Acridine Orange Fluorescence Staining as a Means of Detecting Sperm-Egg Fusion in Mammals

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Abstract: *The aim of this study was to investigate basically, using the gametes of golden hamster, whether acridine orange (AO) fluorescent dye was efficient for detecting the sperm-egg fusion in vitro, and to evaluate the usefulness of this dye by comparison with other staining methods such as toluidine blue and Giemsa. Before insemination with zona-free hamster mature oocytes (metapase-II), the nuclei of acrosome reacted spermatozoa collected from the cauda epididymis emitted bright green AO fluorescence. Chronologically sperm nuclei changed AO fluorescence from green to red before it began to decondense within the ooplasm. Condensed nuclei attached to the oolemma of GV oocytes and pronuclear stage eggs, which were thought to be fused, were stained red 1 hour after insemination. On the other hand, although the spermatozoa incubated with zona-free, metaphase-II oocytes under calcium-magnesium free conditions had condense shaped nuclei after 1 hour insemination, the AO stained nuclei were completely green. The AO staining indicates the thiol-disulfide status of sperm nuclei, namely, green and red fluorescences mean S-S rich and S-S poor, respectively. Therefore, since nucleoproteins of the sperm nucleus should be reduced after being mixed with the ooplasma during the sperm-egg fusion event, the condense shaped and red nuclei are considered fused with oolemma before nuclear decondensation occurred. The AO fluorescence dye was proven to be effective compared with other staining methods reported previously, and AO staining will be useful for a precise and efficient means of detecting and investigating sperm-egg fusion events in mammals.*

Key words: *Fertilization, Sperm-egg fusion, Acridine orange, Disulfide bonds.*

Received: August 10, 1999
Accepted: September 8, 1999
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In mammals, only acrosome-reacted spermatozoa have the ability to fuse with oolemma [1, 2]. Following capacitation of sperm either *in vivo* or *in vitro*, the plasma membrane and outer acrosomal membrane of a spermatozoon fused during the acrosome reaction after induction of the signal transduction mechanism [3]. The plasma membrane over the equatorial segment of the sperm head becomes fusogenic perhaps due to the action of acrosomal enzymes dispersed during the acrosome reaction [2, 4]. Various methods to detect sperm-egg fusion have been developed [5]. For example, the sperm penetration assay (hamster test) [6] was established to study the fertilizing capacity of ejaculated human sperm. After insemination of zona-free hamster mature (metaphase-II) eggs with capacitated sperm, the presence of decondensed or fully developed male pronuclei in hamster ooplasma under a phase contrast microscope indicates the fertilizing ability of the individuals tested. While sperm- egg fusion evaluated by the electron microscope technique [7, 8] is unequivocal, it is time-consuming. In contrast, egg staining with toluidine blue [9, 10], Giemsa [11], or Hoechst dye [12] is simple and efficient. Although mature intact sperm nuclei cannot be stained by either toluidine blue or Giemsa *in vitro*, nuclei become stained with these dyes after fusion with oolemma. The stainability of fused sperm nuclei is thought to depend on the activity of ooplasma, which reduces the disulfide bonds (S-S bonds) of sperm nuclear chromatin to free thiol (SH of cysteine residues) [10]. However, accurate counting of stained sperm nuclei under an ordinary microscope is hampered by heterogenic staining of sperm nuclei.

AO fluorescent dye has been used for analysis of DNA integrity of somatic cell nuclei and mammalian sperm nuclei [13–17]. The AO stainability of mammalian sperm nuclei was investigated to clarify the

differences of its fluorescent emissions. According to Kosower *et al.* (1992) [18], the stainability of sperm nuclei depends on the number of S-S bonds in sperm nuclear chromatin, and nuclear maturation of sperm can be analyzed using AO fluorescence dye. In short, mature (S-S rich) nuclei emit bright green fluorescence, whereas, immature (S-S poor) nuclei emit orange or red fluorescence.

The aim of the present study was to investigate, using the gametes of golden hamster, whether AO fluorescent dye is an efficient means for detecting sperm-egg fusion and to evaluate the usefulness of this dye in comparison with other dyes (toluidine blue and Giemsa).

