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Integrins are not involved in the process of human sperm-oolemmal fusion

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Integrins are not involved in the process of human sperm-oolemmal fusion

Running title: Integrin and sperm-oolemmal interaction

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Abstract

BACKGROUND: We investigated whether integrins are required for the human sperm-oocyte binding and fusion process. METHODS: The expression of several integrin subunits at the human oocyte plasma membrane was investigated using immunofluorescence microscopy, and the functional role of integrins expressed at the human oocyte surface in sperm-oocyte interaction was studied using a zona-free human oocyte binding and fusion assay. A total of 144 unfertilized oocytes were stained with anti-integrin antibodies and 147 zona-free unfertilized oocytes were inseminated in the presence of various anti-integrin antibodies that were expressed in oocyte plasma membrane. RESULTS: The antibodies of six alpha integrin subunits (α2, α3, α5, α6, αV, αM) and six beta integrin subunits (β1, β2, β3, β4, β5, β6) were bound to the surface of fixed unfertilized oocytes. In contrast, the presence of α1 and α4 subunits could not be verified. The human sperm-oocyte binding was only partially inhibited by blocking antibodies of α2, α3, α5, α6, αV, αM, β1, β2 and β3 with a maximum of 55% inhibition, but antibodies of β4, β5 and β6 showed no effect on sperm-oolemmal binding. Similar reduction of the number of fused spermatozoa was observed. However, the ratio of fused spermatozoa to total spermatozoa (bound and fusion) was not impaired by all integrins antibodies suggesting that integrins had no role in the sperm-oolemmal fusion process.

CONCLUSIONS: These results suggest that one of the binding mechanisms can be inhibited by integrin antibodies but this mechanism does not play an essential role in the human sperm-oolemmal binding and fusion processes. The other mechanisms,

insensitive to integrins, may involve binding and fusion process in human oocytes.

Key words: human gamete fusion / integrin/ oocyte plasma membrane / sperm

Introduction

The molecular events of sperm-egg binding and fusion have extensively been studied, but identification of the molecules involved in sperm-egg interaction remains incomplete. Several candidate molecules for sperm-egg binding and fusion have been proposed. The best candidate of ligands on sperm is ADAM (A Disintegrin and Metalloprotease) protein. In the mouse, antibodies and peptide of the disintegin domain of fertilin β and cyritestin have been reported to strongly inhibit sperm-egg binding and fusion (Primakoff et al., 1987; Yuan et al., 1997; Evans et al., 1998; Bigler et al. 2000; Zhu et al., 2000; McLaughlin et. al., 2001). The evidence that disintegrin-like domains of fertilin β and cyritestin might be responsible for sperm-egg interactions suggests that complementary binding molecules on the oocyte plasma membrane are integrins (Blobel et al., 1992; Myles et al., 1994; Bronson et al., 1999). Indeed, a number of integrin subunits have been detected in the oolemma of mammalian oocytes. It seems likely that one or more integrins are involved in the processes of sperm-egg binding and fusion. Since peptides with a sequence of fertilin β disintegrin domains bound to α6β1 integrin and an anti-α6 integrin function-blocking monoclonal antibody, GoH3, inhibits mouse sperm-oocyte binding and fusion, mouse egg integrin α6β1 could be a primary candidate for a sperm receptor (Almeida et al.,1995). This concept is further supported by the findings that integrin α6β1 has been reported to be associated with CD9 in various cells including mouse oocyte plasma membrane, because recent studies demonstrate that egg CD9 play a key role in sperm-oocyte fusion (Nakamura et al.,1995; Miyado et al.,2000; Kaji et al.,

2000; Le Naour et al., 2000). However, oocytes from mice null α6 integrin subunit were shown to have no reduction in sperm-oocyte binding and fusion, suggesting that α6 integrin is not critical for oocyte-sperm interactions (Miller et al., 2000). The involvement of other subfamilies of integrins, particulally α4 and α9 integrins, in the processes of sperm-oocyte binding and fusion has also been suggested (Zhu and Evans et al., 2002). This may reflect redundancy of α6β1 and other egg integrins, or it may mean that integrins have no role in binding and fusion.

In humans, several integrin subunits have been identified in the oolemma, but controversy still exists (de Nadai et al., 1995; Ji et al., 1998; Fusi et al., 1993; Campbell et al., 1995; Capmany et al., 1998)). The involvement of the RGD-binding subfamily of integrins in the gamete interactions has also been demonstrated by the inhibition of interactions of human sperm with zona-free hamster and human oocytes by RGD peptides (Bronson and Fusi, 1990; Ji et al., 1998). However, limited information is available concerning the role of integrins in human sperm-oocyte interaction.

