b] Exhibiting: G.V.	Cond. G.V.	Promet. I	Met. I	Ana.	Tel.	Chr. Mass	Promet. II	Met. II
Before Ovulation									
12	100	---	---	---	---	---	---	---	---
10	97.4	2.6	---	---	---	---	---	---	---
7	---	70.0	30.0	---	---	---	---	---	---
4	---	---	---	47.6	52.4	---	---	---	---
2	---	---	---	---	---	52.4	---	49.6	---
0	---	---	---	---	---	---	---	---	100
After HCG									
1	100	---	---	---	---	---	---	---	---
3	25	75	---	---	---	---	---	---	---
7	---	---	75	25	---	---	---	---	---
9	---	---	---	---	33.3	66.7	---	---	---
11	---	---	---	---	---	6.3	12.5	81.2	---
12	---	---	---	---	---	---	---	---	100

[a]Material cited from Iwamatsu and Yanagimachi (1975).

[b]G.V., germinal vesicle; Cond. G.V., condensing germinal vesicle; Promet., prometaphase; Met., metaphase; Ana., anaphase; Tel, telophase; Chr. Mass., chromatin mass.

Table 2. Time and sequence of oocyte maturation in adult female hamsters after ovulation[a]

Time after Ovulation (hrs)	Percent of Oocytes[b] Exhibiting:											
	Promet. II	Met. II	Ana. II	Tel. II	2PB+ 1PN	1PB+ 2PN	2PB+ 2-3PN	1PB+ 3-4PN	1PB+ Scat. Chrom.	2PB+ Scat. Chrom.	2 Cell	Uniden.
0	68	32	---	---	---	---	---	---	---	---	---	---
2	---	100	---	---	---	---	---	---	---	---	---	---
6	---	100	---	---	---	---	---	---	---	---	---	---
12	---	94	4.5	---	1.5	---	---	---	---	---	---	---
18	---	15	3	3	45	18	3	3	---	---	---	9
32	---	2	---	---	30	36	---	---	18	14	---	---
52	---	---	---	---	44	24	---	---	---	---	28	4

[a]Material cited from Yanagimachi and Chang (1961).

[b]Promet., prometaphase; Met., metaphase; Ana., anaphase; Tel., telophase; PB, polar body; PN, pronucleus; Scat. Chrom., scattered chromosomes; Uniden., unidentifiable.

to arise, is covered with numerous microvilli (Yanagimachi and Noda, 1970c). Cortical granules, derived from the golgi apparatus in maturing oocytes, are present in the form of a continuous layer several hundred Angstroms below the ovum surface. The existence of a layer of uniformly distributed granules in mammalian ova was first reported by Austin (1956b) in the golden hamster. These granules are characterized by a round or elliptical shape and are 0.2 to 0.5 μ in diameter. They consist of a highly dense matrix surrounded by a single smooth membrane (Zamboni, 1970). Their contents, which are released upon penetration by a sperm, are responsible for initiating the zona pellucida block to polyspermy. Cortical granules break down spontaneously after ovulation. In hamster ova, by 12 hours after ovulation, nearly all cortical granules are absent from the vitellus and there is evidence of cortical granule material in the perivitelline space. This corresponds to the time of loss of fertilizability of the ovum *in vivo* (Yanagimachi and Chang, 1961).

Estrous Cycle and Ovulation

The hamster has a consistent 4-day estrous cycle. According to Orsini (1961), estrus occurs on day 1 with a post-estrous discharge evident on day 2. It is this white, opaque, mucous discharge which is used in identifying the stage of the cycle. Estrus usually begins about dusk on the evening of day 1. Ovulation occurs 8 hours after the onset of estrus (Harvey, Yanagimachi and Chang, 1960). The duration of ovulation is from 1 to 4 hours. It appears that the failure of the corpora lutea to produce sufficient progesterone to inhibit new ovulation is the reason for the short cycles (Kent, 1968).

Bast and Greenwald (1974) studied gonadotropin levels in females under a 0500 hr to 1900 hr light schedule. This 14:10 light/dark schedule is used with the hamster due to its effects in promoting consistent cyclicity and fertility. Days less than 12.5 hours of light often lead to acyclic periods and continuous light can bring about constant estrus (Kent *et al.*, 1968; Turek *et al.*, 1975). Bast found a biphasic serum FSH pattern with levels rising sharply and peaking at 1600 hr on day 1 and then dropping and rising again to high levels on day 2. Luteinizing hormone also rose sharply on day 1, to peak at 1600 hr, but then fell to pre-surge levels for the remainder of the cycle. There is also a preovulatory increase in prolactin at 1500 hr on day 1. These peaks in gonadotropin levels have been confirmed by other workers (Norman, 1975; Bex and Goldman, 1975; de la Cruz, 1976). If the 14:10 light/dark schedule is shifted to 0600 hr to 2000 hr, the peaks of FSH and LH are at 1700 hr (Rani, 1977). Saidapur and Greenwald (1978) have shown that progesterone levels begin to rise in late morning of day 1 and sharply increase at 1400 hr (0500 hr to 1900 hr light). This would be important in promoting the lordosis, or estrous behavior, characterized by the female hamster. Estradiol levels vary inversely with progesterone levels. Estrone levels remain constant. Normal ovulation yields an average of 10 ova per female (Kent, 1968; Chiras, 1978); however, there are twice as many follicles primed to ovulate. Between days 4 and 1 of the cycle, approximately one-half of these follicles undergo atresia. Ovulation can be induced by administering human chorionic gonadotropin (HCG) to the animal. If the animal is given pregnant mare's serum gonadotropin (PMS) to induce follicular development, then HCG, the result will be ovulation

of as many as 70 ova per female. The PMS acts as a follicle stimulator in promoting the growth of not only the follicles that would normally develop, but also those that would normally become atretic. Additionally, another group develops that ordinarily would not have matured (Greenwald, 1962). Best recovery results are obtained when 30 IU of PMS is given on the morning of day 2 of the cycle and followed by 30 IU of HCG on day 4 or day 1. Superovulation can also be induced in immature females by following the same time course (Yanagimachi and Chang, 1964). Precocious ovulation in mature female golden hamsters is induced when HCG is given as early as 48 hours following PMS administration. Enough follicles are ripe at this time to allow for the ovulatory effect of HCG to be effective. The ova obtained by this method (first used by Yanagimachi in 1969 [1969a]) are as equally fertilizable as those from mature superovulated, immature superovulated, and normally ovulated females. Yanagimachi (1969) found ovulation beginning 11 to 14 hours after HCG. Most ova will reach the ampulla by 16 to 17 hours after HCG. They will stay there for 22 to 34 hours (Yanagimachi and Chang, 1961) before passing into the isthmus. Unfertilized and fertilized ova have the same transport time. If it is necessary to recover ova for *in vitro* trials, flushing or dissection of the oviducts is rcommended. Ideally the ova should be in the oviduct, but not too old, so that fertilizability is diminished. As noted previously, ovulation begins about 11 hours after HCG administration, and nearly all ova are in the ampulla by 17 hours. Maximum fertilizability of the ovum seems to occur between ovulation time and the 3 hours following (Barros and Yanagimachi, 1972). The 12-hour fertilizable life of the ova ends as early as 23 hours after HCG. A compromise

must be made between the time after ovulation necessary for obtaining the maximum number of ova and the time of maximum fertilizability of the ova. Within the narrow time range from the beginning of ovulation to the end of ova fertilizability, recovery and insemination must take place. Time must also be allowed for sperm capacitation to occur *in vitro*. With hamster sperm, this generally begins within 2 to 3 hours under proper *in vitro* conditions (Yanagimachi, 1969; Yanagimachi, 1970a; Bavister, 1973).

Sperm Maturation and Structure

The process of maturation in spermatocytes is completed without rest, which is in contrast with the resting stages of ovum maturation in the female (Zamboni, 1970). Uehara and Yanagimachi (1977) found a variety of modification occurring in the spermatids in their course through the epididymis which most likely lend to their change in ability to fertilize an ovum. Hamster epididymal sperm acquire the ability to penetrate ova at the proximal cauda epididymis (Horan and Bedford, 1972). It is at the distal caudal where sperm samples are taken for *in vitro* studies. Horan found 79% penetration when using sperm from this region. Miyamoto (1972) showed that epididymal sperm are as effective as ejaculated sperm for the penetration of ova. The fertilizable life of sperm in the female tract is about 13 hours.

The hamster spermatozoa consist of a head, midpiece and tail like other mammalian spermatozoa. The hamster sperm has a sickle-shaped head which is made up of the plasma membrane, acrosomal cap, acrosomal collar, inner and outer acrosomal membranes, perforatorium and nucleus (Yanagimachi and Noda, 1970b). Yanagimachi and Noda

(1970c) noted that the acrosomal cap is associated with hyaluronidase, which acts to dissolve the hyaluronic acid-protein complex of the cumulus matrix. Zona lysin, thought to be important for traversing the zona pellucida, was located in the acrosomal collar region. Austin (1968) also found evidence for zona lysin in the equatorial segment. Other workers have theorized that zona lysin is present on the inner acrosomal membrane (Bedford, 1968, 1970, 1972; Piko, 1969; McRorie and Williams, 1974). Yanagimachi (1977) noted the possibility of the enzyme situated in either location or both. The perforatorium probably serves simply as a physical wedge enabling the sperm to penetrate the ovum investments. The plasma membrane of the post-nuclear cap region is the initial site of sperm-ovum fusion (Barros and Franklin, 1968; Yanagimachi and Noda, 1970c).

Energy production in the mammalian sperm, in the form of ATP, is produced via the glycolytic pathway or by oxidative phosphorylation. The spermatozoa metabolize glucose, fructose and mannose anaerobically and aerobically by glycolysis. Pyruvate and lactate are metabolized aerobically by the Krebs cycle and oxidative phosphorylation (Mann, 1973).