Materials and Methods

Animals

Golden (Syrian) hamsters were maintained in a temperature-controlled room with a 14 h light phase (05:00–19:00) and a 10 h dark phase (19:00–05:00). The ages of females and males were 2–4 months and 6–10 months, respectively.

Reagents

All inorganic salts were purchased from either Mallinckrodt Chemical Works (St. Louis, MO, USA) or Sigma Chemical Co. (St. Louis, MO, USA). Toluidine blue (Gurr) was obtained from Atomergic Chemicals Co. (Plainview, NY, USA), acridine orange (3,6-bis [Dimethyl amino] acridine, hemi [zinc chloride] salt) from Sigma Chemical Co. (St. Louis, MO, USA), and Giemsa (Gurr R66 solution) from Hoskins and Williams (Essex, England). Other organic compounds were obtained from Sigma unless otherwise stated.

Media

The medium used for isolation of immature ovarian eggs was HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])-buffered TCM-199 medium [19]. A modified Tyrode's solution, m-TALP-3 [19], was used for isolation of mature eggs and fertilized eggs from oviducts, induction of capacitation and acrosome reaction of spermatozoa, and insemination of ovarian and oviductal eggs as well as fertilized eggs.

Preparation of capacitated and acrosome-reacted spermatozoa

Spermatozoa from the cauda epididymis were incubated in m-TALP-3 [20]. The concentration of spermatozoa in the incubation medium was approximately 4–5 $\times$ 10^6/ml. After incubation for 4–5 hours at 37°C under 5%CO_2 in air, 40–80% of spermatozoa in the entire population were spontaneously acrosome-reacted and most of them were hyperactivated.

Preparation of eggs

To stimulate follicular growth, all females were injected with 30 i.u. of pregnant mare serum gonadotropin (PMSG, Sigma Chemical Co., St. Louis, MO,USA) on the morning of ovulation (day 1), which was characterized by the presence of conspicuous vaginal discharge. Fully grown eggs in the germinal vesicle stage (GV eggs) were collected by puncturing preovulatory follicles on the afternoon of day 3 or on the morning of day 4. Both cumulus cells and zona pellucida were removed by treating eggs for 20 min with TCM-199 containing 2.5 mg/ml collagenase (type II, 2025 Kunitz units/mg; Sigma) [19]. Zona-free GV eggs were rinsed with TCM-199 and kept in m-TALP-3 for no longer than 30 minutes before insemination. To obtain mature unfertilized eggs (metaphase II), PMSG-treated females were each injected with 30 i.u. human chorionic gonadotropin (hCG, Organon, Tokyo, Japan) on the evening of day 3. Between 15 and 17 hours after hCG injection, the females were sacrificed and their oviducts were flushed with m-TALP-3. The eggs were freed from cumulus cells and zona pellucida by treating them consecutively with hyaluronidase and trypsin and kept in m-TALP-3. To obtain fertilized eggs at the pronuclear stage, PMSG-treated females were each injected with 20 i.u. of hCG at 14:30 on day 4 and allowed to mate with one or two males of proven fertility on the evening of the same day. The next morning, their oviducts were flushed with m-TALP-3 (10:00–12:00). Zona pellucida were removed by trypsin treatment. Hyaluronidase treatment to remove cumulus cells was unnecessary in most cases, because the cells had already dispersed spontaneously in the oviducts.

