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Origin and biological significance of DNA fragmentation in human spermatozoa

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1. ABSTRACT

The occurrence of DNA fragmentation in mammalian spermatozoa was identified in 1993. In human, sperm DNA fragmentation is particularly relevant in subfertile patients (i.e, those subjects more likely to be treated by assisted reproductive techniques). Thus, concerns have been raised about the possibility that sperm with DNA fragmentation may be involved in the process of fertilization, in particular when invasive techniques (such as intracytoplasmatic sperm injection) are applied. Knowledge of the mechanisms responsible for generation of DNA strand breaks may thus help in disclosing and possibly identifying new therapies for the treatment of male infertility. However, the mechanisms involved in generating sperm DNA anomalies are far from being clarified. In this review, we summarize and critically analyze the main current theories that explain generation of DNA fragmentation in spermatozoa: abortive apoptosis (anomalies in apoptosis that occur normally during spermatogenesis), problems in packaging of chromatin (mainly anomalies in histone to protamine substitution) and generation of reactive oxygen species (that may occur at any level during spermatogenesis, sperm maturation and transit in the male genital tract).

2. INTRODUCTORY REMARKS: WHY STUDY SPERM DNA INTEGRITY?

Sperm DNA fragmentation is a genomic anomaly frequently detected in subfertile patients. Since the first reports on this particular type of sperm damage (1, 2), many studies were published on this topic. There are two main reasons as to why researchers have focused their attention on sperm DNA fragmentation. The first reason is related to the demonstration that the incidence of DNA fragmentation is particularly high in men with poor quality semen (3, 4 and 5). For these patients, assisted reproductive techniques (ART) are often the only therapeutical option to treat their infertility problem. Since protocols of assisted reproduction imply the overriding of several natural barriers to fertilization (if not all, as in the case of intracytoplasmatic sperm injection - ICSI), concerns have been raised about the possibility that a DNA fragmented sperm might participate in the fertilization process and the consequences of such an event for the ensuing conceptus (6). Indeed, increased DNA fragmentation has been related to lower fertilization (3, 7), blastocyst (8) and pregnancy (9) rates after *in vitro* fertilization (IVF), although these data were not confirmed in a different group of patients (10). Since the outcome of IVF is dependent on several

variables, including quality of oocytes and maternal age, it appears clear that large-scale studies are needed to define the impact of sperm DNA fragmentation in IVF outcome.

Secondly, andrologists are presently searching for semen parameters able to predict fertilization potential, clinically and statistically more relevant than those presently available after a routine semen analysis, such as that recommended by WHO. In this respect, sperm DNA fragmentation might represent a good option. Indeed, its occurrence in high levels in subfertile men reflects, although not exactly overlaps, the extent of poor quality sperm (reduced count and motility, abnormal morphology) in semen (3, 4 and 5). In addition, as mentioned above, the percentages of DNA fragmented sperm negatively correlate with the outcome of *in vitro* fertilization (3, 7-9) and a threshold value of DNA fragmentation seems to exist over which the probability of pregnancy is dramatically reduced (11).

3. THEORIES ON THE ORIGIN OF SPERM DNA FRAGMENTATION IN EJACULATES

Several techniques have been used to reveal DNA fragmentation in sperm, including TUNEL (Terminal deoxynucleotidildyl transferase (TdT)-mediated dUTP Nick end Labelling, 3, 5), single cell gel electrophoresis (comet assay, 4), and the SCSA (Sperm Chromatin Structure Assay, 11). Due to the ease and rapidity of executing the assay, TUNEL is one of the more widely used methods to study sperm DNA fragmentation. In somatic cells, the TUNEL assay is considered specific for apoptosis (12-14). Indeed, it has been reported that the template and primer-independent TdT is quite selective in detecting apoptotic DNA degradation whereas template and primer-dependent DNA polymerases preferentially label DNA breaks with other origin, including necrosis and irradiation. Such a difference is explained by the fact that the DNA polymerase template and primer-independent TdT is able to label both single and double stranded fragments at the hydroxylated 3' ends (including blunt-ended or 5' recessed DNA fragments) at variance with DNA polymerases template and primer-dependent which labels single strand breaks only (15). Thus, TdT is more suitable to detect the highly frequent double-strand DNA breaks occurring in apoptosis. Detecting DNA fragmentation in spermatozoa with both types of assays (i.e. using template, primer dependent DNA polymerases and template, primer independent TdT) did not yield to any difference in sperm labelling (15). This result, which could be explained by considering the different chromatin packaging between histone-linked and protamin-linked DNA (15), demonstrated that TUNEL positivity in sperm cannot be considered, by itself, an index of apoptosis (15) and that there is the possibility that other mechanisms exist that lead to sperm DNA breaks revealed by TUNEL (see below). On the other hand, although apoptosis-like features have been detected in sperm (16-18), a clear demonstration of an association between sperm DNA fragmentation, as detected by TUNEL, and these features is lacking (5). Nonetheless in literature, TUNEL positivity is often considered as a sign of sperm apoptosis, generating some confusion.

