Sperm Penetration Through Zona Pellucida and Perivitelline Space in The Hamster

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Abstract

Sperm penetration through zona pellucida was observed in tubal fluid containing eggs and spermatozoa, which was recovered under a layer of paraffin oil previously insufflated with a mixture of 5% CO_2 and 95% air and warmed to 38°C before each experiment. Both Nikkon F 35 mm camera and Bolex Rex-5 16 mm movie camera synchronized with a Xenon-gas flush equipment were used for the recording of such fast phenomena under a microscope with or without phase-contrast apparatus. When 284, 306, 312 and 293 tubal eggs were recovered in the paraffin oil 4, 4½, 5 and 5½ hours after delayed mating, 1, 9, 10 and 1 eggs were respectively penetrated by spermatozoa either attached to or approached by them in vitro. The change in the CO_2 tension of the paraffin oil had no apparent effect on the incidence of in vitro fertilization. Nevertheless, the containment of eggs and spermatozoa in the small space sandwitched in between a cover slip and glass slide, or the dilution of tubal fluid with the liquid medium of 0.1 ml hamster serum and 0.4 ml CDM 199 were both detrimental to the in vitro environment of fertilization. Similar to previous investigations, most spermatozoa penetrated obliquely though the zona pellucida, but very few spermatozoa penetrated directly through it. The duration of time for spermatozoa to traverse the zona pellucida was variable lasting from about 4 minutes to 22 minutes. Although almost all tubal eggs were completely penetrated at the results of fertilization in the oviduct of golden hamsters, a rather high percentage, 36% of spermatozoa penetrated incompletely through the zona pellucida in vitro. Following sperm penetration through the zona pellucida, the vibration of ooplasm was initially induced by the impact of two gametes and then the rotation of ooplasm was subsequently caused by the migration of spermatozoa in the perivitelline space. Thus, the dynamic, characteristic movement and pictures of hamster spermatozoa and eggs at fertilization were observed and photographed in vitro, these gave some results different from what had been proviously held.

Introduction

The manner of spermatozoa penetration through the zona pellucida in mammals has been assumed mostly from the posture of sperm heads lying in the zona pellucid and also from the direction of the slit left in the membrane by the passage of spermatozoa (Sobotta 1895, Sobotta and Burckhard 1910, Austin and Bishop 1958, Dickman 1964, Dickman and Dziuk 1964, Dziuk and Dickman 1965). Rarely the whole process of sperm penetration through the zona pellucida has been directly observed in vitro. The total phenomena of sperm penetration has actually been observed twice in the rabbit (Pincus 1930), once in the human (Shettles 1953) and also once in the hamster (Yanagimachi 1964, 1966) yet there has been some controversy over the direction of sperm passage and also over the duration of time for sperm penetration through the zona pellucida. It is still a matter of question whether these differences of opinion are due to species or individual differences, as the observation of this phenomena has been achieved in such few instances. It is therefore the chief purpose of the present work to increase the number of observations on the process of sperm penetration using improved experimentation methods and microcinematography, so that more detailed and exact information on the phenomena can be obtained.

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Materials and Methods

Mature female golden hamsters weighing from 80 to 150 grams under controlled light from 6 AM to 6 PM and darkness from 6 PM to 6 AM in an air-conditioned room with temperature between 23 to 25° C were used in this study. Under these conditions, most females came into estrus every fourth day between 5 to 10 PM lasting until the early morning of the following day and ovulation occured most frequently about 8 hours after the onset of estrus (Harvey, Yanagimachi and Chang 1961). Females for this experiment were examined initially for the onset of lordosis, and then mated 8 hours later approximately at the time of ovulation by application of the above time relationship. Males for this experiment were selected from these test-mated with females at the time of ovulation and showed apparent fertility when the females were examined 5 hours later. In experimental Groups 1 to 4, females were sacrificed respectively at 4, 4½, 5 and 5½ hours after mating and both Fallopian tubes were removed onto a piece of filter paper, then these tubes were gently rolled on the paper for removal of blood adhering to the tubes. The tubes were then put in the center of a cover slip (18 X 18 mm) which was fastened with epoxyl adhesive to the bottom of a rectangular hole (14 X 14 mm) on an acrylite plate (70 X 25 X 5 mm). Thereafter, the tubes were covered with 0.4 ml paraffin oil (Mineral oil, Squibb & Co.) which was previously insufflated with a mixture of 5% CO_2 and 95% air for 5 minutes and warmed to 38° C before each experiment. The oviducts appeared semitransparent under a streomicroscope and the eggs with cumulus oophorus moving forward and backward according to the peristallic movement of oviducts were located near to ampullary portion of the tube. The tubal fluid containing the eggs, spermatozoa and fragments of epithelia was punctured and carefully pressed out from the oviducts into the bottom of the glassware with a pair of dissecting needles.

In the controls, Groups 5 and 6, females were killed 5 hours after mating, but the tubal contents with the eggs and spermatozoa were recovered in the paraffin oil insufflated with a mixture of 10% CO_2 and 90% air, and with 100% air alone respectively. In the other control, Group 7, females were killed 5 hours after mating, but similar to Yanagimachi's experimental method (Yanagimachi 1966), tubes were put in the center of four vaseline dots on a slide and immediately covered with a drop of paraffin oil. Tubes were then punctured with a pair of dissecting needles to let the tubal contents out of the tubes. After the tubes were discarded, the tubal contents with the eggs and spermatozoa were then covered with a cover slip for observation of sperm penetration. Furthermore, in another group of hamster, Group 8, a mixture of 0.1 ml hamster serum at estrus stage and 0.4 ml CDM 199 was used to dilute the tubal contents following their recovery in the paraffin oil 5 hours after mating.

Tubal eggs with cumulus oophorus in the paraffin oil were then examined under low power magnification by a regular or reversed phase-contrast microscope. Ova with the attachment of spermatozoa or with free spermatozoa moving around them were observed under high magnification under the reversed microscope. The specially designed holed acrylite-plate with a cover slip adhesed to the bottom of if was particularly suitable for the observation under a reversed microscope. When using a regular microscope, the objective had to be inserted into the paraffin oil for high magnification. The whole set of microscope was set in an incubator regulated between 37° C and 38° C with the ventilation, 500 ml per minutes, of different ratios of the mixture of CO_2 and air according to different experimental conditions.