Insemination and examination of eggs (AO staining)

Zona-free eggs were placed in 300 μl of m-TALP-3 which had been previously placed under mineral oil (Squibb and Sons, Princeton, NJ, USA) in a plastic Petri dish. Insemination was performed by adding a drop of sperm suspension containing actively motile, acrosome-reacted spermatozoa. The final concentration of spermatozoa was approximately 3.0 $\times$ 10^6/ml. The dishes with inseminated eggs were placed in a CO_2-incubator (37°C, with gas phase of 5% CO_2 in air). Initially, 5 minutes after insemination, eggs with attached spermatozoa were transferred to other dishes each containing 300 μl of fresh m-TALP-3 and incubated further. At various intervals of time, eggs were removed from

dishes and placed (fixed) in acetic alcohol (1 part glacial acetic acid plus 3 parts 100% methanol) which was prepared immediately before use. After fixation at room temperature for 5 minutes or over 2 hours (overnight in most experiments), the eggs were transferred, a few at a time, onto clean slides and air-dried. The eggs were then stained with acridine orange, toluidine blue or Giemsa (Table 1). After thorough washing and mounting in distilled water, the eggs were examined to determine the morphology and staining status of sperm nuclei under a fluorescence microscope.

In a series of experiments, mature oviductal eggs were freed from zona pellucida and inseminated in calcium-magnesium free m-TALP-3. Control eggs were inseminated in the regular m-TALP-3. Acrosome-reacted spermatozoa used in these experiments were washed once by centrifugation (350 g, 5 minutes) in 0.9% NaCl supplemented with 3 mg/ml bovine serum albumin (Fraction V, Sigma), 25 mM $NaHCO_3$, and 50 μM EGTA ([Ethylene-bis(oxyethylenenitrilo)] tetraacetic acid) (pH 7.5) prior to insemination. Inseminated eggs were kept in the 5% CO_2-incubator for 1 hour before fixation and staining. In some experiments, inseminated eggs were treated with 1 mM diamide (a SH-oxidizing agent, diazenedicarboxylic acid bis (N,N-dimethylamide)) (pH 7.4, in 20 mM Tris-HCl for 15 minutes) or 5 mM dithiothreitol (DTT, a disulfide bond (S-S) reducing agent) before fixation (pH 8.3, in 20 mM Tris-HCl for 30 minutes).

Results

Changes in AO fluorescence of sperm nuclei during fusion event

The nuclei of all spermatozoa prior to insemination exhibited green AO fluorescence (Fig. 1). Condensed nuclei of the spermatozoa which were unable to fuse with eggs in the calcium-magnesium free medium [21] showed complete green AO fluorescence (Table 2). On the other hand, attached and condensed sperm nuclei on/in the eggs inseminated in the regular medium showed partial or complete red AO fluorescence 1 hour after insemination.

Table 3 summarizes chronologically the results of AO staining of mature zona-free eggs inseminated in the regular (calcium-magnesium containing) medium. It is clear from this table that the AO fluorescence of the sperm nucleus changed from green to red before the nucleus began to decondense within the ooplasm. When nuclear decondensation began at the equatorial segment, this region exhibited green fluorescence again, and then the fully decondensed nucleus exhibited uniform green fluorescence. Chronological changes in nuclear AO fluorescence are clearly shown in Fig. 1.

When GV eggs and fertilized eggs at the pronuclear stage were inseminated, none of them contained decondensing sperm nuclei 1 hour after insemination (Table 4, also see [22]). However, the majority of condensed sperm nuclei on/in the eggs exhibited red AO fluorescence (Table 4). When the same eggs were treated with 1 mM diamide for 15 minutes, all of the condensed sperm nuclei exhibited green AO fluorescence. Moreover, we were able to stain the nuclei red again by further treatment with 5 mM DTT (data not shown).

Discussion

As reported by Krzanowska (1983) [9] and Miller and Masui (1982) [11], both toluidine blue and Giemsa staining can be used to detect a drastic change that occurs in the sperm nucleus before its decondensation within the ooplasm. These dyes did not stain the nuclei of free spermatozoa before fusion, but stained them dark blue when the nuclei were within the ooplasm (Fig. 2).

Table 1. Comparison between the staining methods for detecting the fused spermatozoa with the oocytes

Method	Duration of fixation	Staining solution (staining time)	Examination means	Ref.
I	5 min	1% Toluidin blue in distilled water (10–15 min)	Ordinary microscope	Krzanowska (1983) [9]
II	5 min	0.15% Giemsa in 20 mM Tris-buffer pH 7.4 (5 min)	Ordinary microscope	Miller and Masui (1982) [11]
III	2 hours-overnight	0.019% Acridine orange in 76 mM citrate/phosphate buffer pH 2.5 (5 min)	UV microscope*	Tejada et al. (1984) [17]

*Excitation filter 450–490 nm; barrier filter 520 nm.

