

Human sperm chromatin stabilization: a proposed model including zinc bridges

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ABSTRACT: The primary focus of this review is to challenge the current concepts on sperm chromatin stability. The observations (i) that zinc depletion at ejaculation allows a rapid and total sperm chromatin decondensation without the addition of exogenous disulfide cleaving agents and (ii) that the human sperm chromatin contains one zinc for every protamine for every turn of the DNA helix suggest an alternative model for sperm chromatin structure may be plausible. An alternative model is therefore proposed, that the human spermatozoon could at ejaculation have a rapidly reversible zinc dependent chromatin stability: Zn^{2+} stabilizes the structure and prevents the formation of excess disulfide bridges by a single mechanism, the formation of zinc bridges with protamine thiols of cysteine and potentially imidazole groups of histidine. Extraction of zinc enables two biologically totally different outcomes: immediate decondensation if chromatin fibers are concomitantly induced to repel (e.g. by phosphorylation in the ooplasm); otherwise freed thiols become committed into disulfide bridges creating a superstabilized chromatin. Spermatozoa in the zinc rich prostatic fluid (normally the first expelled ejaculate fraction) represent the physiological situation. Extraction of chromatin zinc can be accomplished by the seminal vesicular fluid. Collection of the ejaculate in one single container causes abnormal contact between spermatozoa and seminal vesicular fluid affecting the sperm chromatin stability. There are men in infertile couples with low content of sperm chromatin zinc due to loss of zinc during ejaculation and liquefaction. Tests for sperm DNA integrity may give false negative results due to decreased access for the assay to the DNA in superstabilized chromatin.

Key words: sperm chromatin / zinc / sperm DNA damage / disulfide bridges / sequence of ejaculation

Significance and consequence of the unique sperm chromatin structure

The unique purpose of the spermatozoon to transport a haploid genome unharmed to the egg has led to a chromatin structure that is completely different from that of somatic cells. The chromatin structure is developed to be extremely resistant towards conditions that could harm the DNA. At the same time the chromatin structure must have the property to rapidly make the DNA available to the ooplasm. It is the aim of this review to discuss how a model where zinc bridges rather than disulfide bridges can form the basis for this dual biological property.

The exchange of somatic histones into basic protamines makes the DNA transcriptionally inactive simultaneously with a very compact and almost crystalline packing. This protects the DNA by reducing both the access of a potential source of free radicals (i.e. free water) and water soluble compounds that could contribute to DNA damage. Still the DNA must be readily available at arrival in the oocyte.

Defective sperm chromatin packing could be either reduced compaction or supernormal compaction. Decreased compaction would increase the access to the DNA whereas a supernormal compaction of the sperm chromatin would jeopardize the timing of the rapid delivery of the sperm DNA in the ooplasm. Therefore, all factors affecting the compaction must be considered to understand normal physiology and pathological outcomes.

Chromatin Remodeling

To better understand, and to be able to investigate, the mechanisms behind normal and disturbed sperm chromatin structure, it is essential to identify agents that participate in or interact with the possible mechanisms.

The sperm protamines

Arginines are the predominant component (45–48%) of both protamines (P1 and P2) in human spermatozoa (Gusse *et al.*, 1986). Arginine brings the abundance of positively charged $-NH_3^+$ groups into

the protamines. These groups neutralize the negative charges of the phosphate groups of the DNA backbone and thereby allow a high degree of compaction of adjacent chromatin fibers (Balhorn, 2007).

Histidines bring imidazole groups and cysteines bring thiol ($-SH$) groups. Both these groups are efficient Zn^{2+} binders (Porath *et al.*, 1975) and therefore likely candidates to interact with zinc in the sperm chromatin. These groups are also possible participants in ion bridges involving Zn^{2+} . In classical zinc fingers, a single zinc ion is tetrahedrally coordinated by conserved histidine and cysteine residues, stabilizing the motif.

In the absence of zinc, thiol groups could provide the basis for the formation of disulfide bridges (Kvist *et al.*, 1980; Gatewood *et al.*, 1990; Bianchi *et al.*, 1992, 1994; Bal *et al.*, 2001).