Pictures of spermatozoa in the act of penetrating eggs were usally taken by a motor driven Nikkon F camera synchronized with a Nikkon flush equipment attached to a microscope. Cinemicrography was also performed using a Bolex Rex-5 16 mm movie camera with its shutter movement synchronized to the Xenon gas flush of the Strobex 135N (Chadwick Helmuth Company) with a self-designed synchronizer (Figure 1). All parts of the synchronizer were assembled on the U-shaped steel frame attachable to the anterior body of the MCE-17B electric motor for the movie camera. The movie camera (1), the electric motor (2) and the U-shaped steel frame (3) were well adjusted so that the flexible driving shaft (a) of the motor fitted on to the hand cranking spindle (b) of the camera and a gear

(c) adapted to the extra external shaft of the camera bited into a gear (d) on the internal end of the synchronizer's shaft. A rubber disk (e) with a small magnet (f) fixed to the edge of it was set on the external end of the synchronizer's shaft so that the shutter movement of the camera was coordinated with the magnetic signal induced by the rotating magnet around the shaft. A magnetic switch (g) which was set on the acrylite plate (4) screwed to the U-shaped steel frame, was then adjusted as near to the disk as possible for transducing the magnetic signal to the 6 volts DC signal for triggering Xenon gas flush of the Strobex 135N. Thus by adjusting the disk to the position that the magnet would meet the magnetic switch when the shutter of the camera was opened, the flush light of the Strobex 135N could be synchronized to the opening of shutter movement in the Bolex Rex-5 movie camera. The whole set of cinemicrographic apparatus was adjusted to the microscope in the incubator (Figure 2).

Results

A total of 2249 eggs from 232 females were recovered from 4 to 5½ hours after delayed mating, of which 44 eggs had spermatozoa attached to them and 17 eggs were approached by free spermatozoa. These were observed under a microscope for sperm penetration through the zona pellucida and perivitelline space.

Five eggs namely 1.8% of 284 tubal eggs from 28 females had already been penetrated in vivo by the spermatozoa at the time of observation which was about 4 hours after mating. Feeble movement of the sperm tail was still visible in the perivitelline space of these newly fertilized eggs. Only one egg showed an active spermatozoan attached to it and this took 7 minutes and 25 seconds for the sperm to penetrate through the zona pellucida. This included the rotation of ooplasm in the space encircled by the zona pellucida (Group 1).

Thirty-two eggs, namely 10.5% of 306 eggs from 31 females, had already been fertilized 4½ hours after mating. Sperm penetration through the zona pellucida was seen to take place in 7 cases of 9 eggs to which spermatozoa attached and in 2 eggs to which free spermatozoa had approached at the begining of observation. Nevertheless, in 2 fertilized eggs which were attached by another spermatozoa, the penetration of the second spermatozoa did not succeed and the spermatozoa later escaped from the ova following 2 to 5 minutes of attachment to the eggs (Group 2).

One hundred and fifty-two eggs, namely 48.7% of 312 eggs from 30 females, had been penetrated 5 hours after mating. Ten eggs were found attached by spermatozoa which subsequently penetrated 7 of them. Three eggs were approached by free spermatozoa, they were subsequently attached and later penetrated by the spermatozoa. Failure of sperm penetration was observed in 2 fertilized eggs. Spermatozoa were seen and be attached to these eggs, and later they broke away after 3 or 5 minuetes of attachment. Sperm penetration failed in one case, in which a spermatozoon attached to an unfertilized egg for more than 40 minutes was still not able to penetrate it. Finally the movement of this attached spermatozoon gradually subsided (Group 3).

Two hundred and ninety-three eggs, namely 92.5% of 293 eggs from 29 females, had been penetrated by the time of observation about 5½ hours after mating. Although sperm attachment was found in 2 fertilized eggs and 2 unfertilized eggs, however only one of unfertilized eggs was subsequently penetrated. In 4 fertilized eggs, approached and subsequently attached by free spermatozoa, sperm penetration did not occur (Group 4).

In 4 control groups, tubal eggs were recovered 5 hours after mating, but they were kept under different experimental conditions (Groups 5, 6, 7 & 8). Tubal eggs were recovered in paraffin oil insufflated with a mixture of 10% CO_2 and 90% air (Group 5) and with 100% air (Group 6) respectively. In Groups 5 and 6, similar to insufflation with 5% CO_2 and 95% air (Group 3), moderate incidences of sperm penetration were observed. Nevertheless, in Group 7 when tubal eggs were recovered in the paraffin oil insufflated as usual with 5% CO_2 and 95% air, but sandwitched between a cover slip and slide glass for observation according to Yanagimachi's method, sperm penetration was rarely observed. This agrees with his previous investigation results (Yanagimachi 1966). In Group 8, when 20% hamster serum and 80% CDM 199 were used in the medium for dilution of the tubal contents, sperm penetration was not observed although 8 tubal eggs were

either attached or approached by spermatozoa which moved vigorously toward eggs.

In general ,one spermatozoan moved to and attached to an egg and subsequently monospermic fertilization occured. Nevertheless, in 6 instances, eggs were simultaneously attached by 2 or 3 spermatozoa of which only one penetrated the eggs and the others kept their attachment to eggs until their sperm movement ceased, or they later became detached.

The average duration of time for penetration of the zona pellucida by sperm heads was 5 minutes 17 seconds. The duration of time was widely distributed from immediately after the observation to 22 minutes and 25 seconds after the commencement of observation in 29 eggs attached by spermatozoa in Groups 1 to 8. In 10 eggs of the same groups, in which sperm penetration was observed from the beginning of attachment to the penetration of the zona pellucida by the sperm head, the average duration of time for the penetration was 7 minutes 3 seconds, and the distribution of the duration of time from 4 minutes 20 seconds to 10 minutes 53 seconds (Table 2).