Knowledge of the mechanism involved in the development of sperm DNA fragmentation in human could favour the design of new therapies for subfertile patients and of treatments for sperm populations used in ARTs. However, up to now, the origin and the cause of the phenomenon are far from being clarified, despite the multitude of studies in the last decade.

In the literature, several theories about the origin of DNA fragmentation have been proposed. The first theory originates from studies obtained in rodent models (19, 20) and then also confirmed in human (21). These studies (19-21) reported that DNA breaks occur and later on disappear, during the spermiogenesis. The enzyme topoisomerase II might be involved in the re-ligation of the DNA nicks (22) and the transient DNA breaks might have an important role in chromatin remodelling (21) as suggested by the temporal coincidence between the appearance of DNA breaks and the occurrence of histone H4 hyperacetylation (21). Based on these observations, the DNA fragmented spermatozoa present in ejaculates could be interpreted as cells that failed to complete maturation and in particular to complete the correct packaging of chromatin. Some findings support this speculation: i) a close correlation has been reported between DNA breakage and both poorly protaminated chromatin (15) and increased sensitivity of DNA to denaturation (an index of less stable and resistant chromatin) (23) and ii) DNA fragmented sperm often display persistent cytoplasmic residues, as detected by electron microscopy (5).

In 1999 Sakkas reported the occurrence of a high amount of sperm expressing FAS receptor in ejaculates, especially those from patients with abnormal semen parameters (25). In a similar group of patients, the presence of ultrastructural features resembling somatic apoptosis (16-18) has been reported in ejaculated sperm and such an occurrence appears to be partially reversed by a short term treatment with FSH (17). The occurrence of both ultrastructural apoptotic signs and Fas expression in spermatozoa from subfertile patients, prompted Sakkas *et al* (26) to hypothesize that the presence of DNA fragmented sperm in human ejaculates could be explained by the occurrence of a phenomenon denominated by Sakkas *et al* as "abortive apoptosis" (26). Abortive apoptosis is an apoptotic process that begins in the testis but fails to be completed because of an impairment in the program of cell death or mismatching with spermatogenesis. Thus cells committed to death cannot be completely deleted and can be observed in the semen together with other abnormal sperm (26). In the period in which the "abortive apoptosis" theory was proposed, it was widely used to summarize and integrate most of the finding reported until then. Indeed, apoptosis had been observed in human and animal testis (27-29), where it seems to have the important role of matching the number of germ cells with the amount of the supportive Sertoli cells (26) and of deleting injured cells (30-32). In addition, at the time that this theory was developed, germ cell apoptosis was considered to be triggered by an interaction between FAS receptor (expressed in germ cells surface) and Fas ligand (secreted by Sertoli cells) (29, 33), although, more recently, it has been demonstrated that this may not be the case (34, 35). Moreover, many studies have shown that apoptosis in

seminiferous epithelium is controlled by hormones, in particular FSH (27, 28), consistent with the partial reversion of apoptosis-like ultrastructures in ejaculates of patients after short term treatment with this hormone (17). Data supporting the occurrence of abortive apoptosis have been implemented after the time of first report (26). Other signs of apoptosis (as found in somatic cells) have been detected in ejaculates of subfertile patients, including the expression of the apoptotic markers p53 and bcl-x (36) and the occurrence of caspases (key apoptotic enzymes, 37-39). Caspases are members of a family of aspartic acid-directed cysteine proteases that act either as initiators (caspases 8, 9 and 10) or as effectors (caspases 3, 6 and 7) in apoptosis (40-42). Both types of caspase have been described in human sperm (37). In addition, our laboratory has recently described the occurrence in human ejaculates of round bodies, virtually devoid of chromatin material, that resemble somatic apoptotic bodies (43). Such bodies are stainable with merocyanine 540 (a probe that detects apoptosis in somatic cells, 44) and are present in high amount in ejaculates of oligoasthenoteratozoospermic patients (43), the same category of subjects in which Sakkas *et al* (25) found a high expression of FAS.