Capacitation, Acrosome Reaction

There are changes in the sperm structure associated with the acrosome reaction (Yanagimachi and Noda, 1970c). The acrosome reacted hamster spermatozoa have lost the anterior part of the acrosome, the acrosomal cap. Extensive membrane vesiculation between the overlying plasma membrane and the outer acrosomal membrane leads to this loss. The acrosomal collar shows varying degrees of membrane vesiculation at this stage. When the acrosome

reaction is complete, the outer acrosomal membrane and the overlying plasma membrane in the anterior part of the sperm head will have disappeared. As a consequence, the perforatorium becomes the anterior-most segment of the sperm. While the acrosome reaction is a visible event, the molecular basis of capacitation is still not understood. Capacitation does not seem to involve visible changes in the sperm, but is a prerequisite for the acrosome reaction. It is theorized that capacitation involves some modification of the sperm surface which enhances the sperm's ability to bind the ovum (Yanagimachi, 1977). Spermatozoa undergoing capacitation exhibit a distinct change in the pattern of motility, commonly termed activation (Yanagimachi, 1969b). This activation was observed in sperm known to be capacitated, but never in sperm under conditions unfavorable for capacitation. Accordingly, activation reflects an important change associated with capacitation. Activation is characterized by a larger beating amplitude of the flagellum and increased side-to-side movements of the sperm head. The possible significance of this change may result from an increased ability to propel the sperm through the ovum investments (Yanagimachi, 1970b). Examination of activated sperm showed that the majority have undergone or are in the process of undergoing the acrosome reaction.

Rogers and Yanigamachi (1975) suggested that spermatozoa require lactate and pyruvate in order to become capacitated and undergo the acrosome reaction. The importance of lactate, pyruvate and glucose in some combination for capacitation and the acrosome reaction has been shown by other workers as well (Miyamoto and Chang, 1973; Toyoda and Chang, 1974; Tsunoda and Chang, 1975; Bavister and Yanagimachi, 1977).

Sperm-Ovum Interaction

Barros and Yanagimachi (1972) studied cumulus-free hamster ova mixed with capacitated hamster sperm. They found that the sperm bound immediately to the zona pellucida but remained attached to the surface at least 5 minutes before penetrating. About 15 minutes after sperm attachment, some sperm were seen in the perivitelline space. Yanagimachi and Noda (1970c) used superovulated hamster ova and capacitated hamster sperm to study, using electron microscopy, the process of sperm penetration in the hamster. Profound membrane vesiculation of the acrosomal cap region on the sperm was seen before sperm began to enter the zona pellucida, which confirmed an earlier report (Barros and Austin, 1967). This vesiculation occurs as the sperm passes through the matrix of the cumulus oophorus. It is thought that this process allows hyaluronidase to be released, thus aiding the sperm in its passage through the cumulus layer. Yanagimachi and Noda also saw membrane vesiculation in the acrosomal collar region while the sperm were crossing the zona pellucida. Based on these findings, they proposed that zona lysin was associated with the acrosomal collar region. The membrane vesiculation would allow release of the enzyme which causes the zona pellucida to weaken and thereby allow the sperm to pass through. The sperm passes obliquely through the zona pellucida tangentially to the zona surface and leaves a penetration slit. The perforatorium and the post-nuclear cap region remained visibly unchanged as the sperm traversed these structures. After crossing the zona pellucida, the sperm crosses the perivitelline space and reaches the vitelline membrane in 1 to 2 seconds. Yanagimachi and Noda (1970a) also studied zona-free hamster ova interactions with capacitated hamster

sperm. There is no substantial difference between *in vivo* fertilization and polyspermic *in vitro* fertilization of zona-free ova as far as the early behavior of penetrating sperm is concerned. Once the sperm touches the ovum surface, it attaches laterally and numerous microvilli surround it. This seems to activate the ovum cortex as wavelike protrusions occur within it. Fukada (1978) reported that activation of the ovum by the sperm starts from the breakdown of the cortical granules and is followed by the second maturation division resumption. The head, midpiece and flagellum are incorporated into the ovum cytoplasm (Yanagimachi and Noda, 1970a). The sperm itself plays a passive role in this incorporation.

Barros and Yanagimachi (1972) studied the interaction between sperm and ova, at the penetration stage and beyond, utilizing zona-free hamster ova. Many capacitated sperm attach to the ovum and their flagellar motion soon ceases. Yanagimachi (1978) noted that within 15 to 25 seconds after contact with the ovum, the sperm becomes motionless. Barros and Yanagimachi saw cortical granule breakdown within 2.5 to 5 minutes after attachment. Ten minutes after attachment the ovum chromosomes begin anaphasic movements. Thirty minutes later the second meiotic division is complete. At 60 minutes after attachment the whole sperm head is dispersed and developing as the spherical male pronuclei. The female pronucleus is distinct at this time. At 2 hours after initial sperm contact with the ovum, the male and female pronuclei are similar in appearance and the entire midpiece of the sperm is in the ovum cytoplasm. By the time 3 hours have elapsed from initial sperm binding, each pronucleus has 1 to 3 large nucleoli and the sperm tail is almost completely within the cytoplasm. This stage is discernible using

phase-contrast optics and is considered a positive sign of sperm penetration.

In vitro Conditions for Sperm-Ovum Union

When utilizing a system involving the interaction of zona-free ova with capacitated sperm, it is necessary to prepare the zona-free ova and provide proper conditions for sperm capacitation and ovum penetration. The use of hyaluronidase at a 0.1% to 0.2% concentration to dissolve the cumulus cells and trypsin or pronase at a 0.1% concentration to dissolve the zona pellucida of hamster ova dates back to Yanagimachi's original observations (1972). Hirao (1978a) studied the effects of a variety of enzyme treatments on ova upon subsequent penetration by sperm. Treatment with 0.1% trypsin had no adverse effect on penetration, even with incubation periods up to 30 minutes. The only difference observed was the degree of polyspermy. This lack of adverse effect of enzymes allows zona-free ova to be prepared by enzymatic removal of the zona rather than mechanical removal, which can damage the ovum.

Hamster spermatozoa seem to require a sperm motility factor, protein, and energy substrate(s) in order to become acrosome reacted and to fertilize ova *in vitro* (Bavister and Yanagimachi, 1977). Yanagimachi (1970b) suggested a sperm capacitation factor made up of 2 separable components, one high molecular weight factor and one of low molecular weight. The sperm motility factor seems to be that low molecular weight component. It is found in blood serum, adrenal extracts and follicular fluid. In order for blood serum to be used in sperm culture, spermicidal factor(s) must be destroyed by heat. The cumulus oophorus cells, their matrix, the corona radiata, and

the ova themselves have no part in inducing capacitation of the sperm (Yanagimachi, 1969a). The high molecular weight component appears to be albumin (Yanagimachi, 1970b). It is responsible for promoting the occurrence of the acrosome reaction. The energy substrates are necessary for sperm metabolism and survival.

The proper medium for sperm survival and capacitation should attain a final measured osmolality of 270 to 402 mOSm, according to Miyamoto (1973). Over this range, 86 to 96% fertilization occurs. Miyamoto thus concluded that the proper medium for *in vitro* fertilization should have similar or slightly higher osmolality than blood serum (308 mOSm). Miyamoto used a medium of heated hamster serum plus Tyrode's solution (1:2) for his study. In 1974, Miyamoto used one portion of heated hamster serum to three portions of Tyrode's solution for hamster gametes. He found an optimal pH range for sperm penetration of 6.8 to 8.2. Mahi (1973), using a medium of Tyrode's solution and agamma human serum (1:1), tested the effect of osmolality, temperature and pH on sperm activation, acrosome reaction and survival. The best activation of sperm occurred at measured osmotic pressures of 268 to 343 mOSm. The optimum temperature was found to be 37°C and an initial pH of between 7.0 and 8.3 gave good activation. More than 50% of motile sperm had an acrosome reaction when the initial pH was 7.2 to 8.3. Sperm survival was best at pH 7.0 to 7.8. Bavister (1969) reported a pH range of 7.2 to 7.8 as best for fertilization utilizing a gas-equilibrated, modified Tyrode's solution containing sodium bicarbonate, bovine serum albumin and sodium pyruvate. Hirao (1978a) used a modified Krebs-Ringer solution (Biggers *et al.*, 1971) commonly referred to as BWW medium. To BWW medium was added heat-inactivated human serum

in a 2 parts BWW to 1 part serum mixture. He did not note the pH or osmolality of the mixture but did obtain excellent penetration results. Hirao and Yanagimachi (1978b,c) again used the BWW medium and human serum mixture for studies on sperm-ovum fusion and the effects of visible light on meiosis of ova. Exposure of unfertilized hamster ova to short wavelength visible light from ordinary light sources disturbed the completion of normal meiosis after ova were pentrated by spermatozoa. Fluorescent light was found to be more harmful than light from incandescent lamps.

Generally, a 5% CO_2 in air mixture is used for incubation with BWW medium, while air is used for Tyrode's solution. Whittingham (1971) recommended the use of 3-times glass-distilled water in preparing culture media in order to provide sufficient purity.

The literature varies somewhat on the concentration of sperm used in cultures. Talbot (1974) studied the effect sperm concentration had on the acrosome reaction and ovum penetration *in vitro*. Utilizing sera from several species, Talbot found that a 2×10^7/ml concentration produced the best results for both the acrosome reaction and penetration, regardless of the serum used. Hirao (1978a) used a concentration of 4×10^7/ml to promote capacitation of sperm.