Spermatozoa regularly penetrated through the zona pellucida at sharp oblique angle, however a very few spermatozoa penetrated through the membrane at nearly right angle. The spermatozoa were obliged to take such direction in very few instances by the adhesion between the sperm tail and the surrounding granulosa cells or the surface of glassware.

When sperm penetration of the zona pellucida reached near to the perivitelline space, a rebounding of the inner surface of zona pellucida to the movement of sperm head was observed, the sperm head then dashed into the space inducing a concomitant vibration and rotation of the ooplasm for few seconds by the impact of two gamates and the migration of spermatozoa in the perivitelline space (Figures 3 to 4 and 5 to 6). Such kind of ooplasmic rotation might prolonged as long as one or three minutes in 2 special cases, in which spermatozoa wandered here and there in the perivitelline space for rather long period before the sperm head fixed on a portion of ooplasm and subsequently stopped the vigorous movement of the spermatozoa. In a case of incomplete sperm penetration into the perivitelline space, the ooplasmic rotation was very limited, or only ooplasmic vibration was induced by the impact of sperm head with ooplasm (Figures 7 to 8).

In the golden hamster, although tubal eggs fertilized in tubal environment in vivo were rarely penetrated incompletely by the sperm flagellum (3 instances in 894 fertilized eggs), a rather high percentage (35.9%) of eggs fertilized in vitro in this experiment resulted in incomplete penetration with a part or a large part of the sperm flagellum remaining outside of the zona pellucida (Table 3).

In general, the sperm head was not fixed and embedded at the primary site of its impact with ooplasm, but crawled for a variety of distances before it became completely fixed to a portion of ooplasm. Subsequently the vigorous movement of spermatozoon and the rotation of ooplasm ceased. Thereafter, a feeble movement of the sperm flagellum continued for about one hour before it completely subsided. The fertilization cone was usually not observed except in one particular case, in which the protrusion of a part of ooplasm actually occured immediately after the fixation of sperm head to a portion of ooplasm.

Discussion

It has been demonstrated that in the hamster the ejaculated spermatozoa arrived at the ampulla of the oviduct about one hour after the mating (Yanagimachi and Chang 1963) but the spermatozoa began to penetrate tubal eggs approximately 4 hours after the delayed mating (Strauss 1956, Yanagimachi and Chang 1961). This 4 hours time interval was suggested as the necessity of capacitation of spermatozoa for fertilization (Austin 1952, Austin and Walton 1960). In the current experiment, the percentage of eggs fertilized in vivo was extremely few 4 hours after the delayed mating, however these percentages were sharply increased from $4\frac{1}{2}$ to 5 hours, and nearly all eggs were fertilized $5\frac{1}{2}$ hours after the mating. It is, therefore, ideal to intercept the scene of sperm penetration from $4\frac{1}{2}$ to 5 hours post coitum, and actually the incidence of fertilization in vitro was most frequently observed in this period (Table 1).

The results obtained by using our open method with paraffin oil exposed to gas

ventilation was much superior for observing fertilization in vitro to the closed method of Yanagimachi in which the paraffin oil was held between the cover slip and glass slide. Since the ratio of the CO_2 in the mixture to insufflate the paraffin oil did not markedly change the incidence of fertilization in vitro, it appears that probably other factors such as the mechanical interference of sperm movement by contact between spermatozoa and the surface of the glassware in the limited space between a cover slip and glass slide might be responsible for the low frequency of fertilization in vitro (Group 7).

Although sperm penetration through zona pellucida has been observed in liquid media containing rabbit plasm and embryonic extract in the rabbit (Pincus 1930) and also in the follicular fluid in the human (Shettles 1953), other liquid medium (20% hamster serum and 80% CDM 199) added to tubal contents in the current experiment (Group 8) was detrimental to the progress of fertilization in vitro. It is very possible that following the dilution of tubal contents with liquid midium, the critical environment for fertilization might have been greatly altered so that the process of sperm penetration was interrupted. The reverse of this hypothesis might also account for why the addition of paraffin oil to cover the tubal contents was not harmful for fertilization.

The duration of time for sperm penetration through zona pellucida had been suggested to be a very rapid process, judging from the infrequency with which eggs were recovered with spermatozoa in the act of penetrating the membrane (Austin 1953). On the other hand, the time required for the sperm head to traverse the zona pellucida and the perivitelline space was 3-4 minutes and 1-2 seconds, respectively in the hamster (Yanagimachi 1964, 1966). The current investigation however demonstrated a rather wide distribution in the duration of time for sperm penetration in the hamster (Table 2).

The passage of spermatozoa through the zona pellucida has been suggested to be in an oblique direction, judging from the obliquely lying sperm heads in the zona pellucida and also from the slant slits left in the zona pellucida by penetrating spermatozoa around the time of fertilization in the mouse (Sobotta 1895), rat (Sobotta and Burckhard 1910), hamster and libjan-jird (Austin and Bishop 1958), rabbit (Dickman 1964), pig (Dickman and Dziuk 1964) and sheep (Dziuk and Dickman 1965). The current observation and early investigation (Yanagimachi 1964, 1966) on the sperm penetration in the hamster confirmed the previous assumption on the oblique penetration of spermatozoa through the zona pellucida. Probably the vertical penetration of spermatozoa into eggs observed in the rabbit (Pincus 1930) and human (Shettles 1953) were exceptional cases which were also observed in rare instances, in the present experiment, when spermatozoa were obliged to take such a posture because of adhesion of the sperm tail to granulosa cells or to the surface of glassware.

In the Chinese hamster, incomplete penetration of the zona pellucida by sperm flagellum was found in 17% of eggs fertilized in vivo (Pickworth, Yerganian and Chang 1968), such phenomena has been rather rarely seen in the golden hamster eggs fertilized in vivo (Table 3). Nevertheless, in the current experiment, a great proportion (35.9%) of golden hamster eggs fertilized in vitro under microscopical observation were penetrated incompletely by sperm flagellum (Table 3). Weakness of sperm movement and adhesion of sperm tail to granulosa cells or to the surface of glassware in vitro were probably the main reason for incomplete sperm penetration.