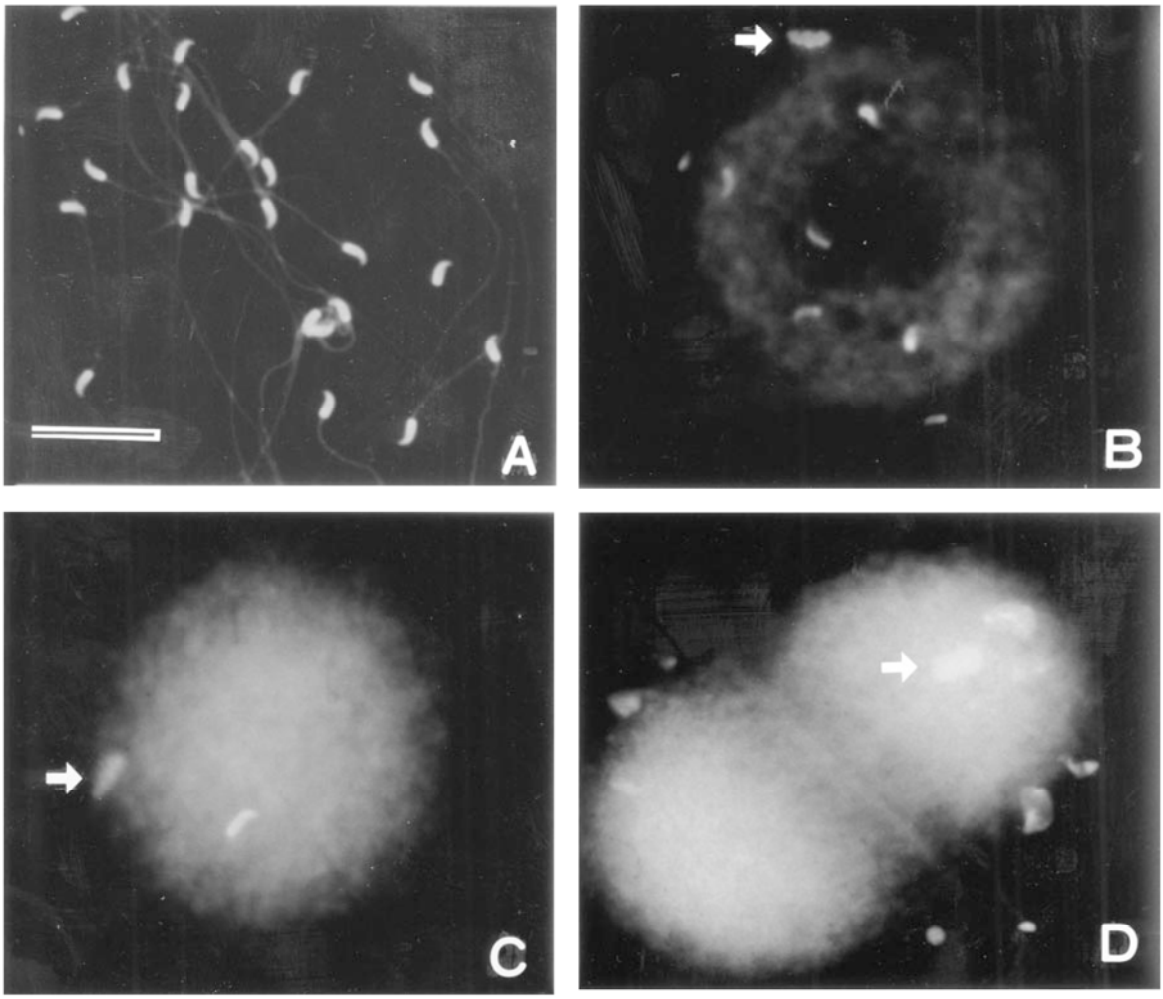

Fig. 1. AO fluorescence of hamster cauda epididymal spermatozoa and the chronological changes of the fluorescence during sperm-egg fusion after insemination. (A) Nuclei of spermatozoa collected from cauda epididymis exhibited bright green fluorescence after being stained with AO dye; (B–C) photographs of inseminated eggs after 10 minutes and 20 minutes, respectively. Intact, red nuclei could be observed around this time; (D) partially decondensed nuclei could be seen 30 minutes after insemination. White arrows indicate metaphase-II female chromosomes in the ooplasma. Scale bar represents 50 μm (× 400).

Table 2. Acridine orange staining reaction of sperm nuclei one hour after insemination of mature oocytes in regular and calcium-magnesium free media

Inseminated in m-TALP-3	No. of oocytes (No. exp.)	No. of sperm exam.	% Sperm nuclei			
			Condensed*		Partially decondensed	Completely decondensed
			G	(G/R)+R		
Regular	25 (2)	158	39	21	12	28
Ca-Mg-free	22 (2)	122	100	–	–	–

