Scanning electron microscopy of the zona pellucida of human oocytes during intracytoplasmic sperm injection (ICSI)

P.Schwartz¹, C.Magerkurth¹ and H.W.Michelmann²,³

¹Department of Anatomy and ²Department of Obstetrics and Gynaecology, University of Goettingen, Robert-Koch-Str 40, 37075 Goettingen, Germany

To whom correspondence should be addressed

During intracytoplasmic sperm injection (ICSI) approximately 10% of all injected oocytes degenerate. The reason for this process is unknown. It has been speculated that the mechanical procedure of the insertion of the ICSI needle induces injuries to the zona pellucida which lead to the death of the cell. By scanning electron microscope (SEM), it could be shown that the surface structure of mature oocytes is extremely elastic so that the injection needle penetrates the zona pellucida without destroying the mesh-like or more compact surface. No tissue pieces or zona fragments were detectable. After a culture time of 15 min the penetration site on the zona was no longer easily visible. We believe that oocyte degeneration is not caused by the penetration of a glass needle into the ooplasm but by an injury to the meiotic spindle or by an excessive dose of fluid [polyvinylpyrrolidone (PVP) or medium] during sperm injection.

Key words: intracytoplasmic sperm injection/ICSI/scanning electron microscope/SEM/zona pellucida

Introduction

ICSI is the injection of a single spermatozoon into the ooplasm under a microscope with the help of a micromanipulator. Because this technique requires only one living spermatozoon, it can be performed with immotile, tailless or morphological aberrant spermatozoa, which may or may not have undergone the acrosomal reaction (Tournaye et al., 1994). It is possible to achieve fertilizations even with immature spermatozoa which, however, must at least have developed to the stage of late spermatid. When ICSI is used there is no longer a correlation between the severity of the andrological subfertility and the fertilization and pregnancy rates. Furthermore, it is irrelevant whether the spermatozoa are from fertile or infertile men (Tournaye et al., 1994).

Nevertheless, many people have reservations about this method of 'artificial' fertilization, mainly because they believe that bypassing the barrier of the zona pellucida eliminates a natural selection tool. However, the zona pellucida does not in any way serve as a selection barrier against genetically aberrant spermatozoa. A selection against genetically based defects occurs after fertilization during both embryonic and fetal development.

Although ICSI does not result in an increase in the number of genetically based diseases or infertile males in the next generation (Engel and Schmidt 1995), it has been shown that approximately 10% of oocytes show signs of degeneration after microassisted insemination. Therefore it was of interest to discover what happens to the zona pellucida during and after the insertion of the micro needle. Does this mechanical procedure, if improperly carried out, induce severe injuries which then lead to the degeneration of the oocyte? It was the aim of this study to determine the reaction of the zona pellucida to the injection needle by scanning electron microscopy (SEM) pictures and to analyse the time span of the self-healing procedure.

Material and methods

A total of 26 mature oocytes which remained unfertilized following ICSI at the Department of Obstetrics and Gynaecology, Study Group of Reproductive Medicine, University of Goettingen were used for SEM 48 h after oocyte retrieval. The samples had been obtained from hyperstimulated cycles. Hormonal stimulation was performed using human menopausal gonadotrophin (HMG) and human chorionic gonadotrophin (HCG). HMG was administered from cycle day 3 and continued until the dominant follicle reached a diameter of at least 16 mm, and was followed by HCG administration (10 000 IU) 36 h before follicular puncture took place.

Micromanipulation procedure

During the routine ICSI procedure all oocytes were injected at a position in which the polar body was at 6 o'clock. For research purposes those oocytes which remained unfertilized were again placed in microdroplets under oil and reinserted. This time, however, the polar body was located at 12 o'clock to minimize the possibility of puncturing the same area of the oocyte twice. Micromanipulation was performed using a micromanipulator built by the Luigs & Neumann Company, Ratingen, Germany (Figure 1). It is not a hydraulic system but is run by electronic step motors which are controlled by one multifunction control panel on each side of the microscope. All functions can be used without changing hand position. Thus, the use of different joysticks and the screw of the microsyringe is not necessary. The movement of the eight-step motors (four on each side) is controlled by a trackball with a technology similar to that of ultrasound machines. By using a 'home-function' the manipulators store their last position and move out of the working area. Later, by reactivating this function, the capillaries return to the stored position with total precision.