The duration of time for a spermatozoa to traverse the perivitelline space prior to the primary contact with ooplasm was shown to be between one and two seconds in the hamster (Yanagimachi 1966). The time was observed to be immeasurably short, since the spermatozoa dashed through the space so quickly to impact on the ooplasm in the instant of their penetration through the zona pellucida. After sperm penetration, ooplasmic vibration was instantly induced by the impact of two gamates and ooplasmic rotation was subsequently resulted from the migration of spermatozoa in the perivitelline space. The duration of time for ooplasmic rotation varied from few seconds to 3 minutes depending on the degree of sperm penetration (complete or incomplete penetration) and the duration of time for sperm migration in the perivitelline space. On the other hand, the direction of ooplasmic rotation was usually changing since spermatozoa moved zigzagly and turned frequently in the space.

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Table 1. Sperm penetration observed in hamster eggs of various ages and under different experiment controls.

Group No.	No. of hamsters	Age of eggs (hrs)	No. of eggs Recovered	No. of eggs Fertilized in oviducts (%)	No. of eggs attached by sperm Total	No. of eggs attached by sperm Penetrated in vitro	No. of eggs approached by sperm Total	No. of eggs approached by sperm Penetrated in vitro
1	28	4	284	5 (1.8%)	1	1	0	0
2	31	4½	306	32 (10.5%)	9	7	2	2
3	30	5	312	152 (48.7%)	10	7	3	3
4	29	5½	293	271 (92.5%)	4	1	4	0
5	28	5	291	155 (53.3%)	7	7	3	2
6	30	5	302	136 (45.0%)	6	5	3	3
7	27	5	278	132 (47.5%)	2	1	0	0
8	29	5	283	147 (51.8%)	5	0	2	0

Groups 1 to 4: Eggs were recovered in paraffin oil insufflated with 5% CO_2 and 95% air.
Group 5: Eggs were recovered in paraffin oil insufflated with 10% CO_2 and 90% air.
Group 6: Eggs were recovered in paraffin oil insufflated with the air alone.
Group 7: Eggs were recovered as usual as in Groups 1 to 4, but the paraffin oil with eggs was then sandwitched between a cover slip and glass slide.
Group 8: Eggs were recovered in the medium containing 0.1 ml hamster serum and 0.4 ml CDM 199.

Table 2. Duration of time for penetration of the zona pellucida by sperm heads.

Eggs at the onset of observation	Total no. of eggs	Mean duration of time	Duration of time* for sperm penetration in minutes and no. of eggs 0-2	2-4	4-6	6-8	8-10	10-20	20-30 (min.)
Attached by sperm	29	5 min. 17 sec.	5**	7	8	4	3	1	1
Approached by sperm	10	7 min. 3 sec.	-	-	4	4	1	1	-

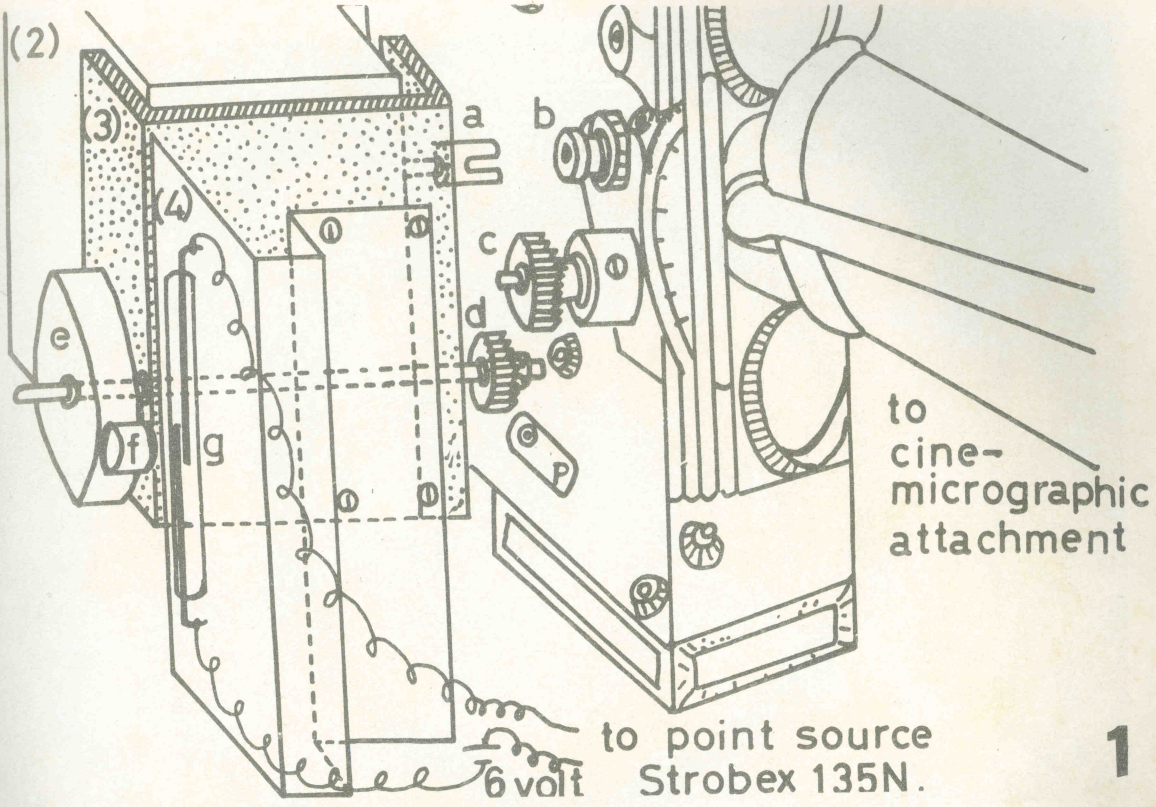
* From the commencement of observation to the sperm penetration in the eggs attached by spermatozoa, or from the beginning of sperm attachment to the sperm penetration in the eggs approached by spermatozoa.

