Presence and localization of oscillin in human spermatozoa in relation to the integrity of the sperm membrane

M. Montag[a,*], J. Parrington[b], K. Swann[b], F.A. Lai[c], H. van der Ven[a]

[a] *Department of Endocrinology and Reproductive Medicine, University of Bonn, 53105 Bonn, Germany*
[b] *Department of Anatomy and Developmental Biology, University College, London, UK*
[c] *MRC National Institute for Medical Research, Mill Hill, London, UK*

Received 17 December 1997; revised version received 19 January 1998

Abstract We investigated the presence and localization of oscillin in human spermatozoa in relation to the integrity of the sperm membrane, which was assessed by the hypo-osmotic swelling (HOS) test. We found no gross differences in the presence of oscillin in semen samples from men who presented with 70%, 40%, 25% or 2% of membrane-intact spermatozoa. By immunofluorescence, membrane-intact (HOS-positive) spermatozoa showed staining of a single band at the equatorial region, whereas over 80% of HOS-negative spermatozoa consistently showed a diffuse distribution of oscillin over the sperm head. However, some individuals presented with up to 50% of HOS-positive spermatozoa showing an aberrant localization of oscillin. We found a significant correlation rate ($r = 0.70$, $P < 0.05$) between the percentage of HOS-positive spermatozoa with an equatorial oscillin localization and the fertilization rates achieved after intracytoplasmic sperm injection. These data suggest that the localization of oscillin in human spermatozoa might have an impact on egg activation and fertilization rates.

Key words: Oocyte activation; Hypo-osmotic swelling test; Intracytoplasmic sperm injection; Oscillin; Sperm cytosol; Human

1. Introduction

The intracytoplasmic sperm injection (ICSI) technique has become the most successful treatment for male subfertility [1–3]. However, even those invitro fertilization (IVF) centers with a long experience of ICSI only achieve maximum fertilization rates of 70% on average [4]. Several groups have reported a high number of failed-fertilized oocytes following ICSI or IVF, which were found to be penetrated by a sperm cell but failed to become properly activated [5,6]. The successful activation of the oocyte is an initial element of mammalian fertilization and it is known that this process is initiated by the sperm cell via its ability to induce intracellular Ca^{2+} oscillations [7]. Furthermore a Ca^{2+} oscillation-inducing and oocyte-activating capacity is present in a sperm cytosolic fraction of rabbit, hamster, pig, rhesus monkey and human spermatozoa [6,8–12]. Recently, a candidate sperm factor protein has been isolated from hamster spermatozoa and a corresponding cDNA clone was identified and named oscillin because it correlated with the ability of extracts to trigger Ca^{2+} oscillations upon injection into mammalian eggs [13]. Oscillin may therefore be a key component of the sperm cytosolic activation factor which seems to be necessary for proper oocyte activation [14]. Immunocytochemical data support the presence of an oscillin-like protein in human spermatozoa which is localized to the intracellular side of the equatorial region [13,14]. Consequently, human oscillin may hold the key to fertilization in ICSI and IVF and it was proposed that its absence may explain, at least partially, the finding of failed-fertilized oocytes [15].

The aim of this study was to investigate the presence and localization of an oscillin-like protein in human spermatozoa in semen samples from fertile and subfertile males by immunoblotting and immunocytochemistry techniques. Because oscillin is a soluble protein, we further addressed the question of whether its presence and localization are dependent on the integrity of the sperm plasma membrane. Membrane integrity was assessed by the hypo-osmotic swelling test (HOS test) [16], which allows for the detection of membrane-intact, viable spermatozoa.

2. Material and methods

2.1. Collection of sperm samples and HOS test

Spermatozoa were derived from male patients attending the fertility unit at the University of Bonn. Following liquefication and analysis of sperm parameters, one part of the semen samples was processed for ICSI treatment, which was performed as previously described [17]. The remaining part was washed once in culture medium (Gamete-100, Scandinavian IVF Science AB, Göteborg, Sweden). An aliquot of the resuspended sperm pellet was mixed with 100 μl of hypo-osmotic swelling medium (Hypo-10, Scandinavian IVF Science) and the percentage of HOS-positive and -negative spermatozoa was assessed using an inverted microscope equipped with phase optics (DMIRB, Leica, Bensheim, Germany). Sperm solutions were further processed for immunofluorescent analysis and, when enough spermatozoa were present, for Western blot and slot blot analysis.