Serines and threonines can be phosphorylated, i.e. bind negatively charged phosphate groups. Thus, serines and threonines provide the basis for negative charged repulsive forces when phosphorylated and allows compaction of adjacent chromatin fibers when dephosphorylated. Phosphorylation is therefore likely to provide an important mechanism to induce a rapid decondensation by repulsion of chromatin fibers whereas unpacking the DNA in the oocyte. Sperm decondensation in the oocyte requires glutathione. If glutathione is blocked, pretreatment of the oocytes with dithiothreitol (DTT) made sperm chromatin decondensation again possible (Sutovsky and Schatten, 1997). Besides disulfide-bridge cleaving, both compounds have thiols with affinity for zinc and a mechanism for both zinc-chelation and disulfide-bridge cleavage seems therefore to be present in the mature ooplasm.

Zinc

Zinc is incorporated into the sperm nucleus during spermiogenesis when the compaction of the nucleus starts. The earliest sign of zinc deficiency is an arrest at spermiogenesis with lack of elongated spermatozoa (Barney *et al.*, 1968). The chromatin content of zinc can be calculated to be ~ 8 mmol Zn^{2+}/kg (Kvist *et al.*, 1985). With X-ray microanalysis of the sperm head, up to one zinc atom for every five sulfur atoms is revealed. Since each human protamine contains approximately five sulfur atoms there appears to be one zinc ion for every protamine molecule. One protamine molecule provides positively charged $-NH_3^+$ (in the guanidinium group of arginine) that neutralizes the 20 negatively charged phosphate groups in the 10 base pairs of the DNA, equaling one turn of the DNA-protamine helix (Balhorn, 2007). Thus, the human sperm chromatin contains one zinc ion for each protamine molecule for each turn of the DNA.

Zn^{2+} is an important structural component in different proteins involved in nucleic acid binding or gene regulation (Berg, 1990). These zinc-stabilized structures (usually referred to as zinc fingers) lock the tertiary structure and thereby reduce the number of accessible conformations of the protein. This renders the protein a conformational stability which is suitable for interaction with other macromolecules like DNA or other proteins (Banecki *et al.*, 1996). In the classical zinc-finger a single zinc ion tetrahedrally coordinates conserved histidine and cysteine residues. Mostly zinc-fingers are composed of 2 HIS and 2 CYS. However, variation in the number of HIS and CYS has been reported. Furthermore, Zn^{2+} can also contribute to the quaternary structure by forming stable inter-molecular zinc-bridges. An example of an inter-molecular zinc-bridge, is in the

active enzyme nitric-oxide synthase (NOS), where one zinc coordinates two cysteine-residues in each monomer into an active dimer $(CYS)_2-Zn^{2+}-(CYS)_2$ (Raman *et al.*, 1998). Because zinc ions show no tendency for oxidation or reduction in biological systems they may to a certain degree protect thiols from oxidation into disulfide bridges and thereby act as reversible inhibitors of sites requiring free thiols (Chvapil, 1973; Chesters, 1978). However, experimental oxidative challenges inactivate the NOS-enzyme by releasing zinc and thiols then become oxidized into disulfide-bridges (Zou *et al.*, 2002).

It is therefore reasonable to assume that zinc ions can contribute to the DNA-protamine structure by linking protamines with zinc bridges, where Zn^{2+} ionically binds thiol groups of cysteine and possibly imidazole groups of histidine, respectively (Porath *et al.*, 1975; Gatewood *et al.*, 1990; Bianchi *et al.*, 1992, 1994; Kjellberg, 1993; Raman *et al.*, 1998; Bench *et al.*, 2000) (Fig. 1).

The chromatin of ejaculated spermatozoa

At ejaculation the chromatin of human spermatozoa can be made to decondense rapidly *in vitro* by mere zinc removal EDTA (ethylene diamine tetra acetate; chelates bivalent metal cations) (Kvist, 1980a, b; Roomans *et al.*, 1982) concomitant with exposure to the anionic detergent SDS (sodium dodecyl sulfate; removes membranes and imposes chromatin fiber repulsion) (Figs 2–4) (Björndahl and Kvist, 1985). Within 1 h after ejaculation exposure to 6 mM EDTA extracts 86% of the sperm chromatin zinc as revealed by X-ray microanalysis (Roomans *et al.*, 1982). This means that at ejaculation the sperm chromatin appears to have a zinc dependent chromatin stability.