** Number of eggs.

Table 3. Incidence of incomplete sperm penetration observed *in vitro* and that occuring *in vivo*.

Experimental conditions	Total number of eggs	Incomplete sperm penetration with		Complete sperm penetration
		Sperm head alone	Sperm head and a portion of flagellum	
Observed *in vitro*	39	2*	12	25
Fertilized *in vivo*	894	0	3	891

* Number of eggs observed.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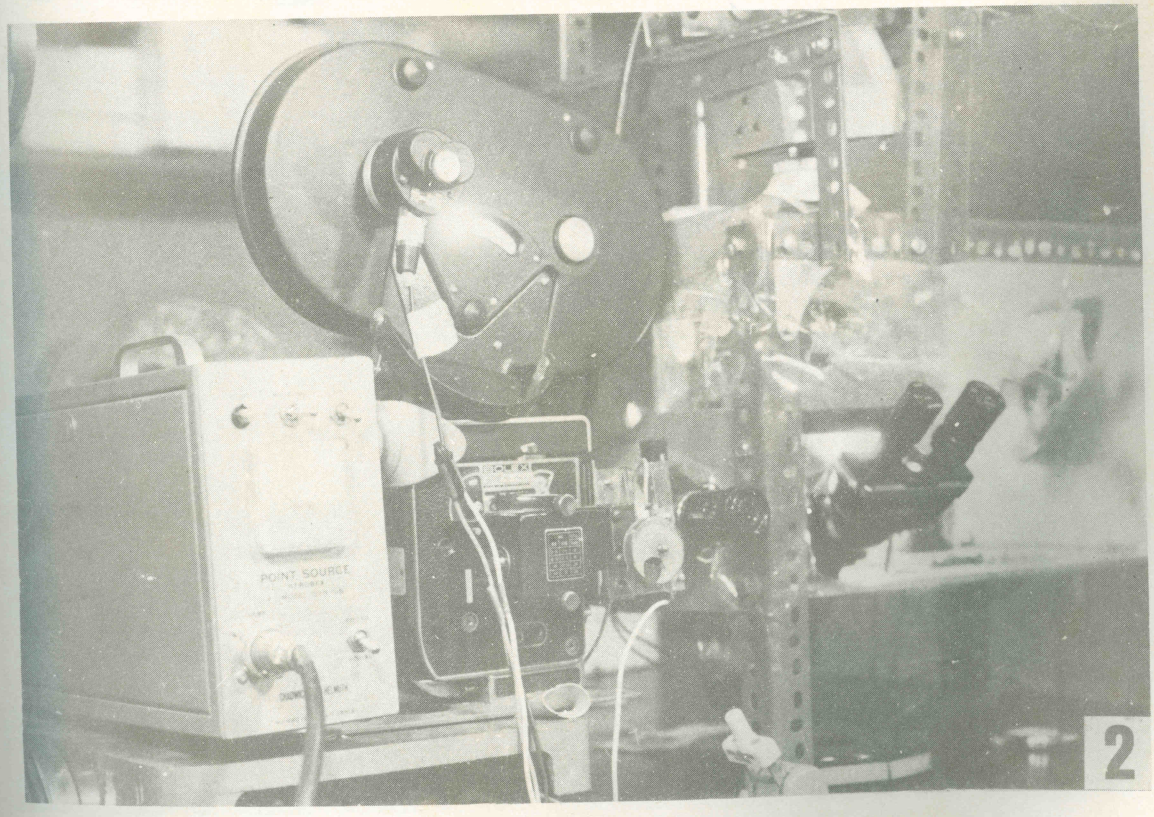
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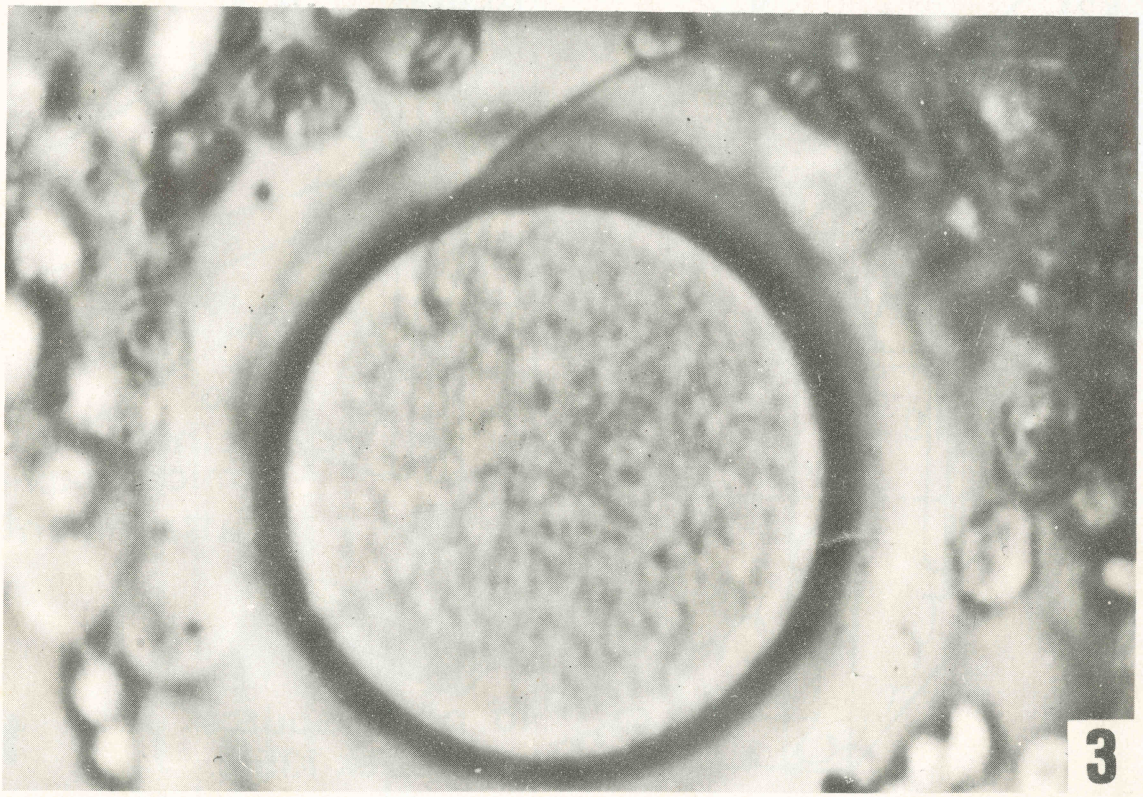
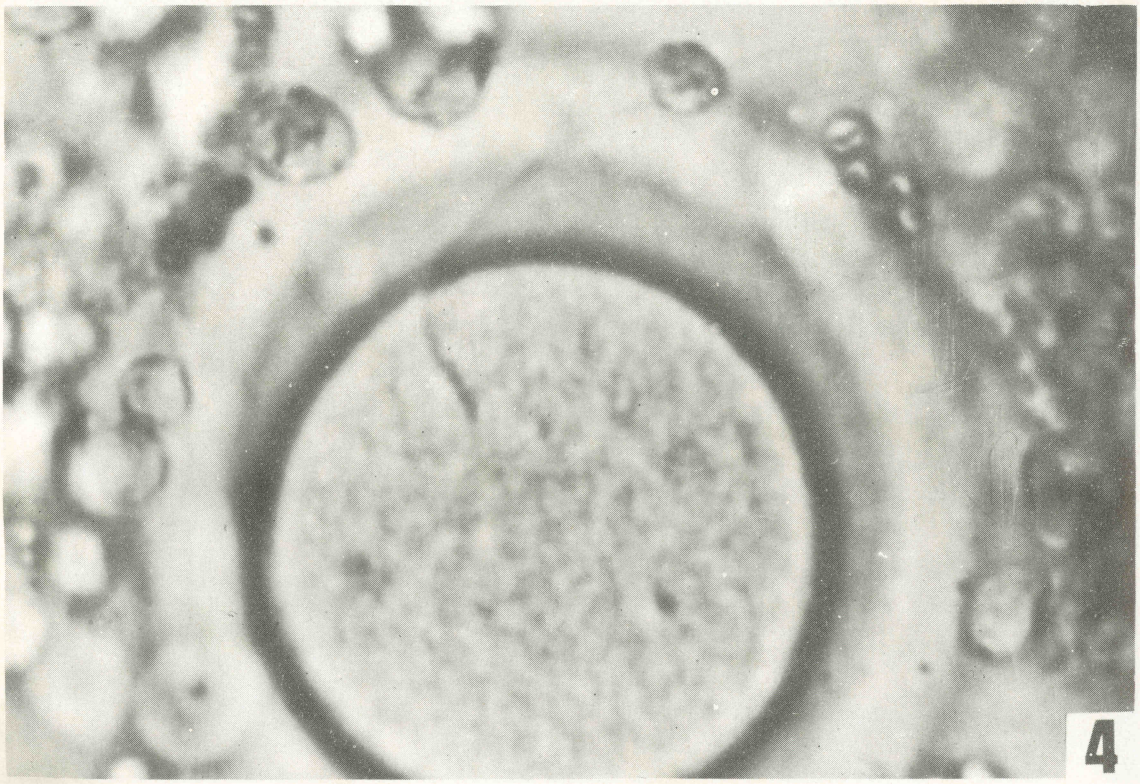
Plate 1