2.2. Isolation of soluble sperm proteins and Western/slot blot analysis

For Western and slot blot analysis of oscillin, sperm cytosol was prepared from 10×10^6 spermatozoa. Spermatozoa were washed once in D-PBS and then dissolved in 20 μl of D-PBS, plunged into liquid nitrogen and subjected to two freeze-thaw cycles. Samples were immediately centrifuged at $16\,000 \times g$ in a microfuge (Biofuge, Heraeus, Osterode, Germany) for 10 min, followed by a further clarification of the supernatant for another 5 min. With this protocol we saw no major difference in oscillin recovery when compared to the more commonly applied ultraspeed centrifugation at $100\,000 \times g$. This protocol suited best the isolation of oscillin from semen samples of males with oligoasthenozoospermia (OAT), where only a low number of spermatozoa is available for cytosol preparation on a small scale.

For Western blot analysis, an equal amount of 2×Laemmli buffer was added to the clear supernatant and soluble sperm proteins (SSP) from $1–5 \times 10^6$ spermatozoa were subjected to 10% SDS-PAGE [18] (Blue Vertical 100C, Bioproducts, Heidelberg, Germany). A reference lane of the gel was stained with Coomassie brilliant blue. Proteins were blotted onto nitrocellulose (Protran BA85, Schleicher and Schuell, Dassel, Germany) using a wet blot device (Blue Blot Wet 100, Bioproducts) and efficiency of transfer was assessed by staining

*Corresponding author. Fax: (49) (228) 287 4651/5795.
E-mail: vdven@mailer.meb.uni-bonn.de

PII S0014-5793(98)00107-0

the nitrocellulose with Ponceau S (Sigma, St. Louis, MO, USA). Blots were blocked in 5% skim milk powder (Merck, Darmstadt, Germany) in TBST (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) for at least 2 h and incubated with diluted primary antibody (Ab126, a rabbit anti-oscillin antibody, 1:500) overnight, followed by alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG, 1:5000; Sigma). The rabbit Ab126 anti-oscillin antibody was raised against the C-terminus region of the cloned hamster oscillin sequence ([13], Parrington and Lai, unpublished). Alkaline phosphatase detection was performed as described [19]. For slot blot analysis, sperm cytosol from 1×10^5 and 1×10^4 spermatozoa was diluted in 100 μl TBS and blotted onto nitrocellulose using a slot blot device (Hoefer, Pharmacia, Freiburg, Germany) and oscillin detection was performed using Ab126 as described above.

2.3. HOS test and immunofluorescent localization of oscillin

Immunocytochemistry was performed using the J4 mouse monoclonal antibody to hamster sperm 33 kDa oscillin protein as described earlier [13], except that 20–50 μl of sperm suspension was subjected to the HOS test for 10 min prior to the addition of fixative. The HOS test was performed as previously described [20]. For the primary antibody reaction, the J4 monoclonal anti-oscillin antibody (diluted 1:300) was added to the fixed spermatozoa, together with a mouse anti-acetylated tubulin antibody (1:150; Sigma). Unbound primary antibodies were washed off with D-PBS and remaining bound antibodies were detected using a fluorescein-conjugated goat anti-mouse secondary antibody (1:100; Sigma). After five more washing steps, another tubulin antibody (anti-β-tubulin conjugated to Cy3, 1:150; Sigma) was applied for 30 min. The preparations were washed again and the DNA was counter-stained with Hoechst 33342 (Sigma). Immunofluorescence microscopy was performed using an inverted microscope (DMIRB, Leica) equipped with a 100× fluotar objective and appropriate filter sets for UV and combined blue/green excitation. For each patient at least 200 randomly selected spermatozoa were evaluated. The simultaneous visualization of oscillin fluorescence (localized to the sperm head; in green) and tubulin fluorescence (localized to the sperm tail; in yellow) facilitated the correlation of HOS reactivity and localization of oscillin. HOS-positive spermatozoa showed a swelling of the sperm tail in the hypo-osmotic medium and were further classified as described [16]. The swelling of the sperm tail, indicative of the presence of intact membranes, was easily detected by tubulin fluorescence. HOS-negative spermatozoa showed no reaction at all due to damage of the sperm membrane.

