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Review

In Vivo and *In Vitro* Decondensation of Human Sperm and Assisted Reproduction Technologies

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Abstract. *For a successful fertilization and pronucleus formation to occur, not only a properly condensed sperm nucleus, but also decondensation ability in the oocyte is important. The sperm abnormalities causing failure of sperm decondensation in the oocyte are unrecognizable by conventional semen analysis and different methods are used. The chromatin decondensation ability of the human spermatozoa in vivo and in vitro and its association with infertility and assisted reproduction techniques (ART) are clearly discussed in this paper. The factors affecting the decondensation ability of the human sperm are also mentioned. It is suggested that the methods currently used to assess sperm chromatin decondensation are of limited value in assessing fertilization and pregnancy rates after ART.*

Spermiogenesis results in the segregation and packaging of the haploid sperm nucleus into the compact and inactive nucleus, in addition to the loss of most cytoplasmic organelles, with the exception of mitochondria which are essential for motility (1). In a successful fertilization, on entering the ooplasm, the spermatozoon undergoes a series of processes, namely chromatin decondensation, pronucleus formation, DNA replication, chromosome condensation and entry into the mitotic phase (2). During the process of fertilization, matured sperm nucleus is thought not to be damaged because of its stability to physicochemical exposure (3-5).

Fertilization, in humans, is the process of the union of the parental genomes of markedly different chromatin within the activated oocyte. For successful fertilization and pronucleus formation to occur, not only a properly condensed sperm nucleus, but also decondensation ability in the oocyte is important. Defective chromatin decondensation has been found to be associated with infertility (6-9). Structural or biochemical defects in chromatin packaging during spermatogenesis are associated with failure in sperm decondensation in the oocytes (10). In this review, the studies performed to investigate the prevention or the delay of chromatin decondensation in relation to infertility, and assisted reproduction techniques are clearly discussed. Particular emphasis is given to the *in vivo* and *in vitro* chromatin decondensation ability of human sperm and their relation to *in vitro* fertilization.

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In vivo condensation of human spermatozoa

During spermiogenesis, the haploid phase of spermato-genesis, inside the seminiferous tubes of the testis, the transition proteins and protamines are responsible for sperm chromatin condensation (11, 12). First, the somatic histones are replaced with transition proteins (TP1 and TP2) in haploid round spermatids and then in elongating spermatids, the protamines (P1 and P2) replace transition proteins (13, 14). Protamines contain a large amount of cysteine, initially present as thiols (15). During sperm maturation and the passage through the epididymis, the thiols (-SH) of cysteines in the protamine molecules are oxidized to disulphide bonds (S-S) (16, 17). The compacted DNA is held in place by disulfide bonds, formed by the oxidation of sulphhydryl groups present on protamines (18-20). After ejaculation, chromatin stability is further increased by seminal plasma (15). Zinc, which is present in the prostatic fluid, seems to play an important role in this process, entering into the chromatin and binding to free thiol groups, further increasing the stability (15, 21-23).

In vivo decondensation of human spermatozoa

During natural fertilization, following fusion of the two cell membranes, the naked sperm nucleus is incorporated into the ooplasm. Since the oocyte is arrested prior to sperm incorporation and also the sperm enters the oocyte with a highly condensed and inactive nucleus, sperm-oocyte membrane fusion leads to decondensation and remodelling of the parental genomes (1). The oocyte is simultaneously activated following sperm entrance and sperm membrane permeabilization (1). The maturation of the nuclear and cytoplasmic components of the oocyte are required for these processes. One of the criteria for oocyte cytoplasmic maturation is the ability to decondense the sperm chromatin following sperm entrance and to transform it to a male pronucleus (24). After sperm-oocyte fusion, demembranated sperm in the ooplasm becomes accessible to ooplasmic factors, especially thiol-reducing agents (25). Glutathione, present in the oocyte, is a disulphide bond reducer (26). The decondensation of the DNA utilizes glutathione to reduce the protamine disulphide bonds (25, 26). Disulphide bond reduction in protamines is the first step of human sperm nuclei decondensation. As the links are cleaved, the coiled chromatin loops unfold and enable egg factors to further decondense the chromatin (27). The decondensation of sperm nucleus is considered to occur under the influence of a cytoplasmic factor called sperm decondensation factor (SDF) (28). The synthesis of SDF is induced during normal oocyte maturation (29). Although the role and nature of SDF in the oocyte cytoplasm is obscure, experimental evidence suggests that it appears after germinal vesicle breakdown in most mammals (30). Oocyte chromatin and spindle formation are governed by maturation-promoting factor (MPF) (31-34). High levels of MPF activity are present in the cytoplasm of MII oocytes (35-37). The decrease in MPF activity triggered by sperm entry supplies the cytoplasmic conditions required for sperm decondensation and also female pronucleus formation (38-41). The decondensation of the sperm nucleus is completed after the protamines are replaced with histones of the oocyte (27, 42-46).