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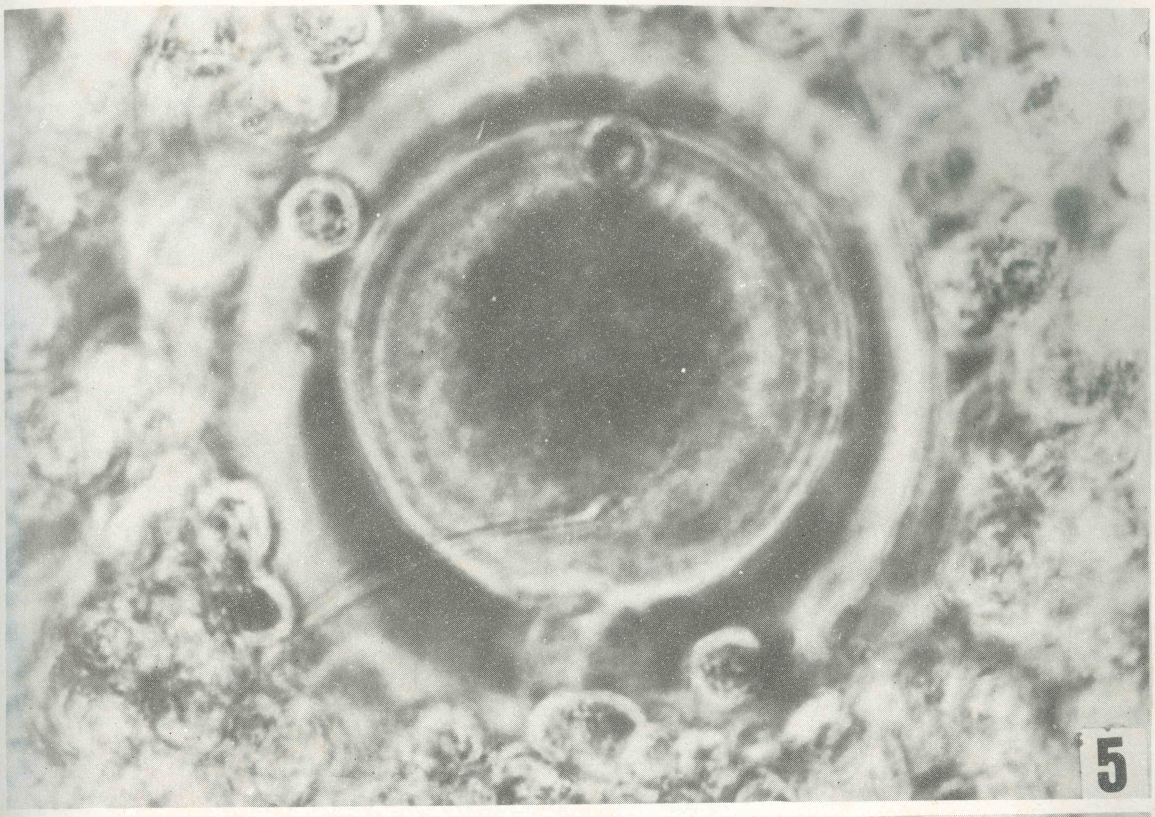