Kuehl and Dukelow (1975), while working with *in vitro* fertilization of *Saimiri sciureus* follicular oocytes, found significantly better results for fertilization in chamber slides over the more conventional culture dish utilizing a light viscosity silicone oil cover. They noted the advantage of the 8 chambers, which allow for varied culture conditions for *in vitro* work. Several investigators (Barros and Austin, 1967; Hanada and Chang, 1972; Yanagimachi and Chang, 1961) recommend transferring cultured ova to a glass slide

in a small volume of medium following culture. The ova are situated between 4 petroleum jelly dots and a coverslip is gently placed over the droplet containing the ova. This allows for better viewing, which increases the probability of detecting evidence of penetration. Hirao (1978a) observed that not all sperm nuclei incorporated into the ovum cytoplasm necessarily decondense. At light microscopic level it is often difficult or impossible to distinguish sperm heads incorporated into the ovum, but not decondensing, from those outside (Usui and Yanagimachi, 1976). Thus, the absence of sperm heads in the ovum cytoplasm does not give definite indication of a failure of the sperm to penetrate the ovum. The presence of a swollen sperm head or heads, however, does give proper evidence of successful sperm-ovum fusion.

Zona-Free Ovum System

In the proper culture conditions, if the zona pellucida of a hamster ovum is removed, one or more capacitated hamster spermatozoa are capable of penetrating the vitelline membrane. Yanagimachi (1972) tested guinea pig spermatozoa in the presence of hamster zonal ova. The incubation medium used was BWW medium. When uncapacitated guinea pig sperm were incubated with zona-intact hamster ova, none attached permanently. The results were the same when zona-intact ova were inseminated with capacitated guinea pig sperm. With guinea pig sperm, loss of the acrosomal cap is indicative of capacitation. When zona-free hamster ova were mixed with the uncapacitated guinea pig sperm, some attached to the vitelline membrane but no ova showed signs of activation or sperm penetration. However, when capacitated guinea pig sperm were added to a culture of zona-free

hamster ova there was almost immediate attachment of several spermatozoa to the ovum surface. Vigorous tail movement of some diminished quickly. By 40 minutes after insemination, most ova were devoid of cortical granules, indicating activation of the ovum cytoplasm. The sperm heads were distinctly swollen and some transformed into male pronuclei. The number of spermatozoa having entered the ovum vitellus varied from 1 to 6. All ova examined had been penetrated. Yanagimachi also observed that while capacitated guinea pig spermatozoa readily penetrate guinea pig zona pellucida *in vitro*, they failed to do so with the hamster zona pellucida. This indicates that at the level of the zona pellucida, in the hamster, there is present a species-specific mechanism which prevents non-homologous spermatozoa from penetrating. Once the vitelline surface is exposed to the heterologous capacitated sperm, this species-specific blockage appears to be lost. Hanada and Chang (1972) found that rat and mouse spermatozoa are able to penetrate the vitelline membrane of zona-free hamster ova. The culture medium consisted of an equal volume of Tyrode's solution and either bovine follicular fluid or serum. Only enlargement of the sperm head was noted with rat sperm. Ovum activation and development of both male and female pronuclei was common with mouse sperm. Polyspermy was frequently observed. Noting Hanada and Chang's work, Yanagimachi proposed that if the rat and mouse sperm required capacitation before penetrating the zona-free hamster ovum, then this ovum might be used to study sperm capacitation in these and other species. It was not until later that Hanada and Chang (1976b) showed that capacitation of both rat and mouse spermatozoa was required before they could penetrate the zona-free hamster ovum. They found penetration occurring only after

preincubation of sperm for known *in vitro* capacitation times. In the same year, Yanagimachi *et al.* (1976) found that human sperm would penetrate the zona-free hamster ovum after an incubation of several hours in BWW medium with 0.3% human serum albumin. All of the sperm which had fused the ovum had undergone acrosomal changes. These changes were only observed by electron microscopy. It appeared that only sperm with non-intact acrosomes were capable of fusing with the eggs. The acrosome reaction is thought to proceed only after sperm have undergone capacitation (Austin, 1968, 1973; Bedford, 1970; Yanagimachi and Usui, 1974). Based on this reasoning, the human sperm which fused with and penetrated the vitellus of the zona-free hamster ovum must have been capacitated. The penetrating sperm were visible as either swollen heads or pronuclei. The workers also noted that the sperm did not bind the ova until they were capacitated (3 to 4 hours in culture). The number of sperm bound increased over time from 3 to 4 per ovum to 10 to 20 after longer incubations. One to seven sperm were found in the ovum. This work added a primate species to the list of rodent species whose sperm had been shown to penetrate the ova after sperm capacitation *in vitro*. A domestic animal species was added to the list when Imai *et al.* (1977) showed penetration of hamster zona-free ova with boar spermatozoa. The boar sperm had only very limited success in capacitating *in vitro* in a modified Krebs-Ringer bicarbonate solution. Better success resulted from preincubation of the sperm in the reproductive tract of a gilt or sow. Enlarged sperm heads and some male and female pronuclei were observed. Polyspermy was common when penetration did occur.

These studies have shown not only the loss of species specificity at the vitelline membrane of hamster ovum, but also that spermatozoa from different taxonomic orders are able to penetrate the ovum once the zona pellucida is removed.

In the intact hamster ovum, the zona pellucida functions as the major block to multiple sperm entry into the ovum cytoplasm. The release of cortical granule material following sperm attachment to the ovum surface leads to the zona reaction. Austin (1956c) and Austin and Bishop (1958) found less than 1% occurrence of polyspermy in *in vivo* fertilized ova, and these sperm were only seen in the perivitelline space. It appeared from this work that there is also a vitelline surface block to polyspermy. Yanagimachi and Chang (1961) found the zona reaction strongest in the recently ovulated ovum (0 to 3 hours post-ovulation). As fertilizability of the ovum decreases, up to 12 hours post-ovulation, the zona reaction weakens. They noted certain changes in the cortex occur that prevent sperm entry, and this leads to the loss of fertilizability. At 12 hours post-ovulation, the zona pellucida maintains a weakened block while the vitelline block is strong. Both surface blocks are intact by 18 hours after ovulation. A change in the zona pellucida is also indicated since no sperm will attach to it at this time. In the *in vitro* environment, the ovum devoid of the zona pellucida has been shown to allow polyspermic penetration from capacitated sperm of various species. Barros and Yanagimachi (1972) noted that the vitelline surface block normally takes 2 to 3.5 hours to develop once the ovum is fertilized. Obviously, this delay leaves the zona-free hamster ovum devoid of any major blocking mechanisms during the first few hours of exposure to capacitated sperm. In the *in vitro* system

there are several factors which also may contribute to the polyspermy observed. The culture environment, the possibility of immature or aged ova, and the increased numbers of capacitated spermatozoa may also lend to the high incidence of polyspermy.

The Male Squirrel Monkey

The male squirrel monkey (*Saimiri sciureus*) averages 900 g in weight and 30 cm in body length (Bennett, 1967). Bennett was the first in obtaining semen samples from squirrel monkeys by electro-ejaculation. He characterized the ejaculate as consisting of 2 fractions: a colorless sperm-free coagulum and a sperm-rich fluid. Kuehl and Dukelow (1974) developed a restraining device which allowed ease of handling without tranquilizing the animal.

The literature on the spermatozoa characteristics of the male squirrel monkey is limited in comparison to that of the hamster and other rodent species. Much of the work involves *in vitro* fertilization studies utilizing squirrel monkey oocytes (Johnson *et al.*, 1972; Cline *et al.*, 1972; Gould *et al.*, 1973; Kuehl and Dukelow, 1975). Successful *in vitro* penetration of squirrel monkey oocytes by ejaculated squirrel monkey sperm was accomplished in a medium consisting of 80% TC199 and 20% heated agamma newborn calf serum (Cline *et al.*, 1972; Gould *et al.*, 1973; Kuehl and Dukelow, 1975). Kuehl obtained the best results for fertilization in this medium, plus 70 µg/ml pyruvate, 100 U/ml penicillin-streptomycin, and 1 U/ml heparin. The literature cites little regarding sperm survival parameters, attachment to the ovum, time of penetration, capacitation and the acrosome reaction. However, it is known that squirrel monkey sperm acquire the ability to penetrate squirrel monkey oocytes in an

in vitro environment. Bedford (1977) incubated squirrel monkey sperm obtained from the ducts of the cauda epididymis and vas deferens with hamster zona-intact oocytes in TC199 on Bavister's medium. At sperm concentrations of 0.8 to 4.0 x 10^6/ml, he found active attachment (not disrupted by strong field currents created on the slide) of squirrel monkey sperm to the zona pellucida of the hamster oocytes.

Since the hamster zona-free ovum has been shown to be susceptible to penetration by heterologous sperm, including a primate species (human), the squirrel monkey sperm would be an interesting model for testing the ability of non-human primate sperm to interact with the hamster zona-free ovum. If normal sperm is able to penetrate the ovum, then it may be possible to assess the capacitation time of squirrel monkey sperm as well as the fertility of particular males.

MATERIALS AND METHODS

Animal Care

The golden hamsters used were either locally bred or obtained from an animal supplier (Charles River Breeding Laboratories, Wilmington, Massachusetts). The animals were housed in plastic cages with attachable stainless steel cage tops. The bedding in the cages was a ground corncob mixture (San-I-Cell, Paxton Processing Co., Paxton, Illinois). The animals were kept on a 14:10 light/dark schedule (lights on 0600 hr to 2000 hr). The temperature was maintained at 21.1°C. The food supplied was a commercial preparation (Wayne Lab-Blox, Allied Mills, Chicago, Illinois). Males and females were housed separately before reaching maturity. The male squirrel monkeys used in these studies were of the Bolivian type (Primate Imports Corp., Port Washington, New York). The males were housed in groups of 4, in a stainless steel, flush-type cage. Commercial monkey feed (Wayne Monkey Diet, Allied Mills, Chicago, Illinois) was fed along with supplemental fruit. The animal quarters were maintained on a 12:12 light/dark cycle using fluorescent lighting (0600 hr to 1800 hr). The temperature was kept at 21 $\pm$ 2°C. Relative humidity varied from 40 to 60% dependent on the season.