Another hallmark of somatic apoptosis is the translocation of the membrane phospholipid phosphatydilserine (PS) from the inner to the outer leaflet of the plasma membrane. Many papers report the occurrence of variable amounts of live PS exposing spermatozoa in human ejaculates (37, 45, 46). This feature has been considered as evidence of (abortive) apoptosis of germ cells. However the meaning of PS externalization in live sperm is still controversial. De Vries *et al* (47), reported that translocation of PS is a physiological membrane modification occurring during human sperm capacitation, similarly to other mammalian species (48). In addition, it is known that PS exposure in live sperm can be induced by different noxious stimuli, including ROS (49). However, in contrast to somatic cells (50), the noxious action of ROS does not seem to act via an apoptotic program in sperm, at least it does not involve or only partially involves caspase activation (51, 52). Furthermore, our group has shown that although spontaneous PS exposure in live sperm is linked to the development of *in vitro* DNA fragmentation, the mechanism of DNA cleavage does not appear to involve a nuclease activity (53). Indeed, treatment with a wide spectrum nuclease inhibitor, such as auryltricarboxilic acid, does not prevent the development of DNA damage *in vitro* (53).

Even if abortive apoptosis actually seems to occur in the testis in certain conditions, whether such phenomenon is the cause of DNA fragmentation in ejaculated sperm is not yet univocally demonstrated. Simultaneous detection of DNA fragmentation and the hallmarks of apoptosis, revealed that only a weak overlapping is present between DNA fragmented sperm and sperm exhibiting p53, bcl-x and FAS expression (36). Furthermore, no association has been detected between apoptosis-like ultrastructures and the amount of sperm DNA fragmentation (5). On the contrary, levels of sperm caspases seem to overlap the distribution of DNA fragmentation. Indeed, expression of these enzymes are higher in subfertile patients than in healthy donors (54) and in immature sperm fractions than in mature ones (55, 56). Studies on the relationship between caspases and DNA fragmentation in sperm are very important since apoptotic DNA degradation is dependent from their activation in many somatic cell types (57) . However a clear cause-effect relationship between caspase activity and sperm DNA fragmentation has not been clearly demonstrated so far. For instance, Weng *et al* (37), showed that a positive correlation occurs between the active form of caspase-3 (the main apoptotic executor) and the amount of DNA fragmentation. However, the percentage of sperm expressing the enzyme (up to 5%) resulted much lower than that of DNA fragmented sperm (up to 35%). Hence, the authors concluded that it cannot be ruled out that sperm DNA fragmentation is a caspase-3 independent process. Alternatively, they proposed the involvement of other types of caspases or a temporal dissociation between caspase activation and DNA degradation (37). Taylor *et al* (52) showed recently that caspase activation in ejaculated sperm is not associated to other hallmarks of apoptosis (such as for instance PS externalization), suggesting that, in ejaculated sperm, caspases may serve functions different from apoptosis (52).

Recently Sakkas *et al* (58) proposed a modification of the abortive apoptosis theory, according to which, the lack of a sharp association between DNA fragmentation and apoptotic markers in ejaculated sperm (5, 36) might be due to the fact that DNA damage and persistence of apoptosis markers are generated by independent, albeit interacting processes. Apoptotic features as the FAS receptor, bcl-x and p 53 expression found in ejaculates, would be the result of a failure of the testis apoptosis triggered before the intensive nuclear and cytoplasmatic remodelling of spermatids (58). Presence of DNA nicks would be the result of the failure of re-ligation normally occurring during the nucleus remodelling in spermiogenesis. However, nuclear and cytoplasmic remodelling might derail the normal course of apoptosis and thus provoke the escaping of apoptotic cells from their elimination (58). On the other hand, executing the apoptotic process might impair the re-ligation of DNA nicks. The net result would be a heterogeneous sperm population in which, besides normal cells, DNA nicks and apoptotic markers can or cannot coexist (Figure 1, 58).

Another proposed cause of DNA strand breakage in human ejaculates is oxidative stress. A large variety of semen factors are known to generate ROS, including spermatozoa themselves (59). Small and time regulated ROS production by sperm has an important role in sperm capacitation and the acrosome reaction processes (59). However, an excessive level of these aggressive compounds may be responsible for cellular damage and, in particular, DNA damage (59, 60). Endogenous (61) and exogenous (62) free radicals are known to attack sperm DNA. A high amount of ROS in seminal plasma has been associated with poor sperm parameters (63, 64) and infertile patients show increased amounts of 8-hydroxy-deoxy-guanosine (a biomarker of oxidative DNA damage) (65). Moreover, a positive correlation has been reported