3. Results

3.1. Presence of oscillin in semen samples from different males

In a first experiment, we investigated whether human spermatozoa contain a protein which could be detected by an antibody, Ab126, that was raised against a peptide that was specific to the C-terminal region of the cloned hamster oscillin sequence ([13], Parrington and Lai, unpublished). As shown in Fig. 1A, SDS-PAGE and Western blot analysis revealed the presence of a protein which migrated at an apparent molecular weight of 33 kDa. This is identical to the 33 kDa oscillin band previously detected in hamster sperm cytosol [13], indicating it was most likely the human homologue of hamster sperm 33 kDa oscillin. Using Ab126, we then employed slot blot analysis to investigate the presence of oscillin in human semen samples from four different individuals. These individuals were selected to represent a range of different degrees of membrane-intact spermatozoa. The degree of membrane-intact spermatozoa in different samples was first determined using the HOS test and this showed 70%, 40%, 25% and 2% HOS-positive spermatozoa. Fig. 1B shows the overall presence of oscillin in individual samples. Although there is a minor loss of oscillin concentration, those with 2% and 25% intact spermatozoa had only slightly less oscillin than samples with 40% or 70% intact spermatozoa. These results were verified in triple runs and were consistent. From these data we concluded that, although oscillin is a soluble sperm protein, it

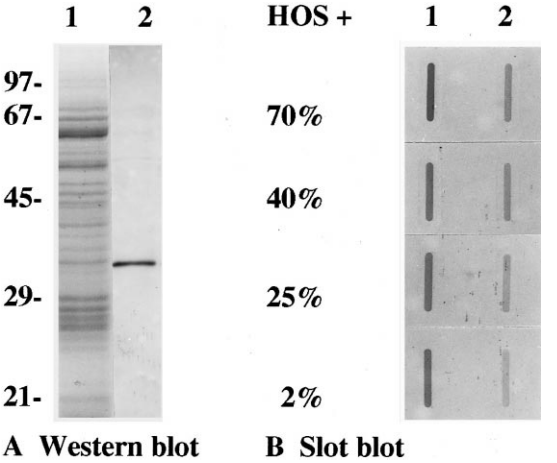

Fig. 1. Presence of oscillin in human spermatozoa. Lane 1 shows Coomassie-stained human sperm cytosol proteins from 5×10^6 spermatozoa. Using Western blot analysis of cytosol from 1×10^6 spermatozoa, a single band with a molecular weight of 33 kDa was detected with an anti-hamster oscillin antibody (lane 2) (A). The amount of oscillin detected by slot blot analysis and immunocytochemistry from semen samples with 70%, 40%, 25% and 2% membrane-intact (HOS-positive) spermatozoa shows no gross differences. Cytosol from 1×10^5 spermatozoa was applied to the left row (1) and from 1×10^4 to the right row (2) (B).

Table 1
Evaluation of HOS reactivity, localization of oscillin and fertilization rate in 13 patients

Patient	HOS-positive spermatozoa (%)	HOS-positive spermatozoa showing equatorially localized oscillin (%)	HOS-negative spermatozoa showing equatorially localized oscillin (%)	Fertilized oocytes (%)
Os1/97	15	91	16	100
Os2/97	45	80	4.5	77.8
Os3/97	65	67	20	64.7
Os4/97	40	79	15.8	68.7
Os5/97	33	66.7	22	63.6
Os6/97	45	63	10	55.2
Os7/97	72	58.8	10	75
Os8/97	54	36.6	18	57.1
Os9/97	60	65.4	19	75
Os10/97	70	70.8	10	85.7
Os11/97[a]	99	61	n.d.	28.6
Os12/97[a]	97	56	n.d.	31.6
Os13/97[a]	98	71.4	n.d.	63.2

[a]For these patients only motile spermatozoa prepared by swim-up technique were analyzed.

is likely to be retained in the sperm head, independent of the integrity of the sperm plasma membrane as determined by the HOS test.