The aim of the present study was to investigate whether integrins are required for human sperm-oocyte binding and fusion process. The expression of several integrin subunits at the human oocyte plasma membrane was investigated using immunofluorescence microscopy, and the functional role of integrins expressed at the human oocyte surface in sperm-oocyte interaction was studied using a zona-free human oocyte binding and fusion assay.

Materials and Methods

Unfertilized oocytes in this study were obtained from patients undergoing IVF. Written informed consent was obtained from all patients and this study was approved by the local ethical committee. Follicular stimulation and IVF protocols were described previously (Sengoku et al., 1995). Briefly, follicular stimulation was achieved with a desensitizing protocol using a combination of GnRH agonist (buserelin acetate; Spurecur, Hoechst, Tokyo, Japan) and hMG (Pergonal; Teikokuzouki, Tokyo, Japan). Human chorionic gonadotropin 10000 IU (hCG mochida; Moshida Pharmaceutical, Tokyo, Japan) was administered when leading follicles were > 16 mm in diameter. Oocyte recovery was performed 34-36 hr after the hCG injection using transvaginal ultrasound-guided aspiration. The oocytes were examined for fertilization 16-18 after insemination. Oocytes with no signs of fertilization and apparently normal morphology were included in this study.

Immunohistochemistry

One-day old unfertilized oocytes were transferred to PBS. The zona pellucida was removed by a brief exposure to acid Tyrode's solution. After 3 h of incubation (recovery time) in human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific), zona-free unfertilized oocytes were fixed in 2% paraformaldehyde in PBS for 30 min. After washing in PBS with 0.3% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and 100 mM glycine

(blocking solution), the unfertilized oocytes were incubated for 1 h with various anti-human integrin antibodies diluted in PBS containing 3% fetal calf serum (to mask non-specific binding sites). Mouse monoclonal antibodies against human integrin subunits α1(FB12), α2 (P1E6), α3 (P1B5), α4 (P4G9), α5 (P1D6), αV (CLB706), αM(ICRF44), β2(P4H9), β3(PM6/13), β4(ASC3), β6(CSb6), and rabbit polyclonal antibodies against β5 were supplied by Chemicon International (CA, USA). Anti-human integrin subunit α6(GoH3) monoclonal antibody was raised in the rat (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) and mouse monoclonal anti-human integrin subunit β1(6S6) was purchased from UBI (Upstate Biotechnology Inc., Lake Placid, NY,USA). They are all used at a 1:20 (v/v) dilution in PBS. The specimens were washed by several transfers to blocking solution and incubated with one of the following fluorescent conjugates for 45 min. The conjugated secondary antibodies (raised in mouse, goats or rabbit) were used at a dilution of 1:200 in PBS-BSA.

When staining was not detected, the detection was amplified by a biotinylated anti-mouse, anti-rat or anti-rabbit IgG and streptavidin-fluorescein isothiocynate (FITC). The unfertilized oocytes labeled by integrin antibodies were incubated for 45 min in a solution containing biotinylated goat anti-mouse, anti-rat or anti-rabbit IgG at a dilution of 1:200 in PBS-BSA (Sigma Chemical Co) and then reincubated for 30 min in PBS with Streptavidin -FITC (at a dilution of 1:150, Sigma Chemical Co.; Ji et al, 1998).

Negative controls were obtained by substituting the incubation in primary antibody for an incubation in PBS containing 3% fetal calf serum. Moreover, negative controls were established during each staining procedure to confirm that the fluorescence observed was

not attributable to nonspecific binding of the secondary antibody.

Labelled specimens were mounted on slides in PBS supplemented with 25 mg/ml 1,4 diazabicyclo-(2.2.2) octane (DABCO; Sigma) and photographed on an Olympus BX60 fluorescent microscope (Olympus Optical Co., Tokyo, Japan). Overlays of captured images were processed with Adobe Photoshop 7.0.

The possibility of penetration of spermatozoa into unfertilized oocytes, and the possible activation of oocytes were confirmed by Hoechst 33342 (10 μg/ml) staining. Only metaphase II stage oocytes were included in this study.