However, *in vitro* the inherent, zinc dependent chromatin stabilization is rapidly lost (Fig. 2) and superseded by another type of stability (Fig. 4) (Kvist and Björndahl, 1985). This second type of stability calls for disulfide breaking agents to allow unpacking of the chromatin. This change of stabilization is enhanced when zinc is withdrawn from sperm *in vitro*, and can to a large extent be counteracted by storing sperm in an environment with high availability of Zn^{2+} .

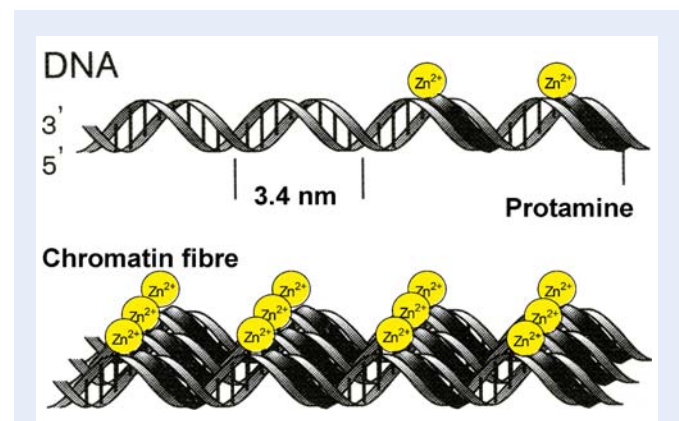


Figure 1 Schematic outline of the sperm DNA double helix and its relation to the protamine polymers illustrating a possible role of zinc as an agent coordinating e.g. thiols in protamine monomers into protamine polymers: one zinc per protamine molecule for each 10 base pairs of the DNA. Neutralization of negatively charged phosphate groups of the DNA by protamines allows a tight packing of the sperm DNA chromatin fibers.