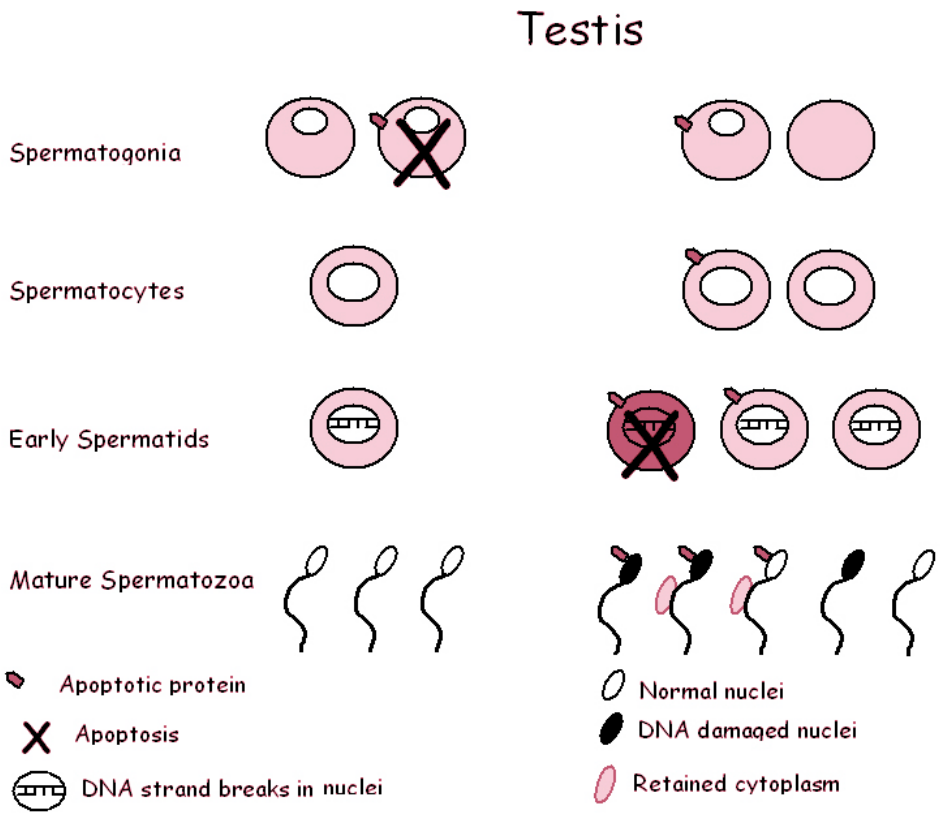

Figure 1. Hypothetical model explaining the association between cell immaturity, apoptotic marker proteins and DNA damage in human sperm. Modified from Sakkas *et al*, 2004 (58).

between sperm DNA breakage and sperm ROS generation (66). Importantly, *in vivo* studies have shown that treatment with antioxidants diminishes sperm DNA damage (revised in 67). Excessive ROS production by sperm is associated to a decreased degree of cell maturity, in particular to an abnormal retention of cytoplasm (68) and thus of the cytosolic enzymes responsible for production of free radicals, such as glucose-6-phosphate deydrogenase and a putative NADPH oxidase (69). In turn, immaturity is associated to abnormal morphology (70, 71), consistent with the reported negative relationship between DNA fragmentation and poor seminal morphology (3-5).

One of the main differences between the ROS theory and others, is the site of the origin of DNA fragmentation. Both DNA degradation in abortive apoptosis and the failure of DNA breaks to re-ligate would originate in the testis, whereas oxidative DNA damage could originate in testicular as well as post testicular sites (Figure 2). Our group has demonstrated that spontaneous DNA fragmentation in sperm continues after ejaculation (53), indicating that the cause of the phenomenon is not necessarily located in the testis. Accordingly, data from these studies suggest that endogenous ROS production from sperm is responsible for the development of de novo DNA damage (53). In particular, the finding that de novo DNA fragmentation develops mainly in morphologically abnormal sperm (53), is in agreement with the association between abnormal morphology, immaturity and excessive ROS production of these cells (68, 70, 71). Interestingly, development of *de novo* DNA fragmentation in ejaculated sperm does not occur in spermatozoa from healthy donors (72).

4. ARE MATURE SPERMATOZOA ABLE TO UNDERGO APOPTOSIS?

It is important to stress that abortive apoptosis is a theory which attempted to explain the occurrence of sperm DNA fragmentation in semen but it does not give any information about the possible occurrence of a putative apoptosis in fully mature sperm. The latter process implies a different site of origin and possibly different pathways of signalling and execution. So far, it has not been demonstrated that mature sperm are able to die via apoptosis. However, a nuclease activity in ejaculated mammalian sperm, cleaving chromatin at the bases of DNA loop domains into large fragments (about 50 kb) has been shown (73) and speculation about the occurrence of apoptosis in fully mature sperm is attractive. Apoptosis is a ubiquitous mechanism of death in mammalian cells. Several examples show that its physiological meaning is to massively delete damaged or useless cells and/or to regulate tissue homeostasis without provoking inflammation. Hence, it is reasonable to speculate that a similar mechanism could be involved in the elimination, after fertilization, of the many sperm deposed in female genital tracts of which only one is committed to fertilize the