Assessment of sperm-oocyte interaction

The dye transfer technique (Hinkley et al., 1986) was used to assess the sperm-oocyte interaction. Zona pellucida-free unfertilized oocytes were incubated in HTF medium containing 0.1 μg/ml Hoechst 33342 (Sigma) for 30 min, and then rinsed thoroughly in PBS over 15 min. Oocytes were preincubated with the 25μg/ml anti-integrin antibodies for 1 h, washed free from unbound antibodies and then inseminated with 100,000/ml spermatoza in fresh HTF medium. The Control group contained mouse immunoglobulin G (IgG; Sigma). Two hours after insemination, the oocytes were washed using a narrow bore pipette to remove loosely adhering spermatozoa. Then, oocytes were fixed with 2.5% glutaraldehyde in PBS at pH 7.4 for 30 min, rinsed with PBS and mounted for observation under an Olympus BX60 fluorescent microscope. Spermatozoa were considered fused when fluorescent-positive condensed or decondensed sperm head were

observed on the egg surface. Spermatozoa attached to the egg surface which could be seen by light microscope, without fluorescence, were designated as binding spermatozoa.

To confirm the validity of this dye transfer technique, we preloaded zona-free human oocytes with 0.1 μg/ml Hoechst dye and then inseminated with uncapacitated spermatozoa (cultured in Ca2+-free HTF medium). When oocytes were inseminated with uncapacitated spermatozoa, none of the attached spermatozoa showed fluorescent-positive condensed sperm nuclei. Therefore, the present experiment in which 0.1 μg/ml Hoechst dye was preloaded made it possible to distinguish fused from unfused spermatozoa.

Statistical Analysis

Statistical significance of the data was determined by Student's t-test and the chi-squared test, as appropriate. Differences were considered significant at $P < 0.05$.

Results

A total of 144 unfertilized oocytes were stained with anti-integrin antibodies. The detection was amplified by a biotinylated anti-mouse, anti-rat or anti-rabbit IgG and streptavidin-fluorescein isothiocynate (FITC) in six integerin subunits (α 1, 2, 4, 5, M, β 1 3, 6). Antibodies of six alpha integrin subunits (α2, α3, α5, α6, αV, αM) and six beta integrin subunits (β1, β2, β3, β4, β5, β6) were bound to the surface of fixed

unfertilized oocytes. In contrast, the presence of α1 and α4 subunits could not be verified. The heterogeneity of the intensity or distribution of fluorescence was found among oocytes. In some oocytes, surface staining was unevenly distributed, but the most typical pattern was intense and uniformly distributed at the oocyte surface. Furthermore, some integrin subunits were inconsistently expressed in unfertilized oocytes. Negative controls substituting the incubation in primary antibody for an incubation in PBS containing 3% fetal calf serum showed no immunofluorescence labellings. The representative patterns of staining are shown in figure 1, and a summary of these patterns is shown in table I and II.

These immunocytochemical findings indicated a potential role for α and β integrin subunits in the gamete binding and fusion process. We investigated whether integrins could be involved in binding and/or fusion of human gametes during fertilization. One hundred forty seven zona-free unfertilized oocytes were inseminated in the presence of various anti-integrin antibodies that were expressed in oocyte plasma membrane, and 135 zona-free oocytes were served as the control. Any antibodies used in this study, except β4, β5 and β6, partially inhibited binding process by ~55% as compared with controls. Similar reduction of the number of fused spermatozoa was observed by anti-integrin subunits. No apparent effect on binding and fusion was observed in the presence of β4, β5 and β6 integrin subunits. However, the ratio of fused spermatozoa to total spermatozoa (bound and fusion) was not impaired by all integrin antibodies indicating no effect of integrins on the sperm-oolemmal fusion (Table III and IV). A dose-dependency inhibition was not done due to limited number of samples and higher

concentrations of antibodies caused damage to the gametes. This inhibition was specific since mouse IgG control antibody had no effect on binding and fusion.

Discussion

To investigate the expected role for integrins in human sperm-oocyte binding and fusion, we studied the expression of integrins on the human oocyte surface and whether antibodies against integrin subunits inhibit human sperm-oocyte membrane binding and fusion.

In this study, α2, α3, α5, α6, αV, αM, β1, β2, β3, β4, β5 and β6 subunits were detected in human oocyte plasma membrane, however, exposed surface localization of the α1 and α4 subunits could not be verified. These results extend previous observations of integrin expression on the human oocyte surface, although some controversy still exists. Integrin subunits α2, α3, α4, αV, αL, β1, β2, β3, β4, β5 and β7 have been demonstrated in human oocytes by Campbell et al. (1995). Using a rosetting technique, Fusi et al. (1992, 1993) demonstrated that α2, α5, α6, αV, β 1 but not α4 were detected on human oolemma. It has also been reported that α2,α5 and α6 were detected by immunofluorescense labellings, but α4 and β1 were not detected on the surface of human oocytes (de Nadai et al.,1996).