In vitro decondensation of human sperm

Evaluation of condensation and decondensation of the human sperm nuclei. In order to evaluate the chromatin of the spermatozoa, many dyes and fluorochromes have been used. Some of the dyes for the evaluation of sperm nucleus condensation are tolidine or aniline blue, acridine orange, ethidium bromide and propidium iodide (47). The analysis of the quantity of fluorochromes (acridine orange, ethidium bromide, propidium iodide) bound by the sperm nucleus, using either fluorescence microscopy (48) or fluorescence flow cytometry (49, 50), is believed to reflect the degree of nucleus condensation (51, 52). The degree of chromatin decondensation can be evaluated by phase-contrast microscopy (53, 54), electron microscopy (8) or by evaluating the uptake of intercalating compounds (55, 56) after treating the spermatozoa with different chelating, *e.g.* EDTA, or disulphide-reducing agents, *e.g.* dithiothreitol (DDT) (18, 57), or anionic detergents, *e.g.* sodium dodecyl sulphate (SDS).

In the presence of physiological concentrations of heparin and glutathione, human sperm can be decondensed *in vitro* (58, 59). Heparin receptors are present on the sperm plasma membrane (60-62). Destabilization of the sperm plasma membrane, after heparin binding to its receptor, has been proposed as the mechanism allowing the incorporation of glutathione-like molecules into the sperm nucleus (60). However, the ability of heparin to decondense human sperm *in vitro* is still under debate. Romanato *et al.* (63) suggested that the decondensing ability of heparin *in vitro* is related to the structural characteristics of the molecule and, among different glycosaminoglycans tested in their study, only heparan sulphate, a structural analogue of heparin, possessed sperm nuclear-decondensing ability *in vitro*. Morover, they also mentioned that heparan sulphate, but not heparin, has been found in the oocyte-cumulus complex (64-66).

In vitro decondensation of the human sperm and the oocyte-related factors affecting sperm decondensation. The fusion between the plasma membranes of the sperm and oocyte starts a cascade of events, which initiate the metabolic activation of the arrested oocyte (1). The activation of the human oocyte after intracytoplasmic sperm injection (ICSI) was found to be induced by spermatozoon-associated oocyte activating factor (67). Furthermore, the decondensation of the sperm nucleus requires SDF found in the ooplasm of the oocyte (28). The evaluation of these processes by Dozortsev *et al.* (30) showed that the swollen sperm nuclei, found in unfertilized oocytes after ICSI, was probably an initial step of the true decondensation, which is not dependent on oocyte activation (30). This initial swelling is believed to be a reaction to SDF (68). However, the complete decondensation of the human sperm nucleus after ICSI was reported as oocyte activation-dependent (30). Tesarik *et al.* (69) suggested that penetration of the oocyte without a following decondensation of the sperm nucleus may indicate the inability of the ooplasm to promote its decondensation.