Preparation of oocytes for SEM

This was performed at different time points:

1. During the insertion of the microneedle
Results

The zona pellucida surface of the investigated oocytes showed a variety of morphological structures. Despite the fact that all oocytes were mature (stage of the first polar body) and were treated in the same way (denuding of the cumulus mass with hyaluronidase (Van Steirteghem et al., 1995) the outermost surface of the zona showed either numerous fenestrations and a spongy appearance (Figures 2–6) or revealed a more compact structure with small fenestrations which may have led to a completely smooth surface (Figures 7–9).

Figure 2 demonstrates how the microneedle, which had an outer diameter of 7 μm, penetrated the zona pellucida from which the surrounding granulosa cells had been removed. As already demonstrated from photographs taken by light microscope, the zona is extremely elastic and can be pushed by the tip of the needle a considerable depth into the ooplasm before breaking. After the needle has penetrated the oocyte the zona bounces back and tightly surrounds the needle in a crater-like structure (Figures 3 and 4). To determine the subsequent response of the zona pellucida to puncture, the oocyte was cultured for 5 min with the needle remaining inserted into the ooplasm. During this time the very elastic material of the zona totally surrounded the shank of the needle, forming a ring-like structure. Leaking ooplasmic material might also have contributed to this structure (Figures 5 and 6). By the time the needle was retracted the zona contained a hole with clean sharp edges. No destroyed tissue or zona
Scanning electron microscopy during ICSI

Figure 5. Appearance of the zona pellucida 5 min after needle penetration

Figure 6. Five minutes post-injection ring-like structure of the zona pellucida formed around the shank of the needle.

Figure 7. Appearance of the zona pellucida immediately following retraction of the needle. Oocytes in Figures 7 and 8 were of compact appearance with small fenestrations.

...fragments were detectable (Figure 7). After a culture time of 15 min in Ham's F-10 medium, the penetration site in either a spongy or a compact zona was barely detectable. Even the fenestrations of the spongy zona type had become fully reconstructed. Only a small dent indicated the point where the injection needle had penetrated the outer layer of the zona (Figure 8). The zona morphology at the injection site had been reconstructed, with no lesions or further injuries, and resembled the zona of a non-injected oocyte (Figure 9).

Discussion

The injection of a 7 μm glass needle into an oocyte of 100–150 μm in diameter appears rather destructive. However, the zona is highly resilient because of its mesh-like structure. Therefore the needle can be pushed forward through the oocyte until it nearly touches the inner part of the zona pellucida of the opposite side. The moment the zona is punctured the needle forms a clearly visible injection canal direct through the centre of the oocyte. This is the cavity in which the single spermatozoon is injected. After injection the needle is retracted out of the ooplasm.

Although the oocyte is mechanically affected to a great extent, the extraordinarily good results of ICSI with regard to fertilization and pregnancy rates make it obvious that this...
damage is of minor consequence for the survival and developmental potential of the oocyte. However, the ability of zona pellucida to heal itself quickly is of interest.

To date, only a few SEM observations on the human zona pellucida before and during fertilization have been made. The zona pellucida is very difficult to preserve for SEM examination, since it is a highly hydrated structure which might be deformed by dehydration or drying. In fact, the material of the zona, due both to the presence of a large amount of surrounding extracellular amorphous material and to the hydrophilic peculiarity of its components, is particularly hard to define through SEM observations without appropriate techniques to unmask and stabilize its components.

A modified technique which enables tiny specimens to be prepared for scanning electron microscopy has made it possible to obtain very good quality photos of objects as small as human oocytes at all stages of the ICSI procedure. These photographic images have an extremely high resolution factor which renders visible components that are in a nanometer order of magnitude. All pictures obtained at different stages of the ICSI procedure clearly show that the mechanically created injury to the zona caused by the injection needle is only very minor. The ooplasm and the surface of the zona pellucida are extremely flexible. This allows the funnel-like injection channel to close immediately following the withdrawal of the micro-needle. Fifteen minutes later, the point of injection is no longer visible. Because this time span is too short for any growth processes, the restored mesh-like outer structure of the zona pellucida must be due to its extreme flexibility.

As reported by Van Steurteghem et al. (1995), the injection of 29,415 oocytes led to the degeneration of 10.8%. We believe that any degeneration is not the result of damage to the zona pellucida but either of damage to the meiotic spindle which is located close to the first polar body or of an excessive dose of fluid during sperm injection. Furthermore a short hormonal stimulation with low serum oestradiol concentrations may result in oocytes with fragile membranes and a higher rate of oocyte damage after ICSI (Palermo et al., 1996). This hypothesis requires further investigations by scanning and transmission electron microscopy.

References