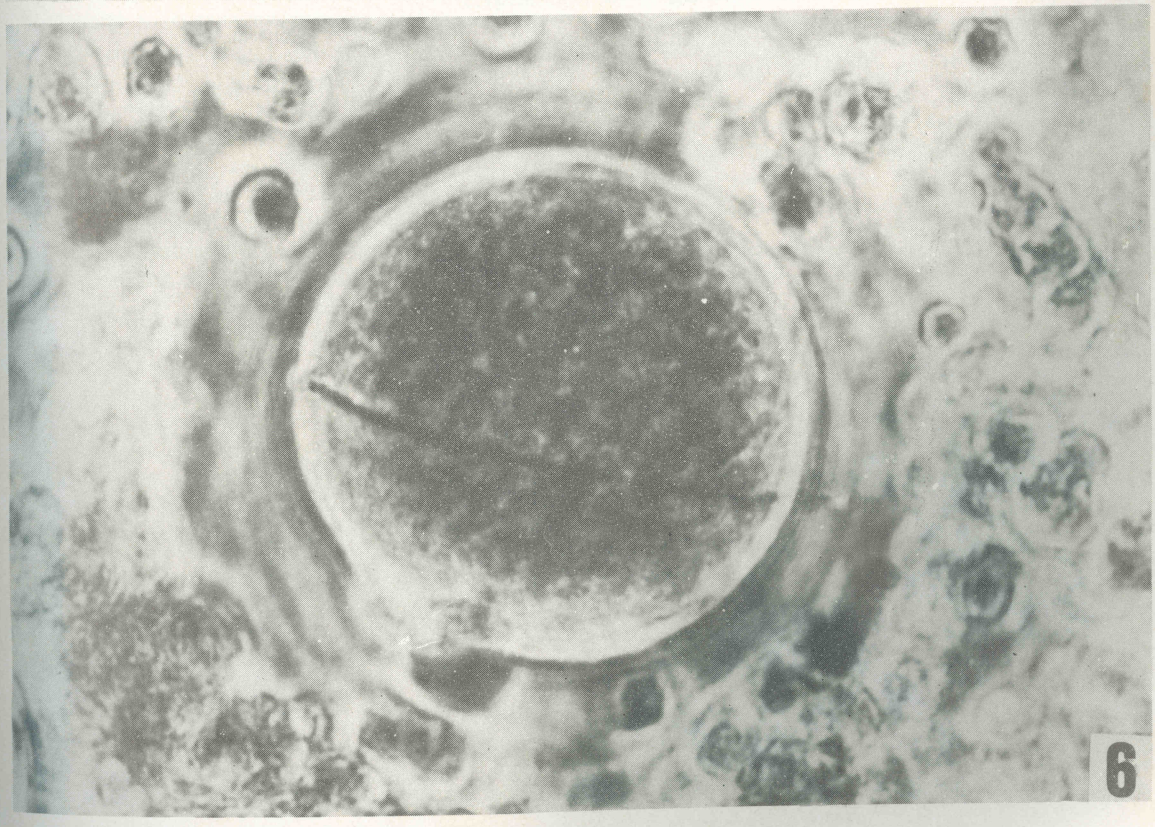

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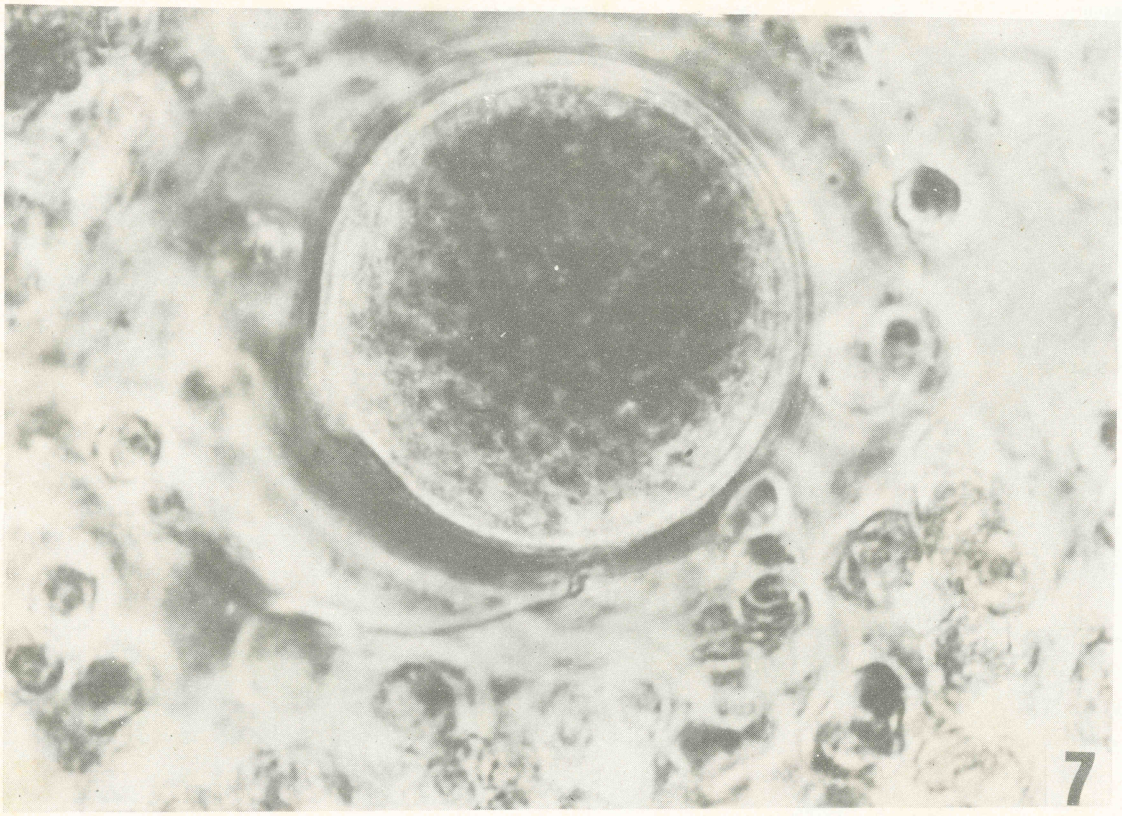