Culture Preparation

Media

Two basic culture media were utilized in this study. BWW medium (Biggers, Whitten and Whittingham, 1971) was prepared according to the original paper, with the exception that 1.71 mM $CaCl_2$ was substituted for 1.71 mM Ca-lactate. Three-times glass-distilled water was used as the solvent. The second culture medium consisted of a commercial preparation, TC-199 (Grand Island Biological Co., Grand Island, New York), containing HEPES buffer, Earle's salts and L-glutamine. Additionally, 70 µg/ml of sodium pyruvate was included. Hereafter, the solution will be referred to as TC199. Each medium was passed through a 0.45 µ Millipore filter into sterile vials. These media preparations were used fresh or stored at -20°C until use. When needed, the medium was thawed and placed into a 5% CO_2 in aire atmosphere for a period of 1 hour. Following this, each medium pH was measured. An initial pH range of 7.35 to 7.45 was desired and, if necessary, adjusted with either 1M NaOH or 1M HCl.

Sera

The human, monkey and hamster sera were obtained from donors. The newborn calf serum and the agamma fetal bovine serum were commercial preparations (Grand Island Biological Co., Grand Island, New York). The female hamster blood was obtained while under light ether anesthesia by capillary tube puncture of the orbital sinus. The female squirrel monkey blood was recovered, while under sodium pentobarbital (Nembutal Sodium, Abbott Laboratories, North Chicago, Illinois) anesthesia, through the femoral vein. Human male blood was drawn from the median cubital vein. The blood was transferred

to test tubes and allowed to clot at room temperature, then centrifuged at 1540 g for 20 to 50 minutes. The serum was passed through a 0.45 μ Millipore filter for sterilization. Both the newborn calf serum and the agamma fetal bovine serum, which had been kept at -20°C, were thawed and passed through the Millipore filter as well. All sera were placed into sterile vials following filtration. These samples were used fresh or kept at -20°C until needed. The sera were thawed and then heated (56 to 60°C, 35 to 60 minutes) immediately prior to use. Samples of the sera and selected medium-to-serum ratios were tested for determination of the pH values of the initial cultures. The glassware was rinsed with 3-times glass-distilled water and sterilized at 150°C for 90 minutes. No fluorescent lighting was utilized.

Procedure to Obtain Hamster Ova

Mature (75 g and over) and immature female hamsters were subjected to a gonadotropin regime in order to obtain superovulation. An intraperitoneal injection of 30 IU of PMS (Gestyl, N.V. Organon, Oss, Holland, or Serotropin, Teizo Pharmaceutical Co., Tokyo, Japan) was administered between 0900 hr and 1200 hr on the day of the post-ovulatory discharge (day 2). The PMS injection was followed 52 to 64 hours later by an intraperitoneal injection of 30 IU HCG (A.P.I., Ayerst Laboratories, New York, New York). The animals were killed by ether overdose 14 to 17 hours after HCG administration. The oviducts were removed, flushed with 0.4 ml of BWW medium, and minced. The resultant mixture was subjected to 0.1 to 0.2% (w/v) hyaluronidase (ovine testicular hyaluronidase, 720 units/mg, Calbiochem, San Diego, California) at 22 to 25°C for removal of the cumulus cell layers.

The ova were then washed once in BWW medium. If zona pellucida removal was indicated, the ova were transferred to 0.1% trypsin (2X cryst., salt free, 2910 NF units/mg, General Biochemicals, Chagrin Falls, Ohio) at 22 to 25°C for 1 to 2 minutes. The ova destined for culture in TC199 were washed in that medium prior to addition to the culture.

Procedure to Obtain Hamster Sperm

Mature male hamsters, over 100 g, were ether-killed to obtain the epididymides. Once recovered, the epididymides were placed on tissue paper to remove extraneous blood. The sperm sample was obtained by clamping near the proximal caudal region and puncturing the distal caudal region several times with a sterile needle. A drop of sperm suspension was placed onto a 3-well glass slide. To this was added 0.8 ml of BWW medium. In 3 to 4 minutes, the sperm dispersed and the most concentrated sample was removed by pipette. The remaining solution was stirred to obtain a homogeneous mixture. Part of this mixture was diluted with BWW medium, usually at a 1:9 ratio, for determination of the concentration of sperm in the solution. The ratio of sperm solution to sperm-free medium to serum was altered to maintain the sperm concentration as constant as possible in the different culture chambers.

Procedure to Obtain Squirrel Monkey Sperm

Adult male squirrel monkeys were restrained without anesthesia according to a method of Kuehl and Dukelow (1974). Electroejaculation was used in order to obtain a sperm sample. Although part of the ejaculate is liquid immediately upon discharge, the entire sample coagulates within 1 minute. Washing of the sample is unnecessary

since the sperm are released upon incubation in medium. The ejaculate, 0.1 to 0.5 ml, was divided into 2 parts for culture in either BWW medium or TC199 medium. After division of the ejaculate, 0.25 ml of the appropriate culture medium was added to the sample. A period of 15 minutes was allowed between the addition of the culture medium to the ejaculate and the initial assessment of motility and concentration of the sample. If the concentration of the sample was very high, further dilution was used as an aid in counting the sperm. The final concentration of sperm was generally above 10^6/ml but varied between males, ejaculates, and the 2 culture media. Attempts were made in each trial to equalize the sperm concentrations.

Culture of Gametes

Preparation of sperm and ova cultures was carried out at 22 to 25°C. Collection and culture of the sperm samples preceded ova recovery. After preparation, the ova were added to viable sperm cultures. Chamber slides (tissue culture chamber/slide, No. 4808, Lab-Tek Products, Naperville, Illinois) with 8 chambers were used for the incubation. A 5 µl micropipette was used when preparing the cultures of various medium to serum ratios. The final sperm culture volumes ranged from 20 to 50 µl. The ova were added to the sperm cultures in a volume of 1 to 2 µl of medium. Once added, the sperm and ova were incubated at 37°C in a saturated 5% CO_2 in air atmosphere. Cultures were periodically checked for sperm motility, activation, binding, ovum penetration and physical condition. An inverted tissue culture microscope (Wild) with phase-contrast optics was utilized for viewing the cultures. Ova were removed from culture. Once removed, the ova were placed in 0.15 M NaCl and washed to remove

extra or weakly attached sperm. A small volume (1 to 2 µl) of saline with ova was placed on a glass slide. The ova were then surrounded by petroleum jelly dots placed in 4 corners on the glass slide. A coverglass was gently placed upon the dots and lightly pressed until the coverglass made contact with the surface of the saline droplet. This procedure allowed the ova to be examined while still uncompressed. Photographs were taken at that time. Each culture, containing the sperm of one male (hamster or monkey) and the ova from one to three females was listed as a single trial. The percentage of sperm exhibiting any form of movement was recorded as motility. Rates of movement were classified on six levels: minimal (slight wiggling), very low, low, medium, high, and very high (sperm crossed field at 225X in $\leq$ 1 sec.). Hamster sperm activation was recognizable as an increase in three sperm characteristics: amplitude of tail waves, side-to-side movement of the head, and motility rate. The percentage of sperm exhibiting this activated movement was recorded. Squirrel monkey sperm do not show signs of activation. The number of sperm bound per ovum, after removal from culture and washing, was noted. Ovum penetration was classified according to the evidence of sperm entry: swollen sperm heads, tails leading to the swollen heads and in the vitellus, and pronuclei. The presence of two polar bodies was not used as a criterion of penetration since the first polar body was frequently lost upon zona pellucida removal.

Mean values are expressed as $\pm$ standard errors.

RESULTS

A summary of much of the data resulting from these trials is listed in Tables 3 through 7.

A main purpose of the study was to develop an *in vitro* system in which hamster sperm would penetrate zona-free hamster ova and to test the ability of squirrel monkey sperm to bind and penetrate the zona-free hamster ovum. Based upon these major aims, other sperm and ova characteristics in culture would be examined.

Failure of induction of follicular growth in early trials led PMS to be implicated as the cause of an ineffective superovulation regimen. Other problems involving animals and gonadotropin concentrations arose. Occasionally, superovulation did occur and yielded 20 to 50 ova per female. Since several trials were run concurrently, the ova which were recovered had to be divided up into the various culture chambers. Ova were not added to some cultures when sperm activation was questionable. Both zona-free and zonal hamster ova were utilized, although not always in the same culture system.

The pH values of the sera were: squirrel monkey, 8.13 $\pm$ 0.04; hamster, 7.89 $\pm$ 0.01; human, 8.22 $\pm$ 0.02; newborn calf, 7.38 $\pm$ 0.02; and agamma fetal bovine, 7.04 $\pm$ 0.00. When initially mixed with the media in ratios of 1 part medium to 1 part serum and 4 parts medium to 1 part serum, the pH values were: BWW:squirrel monkey, 8.17, 8.04; TC199:squirrel monkey, 7.69, 7.65; BWW:hamster, 7.92, 7.88;

TC199:hamster, 7.67, 7.54; BWW:human, 8.17, 7.97; TC199:human, 7.75, 7.69; BWW:newborn calf, 7.54, 7.69; TC199:newborn calf, 7.50, 7.50; BWW:agamma fetal bovine, 7.29, 7.45; and TC199:agamma fetal bovine, 7.42, 7.50. The calculated osmolality of BWW medium and the average osmolality of blood serum is 308 mOSm. The osmolality of TC199 is approximately 300 mOSm, so that any combination of medium and serum would give an osmolality well within the ranges proven successful in previously reported work (270-402, 268-343 mOSm).