3.2. Localization of oscillin in relation to sperm membrane integrity

We next assessed the subcellular localization of oscillin in HOS-positive versus HOS-negative spermatozoa in semen samples from different males. For that purpose we performed a simultaneous immunofluorescent detection of oscillin and tubulin in order to correlate the type of HOS reactivity and the localization pattern of oscillin. In general, we identified two predominant types of oscillin localization, namely, (i) spermatozoa with a bright oscillin fluorescence at the equatorial segment (Fig. 2A,B) and (ii) spermatozoa with a diffuse, sometimes spotted fluorescent pattern at the pre-equatorial part of the sperm head (Fig. 2C–F). Both staining patterns were observed in all the different types of HOS-reacted spermatozoa and the examples given illustrate HOS-positive spermatozoa (type G: Fig. 2A,C; type B: Fig. 2B,D), as well as HOS-negative spermatozoa (type A: Fig. 2E,F) (equatorial localization for type A not shown).

The evaluation of 10 different semen samples derived from subfertile males with oligoasthenoteratozoospermia (OAT) is shown in Table 1 (Os1/97–Os10/97). For all semen samples, the portion of HOS-negative spermatozoa with an equatorial localization of oscillin was 10–22% and over 80% of HOS-negative spermatozoa showed a diffuse staining of the sperm head (compare Fig. 2E,F). For HOS-positive spermatozoa we observed a high variability among individual patients. In one patient (Os1/97) 91% of HOS-positive spermatozoa showed an equatorial oscillin fluorescence, whereas in most patients 55–80% of HOS-positive spermatozoa presented with equatorial oscillin and in one case (Os7/97) this was only 36%. Also, the presence of a high or a low percentage of HOS-negative spermatozoa in the whole ejaculate was not a predictor of the quality of the localization of oscillin in HOS-positive spermatozoa. In all samples we could detect HOS-positive and HOS-negative spermatozoa which showed no signals after immunostaining. When we carried out a similar examination on motile spermatozoa from three normozoospermic samples (Table 1: Os11/97–Os13/97), we found 71%, 61% and 56% of HOS-positive spermatozoa with an equatorial localization of oscillin. In one sample (Os13/97) we investigated the proportion of HOS-positive spermatozoa with an equatorial oscillin localization at certain time intervals. No differences were found for spermatozoa which were fixed immediately after sperm washing or 1 or 24 h later (data not shown).