*G; condensed green nuclei, G/R; condensed green nuclei with partially red fluorescence, R; condensed red nuclei.

Table 3. Changes in the acridine orange staining reaction of sperm nuclei after insemination of mature oocytes

Time after insem. (min)	No. of oocytes exam. (No. exp.)	No. of sperm nuclei exam.	% Sperm nuclei			
			Condensed*		Partially decondensed	Completely decondensed
			G	(G/R)+R		
5	11 (2)	25	100	0	0	0
10	17 (3)	51	59	41	0	0
20	30 (3)	74	22	78	0	0
30	25 (3)	82	8	69	23	0
45	15 (2)	32	9	31	54	6
60	12 (2)	33	0	3	0	97

*G; condensed green nuclei, G/R; condensed green nuclei with partially red fluorescence, R; condensed red nuclei.

Table 4. Changes in acridine orange staining reaction of sperm nuclei after insemination of GV oocytes and pronuclear eggs

Time after insem. (min)	No. of eggs exam. (No. exp.)	No. of sperm nuclei exam.	% Sperm nuclei		
			Condensed*		Decondensing
			G	R	
GV oocytes					
30	17 (3)	65	28	72	0
60	36 (3)	182	2	98	0
Pronuclear eggs					
30	26 (2)	128	77	23	0
60	5 (1)	21	5	95	0

*G; condensed green nuclei, R; condensed red nuclei.