Hamster Sperm Cultures

Both TC199 and BWW medium, when used in combination with sera, promote hamster sperm motility. Some combinations of medium and serum led to sperm activation and occasionally to penetration. When TC199 was combined with squirrel monkey serum (2:1) and human serum (2:1, 4:1), sperm activation occurred (Table 3). Sperm penetration was recorded in the TC199 and squirrel monkey serum trial (2:1) (Table 4). Sperm binding to ova was observed in cultures not exhibiting signs of penetration and in the one example of penetration. Trials with TC199 medium led to a mean sperm motility survival time of 4.15 $\pm$ 0.83 hr. Emphasis was placed on trials with BWW medium based on the results of other workers as cited in the Literature Review and the success of sperm culture prolongation in preliminary trials. Trials with BWW medium led to a mean sperm motility survival time of 4.95 $\pm$ 0.27 hr. When BWW and hamster serum were mixed (1:1, 2:1, 3:1, 4:1), the mean sperm motility survival time was 5.10 $\pm$ 0.37 hr.

Hamster sperm trials never lasted over 9 hours. Motility either diminished within the first 2 hours and rose again only upon

Table 3. The number of trials run, sperm motility, and sperm activation parameters of TC199 medium and serum mixtures

Sera		TC199 Medium to Serum Ratios								
		Squirrel Monkey Sperm					Hamster Sperm			
		5:1	4:1	3:1	2:1	1:1	4:1	3:1	2:1	1:1
	TR[a]				2				1	
Hamster	MOT				8.9±0.1				5.5	
	ACT				N.A.[b]				0%	
	TR				3				1	
Squirrel Monkey	MOT				9.0±0.6				5.5	
	ACT				N.A.				30%	
	TR	2	17	1	1	1	2	1	3	1
Human	MOT	7.5±0.0	7.2±0.9	3.0	2.0	1.0	3.2±0.0	<1.0	4.9±1.7	<1.0
	ACT	N.A.	N.A.	N.A.	N.A.	N.A.	0-1%	0%	0-1%	0%
Agamma	TR								1	
Fetal	MOT								1.0	
Bovine	ACT								0%	

[a]TR (trials) - number of trials; MOT (motility) - mean sperm motility duration in hours; ACT (activation) - hamster sperm activation, percentage of total sperm exhibiting activated movement.

[b]N.A. (not applicable) - monkey sperm do not exhibit the activation phenomenon.

Table 4. Sperm culture characteristics (binding to ova, penetration, concentration) of TC199 medium and serum mixtures

Sera		TC199 Medium to Serum Ratios								
		Squirrel Monkey Sperm					Hamster Sperm			
		5:1	4:1	3:1	2:1	1:1	4:1	3:1	2:1	1:1
Hamster	BIN[a]				0-10				N.A.[b]	
	PEN				none				N.A.	
	CON				3.3-10.4				27.0	
Squirrel Monkey	BIN		0						50+	
	PEN		none						XX,SH,T	
	CON		3.2-10.8						27.0	
Human	BIN	0-20	0-50+	1-10	1-10	1-10	10-50	N.A.	0-50	N.A.
	PEN	none	none	none	none	none	none	N.A.	none	N.A.
	CON	1.1-28.0	0.9-24.0	1.8	1.2	1.8	6.0	3.8	5.0	3.8
Agamma Fetal Bovine	BIN								N.A.	
	PEN								N.A.	
	CON								27.0	

[a]BIN (binding) - number of sperm bound to each ovum; PEN (penetration) - none, XX (3+ sperm), SH - swollen heads, PN - pronuclei, T - tails; CON (concentration) - range of sperm concentrations for all trials x 10^6/ml.

[b]N.A. (not applicable) - no ova added to these cultures.

sperm activation, or it slowly dropped over the 5-hour average life of the culture. Motility judgments were based upon any form of movement within the sperm cultures since progressive motility dropped quickly or was absent. Agglutination of the sperm was a prime reason for this lack of progressive motility. Based on trials of media alone (only slight wiggling of sperm within 1 hr), it is apparent that the addition of serum is necessary for sustained sperm motility and activation.

There was evidence of sperm activation in 12 (80%) of the BWW medium cultures (Table 5). Only agamma fetal bovine serum failed to promote activation of sperm; however, this is based on a single trial.

Sperm penetration of ova occurred in 6 (40%) of the cultures (Table 6). In the hamster serum cultures (1:1, 2:1, 3:1), ova were penetrated, as evidenced by several (3 to 10 or more) swollen sperm heads. In cultures of BWW medium and human serum (1:1, 2:1), sperm penetration also occurred. In many of the trials (54%), sperm motility would be drastically decreased (only slight wiggling present) and then increase at the onset of activation. In other trials (46%), sperm would sustain good motility until and through activation. At the beginning of most culture trials, there was a high degree of sperm agglutination (10 to 100+), which decreased over time. No differences were apparent due to the effect of heterogeneous sera. There was no agglutination involving the activated sperm. Never in any trial did a majority of the sperm reach an activated state. The largest proportion of activated sperm was 30% of the total sperm. This occurred with BWW medium diluted 3:1 and 2:1 with hamster serum and with TC199 diluted 2:1 with squirrel monkey serum. It is

Table 5. Number of trials run, sperm motility, and sperm activation parameters of BWW medium and serum mixtures

Sera		BWW Medium to Serum Ratios								
		Squirrel Monkey Sperm					Hamster Sperm			
		5:1	4:1	3:1	2:1	1:1	4:1	3:1	2:1	1:1
Hamster	TR[a]		3	2	7	2	5	5	15	6
	MOT		2.9±1.1	4.0±0.0	6.4±1.3	4.0±0.0	4.9±1.9	4.8±0.6	5.3±0.3	4.3±0.4
	ACT		N.A.[b]	N.A.	N.A.	N.A.	0-10%	0-30%	0-30%	1-10%
Newborn Calf	TR		1	1			3	1	4	3
	MOT		4.0	2.0			4.9±0.2	6.0	5.9±0.4	1.7±0.7
	ACT		N.A.	N.A.			0-10%	<1%	0-<1%	0%
Squirrel Monkey	TR		2				1		2	
	MOT		9.7±0.0				6.3		4.9±1.4	
	ACT		N.A.				<1%		0-<1%	
Human	TR	2	15	2	1	1	5	4	14	5
	MOT	7.5±0.0	8.8±1.3	4.5±0.5	2.0	1.0	6.4±0.2	3.8±0.0	4.9±0.7	2.7±1.0
	ACT	N.A.	N.A.	N.A.	N.A.	N.A.	0-1%	0%	0-1%	0-1%
Agamma Fetal Bovine	TR		1		1				1	
	MOT		2.5		2.5				1.0	
	ACT		N.A.		N.A.				0%	

[a]TR (trials) - number of trials; MOT (motility) - mean sperm motility duration in hours; ACT (activation) - hamster sperm activation, percentage of total sperm exhibiting activated movement.

[b]N.A. (not applicable) - monkey sperm do not exhibit the activation phenomenon.

Table 6. Sperm culture characteristics (binding to ova, penetration, concentration) of BWW medium and serum mixtures

Sera		BWW Medium to Serum Ratios								
		Squirrel Monkey Sperm					Hamster Sperm			
		5:1	4:1	3:1	2:1	1:1	4:1	3:1	2:1	1:1
Hamster	BIN[a]		1-10	1-10	1-10	N.A.	10-50	50+	0-50+	10-50+
	PEN		none	none	PN,X	N.A.	none	none-SH,XX	none-SH,XX	none-SH,XX
	CON		0.1-1.08	0.8-0.9	0.7-3.3	0.5-0.7	9.0-20.0	7.5-18.8	10.0-33.0	7.5-23.0
Newborn Calf	BIN		0	0			1-10	10-50	10-50	10-50
	PEN		none	none			none-PN,X	none	none	none
	CON		3.5	0.9			1.8-20.0	17.5	17.5-20.0	17.5
Squirrel Monkey	BIN		0				N.A.		1-20	
	PEN		none				N.A.		none	
	CON		3.5				20.0		20.0-33.0	
Human	BIN	1-10	0-50+	10-50	1-10	N.A.	0-50+	N.A.	10-50+	0-50+
	PEN	none	none-SH,XX,T	none	N.A.	N.A.	none	N.A.	PN,SH,T,XX	none-SH,XX
	CON	1.0-28.0	1.1-13.2	0.75-0.93	0.7	0.5	9.0-20.0	7.5-17.5	9.3-33.0	7.5-17.0
Agamma Fetal Bovine	BIN		10-50		1-20				N.A.	
	PEN		none		none				N.A.	
	CON		2.8		2.3				33.0	

[a]BIN (binding) - number of sperm bound to each ovum; PEN (penetration) - none, X (1-2 sperm), XX (3+ sperm), SH - swollen heads, PN - pronuclei, T - tails; CON (concentration) - range of sperm concentrations for all trials x 10^6/ml.

[b]N.A. (not applicable) - no ova added to these cultures.

significant that even in some trials in which activated sperm were less than 1% of the total sperm population, there was evidence of sperm binding and penetration. The mean concentration of sperm in those trials in which activation occurred was $1.94 \pm 0.07 \times 10^7$/ml (30 trials). The concentration in trials including penetrated ova had a mean of $1.87 \pm 0.11 \times 10^7$/ml (10 trials).