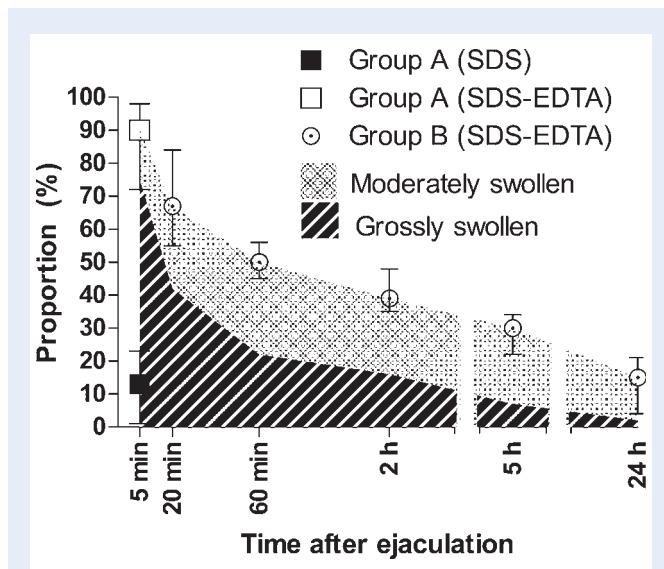


Figure 2 Zinc-dependent sperm chromatin stability is rapidly lost after ejaculation. Two groups of patients were studied. In group A aliquots of semen from five men were exposed to sodium dodecyl sulphate (SDS) with or without 6 mM EDTA for 60 min at 37°C. On the y-axis is shown the proportion of spermatozoa that underwent decondensation. When studied within 5 min only 13% of the spermatozoa decondensed in SDS only i.e. 87% were unreacted in SDS. In contrast, when exposed to EDTA (ethylene diamine tetra acetate that chelates bivalent metal cations and shown to delete 86% of sperm head Zn^{+2} at 1 h after ejaculation, Roomans *et al.* (1982) 90% of the sperm heads underwent decondensation. In experiment Group B sperm from five donors were exposed to only SDS-EDTA at various times from 20 min to 24 h after ejaculation. Results are expressed as proportion (mean, range) of decondensed sperm heads and the area under the curve depicts grossly swollen = complete decondensation (cf. Fig. 4) and moderately swollen = partial decondensation. The proportion of sperm heads with ability for complete decondensation in SDS-EDTA decreased rapidly with time after ejaculation (Björndahl and Kvist, 1985).

A plausible model that can explain that zinc both can stabilize the chromatin and prevent the development of a disulfide bridge dependent chromatin stability, is that zinc forms salt bridges with protamine thiols and potentially also imidazole groups of histidine (Fig. 5). A salt bridge involving zinc, thiols and imidazole groups is as strong as a covalent disulfide bridge and can therefore serve the purpose as a reversible and temporary stabilizer of the sperm chromatin. Removal of zinc without repulsion of chromatin fibers would allow freed thiols to oxidize into disulfide bridges. Moreover, removal of zinc in the ooplasm would allow a rapid unraveling of the chromatin fibers.

Further evidence that interaction between zinc and protamine thiols constitute a base for a stable but still rapidly reversible chromatin stability lies in the finding that the detectable amount of chromatin thiols decrease during sperm maturation in the epididymis and reappear after sperm exposure to DTT. The original interpretation of these findings was that thiols become committed into S–S bridges, due to that DTT is a compound that can break the strong covalent disulfide bridges (Calvin and Bedford, 1971). However, that an alternative

interpretation is valid has not been generally acknowledged; i.e. namely also zinc interacting with thiols in for instance salt bridges could make the thiols undetectable. Also exposure of the sperm chromatin to DTT would chelate zinc and therefore allow free thiols to be detected after DTT exposure. In favor of this is the observation that epididymal spermatozoa reveal more thiols if pre-exposed to acid or EDTA treatment, which both acts zinc chelating agents (Calvin *et al.*, 1973; Calvin and Bleau, 1974; Kvist and Eliasson, 1978). Another observation is that the decondensation of the chromatin of ejaculated human spermatozoa in liquefied semen can be induced by as low concentration as 40 μ M DTT simultaneously to EDTA exposure (unpublished data). Furthermore, spermatozoa exposed to DTT are deprived of zinc but not magnesium (Kvist and Eliasson, 1978).

In conclusion these diverse observations can be explained by the interplay between zinc and protamine thiols:

- (i) At ejaculation the chromatin is stabilized by salt bridges in which zinc interchelates between thiols and possibly imidazole groups of histidine. This stabilization resists exposure to SDS *in vitro*.
- (ii) Zinc binding to thiols prevents their oxidation into disulfide bridges.
- (iii) Partial or premature removal of zinc would increase the access to DNA leaving the DNA more exposed to agents that can damage the DNA.
- (iv) Removal of zinc in the ooplasm, or as a part of an *in vitro* experiment close to ejaculation, makes a rapid decondensation possible if forces causing chromatin fiber repulsion are present simultaneously (e.g. *in vivo* phosphorylation or ooplasm heparins, and *in vitro* SDS).
- (v) Removal of zinc without simultaneous repulsion of chromatin fibers may result in oxidation of freed thiols into disulfide bridges (S–S dependent chromatin stability). This could cause delay in the delivery of DNA in the oocyte and thereby result in a defect zygote development. Thus, sperm chromatin zinc deficiency induced during sperm collection and processing may be one factor jeopardizing the outcome of ART procedures. Moreover, the increased inaccessibility of the sperm chromatin due to excess formation of disulfide cross-linking is also likely to hinder detection of DNA breaks by assays like Comet, Tunel, SCSA techniques and give false negative results (From Björk *et al.*, 2009, Pettersson *et al.*, 2009, Tu *et al.*, 2009).