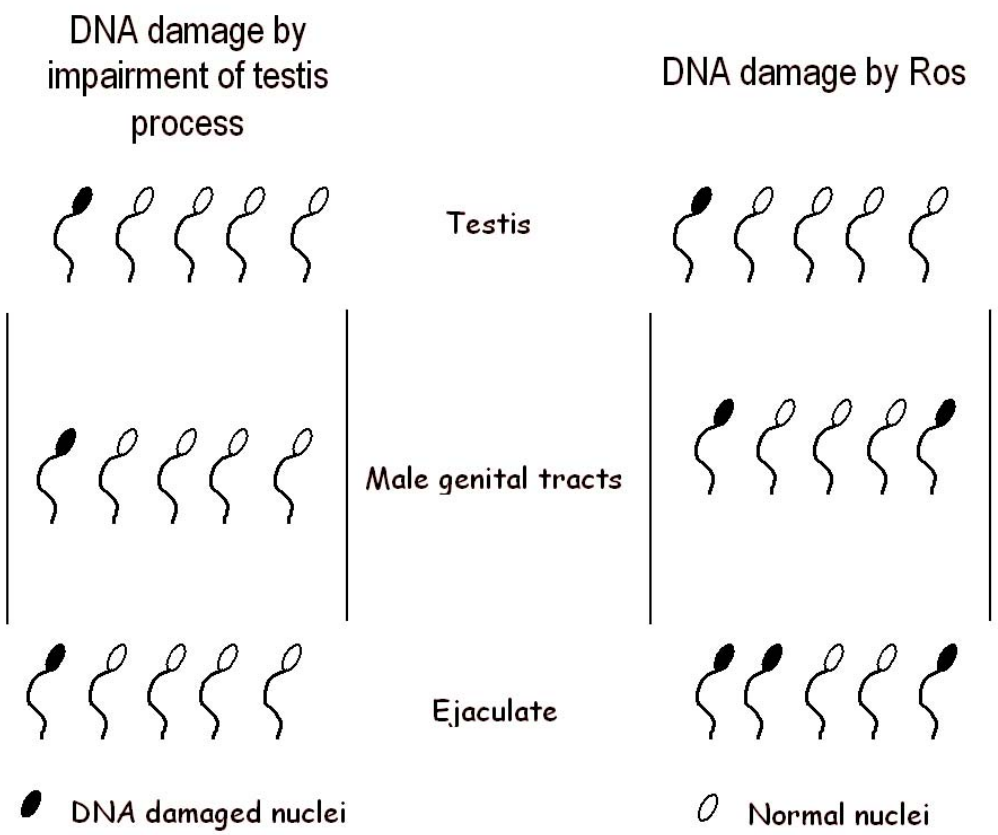

Figure 2. Putative different sites of origin of DNA damage in sperm according to the different theories on the origin of sperm DNA fragmentation. According to the theory attributing DNA fragmentation to impaired testis processes, sperm DNA damage can originate only in the testis. According the "ROS theory", DNA damage may originate in the testis as well as in post testicular sites.

oocyte. At the same time, apoptosis in post testicular sperm might represent a route to eliminate damaged cells and to regulate the homeostasis of the number of sperm in the male genital tract.

Whether mature male gametes retain the machinery of apoptosis has not been verified up to now. The finding that apoptotic markers are associated with characteristics of immaturity, including high levels of Creatinine Phosphokinase and low levels of Heat Shock Protein A2 (74), suggested that the apoptotic machinery is committed to be lost as a consequence of sperm maturation. Accordingly, Blanco-Rodriguez and Martinez-Garcia (75) showed that, in rat spermatids, the apoptotic signalling molecules are restricted to a specific cytoplasmic region, detaching from the cell as residual bodies. However, it cannot be excluded that unique cells, such as mature sperm, may have a program of apoptosis different from both somatic and testis germ cells.