Hamster Sperm-Ova Interaction

When zona-free ova were added to sperm cultures in TC199 or BWW medium, binding of sperm to the vitelline membrane was always observed, regardless of serum used. Zonal ova were rarely bound in cultures devoid of activated, and thus capacitated, sperm. The numbers of zonal ova used were low (maximum of 1 per trial), however. When zona-free and zonal ova were present together, uncapacitated sperm bound in greater numbers to the zona-free than the zonal ova (6+ vs. 0-5). In cultures exhibiting capacitated sperm, zona-free ova were bound with equal or greater numbers of sperm than zonal ova (50+ vs. 5-50+). Table 7 lists the specific trials in which penetration was observed. Generally, 3 common factors were evident in these cultures: 1) large numbers of sperm fused to the ovum (50+), 2) activation of the sperm, 3) polyspermy. When all of these elements were present, a high percentage of penetration was also evident (66 to 100%). There were 2 trials which had lower concentrations of sperm bound ($\sim$10). These trials also differed in that sperm nuclei were clearly within the vitellus and had assumed a spherical shape and had transformed into pronuclei. Sperm tails were evident within the vitellus. In the other successful penetration trials, due to the large numbers of sperm bound, it was

Table 7. Sperm culture trials exhibiting penetrated hamster zona-free ova

	Medium, Serum, Ratio											
	Monkey Sperm		Hamster Sperm									
	BWW hamster 2:1	BWW human 4:1	BWW hamster 1:1	BWW hamster 2:1	BWW hamster 2:1	BWW hamster 3:1	BWW n.calf 4:1	BWW human 1:1	BWW human 2:1	BWW human 2:1	BWW human 2:1	TC199 monkey 2:1
No. sperm bound	1	10-20	50+	50+	50+	50+	N.A.	50+	10	50+	10	50+
Evid. penet.	2PN[a]	2SH,2T	XX,SH,T	3-4PN, 1T	XX,SH,T	XX,SH,T	XX,PN	XX,SH,T	XX,PN, T	XX,SH, T	2PN	XX,SH
Penet. %	14 (1/7)	17 (1/6)	67 (2/3)	100 (1/1)	67 (4/6)	75 (3/4)	50 (1/2)	100 (2/2)	33 (1/3)	100 (3/3)	14 (1/7)	100 (1/1)
Activ. began (hr)			1.0-2.0	none	1.0-2.2	1.0-2.0	1.0-2.3	1.2-2.0	1.5-3.0	1.3-2.0	none	1.8-2.5
Sperm culture time (hr)	9.6	7.8										

[a]PN - pronuclei, SH - swollen heads, T - tails, XX - 3+ sperm.

difficult to discern if sperm tails were deep within the vitellus of the penetrated ova. These ova were characterized by swollen heads and tails leading to them just below the vitelline surface. The time at which activation was first noted varied between trials. In the trials in which penetration occurred, the range of time at which activation began was 1.5 to 2.0 hours. There were 2 trials which included penetrated ova but no sperm activation was observed. Sperm activation was always of limited duration, lasting from 1.0 to 3.5 hours dependent on the trial.

Squirrel Monkey Sperm and Hamster Ova

There were no appreciable differences in the ability of either TC199 or BWW medium, plus serum, in maintaining squirrel monkey sperm motility. When the media were used alone for culture, TC199 proved better for sperm motility survival at 2.5 hours of culture (90%, low-medium rate, vs. 1%, very low rate), but both cultures were immotile by 9.5 hours. Sperm bound to ova in these cultures, whether zona-free or zonal ova (2 to 20 sperm per ovum) were used. The cultures which included serum produced better overall results in motility duration. Many trials were terminated after 9 hours of sperm culture and had maintained decent mean sperm motility (30%, very low to medium rate). In trials lasting 11 hours, 35% were motile with 30% progressive motility. One trial had 50% motile with a low motility rate at 9 hours and 20% motile with a low to medium motility rate at 23 hours. Human, hamster and squirrel monkey serum proved capable of preserving sperm survival when mixed with either TC199 or BWW medium (Tables 3 and 5). Newborn calf serum and agamma bovine fetal serum were not tested sufficiently to conclude their

effects on sperm cultures. However, when agamma bovine fetal serum was compared with human and hamster serum, it was found to be a poor stimulator of sperm motility. The agamma serum cultures were immotile at 2.5 hours of culture, while 20 to 40% of the sperm in the human and hamster serum trials were still motile at 9 hours.

The rate of sperm motility consistently began at high to very high levels and decreased over time to low levels at the end of culture. There did not appear to be a direct correlation between the percentage of motile sperm and the rate of motility. Higher concentrations of sperm ($\geq 10^7$/ml) seemed to sustain higher levels of sperm motility and rate better than lower concentrations.

A minority (10 to 30%) of the squirrel monkey sperm would exhibit head-to-head agglutination in some trials (about 30%). The number of sperm bound together ranged from 2 to 10, with a pair of sperm most common. Agglutination began 1 hour or more after sperm were cultured.

While a number of various trials utilizing different media, sera, and medium to serum ratios were conducted, most trials were centered around a 4:1 ratio of medium to serum. Trials showed that low ratios of medium to serum, except for hamster serum, were less effective in promoting sperm survival than the higher ratio trials. Another factor important in determining the proper culture for squirrel monkey sperm is the degree of sperm binding to the ova. Binding was noted in cultures containing agamma fetal bovine serum, human and hamster serum (Tables 4 and 6). There did not appear to be a difference in the ability of sperm to bind to ova in cultures that differed in culture media. Binding appeared to increase over time, but ova were not removed from culture to verify this. The

best cultures for both sperm motility survival and binding to ova were BWW:human serum (3:1, 4:1, 5:1) and TC199:human serum (4:1, 5:1). BWW and TC199 medium, in combination with hamster serum, produced some binding and good sperm motility prolongation. In one case sperm penetration of a zona-free ovum occurred. The only other case of sperm penetration, and a much more conclusive one, was in a BWW:human serum (4:1) trial, and considering the length of sperm culture, squirrel monkey sperm penetration of this hamster zona-free ovum occurred in less than 8 hours *in vitro*. There was no notable increase in motility or other physical changes evident as the monkey sperm cultures aged. Penetration of a hamster zona-free ovum by squirrel monkey sperm occurred at a sperm concentration of 4×10^6/ml in BWW and human serum (4:1). The second possible penetration occurred at a concentration of 2.3×10^6/ml in BWW and hamster serum (2:1). Figures 1 through 4 provide evidence of hamster sperm and zona-free ova interaction under *in vitro* conditions.

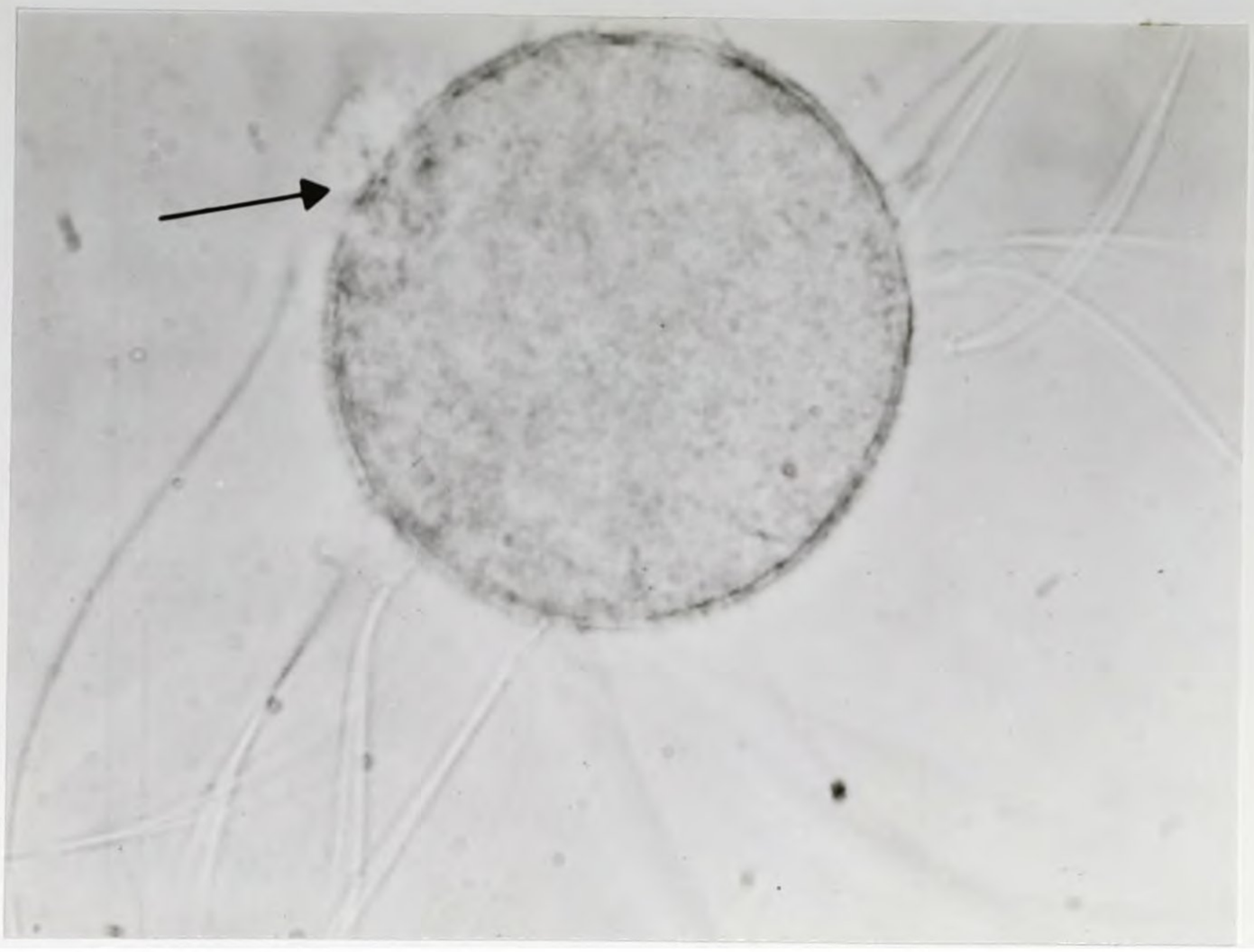

Figure 1. Zona-free hamster ovum with 1 polar body, pronucleus and several sperm attached.