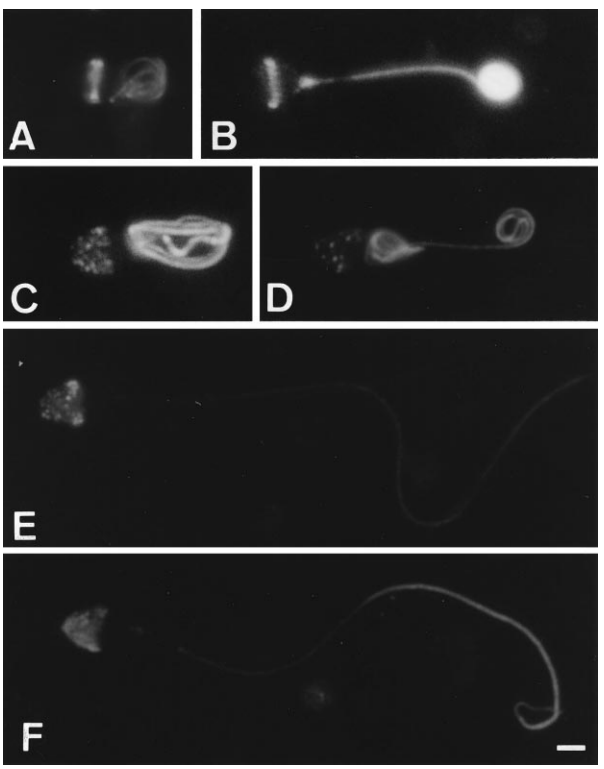

Fig. 2. Immunofluorescent localization of oscillin in the sperm head and tubulin in the sperm tail in HOS-positive and HOS-negative spermatozoa. HOS-positive spermatozoa displayed either an equatorial localization of oscillin (A, B) or a diffuse distribution within the sperm head (C, D). HOS-negative spermatozoa had oscillin distributed over the sperm head (E, F). HOS reactivity was assessed simultaneously by evaluation of the shape of the sperm tail which was stained with anti-tubulin antibodies. HOS-positive spermatozoa are shown in A–D (type G: A, C; type B: B, D) and HOS-negative in E, F (type A: E, F). Identification of different HOS types was accomplished according to the morphological patterns described in the literature [16]. Bar represents 5 μm.

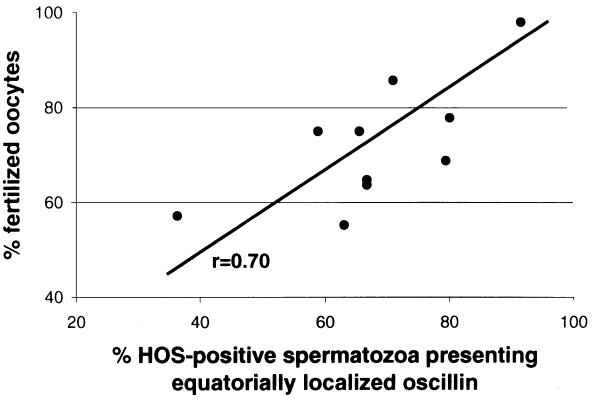

Fig. 3. Relationship between the % HOS-positive spermatozoa presenting equatorially localized oscillin and the % fertilized oocytes. The correlation coefficient is $r = 0.70$. The data are shown for 10 couples which were treated with ICSI due to male subfertility caused by OAT.

3.3. Relationship between the local distribution of oscillin and fertilization outcome

In a further evaluation, we assessed for OAT patients the relationship between the percentage of HOS-positive spermatozoa with an equatorial localization of oscillin and the fertilization rates after ICSI (see also Table 1). We found a high positive correlation coefficient ($r = 0.70$) which was statistically significant ($P < 0.05$). The graphical evaluation is shown in Fig. 3.

4. Discussion

4.1. Human spermatozoa contain oscillin

In this study we have shown that human sperm cytosol contains a 33 kDa protein that is reactive with an anti-hamster oscillin antibody. These data, and also the proven capacity of human sperm cytosol to induce Ca^{2+} oscillations

in oocytes from various species, including humans [10,15], strongly suggest that human spermatozoa also contain oscillin. From our current understanding of egg activation, we can conclude that any sperm-derived factor which is responsible for inducing Ca^{2+} oscillations in the oocyte will be a prerequisite for successful IVF, and also for successful ICSI treatment. Consequently, it has been suggested that the absence or reduction of oscillin might be a possible explanation for some, albeit very rare, cases of human male infertility [13]. One such example is globozoospermia, where activation failures after injection of round-headed spermatozoa have been reported [21,22]. Recently Rybouchkin et al. [23] have shown that oscillin shows a reduced and weak staining in the cytoplasm of spermatozoa from these patients.