Treatment of ejaculated spermatozoa with stimuli able to induce apoptosis in somatic cells does not univocally trigger activation of the apoptotic pathways as it occurs in the latter. Taylor *et al* (52) demonstrated that treatment with staurosporine increases caspase activity in high motile sperm without inducing PS externalization in live sperm. On the contrary, the treatment with Fas ligand or hydrogen peroxide does not activate caspases but increases PS exposure (52). Similarly, betulinic acid (a pro-apoptotic signal transduction molecule acting on mitochondria) induces caspase activation in mature spermatozoa, while FAS ligand does not (76).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the two main theories about the origin of sperm DNA fragmentation, (one pointing to impaired testis processes and the other one to the oxidative stress) lead to the same sperm trait, that is the presence of immature abnormal sperm (in variable amounts) in human ejaculates. In this context, it cannot be excluded that both mechanisms are, to a varying extent, responsible for sperm DNA damage. Based on this consideration, the research on the causes of sperm DNA fragmentation should naturally shift to address why, in certain subjects, the process of spermiogenesis and /or maturation derails and yields to fractions of sperm characterized by abnormal cytoplasmic and/or nuclear remodelling as well as persistence of features of cells committed to die.

Concerning the putative occurrence of apoptosis in mature spermatozoa, as possible cause of sperm DNA fragmentation, studies published so far suggest that, if any apoptosis occurs, it is executed by a different mechanism in respect to somatic cells.

6. REFERENCES

1.Gorczyca W, J. Gong, Z. Darzynkiewicz: Detection of DNA strand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleotidyl transferase and nick translation assay. *Cancer Res* 53, 1945-51 (1993)

2. Bianchi PG, G.C. Manicardi, D. Bizzaro, U. Bianchi, D. Sakkas: Effect of deoxyribonucleic acid protamination on fluorochrome staining and *in situ* nick-translation of murine and human mature spermatozoa. *Biol Reprod* 49,1083-8 (1993)

3. Lopes S, J.G. Sun, A. Jurisicova, J. Meriano, R.F. Casper: Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* 69, 528-32 (1998)

4. Irvine DS, J.P. Twigg, E.L. Gordon, N. Fulton, P.A. Milne, R.J.Aitken: DNA integrity in human spermatozoa: relationships with semen quality. *J Androl* 21, 33-44 (2000)

5. Muratori M, P. Piomboni, E. Baldi, E. Filimberti, P. Pecchioli, E. Moretti, L. Gambera, B. Baccetti, R. Biagiotti, G. Forti, Mario Maggi: Functional and ultrastructural features of DNA fragmented human sperm. *J Androl* 21, 903-12 (2000)

6. Seli E and D. Sakkas: Spermatozoal nuclear determinants of reproductive outcome: implications for ART. *Hum Reprod Update* 11, 337-49 (2005)

7. Sun JG, A. Jurisicova and R.F. Casper: Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. *Biol Reprod* 56, 602-7 (1997)

8. Virro MR, K.L. Larson-Cook, D.P. Evenson: Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* 81, 1289-95 (2004)

9. Henkel R, M. Hajimohammad, T. Stalf, C. Hoogendijk, C. Mehnert, R. Menkveld, H. Gips, W.B. Schill,T.F. Kruger: Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 81, 965-72 (2004)

10. Gandini L, F. Lombardo, D. Paoli, F. Caruso, P. Eleuteri, G. Leter, R. Ciriminna, F. Culasso, F. Dondero, A. Lenzi, M. Spano: Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod* 19, 1409-17 (2004)

11. Evenson DP, K.L. Larson, L.K. Jost: Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 23, 25–43 (2002)

12. Gold R, M. Schmied, G. Giegerich, H. Breitschopf, H.P. Hartung, K.V. Toyka, H. Lassmann: Differentiation between cellular apoptosis and necrosis by combined use of *in situ* tailing and nick translation techniques. *Lab Invest* 71, 219–225 (1994)

13. Nakamura T, T. Sakai, M. Hotchi: Histochemical demonstration of DNA double strand breaks by *in situ* 3'-tailing reaction in apoptotic endometrium. *Biotech Histochem* 70, 33–39 (1995)

14. Fraga CG, P.A. Motchnik, A.J. Wyrobek, D.M. Rempel, B.N. Ames: Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 351, 199-203 (1996)

15. Manicardi GC, A. Tombacco, D. Bizzaro, U. Bianchi, P.G. Bianchi, D. Sakkas: DNA strand breaks in ejaculated human spermatozoa: comparison of susceptibility to the nick translation and terminal transferase assays. *Histochem J* 30, 33-39 (1998)

16. Baccetti B, G. Collodel, P. Piomboni: Apoptosis in human ejaculated sperm cells. (Notulae seminologicae 9). *J Submicrosc Cytol Pathol* 28, 587-96 (1996)

17. Baccetti B, E. Strehler, S. Capitani, G. Collodel, M. De Santo, E. Moretti, P. Piomboni, R. Wiedeman, K. Sterzik: The effect of follicle stimulating hormone therapy on human sperm structure (Notulae seminologicae 11). *Hum Reprod* 12, 1955-68 (1997)