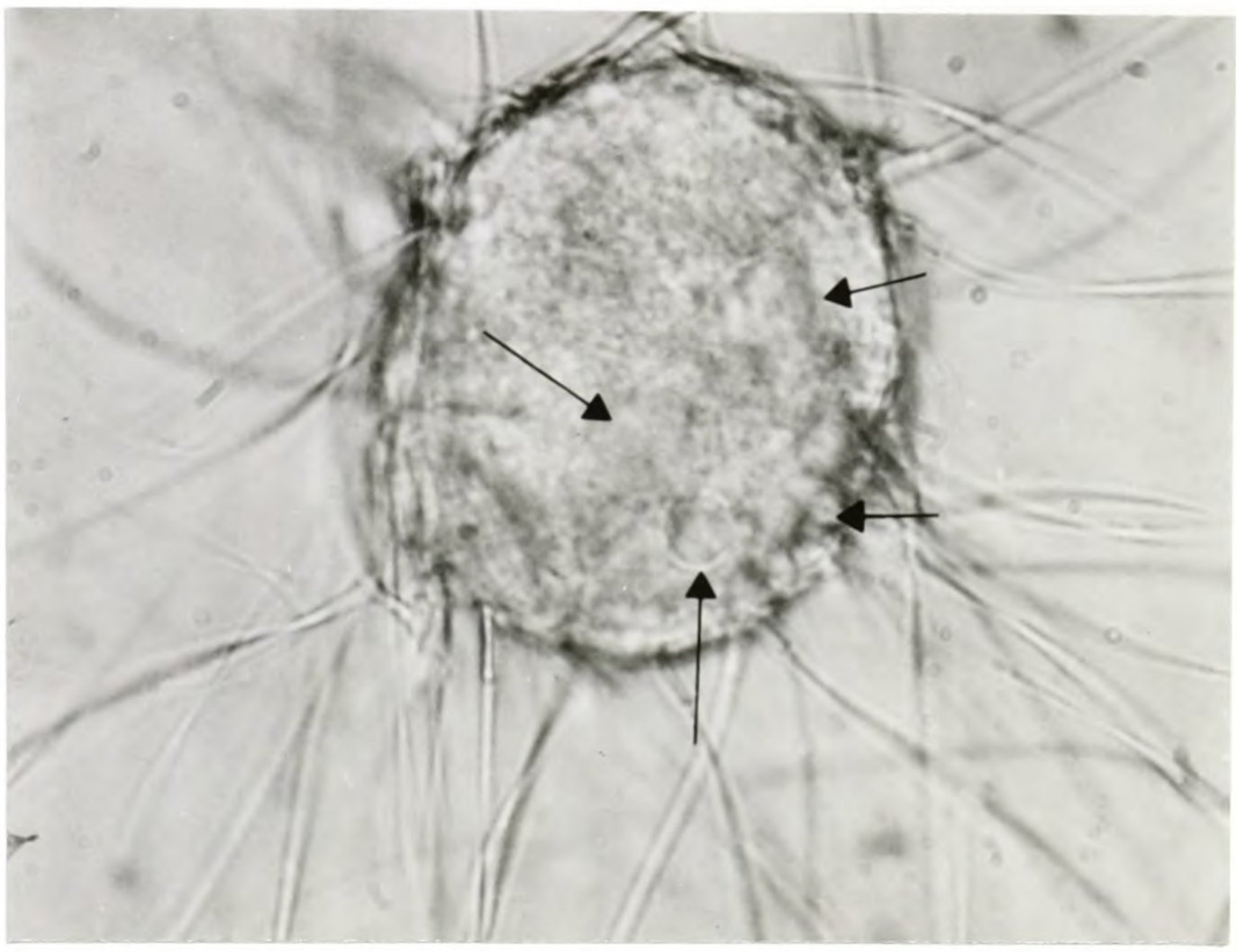

Figure 2. Zona-free hamster ovum with 3 pronuclei and 1 polar body in view. Another pronucleus is not in the plane of the photo.

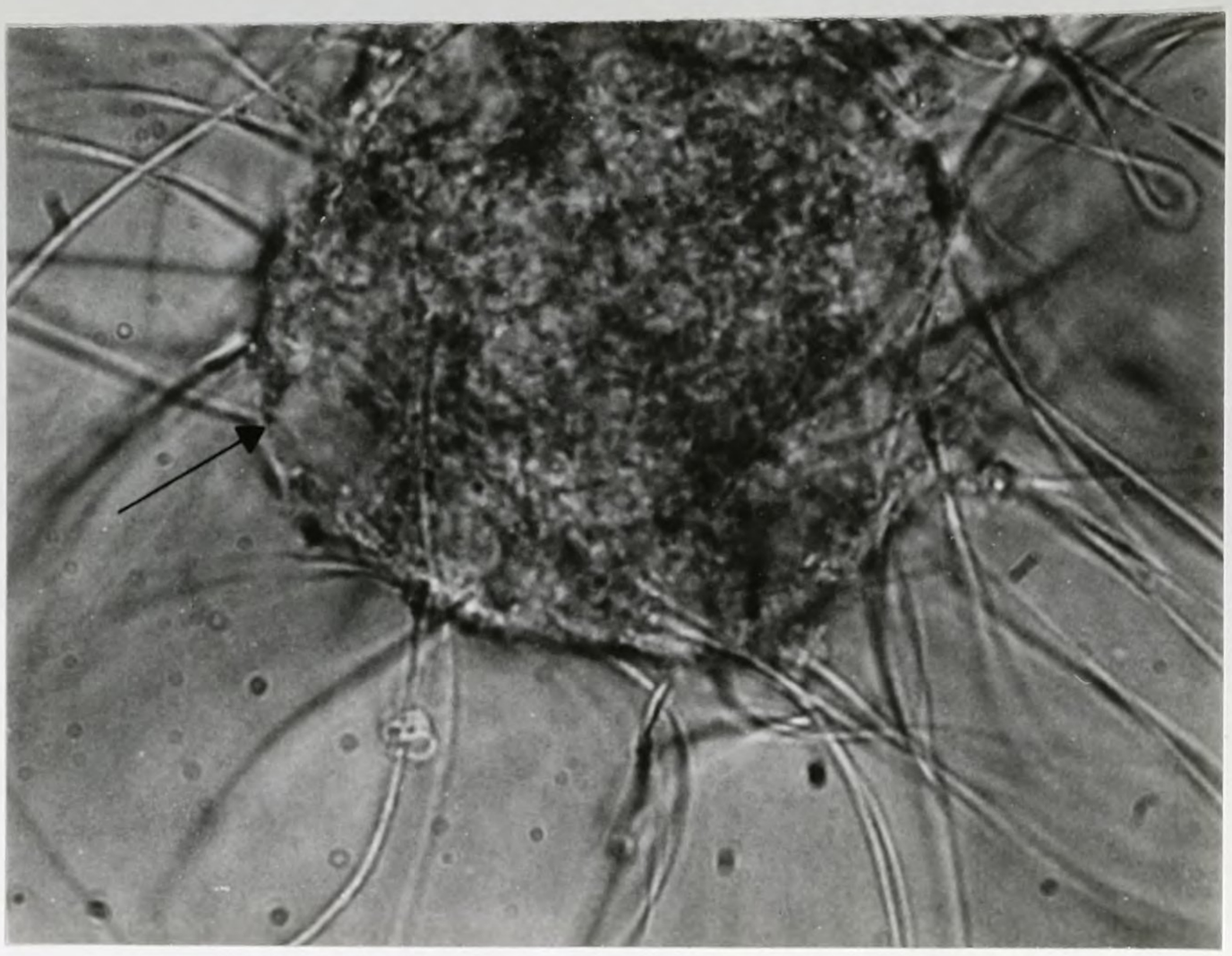

Figure 3. Mid-focal plane photo of zona-free hamster ovum displaying a polar body, swollen hamster sperm head and tail. Many more sperm heads visible out of the plane of photo.