Influences on sperm chromatin stability at and after ejaculation

In the normal course of events, spermatozoa are expelled with the prostatic fluid onto the cervical mucus. Thus the prostatic fluid should be regarded as the physiological vehicle for ejaculated spermatozoa in human. When an ejaculate is collected in one single container for laboratory purposes, also the seminal vesicular fluid is included and allowed to interact with the spermatozoa. The content of zinc in the sperm head and the nature of the chromatin stabilization then become influenced by the more or less random and always changing properties of the 'seminal fluid' which each ejaculated spermatozoon is surrounded by (Björndahl and Kvist, 2003). It is often ignored or overlooked that the ejaculate is not a homogenic and homeostatically regulated fluid like blood plasma; the term seminal plasma is thus

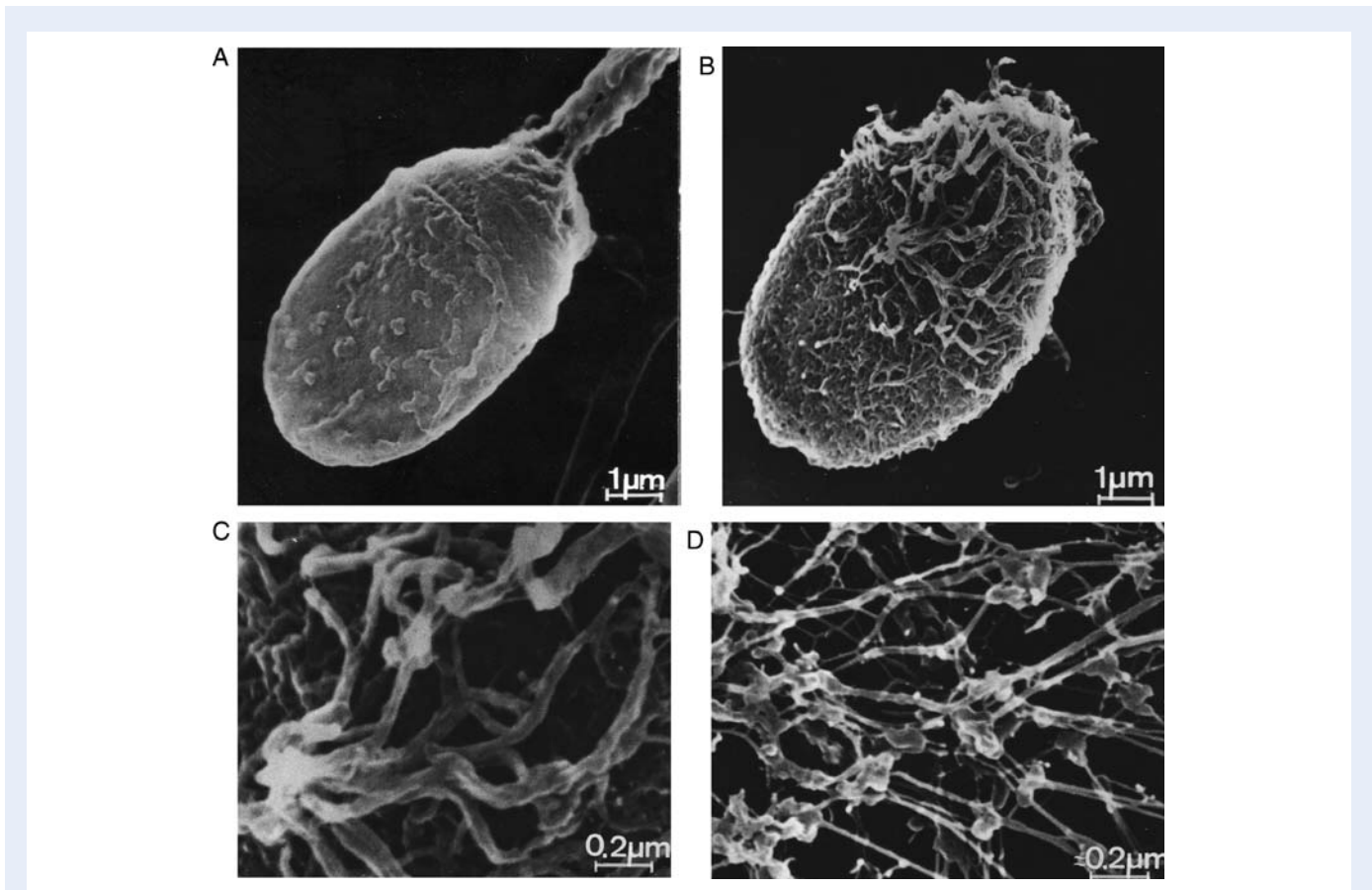


Figure 3 Scanning Electron Micrographs of human spermatozoa: (A) control showing intact plasma membrane; (B) sperm exposed only to SDS (sodium dodecyl sulfate) showing an almost intact sperm nucleus without significant decondensation; (C) same as 'B' at higher magnification showing gross chromatin fibers; (D) sperm exposed to SDS and EDTA showing almost complete chromatin decondensation and unraveled toroids (Kvist, 1980b).

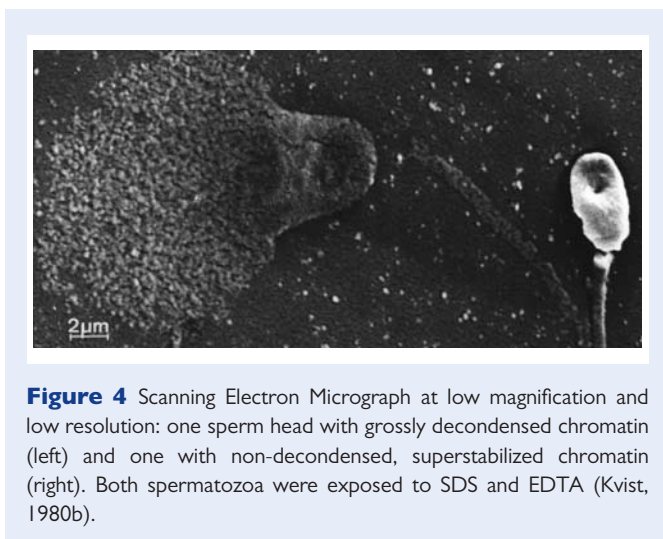


Figure 4 Scanning Electron Micrograph at low magnification and low resolution: one sperm head with grossly decondensed chromatin (left) and one with non-decondensed, superstabilized chromatin (right). Both spermatozoa were exposed to SDS and EDTA (Kvist, 1980b).