Low activation and fertilization rates have been reported after injection of immotile spermatozoa [24]. It was suggested that oscillin might be absent in immotile spermatozoa due to membrane damage and subsequent loss of oscillin [15]. We have investigated the presence of oscillin in samples with a high or low degree of non-viable spermatozoa and found only minor differences with regard to the presence of oscillin, which were not correlated with the loss of membrane integrity. However, we found clear differences in the subcellular localization of oscillin in these spermatozoa, simply by combining immunocytochemistry and the HOS test [16]. We found that in $>80\%$ of HOS-negative spermatozoa, oscillin is no longer localized to the equatorial region. It is not clear how this non-equatorial region localization of oscillin would explain why these spermatozoa are not capable of activating oocytes efficiently after ICSI. However, it is tempting to speculate that impaired sperm membrane integrity promotes the diffusion of oscillin throughout the sperm head and that, as a consequence, oscillin's prospective role in releasing Ca^{2+} and egg activation is impaired. At this stage, however, other explanations cannot be ruled out. Nevertheless, our results may indicate that there is some importance in the equatorial localization of oscillin with regard to the activation capacity of the sperm cell. In addition, the presence of oscillin in HOS-positive but immotile sperm may help to explain the success in achieving pregnancies following intracytoplasmic sperm injection (ICSI) with such spermatozoa [20,25]. Therefore, it should be mandatory in cases of asthenozoospermia to perform ICSI only with viable spermatozoa. This can be easily achieved by using commercially available HOS test media of pharmaceutical grade which do not interfere with subsequent embryonic development [20].

So far we do not know the role of oscillin when less mature forms of germ cells, like spermatids, are injected. This and the appearance of oscillin during spermatogenesis is under investigation.

4.2. The importance of oscillin

It has been reported that following IVF or ICSI a high percentage of seemingly unfertilized oocytes were actually penetrated by a sperm cell but failed to undergo activation [5,6,15]. When such oocytes were injected with human sperm cytosol, a substantial proportion underwent activation and displayed two or more pronuclei [6,15]. The authors concluded that failed fertilization was due to a lack of oocyte activation. The data presented in this paper might help to explain the phenomenon of failed fertilization. In the reports cited above, motile spermatozoa had been used for IVF or ICSI, and it is known that motile spermatozoa are always HOS-positive. We analyzed the localization of oscillin in HOS-positive spermatozoa. In all patients who were investigated thus far, we were able to demonstrate the presence of a subset of HOS-positive spermatozoa which no longer showed an equatorial localization of oscillin. The importance of this became evident when we compared the fertilization rates after ICSI and the percentage of HOS-positive spermatozoa with an equatorial localization of oscillin for 10 OAT patients. We found a statistically significant correlation ($r=0.70$; $P<0.05$) between fertilization rates and the equatorial localization of oscillin. We did not carry out a similar evaluation for IVF patients, as the degree of variation involved in the many events of fertilization is likely to be too high for this technique to be correlated with just one aspect of sperm function.

We are aware that it is impossible to trace failed fertilization to a single event, especially as our observations are based on ICSI where the injection procedure itself might have been deleterious, or where the cytoplasmic maturity of the oocyte may not be optimal. However, given an inconsistent localization of a potential activating factor in motile, membrane-intact spermatozoa, our data raise the possibility that it will be impossible to achieve a fertilization rate in ICSI of 100%. The only reports of more than 90% fertilization rates have been published by Tesarik and Sousa [26] who combined ICSI treatment and subsequent calcium ionophore stimulation. In view of our results, these high fertilization rates might be explained by the fact that these authors have overcome the lack of sperm factor activity in those spermatozoa presenting an aberrant oscillin localization. However, it is clear that the activation stimulus provided by a calcium ionophore or most other parthenogenetic agents is unable to mimic the action of a sperm factor in causing Ca^{2+} oscillations [7]. An improvement in fertilization rates after ICSI may be more effectively achieved by introducing more of an active agent that triggers Ca^{2+} oscillations.

When the ICSI technique was first introduced, it was questioned whether the calcium that was introduced during the injection procedure holds the key to success [27]. We can now extend this question further and ask: Does oscillin hold the one and only key to success in ICSI and if so, what does the lock for this key look like?

Acknowledgements: The authors wish to thank Mrs. Przybilka for expert photographic art work and Mr. Hilgers (Leica, Germany) for his constant support and loan of microscopic equipment. We thank Dr. K. van der Ven for her comments on the manuscript. The expert technical assistance of the IVF laboratory team at the University of Bonn is acknowledged. This work received support by Bonfor (Grant 103/06 to M.M. and Grant 103/10 to M.M. and H.v.d.V.).