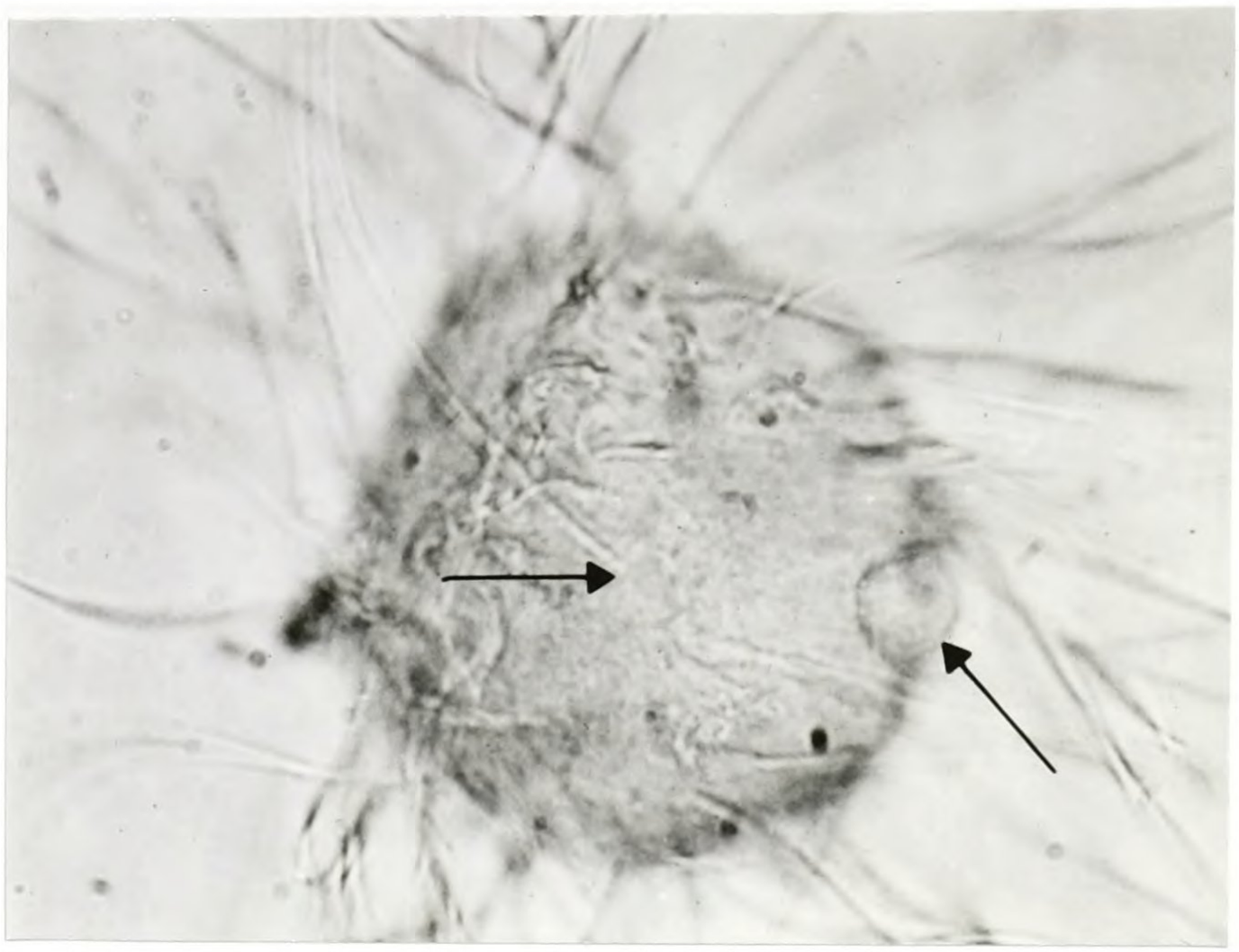

Figure 4. A polar body, swollen hamster sperm head and tail are visible in this zona-free hamster ovum. Many unpenetrated sperm are present in close proximity to the swollen head.

DISCUSSION

Under a variety of culture conditions, capacitated hamster sperm can penetrate zona-free hamster ova. When the conditions were most favorable for sperm capacitation, large percentages (87 ± 6%, range 66 to 100%) of ova were penetrated. The overall penetration percentage was 71 ± 10%, range 14 to 100%. Squirrel monkey sperm survived well in culture under many culture conditions. Two zona-free ova showed evidence of penetration, each from a different culture environment. One such ovum, in BWW medium with human serum (4:1), clearly displayed proof of 2 sperm having passed through the vitelline membrane with their nuclei decondensing within the vitellus. Tails were attached to each of these swollen heads. In a BWW medium with hamster serum (2:1) culture, 2 pronuclei were present in the vitellus of 1 ovum. No corresponding sperm tail was seen, but identification of sperm tails at the pronuclear stage of *in vivo* fertilized ova is very difficult. Even so, this ovum cannot be considered definitive proof of fertilization.

The differences in hamster sperm survival and activation observed between trials utilizing the same culture conditions and similar sperm concentrations may be related to the length of time the sperm spend in culture medium devoid of serum. Both TC199 and BWW media lack the motility factor present in serum. There might be a time limit on the duration allowed for hamster sperm, once diluted,

to remain unexposed to the motility factor. The trials which exhibited good motility characteristics and a high percentage of activation were exposed to the serum sooner. Data regarding time intervals between recovery of sperm from the epididymis and addition of serum to the sperm solution were not consistently recorded; thus, an analysis of this factor was not possible. It may be necessary to compare the procedure utilized in these trials with one involving mixing of the medium and serum to the prescribed ratios prior to sperm dilution.

There always was present a large percentage of sperm which did not show motility throughout the culture. The lowest proportion recorded was 40% immotile. Yanagimachi (1970b) lists initial hamster sperm motility as 70 to 90%. Even at 6 hours there was 50 to 70% motility. He used a Tyrode's solution with agamma human serum (1:1) mixture for the culture. He notes limited activation beginning at 2 hours. By 3 hours the range is from very few activated sperm to 58% of the motile sperm. The comparable trials in the present experiment showed signs of activation at 1.5 to 2 hours. Few sperm were active initially, but as time elapsed 10 to 30% of the sperm were activated.

The female hamster serum proved to be consistently effective in promoting hamster sperm motility survival and activation. Yanagimachi (1970b) found no substantial difference between female and male hamster serum regarding its action on the hamster spermatozoa. Whether this similarity in efficacy of serum between sexes exists in other species is unknown.

Yanamigachi found differences among the sera of various species, whether directly obtained from donors or produced commercially, on

the effect upon sperm cultures. In the present study, while hamster serum plus BWW medium and squirrel monkey serum plus TC199 medium clearly maintained the highest activation percentages, no other substantial differences between sera were apparent. It is evident that the motility promoting factor and acrosome reaction promoting factor are present in all of the sera tested except agamma fetal bovine serum.

Bedford (1970) and Barros (1974) reported that most mammalian sperm show a tendency for head-to-head autoagglutination when mixed with physiological solutions and body fluids. Motile hamster sperm agglutinate in culture and disassociate over time. This action implicates a change in the sperm membrane at this time. Barros states that the agglutination is not immunological, but may be related to surface charge. Possibly, membrane surface changes which lead to deagglutination are important in activation and capacitation.

Trials in which the percentage of capacitated hamster sperm was highest had sperm bound in greater numbers to the ova (50+ vs. ∿10), and the percentage of fertilization was higher (66 to 100% vs. 74 to 33%). Sperm binding and penetration of the ova seemed strongly related to the proportion of activated sperm. Binding of sperm to the vitelline membrane, as recorded in these trials, does not imply fusion of the sperm and ovum. Trials in which extensive binding was present without evidence of penetration lend credence to this. Uncapacitated hamster sperm will bind the zona-free ovum, but the sperm post-acrosomal membrane and ovum plasma membranes will not fuse. Through the processes of capacitation and the acrosome reaction, surface changes occur which allow the sperm to bind the ovum. Washing of the bound gametes after culture will not disrupt

sperm binding, whether sperm are capacitated or not. Microscopic examination of the sperm-bound ovum does not reveal the differences in the degree of binding (simple attachment vs. fusion). Even acrosome-intact sperm, once in contact with the ovum surface, are enveloped by the ovum microvilli (Yanagimachi, 1978). Electron microscopic examination is necessary to differentiate between binding and fusion near the initial time of sperm-ovum contact. Sperm penetration is a sign of capacitated, acrosome-reacted sperm.

Swollen sperm heads, and corresponding tails, were the predominant evidence of penetration. Hamster, human, monkey and newborn calf serum all promoted penetration. Both TC199 and BWW media, in combination with serum, promoted penetration. While it is apparent that homologous and heterologous sera are able to provide sperm with motility and acrosome-reaction stimulatory factors in culture with chemically defined media, there also seem to be differences in the amount, quality or type of factors involved.

The squirrel monkey sperm motility was not hampered by the incubation in medium without serum. Motility was generally high, in both the percentage of sperm moving and rate of movement, at the beginning of culture and decreased steadily over time. Most sperm cultures were still motile when the trials were terminated. Judging the comparable sperm culture characteristics involving TC199 or BWW medium plus serum versus the media alone, the dominant effect of the serum in promoting sperm motility survival is evident. The motility stimulating factor recognized for hamster sperm may be involved in squirrel monkey sperm stimulation as well.

Agglutination among squirrel monkey sperm was evident but was not associated with sperm activation as in the hamster sperm cultures.

Agglutination in monkey trials began after culture had begun (1+ hour). Surface changes may occur in squirrel monkey sperm over time which prompt agglutination in contrast to the hamster sperm characteristic of decreased agglutination. The onset of activation in the hamster sperm cultures was a distinct visual sign of capacitation, something that the monkey sperm cultures lacked. The need for capacitation of squirrel monkey sperm has not yet been proven. Though conclusive proof is lacking, sperm which do not require capacitation should be capable of penetrating ova immediately after attaching to the ovum surface. While this may only be true for homologous ova, the hamster zona-free ovum system offers proof of being subject to penetration by sperm capable of species-specific fertilization. Squirrel monkey sperm in these cultures rarely penetrated the zona-free ova, even though the sperm survived and bound to the ova. Using this reasoning as a case for squirrel monkey *in vitro* capacitation, and based upon the sperm culture time of the penetrated ovum trial, the *in vitro* capacitation time of squirrel monkey sperm in that particular culture was less than 8 hours.

SUMMARY AND CONCLUSIONS

The hamster zona-free ovum system was tested in the present study utilizing two media, a variety of serum sources, and several medium to serum ratios. An 8-chamber culture dish was employed without the conventional method of using a light viscosity oil cover. Along with the hamster sperm, a non-human primate (squirrel monkey) sperm was evaluated regarding its response in this system.

Several sera, in combination with chemically defined media, proved capable of maintaining motility in both hamster and squirrel monkey sperm cultures. Activation of hamster sperm was recorded in a variety of cultures. No comparable activation of squirrel monkey sperm was observed. Binding of zonal and zona-free ova occurred with both hamster and squirrel monkey sperm under most culture conditions. Penetration of ova did occur with sperm of both species. Capacitation times were reported for hamster sperm (1.5 to 2.0 hr), and a time range (<8.0 hr) for aquirrel monkey sperm *in vitro* capacitation was determined.

This system provided conditions favorable for both hamster and squirrel monkey sperm to penetrate zona-free hamster ova *in vitro*.

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