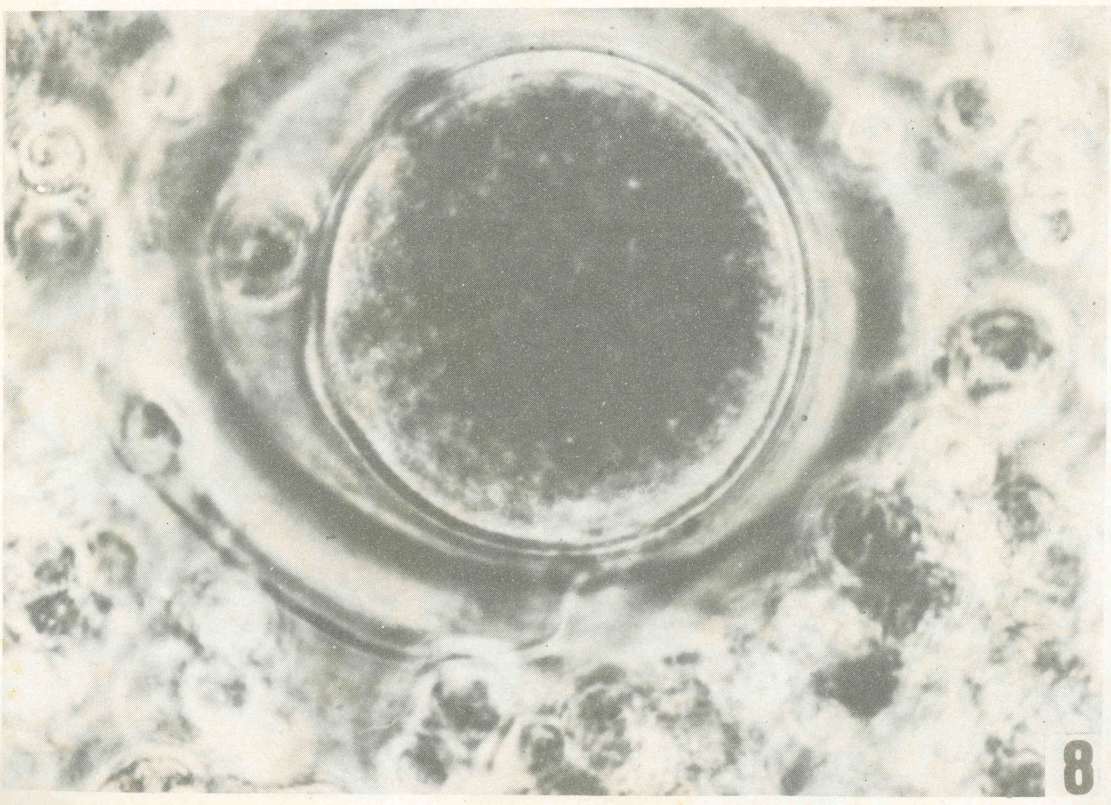

Plate 4

Plate 1

Explantion of Figures

Figure 1. The bolex Rex-5 movie camera (1), MCE-17B electric motor (2) and the self-designed synchronizer (3&4) to be assembled and adjusted for cinemicrography. (a) the flexible driving shaft of MCE 17B motor, (b) the hand cranking spindle of the movie camera, (c) the gear set to the extra external shaft of the camera, (d) the gear set to the internal end of the synchronizer-shaft, (e) the rubber disk set to the external end of the synchronizer-shaft, (f) the small magnet fixed to the rubber disk, (g) the magnetic switch for transducing the magnetic signal to the electric signal; the switch was fixed on the acrylite plate (4) which was screwed to the U-shaped frame (3) of the synchronizer.

Figure 2. Bolex Rex-5 movie camera and other attachments adapted to an inverted microscope in an incubator for cinemicrography.

Plate 2

Explantion of Figures

Figures 3 and 4 demonstrate the pictures of eggs before and after the penetration of sperm head, photographed under a regular microscope. Approximately 900X

Figure 3. A spermatozoan attached to the zona pellucida of an egg at the direction of 11 o'clock immediately after the commencement of observation.

Figure 4. The sperm head penetrated through the zona pellucida and dashed to the direction of 8 o'clock, inducing the ooplasmic vibration and rotation about 6 minutes and 43 seconds after the onset of observation.

Plate 3

Explantion of Figures

Figures 5 and 6 demonstrate the pictures of eggs before and after the penetration of sperm head, photographed under an inverted microscope with a phase-contrast apparatus. Approximately 900X

Figure 5. A spermatozoan attached to the zona pellucida of an egg at the direction of 6 o'clock immediately after the commencement of observation.

Figure 6. The sperm head penetrated and moved to the direction of 3 o'clock in the perivitelline space and then made a big turn of about 150° to the direction of 9 o'clock, inducing the ooplasmic rotation. Approximately 2 minutes and 25 seconds after the onset of observation.

Plate 4

Explantion of Figures

Figures 7 and 8 demonstrate the pictures of eggs before and after the the incomplete penetration of a spermatozoan, photographed under an inverted microscope with a phase-contrast apparatus. Approximately 900X

Figure 7. A spermatozoon attached to the zona pellucida of an egg at the direction of 6 o'clock immediately after the commencement of observation.

Figure 8. The sperm head and small portion of flagellum penetrated through the zona pellucida but nearly all flagellum remained outside of the zona pellucida following the incomplete penetration of the spermatozoan. Ooplasmic vibration but no rotation occured following the penetration. Approximately 3 minutes and 50 seconds after the onset of observation.