References

[1] Palermo, G.D., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Lancet 340, 17–18.
[2] Van Steirteghem, A.C., Liu, J., Joris, H., Nagy, Z., Janssenswillen, C., Tournaye, H., Derde, M., van Assche, E. and Devroey, P. (1993) Hum. Reprod. 8, 1055–1060.
[3] Van Steirteghem, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smitz, J., Wisanto, A. and Devroey, P. (1993) Hum. Reprod. 8, 1061–1066.
[4] Palermo, G.D., Schlegel, P.N., Colombero, L.T., Zaninovic, N., Moy, F. and Rosenwaks, Z. (1996) Hum. Reprod. 11, 1023–1029.
[5] Van Blerkom, J., Davis, P. and Merriam, J. (1994) Hum. Reprod. 9, 2381–2388.

[6] Dozortsev, D., Rybouchkin, A., De Sutter, P., Qian, C. and Dhont, M. (1995) Hum. Reprod. 10, 403–407.
[7] Swann, K. and Ozil, J.P. (1994) Int. Rev. Cytol. 152, 183–222.
[8] Stice, S.L. and Robl, J.M. (1990) Mol. Reprod. Dev. 25, 272–280.
[9] Swann, K. (1990) Development 110, 1295–1302.
[10] Homa, S.T. and Swann, K. (1994) Hum. Reprod. 9, 2356–2361.
[11] Meng, L. and Wolf, D.P. (1997) Hum. Reprod. 12, 1062–1068.
[12] Wu, H., He, C.L. and Fissore, R.A. (1997) Mol. Reprod. Dev. 46, 176–189.
[13] Parrington, J., Swann, K., Shevchenko, V.I., Sesay, A.K. and Lai, F.A. (1996) Nature 379, 364–368.
[14] Swann, K. and Lai, F.A. (1997) BioEssays 19, 371–378.
[15] Palermo, G.D., Avrech, O.M., Colombero, L.T., Wu, H., Wolny, Y.M., Fissore, R.A. and Rosenwaks, Z. (1997) Mol. Hum. Reprod. 3, 367–374.
[16] Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G. and Zaneveld, L.J.D. (1984) J. Reprod. Fertil. 70, 219–228.
[17] Montag, M., Van der Ven, K., Ved, S., Schmutzler, A., Prietl, G., Krebs, D., Peschka, B., Schwanitz, G., Albers, P., Haidl, G. and van der Ven, H.H. (1997) Hum. Reprod. 12 (in press).
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Ved, S., Montag, M., Schmutzler, A., Prietl, G., Haidl, G. and van der Ven, H.H. (1997) Andrologia 29, 241–242.
[21] Battaglia, D.E., Koehler, J.K., Klein, N.A. and Tucker, M.J. (1997) Fertil. Steril. 68, 118–122.
[22] Rybouchkin, A., Dozortsev, D., Pelinck, M.J., De Sutter, P. and Dhont, M. (1996) Hum. Reprod. 11, 2170–2175.
[23] Rybouchkin, A., Swann, K., Parrington, J., Lai, F.A., De Sutter, P. and Dhont, M. (1997) Hum. Reprod. (Abstract Book 1) 12, 36.
[24] Nijs, M., Vanderzwalmen, P., Vandamme, B., Segal-Bertin, G., Lejeune, B., Segal, L., van Roosendaal, Z. and Schoysman, R. (1996) Hum. Reprod. 11, 2180–2185.
[25] Casper, R.F., Cowan, L., Meriano, J.S., Lucato, M.L. and Jarvi, K.A. (1996) Fertil. Steril. 65, 972–976.
[26] Tesarik, J. and Sousa, M. (1995) Fertil. Steril. 63, 343–349.
[27] Edwards, R.G. and Van Steirteghem, A.C. (1993) Hum. Reprod. 8, 988–989.