The decondensation process depends on time, temperature and pH (70). In mammalian oocytes, the sperm decondensation activity lasts for a short time-span after activation (68, 71, 72). Presumably, the human oocyte has also a time-limited decondensation ability (73). The decondensation of the human sperm starts as early as 60 minutes after insemination (74). The sperm nuclear

decondensing activity of the oocytes may be exhausted after a particular time interval after the onset of sperm-induced or parthenogenic activation (68, 71). Goud *et al.* (2), who performed ICSI of human spermatozoa into hamster oocytes, reported that when the oocyte is activated parthenogenically, its sperm nuclear decondensing factors may be exhausted and, as a result, sperm is either minimally or partially decondensed. ICSI of hamster oocytes with human spermatozoa results in high rates of failure in male pronucleus formation due to injection-related parthenogenic activation of the hamster oocytes, which interferes with sperm nuclear decondensation (2). Another study on hamster oocytes ICSI with whole human spermatozoa confirmed the low decondensation rate (75).

The presence of oocyte chromosome condensation factors may cause the sperm nucleus to undergo chromatin condensation prematurely (76). If the oocyte is not activated after IVF and sperm nucleus is present in the ooplasm, MPF activity induces the formation of premature chromosome condensation (PCC) (73). The incidence of premature chromosome condensation (PCC) in unfertilized oocytes was reported as ~28% after ICSI and ~10% after IVF (77). *In vitro* insemination of MI oocytes leads to PCC of male chromatin (78, 79), but intact sperm heads in the cytoplasm of MII-arrested oocytes were not described after *in vitro* fertilization (IVF) (80-82). After IVF, the arrest of sperm heads at a condensed and early PCC stage was suggested to be related to oocyte immaturity (83). On the other hand, others suggested that, after ICSI, the PCC in non-activated MII oocytes might be due to whole sperm entrance in ICSI cases (73).

In vitro decondensation of the human sperm and infertility and ART. The evaluation of sperm characteristics is the first step in male partner examination of an infertile couple. The clinical diagnosis and management of male infertility depends on the total sperm count, concentration, abnormal forms, motility and seminal factors. However, the sperm abnormalities causing failure of sperm decondensation in the oocyte are unrecognizable by conventional sperm analysis (8). The studies performed to evaluate the correlation of sperm parameters and sperm chromatin decondensation ability have different study design (85-88). The decondensation ability of the spermatozoa tested with SDS/DTT were found to be negatively correlated with the percentage of motility of spermatozoa, while concentration and morphology were not correlated (85, 87). The study performed by acridine orange staining also showed that there is no correlation between the chromatin decondensation and sperm concentration, sperm morphology or the percentage of spermatozoa with condensed chromatin (88). Incomplete chromatin decondensation was reported as independent of various causes of infertility, such as teratozoospermia, oligoospermia and asthenospermia (87).

Several authors suggested that defective chromatin decondensation can be found in some cases of infertility (6-9). Chromatin decondensation after SDS treatment or excess decondensation after SDS+EDTA at 45 minutes post-ejeculation is associated with infertility (53, 54, 56, 89, 90). The data in the literature confirms that sperm samples showing a decondensation of >70% with SDS/EDTA can fertilize the oocyte, but no fertilization takes place with a decondensation rate of <70% (91). The study performed with *Xenopus laevis* oocytes showed that men with asthenozoospermia decondense slowly and partially compared with normozoospermic men (92).

The participation of sperm decondensation in fertilization failure has been less well studied. Chromatin condensation is directly related to the capacity of sperm to fertilize the ovum (93). Human sperm, in which the chromatin is not completely condensed, fail to fertilize, even after injection into the ovum (94). Faulty protamine deposition during spermatogenesis is one of the major factors leading to abnormal chromatin packaging (9). Abnormal chromatin packaging can arise due to deficient histone to protamine replacement during spermatogenesis (95-97). Infertility patients more often show sperm chromatin anomalies related to the deposition of protamines (95, 96, 98). After ICSI, normal or only slightly abnormal chromatin may result in fertilization of the oocytes, but a high level of chromatin abnormality of spermatozoa may impede the completion or initiation of decondensation, even if the oocyte possesses the necessary mechanism to initate decondensation (9). Sakkas *et al.* (9) also mentioned that spermatozoa with abnormal chromatin could either fail to decondense due to physical or mechanical inability, or may fail to trigger regulatory mechanisms leading to sperm decondensation.