misleading. The ejaculate fluid is just a mixture of various discharged secretions and the composition of the 'seminal plasma' varies during ejaculation, during liquefaction, after ejaculation and it varies

between different men and between different ejaculates from the same man. Therefore collection of the entire ejaculate in one single container, according to the golden standard for semen laboratories (World Health Organization, 1999), will introduce increased heterogeneity of the sperm chromatin stabilization.

To understand the dynamics *in vitro* of the sperm chromatin stabilization after ejaculation it is essential to be aware of the sequence of ejaculation and how the sperm chromatin zinc content is influenced by prostatic fluid and seminal vesicular fluid (Björndahl and Kvist, 1990; Björndahl *et al.*, 1991).

In the normal ejaculation most spermatozoa are expelled in the first one-third of the ejaculate together with the slightly acidic, zinc rich prostatic secretion. The prostatic fluid has a high biological availability of zinc which prevents a loss of chromatin zinc, as measured by X-ray microanalysis (Kvist *et al.*, 1985; Björndahl and Kvist, 1990) (Fig. 6). The last two-third of the ejaculate contains mainly seminal vesicular fluid. Spermatozoa collected with split ejaculate technique showed very different content of sperm chromatin zinc as measured with X-ray microanalysis (Kvist *et al.*, 1985; Björndahl and Kvist, 1990). The sperm chromatin zinc content was inversely related to the admixture of seminal vesicular fluid to the ejaculate fraction. The admixture