While α6 not identified by Campbell at al. (1995), other studies including present study have described its presence in human oocytes. These staining results were consistent with the report in which the expression of α5, α6 was detected in a serial analysis of gene

expression (SAGE) in the human oocyte (Neilson et al., 2000)

The differences in labeling protocols and antibodies could explain the discrepancies among studies in the ability to detect specific integrin subunits. However, the consistent absence of labeling with α1 antibody in several studies including our current observation indicates that this subunit is not present or is present in extremely small numbers on the surface of human oocytes.

Recently, it has been suggested that α4/α9 is involved in mouse sperm-egg membrane interaction from studies of ferilin β binding assays to mouse zona-free eggs using the peptides perturbing integrin-mediated interaction (Zhu and Evans 2002). The α9β1 may be a major receptor for ADAMs that lack RGD motifs, since α9β1 specifically binds to the disintegrin domain of fertilin β synthesized in bacteria (Eto et al., 2000).

The α4 subunit was reported to be detected by Campbell et al. (1995), but Fusi et al. (1993) and de Nadai et al. (1996) failed to found the expression of α4 on human oocytes. Although a similar amplification step to Ji et al. (1998) was employed in our study to increase sensitivity of detection system, α4 subunit was not detected. Thus, it seems unlikely that α4 is involved in human sperm-oocyte membrane interactions. The α9 integrin has not yet shown to be present on human oocytes, and we did not investigate the expression of α9, because antibody against α9 was not available in our current study.

These immunocytochemical findings might suggest a potential role for integrin subunits in the human gamete binding and fusion process. Zona-free unfertilized human oocytes were inseminated in the presence of various anti-integrin antibodies that were expressed in human oocyte plasma membrane.

Our observation demonstrated that human sperm-oocyte binding was only partially inhibited by blocking antibodies of α2, α3, α5, α6, αV, αM, β1, β2 and β3with a maximum of 55% inhibition, but antibodies of β4, β5 and β6 showed no effect on sperm-oolemmal binding. In addition, the fusion of oocytes by spermatozoa that had become bound to oolemma was not blocked.

It should be noted that the oocytes used in this study were one-day-old unfertilized oocytes and that alterations produced by the acid Tyrodes treatment used for zona pellucida removal were possible. However, several experiments, including our previous study, show that ageing and acid Tyrodes treatment in vitro do not affect the ability of oocytes to fuse with spermatozoa (Tesarik 1989; Sengoku et al.,1995; Ji et al 1997 ;1998) .

It is also unlikely that these findings were due to use of a suboptimal concentration of the antibodies, since Ji et al. (1998) reported that the inhibition of sperm fusion with oocytes reached a plateau at 25 μg/ml of antibody against β1 integrin. Although we did not investigate dose-dependency of inhibition due to a limited specimens and gamete toxicity of high concentrations of antibodies, concentrations of antibodies used in our study is almost similar to that of their experiment.

It has been demonstrated that blocking anti-human β1 integrin monoclonal antibody, RGD (Arg-Gly-Asp)-containing peptide, or both, did not result in full inhibition of human gamete fusion (Ji et al., 1998). They suggested that β1 integrins are involved in human gamete interaction, but human gamete fusion can bypass the β1 requirement or a cofactor is required for the gamete fusion process. Both RGD containing peptide and

FEE (Phe-Glu-Glu) containing peptide, a putative integrin recognition sequence in human fertilin β, as is the QDE peptide in mice, has been reported to inhibit both adhesion and penetration of human spermatozoa with zona free human oocyte, but it is not complete inhibition (Bronson et al., 1999). They proposed that integrins which recognize fertilin β and RGD containing sperm-associated proteins, such as vitronectin and fibronectin, each play a role in gamete interaction and that they may cooperate, although fertilin α and cyritestin genes were determined to be non-functional pseudogenes in humans (Jury et al., 1997; Grzmil et al., 2001).