Chromomycin A3 fluorescence (CMA3), a guanine-cytosine specific fluochrome, is an indicator of the packaging quality of sperm chromatin which provides indirect visualization of protamine-deficient DNA (9). Normal males present a sperm parameter with a CMA3 fluorescence of less than 30% (9, 99). Sakkas *et al.* (9) assessed CMA3 fluorescence and the presence of DNA damage in the sperm of patients treated with ICSI and examined unfertilized oocytes to determine whether a relationship exits between failure of fertilization and sperm chromatin quality. The results showed that no difference exists in the ability to achieve fertilization when ICSI patients were seperated into two groups according to spermatozoa with CMA3<30% and endogenous nicks <10% and higher than these values (9). When unfertilized ICSI oocytes were examined, it was found that patients with CMA3 fluorescence of >30% and endogenous nicks in

>10% of their spermatozoa had a significantly higher number, 41.2 and 48.9%, respectively, of their unfertilized oocytes containing condensed spermatozoa (9). The data from recent studies showed that the fertilization rate is lower when oocytes are injected with sperm from samples with high CMA3 positivity (100, 101). In the study by Esterhuizen *et al.* (100), who examined 170 non-fertilized human oocytes obtained from 49 couples attending the IVF programme, it was reported that 45% of the oocytes inseminated with spermatozoa of elevated CMA3 fluorescence (>60% staining) did not show any signs of decondensation after IVF. In addition, they found that CMA3 staining >60% resulted in a 15.6-fold increase in the risk of decondensation failure relative to CMA3 staining <44%, while for CMA3 staining ≥44-59%, this increase was 11.5-fold (100). The sperm protamine deficiency, an anomaly of chromatin packaging which takes place during spermatogenesis, might affect decondensation of the chromatin. This anomaly of chromatin packaging is reported as one of the factors related to fertilization failure in assisted reproduction techniques (ART) (100).

Acridine orange reflects chromatin resistance to denaturation (102). When completely matured spermatozoa rich in disulphide bonds are treated with acridine orange, green fluorescence is emitted from the sperm nuclei. Studies in which the analysis of human sperm was performed using acridine orange staining reported inconclusive data. Hoshi *et al.* (103) reported that men who ejaculate >50% spermatozoa rich in disulphide bonds have a significantly higher fertilization ability in conventional IVF compared with men having a high proportion of spermatozoa poor in disulphide bonds. On the contrary, others suggested that acridine orange staining is still inconclusive regarding the fertilization potential of spermatozoa (104). A recent study (105) concluded that more accurate information on sperm nuclei can be obtained through diamide-acridine orange staining, than with acridine orange alone. The results of this study detected a significant positive correlation between the green-type increase ratio (percentage of green-pattern sperm after diamide-acridine orange staining/percentage of green-pattern sperm after acridine orange staining) and the fertilization rate in IVF (105).

Morover, damaged DNA of spermatozoa may contribute to the failure of sperm decondensation after ICSI (9). In the study performed on injected oocytes that remained unfertilized after ICSI, 50% of oocytes without pronuclear formation contained chromatin with damaged spermatozoa (106). The results of this study revealed that a significantly greater proportion of condensed spermatozoa in human oocytes had damaged DNA, compared to decondensed spermatozoa (24.7 compared to 5.9%, $p=0.002$) (106). In other studies, a correlation between abnormal chromatin packaging and DNA strand breaks has been shown to exist (97, 107).

Dozortsev *et al.* (73) evaluated the behaviour of sperm cells by analysing unfertilized and one-pronuclear oocytes following ICSI. These authors reported that successful sperm introduction into the ooplasm of mature MII oocytes is not sufficient for fertilization by ICSI because 93% of the unfertilized oocytes following ICSI were found to have evidence of the presence of spermatozoa inside the cytoplasm (73). Following ICSI, a spermatozoon is exposed to the ooplasm of the oocyte, in contrast to what happens during normal fertilization, in which the demembranated sperm enters the ooplasm. In the study by Goud *et al.* (2), who injected human sperm into hamster oocytes, it was suggested that a whole sperm cell may require some time to permeabilize its membranes *prior* to interaction between the decondensing factors and sperm nucleus and that this may exceed the time for which the oocyte decondensing factors are active following the start of parthenogenic activation. On the other hand, it was reported that the injection of demembranated sperm into hamster oocytes results in a high rate of decondensation (3, 108).