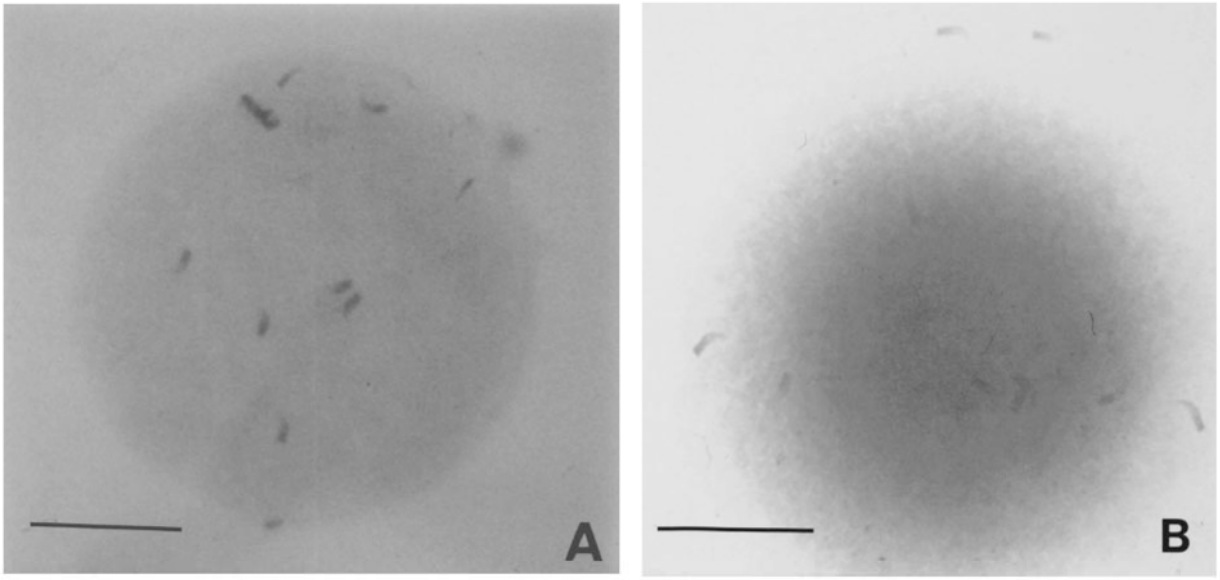

Fig. 2. Photographs of stained eggs 20 minutes after insemination with toluidine blue (A) and Giemsa (B) staining. Bars represent 30 μm (× 400). Staining procedures are summarized in Table 1.

The affinity of these dyes to sperm nuclei was thought to be increased after reduction of S-S bonds of sperm nuclear chromatin. The change of stainability after fusion is difficult to explain, but using a disulfide bond reducing agent, the stainability of these dyes *in vitro* could be induced easily. Nucleoproteins of the sperm nucleus are reduced after being mixed with the ooplasma during sperm-egg fusion. Therefore, intact nuclei were stained with these dyes before sperm nuclear decondensation occurred. However, the fused nuclei were stained heterogeneously and it was difficult to evaluate staining under an ordinary microscope, because the background (ooplasm) was the same color as the fused sperm nuclei.

In AO staining, it takes more time to fix (rather than to denature sperm nuclear DNA with acid-alcohol) [18] the eggs than in the other staining methods, and an epifluorescence microscope is needed for observation (Table 1). However, AO-stained sperm nuclei changed from green to red during fusion and the change was easy to observe. In cauda epididymal spermatozoa collected from golden hamsters and other mammals but not humans, almost all nuclei emit bright green fluorescence. During sperm-egg fusion, AO-stained nuclei changed gradually to red at the portion over the equatorial segment of the sperm head. It is clear from electromicroscopic observation that sperm-egg fusion begins at the equatorial segment of the acrosome-reacted sperm head [22]. Therefore, the spermatozoa that had intact, partially red nuclei were considered to have begun sperm-egg fusion. Furthermore, the results of AO staining suggested that intact sperm nuclei attached to GV eggs and pronuclear eggs, which had no sperm decondensing factor within the ooplasm, were already fused with oolemma 30 minutes after insemination and that calcium and magnesium were essential for sperm-egg fusion.

The red sperm nuclei observed soon after fusion could be changed to green by treatment with a thiol oxidizing agent (diamide). This indicates that intact, red nuclei still contain sperm-specific cysteine-rich nucleoprotein (protamine) and it is thought that transition of nucleoproteins within the sperm nucleus does not occur during this period (within 20 minutes after insemination). Moreover, this conclusion appears to be supported by experiments showing that green-fluorescing, decondensed or partially decondensed nuclei could not be stained red again after being treated with DTT (data not shown). This is indirect evidence that sperm-specific nucleoproteins are already replaced and do not exist in decondensed or partially decondensed nuclei.

The process of mammalian fertilization, including sperm-egg fusion, remains unclear in many respects. In spermatozoa, capacitation and the acrosome reaction have been considered the most important events during fertilization both *in vivo* and *in vitro*. After those events have occurred, the fusibility of spermatozoa with oolemma is essential for the next steps in fertilization. There have been several studies on methods for detecting sperm-egg fusion, particularly those utilizing zona-free hamster eggs. However, all those methods have certain disadvantages. In the present study, AO fluorescent dye was used to detect sperm-egg fusion, and this method was proven to be effective compared with the other staining methods reported previously. Moreover, AO staining enables sperm-egg fusion events to be easily detected under a fluorescence microscope. It was concluded that AO staining is a precise and efficient means of detecting and investigating sperm-egg fusion events in mammals.

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