One report points to one zinc for every protamine P2 (Bench *et al.*, 2000) and another to one zinc for every protamine (Kjellberg, 1993). To solve this matter it is important to secure that the zinc content of the nucleus is not influenced by the experimental procedures and to include sufficient numbers of observations. Another issue is to establish the exact localization of zinc and its ligands in the intact sperm nucleus. Indirect studies of isolated nuclear players i.e. P1, P2, Zn²⁺ and DNA, always carry the problem that the native structure is lost. Furthermore, the exogenous thiols added (e.g. DTT, mercaptoethanol, cysteamine) to keep the protamine thiols unoxidized can all interfere with the native zinc-thiol status by providing a possibility for a multitude of different mixed zinc-thiolates to be formed. This calls for new methodological approaches that may include experiments in oxygen free environment as well as application of biophysical investigation techniques in order to bypass the need for exogenous thiol compounds.

References

- Arver S. Studies on zinc and calcium in human seminal plasma. *Acta physiologica Scandinavica* 1982a;**507**:1–21.
- Arver S. Zinc and zinc ligands in human seminal plasma. III. The principal low molecular weight zinc ligand in prostatic secretion and seminal plasma. *Acta Physiol Scand* 1982b;**116**:67–73.
- Arver S, Eliasson R. Zinc and zinc ligands in human seminal plasma. II. Contribution by ligands of different origin to the zinc binding properties of human seminal plasma. *Acta Physiol Scand* 1982;**115**:217–224.
- Bal W, Dyba M, Szewczuk Z, Jezowska-Bojczuk M, Lukszo J, Ramakrishna G, Kasprzak KS. Differential zinc and DNA binding by partial peptides of human protamine HP2. *Mol Cell Biochem* 2001;**222**:97–106.
- Balhorn R. The protamine family of sperm nuclear proteins. *Genome Biol* 2007;**8**:227.
- Banecki B, Liberek K, Wall D, Wawrzynów A, Georgopoulos C, Bertoli E, Tanfani F, Zyllicz M. Structure-function analysis of the zinc finger region of the DnaJ molecular chaperone. *J Biol Chem* 1996;**271**:14840–14848.
- Barney GH, Orgebin-Crist MC, Macapinalac MP. Genesis of esophageal parakeratosis and histologic changes in the testes of the zinc-deficient rat and their reversal by zinc repletion. *J Nutr* 1968;**95**:526–534.
- Bench G, Corzett MH, Kramer CE, Grant PG, Balhorn R. Zinc is sufficiently abundant within mammalian sperm nuclei to bind stoichiometrically with protamine 2. *Mol Reprod Dev* 2000;**56**:512–519.
- Berg JM. Zinc fingers and other metal-binding domains. Elements for interactions between macromolecules. *J Biol Chem* 1990;**265**:6513–6516.
- Bianchi F, Rousseaux-Prevost R, Sautiere P, Rousseaux J. P2 protamines from human sperm are zinc -finger proteins with one CYS2/HIS2 motif. *Biochem Biophys Res Commun* 1992;**182**:540–547.
- Bianchi F, Rousseaux-Prevost R, Bailly C, Rousseaux J. Interaction of human P1 and P2 protamines with DNA. *Biochem Biophys Res Commun* 1994;**201**:1197–1204.
- Björndahl L, Kvist U. Loss of an intrinsic capacity for human sperm chromatin decondensation. *Acta Physiol Scand* 1985;**124**:189–194.
- Björndahl L, Kvist U. Influence of seminal vesicular fluid on the zinc content of human sperm chromatin. *Int J Androl* 1990;**13**:232–237.
- Björndahl L, Kvist U. Sequence of ejaculation affects the spermatozoon as a carrier and its message. *Reprod Biomed Online* 2003;**7**:440–448.
- Björndahl L, Kjellberg S, Kvist U. Ejaculatory sequence in men with low sperm chromatin-zinc. *Int J Androl* 1991;**14**:174–178.
- Calvin HI, Bedford JM. Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J Reprod Fertil Suppl* 1971;**13**:65–75.
- Calvin HI, Bleau G. Zinc-thiol complexes in keratin-like structures of rat spermatozoa. *Exp Cell Res* 1974;**86**:280–284.