Intact sperm heads observed in puromycin-induced activated oocytes further suggest that the presence of intact sperm plasma membrane enables sperm decondensation factors to reach the nucleus (73). Succesful sperm injection followed by oocyte activation is not sufficient for male pronucleus formation, as the oocyte activation does not supply appropriate conditions in the ooplasm for the sperm membrane to be disrupted (73). Usually, touching the sperm tail with a needle *prior* to injection, known as the immobilization procedure, during ICSI, damages the sperm membrane (109, 110). This makes the sperm chromatin accessible for decondensing factors (73, 110). Sperm cells with an intact membrane usually fail to decondense in ICSI. As the sperm decondensation ability of the oocytes is time-limited, immobilization procedure-related damage of the sperm membrane is not sufficient for timely access of decondensing factors (73). Besides, human oocyte activation after ICSI requires spermatozoon-associated oocyte-activating factor (67) and the release of this factor into the ooplasm is only possible after the sperm plasma membrane is removed or damaged (111).

The examination of human sperm nuclei following ICSI into hamster eggs provided evidence that decondensation of human sperm nuclei after ICSI is atypical, initially occurring from the basal lesion while the apical portion of the sperm nuclei is still condensed (112). However, decondensation of the sperm after *in vitro* insemination into zone-free hamster eggs was homogenous (112). This was suggested to be due to lack of sperm modification, which naturally occurs during sperm entry at cumulus cells, zona pellucida and oolemna (112).

The ability and rate of spermatozoa to decondense *in vitro* was compared with their ability to fertilize human

oocyte *in vitro* after ICSI (87). No correlation was found between the mean percentage of spermatozoa chromatin decondensation at various time-intervals (10, 30, 60, 120 min and 24 h) or the decondensation rate and fertilization rate in both groups treated with SDS+EDTA or SDS+heparin for decondensation (87). The results of another study by the same group of authors (88), undertaken to identify the relationship between sperm chromatin decondensation *in vitro* after incubation with folicular fluid at various points in time (30, 60 and 120 min and 24 h) and fertilization and pregnancy rates after ICSI, in which chromatin decondensation was evaluated by acridine orange staining, showed that no correlation between chromatin decondensation at various points of time and fertilization rate exists (88). These authors concluded that chromatin decondensation *in vitro* cannot be recommended for predicting the fertilization potential of spermatozoa and pregnancy rates in the ICSI program. Confirming these results, others also found no correlation between the fertilization rate and the mean percentage of decondensed spermatozoa *in vitro* (85, 86). The study of Razavi *et al.* (101), conducted with CMA3, aniline blue, SDS and SDS/EDTA, also supported this data, reporting no correlation between these tests and fertilization rate.

Conclusion

The treatment of male factor infertility has been improved after ICSI. ICSI bypasses the natural mechanisms of sperm selection for fertilization, because a single spermatozoon, chosen by the operator, is introduced into the cytoplasm of the oocyte. This makes the sperm chromatin and decondensation ability of the oocyte more important for pronucleus formation and successful fertilization. The conventional semen analysis neither provides information about sperm chromatin condensation, nor about the ability of the spermatozoa to decondense after ICSI. *In vitro* decondensation tests may contribute to a better evaluation of the fertilization potential of semen and to understanding the causes of fertilization failure after ICSI, which will result in better outcomes with ART.

The failure of decondensation of the sperm nucleus is the cause of fertilization failure in some cases of ART. Unfortunately, the methods used today to assess sperm chromatin decondensation are of limited value in assessing fertilization and pregancy rates in ART. It is not possible to identify, with the tests used today, semen samples which will not fail to decondense after injection. Further research may reveal methods for the selection of sperm according to the quality of condensation that can be used in ART cycles, especially when intracytoplasmic sperm injection is being considered as a therapeutic option.

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