Similar results were reported in the heterologous system (human spermatozoa-hamster oocytes). RGD containing peptides can inhibit the adhesion and penetration of zona-free hamster eggs by human spermatozoa suggesting that RGD-dependent integrins, such as α5β1 and, αVβ1, αVβ3 are involved in the process of fertilization (Bronson and Fusi 1990). Furthermore, fibronectin and vitronectin have been expressed on the surface of capacitated human spermatozoa (Fusi and Bronson, 1992; Fusi et al.,1992). It has also been reported that antibody against α2 and α5 integrins inhibited both attachment and fusion of human spermatozoa with hamster oocyte by about 50% (de Nadai et al., 1996)).

Echistatin, a disintegrin inhibits the binding of vitronectin and fibronectin to integrins αVβ3 and αVβ1, inhibited the binding of human spermatozoa to the oolemma of zona-free hamster eggs. Although oolemmal adhesion of spermatozoa was reduced markedly by echistatin, it was not inhibited completely, and it had no apparent effect on egg penetration by sperm that did bind (Bronson et al.,1995). The authors suggested that

oolemmal integrins facilitates sperm adherence to the egg surface but is not required for sperm penetration.

In mice, it has been reported that several lines of mice null for integrin subunits (α6, α7, β3 and β5) are normally fertile (Miller et al., 2000; Hodivala-Dilke et al., 1999; Huang et al., 2000; Mayer et al., 1997). Recently, it has been demonstrated that α3 null eggs and β1 integrin null eggs function normally in sperm-egg binding and fusion suggesting that none of the integrins known to be present on mouse eggs are essential for sperm-egg binding and fusion (He et al., 2003).

Taken together including our findings of the partial inhibition binding and no apparent effect of fusion process by several antibodies against integrins, it seems likely that integrins involve in human sperm-oolemmal interaction, but are redundant with each other or may play an only marginal role, and that the membrane fusion is a separate event which is independent of integrin receptors.

CD9 has been reported to be essential for sperm-egg fusion in mouse (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), although the role of CD9 in gamete interaction in human has not been clearly determined.

It seems likely that an egg surface tetraspanin web involving β1 integrin and integrin associated proteins may define or help maintain a site for sperm fusion (Takahashi et al., 2001) because α6β1 and CD9 reported to be coimmunoprecipitated from mouse eggs (Miyado et al., 2000). However, the combined evidence demonstrating that mouse egg lacking α6β1 fuse normally with sperm (Miller et al., 20000) and that the human sperm oolemmal fusion process was not impaired by antibodies against several integrin subunits

in this study, would appear to indicate that a CD9 partner other than the integrins could function as sperm receptor to initiate the gameta fusion process.

In conclusion, the small but significant reduction of sperm-oocyte plasma membrane binding by antibodies against the several integrin subunits implies involvement of the integrins in human sperm-oocyte interaction. However, our data support the hypothesis that one of the binding mechanisms can be inhibited by integrin antibodies but this mechanism does not play an essential role in the binding and fusion process. The other mechanisms, insensitive to integrins, might involve binding and fusion process in human oocytes.

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Figure 1

Different patterns of staining of human oocytes with antibodies against integrin subunit. (A) Oocyte surface was equally stained (uniformly distributed pattern: integrin αV subunit); (B) almost of oocyte surface was labeled (unevenly distributed pattern: integrin β1 subunit); (C) oocyte surface was partially stained (unevenly distributed pattern: integrin α5 subunit) (D) negative control.

Table I Staining pattern of human oocytes with antibodies against the integrin subunits alpha

Integrin Subunit	No. oocyte examined	Pattern of staining (no. of oocytes)		
		uniformly distributed	unevenly distributed	not stained
α 1	8	0	0	8
α 2	10	5	3	2
α 3	11	8	3	0
α 4	8	0	0	8
α 5	10	6	3	1
α 6	12	9	3	0
α V	11	7	4	0
α M	10	4	3	3

Table II Staining pattern of human oocytes with antibodies against the integrin subunits beta

Integrin Subunit	No. oocyte examined	Pattern of staining (no. of oocytes)		
		uniformly distributed	unevenly distributed	not stained
β 1	10	7	3	0
β 2	9	7	2	0
β 3	12	7	3	2
β 4	11	8	3	0
β 5	10	6	4	0
β 6	12	8	2	2

Table III Inhibition of sperm-oolemma binding and fusion by antibodies against integrin alpha subunits