- Calvin HI, Yu CC, Bedford JM. Effects of epididymal maturation, zinc (II) and copper (II) on the reactive sulphydryl content of structural elements in rat spermatozoa. *Exp Cell Res* 1973;**81**:333–341.
- Chesters JK. Biochemical functions of zinc in animals. *World Rev Nutr Diet* 1978;**32**:135–164.
- Chvapil M. New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. *Life Sci* 1973;**13**:1041–1049.
- Fisch H, Lambert SM, Goluboff ET. Management of ejaculatory duct obstruction: etiology, diagnosis, and treatment. *World J Urol* 2006;**24**:604–610.
- From Björk M, Björndahl L, Zakeri A, Kvist U. Can sperm chromatin packaging influence results of sperm DNA integrity measures? Results of the TUNEL assay. *J Androl* 2009; 40. March/April Supplement: 34th Annual Meeting of the American Society of Andrology, Philadelphia, USA.
- Gatewood JM, Schroth GP, Schmidt CW, Bradbury EM. Zinc induced secondary structure transitions in human sperm protamines. *J Biol Chem* 1990;**265**:20667–20672.
- Gusse M, Sautiere P, Belaiche D, Martinage A, Roux C, Dadoune JP, Chevallier P. Purification and characterization of nuclear basic proteins of human sperm. *Biochim Biophys Acta* 1986;**884**:124–134.
- Kjellberg S. *Zinc and Human Sperm Chromatin*, Department of Gynecology and Obstetrics. Linköping: University of Linköping, 1993.
- Kvist U. Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man. *Acta Physiol Scand* 1980a;**109**:79–84.
- Kvist U. Sperm nuclear chromatin decondensation ability. An *in vitro* study on ejaculated human spermatozoa. *Acta Physiol Scand* 1980b;**486**:1–24.
- Kvist U, Björndahl L. Zinc preserves an inherent capacity for human sperm chromatin decondensation. *Acta Physiol Scand* 1985;**124**:195–200.
- Kvist U, Eliasson R. Zinc dependent chromatin stability in human ejaculated spermatozoa. *Int J Androl* 1978;**(Suppl 1)**:178.
- Kvist U, Afzelius BA, Nilsson L. The intrinsic mechanism of chromatin decondensation ability and its activation in human spermatozoa. *Dev Growth Differ* 1980;**22**:543–554.
- Kvist U, Björndahl L, Roomans GM, Lindholmer C. Nuclear zinc in human epididymal and ejaculated spermatozoa. *Acta Physiol Scand* 1985;**125**:297–303.
- Lundquist F. Aspects of the biochemistry of human semen. *Acta Physiol Scand* 1949;**19**:1–108.
- Pettersson G, From Björk M, Björndahl L, Kvist U. Can sperm chromatin packaging influence results of sperm DNA integrity measures? Results from Acridin Orange Flow Cytometry. *J Androl* 2009; 108–109. March/April Supplement: 9th International Congress of Andrology, Barcelona, Spain.
- Porath J, Carlsson J, Olsson I, Belfrage G. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 1975;**258**:598–599.
- Raman CS, Li H, Martasek P, Kral V, Masters BS, Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 1998;**95**:939–950.
- Roomans GM, Lundevall E, Björndahl L, Kvist U. Removal of zinc from subcellular regions of human spermatozoa by EDTA treatment studied by X-ray microanalysis. *Int J Androl* 1982;**5**:478–486.
- Sillen LG, Martell AE. *Stability Constants of Metal-ion Complexes*. London: The Chemical Society, 1971. Special publication No 25UK.

- Sutovsky P, Schatten G. Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol Reprod* 1997;**56**:1503–1512.
- Tu J, Björndahl L, Kvist U. Can sperm chromatin packaging influence results of sperm DNA integrity measures? Results of the Comet assay. *Int J Androl* 2009; 42. March/April Supplement: 34th Annual Meeting of the American Society of Andrology, Philadelphia, USA.
- World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interactions*, 4th edn. Cambridge, UK: Cambridge University Press, 1999.
- Zou MH, Shi C, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 2002;**109**:817–826.
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