18. Gandini L, F. Lombardo, D. Paoli, L. Caponecchia, G. Familiari, C. Verlengia, F. Dondero, A. Lenzi: Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod* 15, 830-839 (2000)

19. Mc Pherson SMG and F.J. Longo: Localization of DNAase I hypersensitive regions during rat spermatogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. *Mol Reprod Dev* 31, 268-79 (1992)

20. Sakkas D, G. Manicardi, P.G. Bianchi, D. Bizzaro, U. Bianchi: Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biol Reprod* 52, 1149-55 (1995)

21. Marcon L, G. Boissonneault: Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. *Biol Reprod* 70, 910-8 (2004)

22. Mc Pherson SMG and F.J. Longo: Chromatin structure-function alterations during mammalian spermatogenesis:

DNA nicking and repair in elongating spermatids. *Eur J Histochem* 37, 109-28 (1993)

23. Aravindan GR, J. Bjordahl, L.K.Jost, D.P. Evenson: Susceptibility of human sperm to *in situ* DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp Cell Res* 236, 231-7 (1997)

25. Sakkas D, E. Mariethoz, J.C. St John: Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp Cell Res* 251, 350-5 (1999)

26. Sakkas D, E. Mariethoz, G. Manicardi, D. Bizzaro, P.G. Bianchi, U. Bianchi: Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 4, 31-37 (1999)

27. Tapanainen JS, J.L. Tilly, K.K. Vihko, A.J. Hsueh: Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. *Mol Endocrinology* 7, 643-650 (1993)

28. Billig H, I. Furuta, C. Rivier, J. Tapanainen, M. Parvinen, A.J.W. Hsueh: Apoptosis in testis germ cells: developmental changes in gonado-tropin dependence and localization to selective tubule stages. *Endocrinology* 136, 5-12 (1995)

29. Rodriguez I, C. Ody, K. Araki, I. Garcia , P. Vassalli: An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J* 16, 2262-2270 (1997)

30. Lue YH, A.P. Hikim, R.S. Swerdloff, P. Im, K.S. Taing, T. Bui, A. Leung, C. Wang: Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity *Endocrinology* 140, 1709-17 (1999)

31. Li LH, R.N. Wine, R.E. Chapin: 2-Methoxyacetic acid (MAA)-induced spermatocyte apoptosis in human and rat testes: an *in vitro* comparison. *J Androl* 17, 538-49 (1996)

32. Meistrich ML: Effects of chemotherapy and radiotherapy on spermatogenesis *Eur Urol* 23, 136-41 (1993)

33. Lee J, J.H. Richburg, S.C. Younkin, K. Boekelheide: The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology* 138, 2081-2088 (1997)

34. D'Alessio A, A. Riccioli, P. Lauretti, F. Padula, B. Muciaccia, P. De Cesaris, A. Filippini, S. Nagata, E. Ziparo: Testicular FasL is expressed by sperm cells. *Proc Natl Acad Sci U S A* 98, 3316-21 (2001)

35. Hikim AP, Y. Lue, C.M. Yamamoto, Y. Vera, S. Rodriguez, P.H. Yen, K. Soeng, C. Wang, R.S. Swerdloff: Key apoptotic pathways for heat-induced programmed germ cell death in the testis. *Endocrinology* 144, 3167-75 (2003)

36. Sakkas D, O. Moffatt, G.C. Manicardi, E. Mariethoz, N. Tarozzi, D. Bizzaro: Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 66, 1061-7 (2002)

37. Weng SL, S.L. Taylor, M. Morshedi, A. Schuffner, E.H. Duran, S. Beebe, S. Oehninger: Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod* 8, 984-91 (2002)

38. Paasch U, S. Grunewald, G. Fitzl, H.J. Glander: Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. *J Androl* 24, 246-52 (2003)

39. Paasch U, S. Grunewald, A. Agarwal, H.J. Glandera: Activation pattern of caspases in human spermatozoa. *Fertil Steril* 81, Suppl 1:802-9 (2004)

40. Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 326, 1-16 (1997)

41. Bratton SB, M. MacFarlane, K. Cain, G.M. Cohen: Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. *Exp Cell Res* 256, 27-33 (2000)

42. Earnshaw WC, L.M. Martins, S.H. Kaufmann: Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 68, 383-424 (1999)

43. Muratori M, I. Porazzi, M. Luconi, S. Marchiani, G. Forti, E. Baldi: AnnexinV binding and merocyanine staining fail to detect human sperm capacitation. *J Androl* 25, 797-810 (2004)

44. Laakko T, L. King, P. Fraker: Versatility of merocyanine 540 for the flow cytometric detection of apoptosis in human and murine cells. *J Immunol Methods* 261, 129–139 (2002)