	No. of eggs	No. of total sperm (bound or fused) per egg (±SEM)	No. of sperm bound (not fused) per egg (±SEM)	No. of sperm fused per egg (±SEM)	Ratio of fused sperm to total (bound or fused) sperm
control	54	9.0±1.5	3.7±0.4	5.3±0.8	0.59 (286/486)
α2	11	5.2±0.8b	2.4±0.3b	2.8±0.5b	0.54 (31/57)
α3	12	4.9±0.7a	1.5±0.2 a	3.4±0.6b	0.68(40/59)
α5	12	6.0±0.9b	2.7±0.4b	3.3±0.5b	0.56(40/72)
α6	13	5.5±0.8b	2.7±0.4b	2.8±0.3b	0.51 (37/72)
αV	14	4.8±0.6a	2.2±0.2b	2.6±0.3b	0.54(36/67)
αM	11	5.3±0.7b	2.5±0.3b	2.8±0.4b	0.53 (31/58)

a $p < 0.01$ versus control, b $p < 0.05$ versus control

Table IV Inhibitipn of sperm-oolemma binding and fusion by antibodies against integrin beta subunits

	No. of eggs fertilized/ No. of total eggs	No. of total sperm (bound or fused) per egg (±SEM)	No. of sperm Bound (not fused) per egg (±SEM)	No. of sperm fused per egg (±SEM)	Ratio of fused sperm to total (bound or fused) sperm
control	81	9.0±1.5	3.7±0.4	5.3±0.8	0.59 (430/728)
β1	14	5.9±0.8b	2.7±0.2b	3.2±0.5b	0.54 (45/83)
β2	13	4.6±0.6a	2.1±0.2b	2.6±0.4b	0.57(34/60)
β3	12	4.8±0.6a	1.6±0.2a	3.2±0.5b	0.66(38/57)
β4	13	8.1±1.3	2.8±0.3	5.3±0.9	0.65(68/105)
β5	11	8.4±1.4	3.1±0.4	5.3±0.8	0.57(56/98)
β6	11	8.2±1.3	3.0±0.4	5.2±0.8	0.64(58/90)

a $p < 0.01$ versus control, b $p < 0.05$ versus control

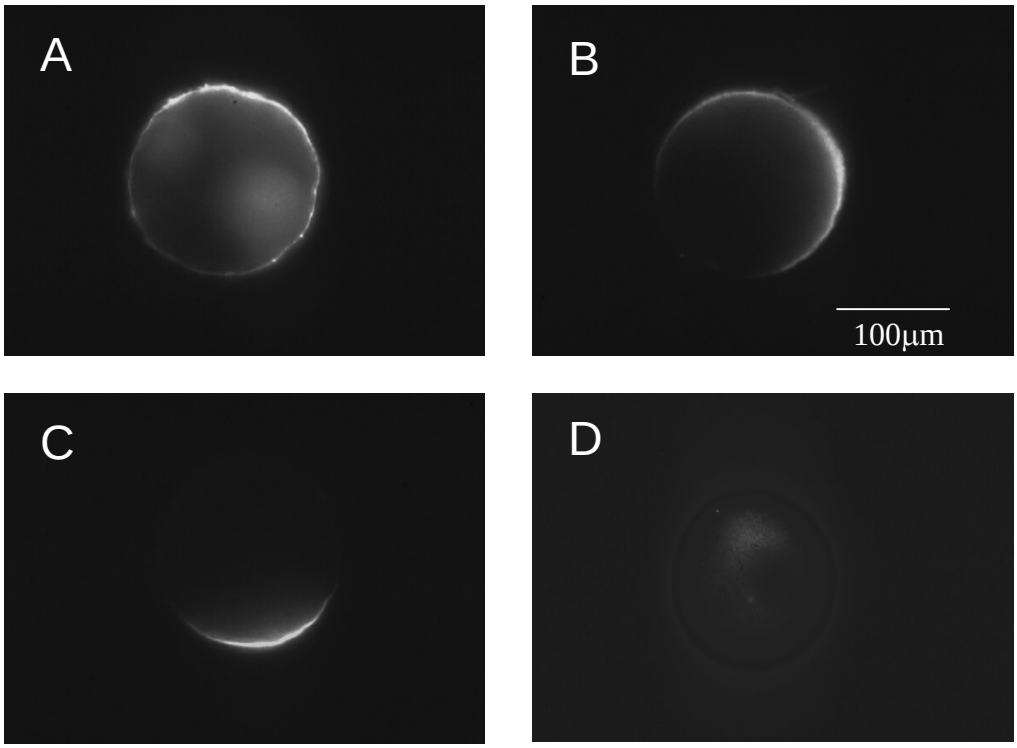

Figure 1