45. Moustafa MH, R.K. Sharma, J. Thornton, E. Mascha, M.A. Abdel-Hafez, A.J. Thomas, A. Agarwal: Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 19, 129–138 (2004)

46. Shen HM, J. Dai, S.E. Chia, A. Lim, C.N. Ong: Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod* 17, 1266-73 (2002)

47. De Vries KJ, T. Wiedmer, P.J. Sims, B.M. Gadella: Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. *Biol Reprod* 68, 2122-34 (2003)

48. Gadella BM and R.A. Harrison: Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the

apical head plasma membrane of boar sperm cells. *Biol Reprod* 67, 340-50 (2002)

49. Duru NK, M. Morshedi, S. Oehninger: Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. *Fertil Steril* 74:1200–1206 (2000)

50. Vinatier D, P. Dufour, D. Subtil: Apoptosis: a programmed cell death involved in ovarian and uterine physiology. *Eur J Obstet Gynecol Reprod Biol* 67, 85–102 (1996)

51. Grunewald S, U. Paasch, T.M. Said, R.K. Sharma, H.J. Glander, A. Agarwal: Caspase activation in human spermatozoa in response to physiological and pathological stimuli. *Fertil Steril* 83 Suppl 1:1106-12 (2005)

52. Taylor SL, S.L. Weng, P. Fox, E.H. Duran, M.S. Morshedi, S. Oehninger, S.J. Beebe: Somatic cell apoptosis markers and pathways in human ejaculated sperm: potential utility as indicators of sperm quality. *Mol Hum Reprod* 10, 825-34 (2004)

53. Muratori M, M. Maggi, S. Spinelli, E. Filimberti, G. Forti, E. Baldi: Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 24, 253-62 (2003)

54. Wang X, R.K. Sharma, S.C. Sikka, A.J. Jr Thomas, T. Falcone, A. Agarwal: Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril* 80, 531-5 (2003)

55. Marchetti C, M.A. Gallego, A. Defossez, P. Formstecher, P. Marchetti: Staining of human sperm with fluorochrome-labeled inhibitor of caspases to detect activated caspases: correlation with apoptosis and sperm parameters. *Hum Reprod* 19, 1127-34 (2004)

56. Paasch U, R.K. Sharma, A.K. Gupta, S. Grunewald, E.J. Mascha, A.J. Jr Thomas, H.J. Glander, A. Agarwal: Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod* 71, 1828-37.(2004)

57. Said TM, U. Paasch, H.J. Glander, A. Agarwal: Role of caspases in male infertility. *Hum Reprod Update* 10, 39-51 (2004)

58. Sakkas D, E. Seli, G.C. Manicardi, M. Nijs, W. Ombelet, D. Bizzaro: The presence of abnormal spermatozoa in the ejaculate: did apoptosis fail? *Hum Fertil (Camb)* 7, 99-103 (2004)

59. De Lamirande E and C. Gagnon: The dark and bright sides of reactive oxygen species on sperm function. In: The Male Gamete: From Basic Science to Clinical Applications. Eds: Gagnon C, *Cache River Press*, IL 455–467 (1999)

60. Aitken RJ and C. Krausz: Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 122, 497-506 (2001)

61. Twigg JP, N. Fulton, E. Gomez: Analisi*s* of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and affectiveness of antioxidant. *Hum Reprod* 13, 1429-1436 (1988)

62. Lopes S, A. Juriscova, J.G. Sun, R.F. Casper: Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Hum Reprod* 13, 896-9000 (1998)

63. Aitken RJ, J.S. Clarkson, T.B. Hargreave, D.S. Irvine, F.C. Wu: Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl* 10, 214-20 (1989)

64. Mazzilli F, T. Rossi, M. Marchesini, C. Ronconi, Dondero F: Superoxide anion in human semen related to seminal parameters and clinical aspects. *Ferti Steril* 62, 862-8, (1994)

65. Kodama H, R. Yamaguchi, J. Fukuda, H. Kasai, T. Tanaka: Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 68, 519-24 (1997)

66. Barroso G, M. Morshedi, S. Oehninger: Analysis of DNA fragmentation, plasma, membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 15, 1338-44 (2000)

67. Agarwal A, K.P. Nallella, S.S. Allamaneni, T.M. Said: Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online* 8, 616-27 (2004)

68. Gomez E, D.W. Buckingham, J. Brindle, F. Lanzafame, D.S. Irvine, R.J. Aitken: Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 17, 276-87. (1996)

69. Aitken RJ, D. Sawyer: The human spermatozoon--not waving but drowning. *Adv Exp Med Biol* 518, 85-98 (2003).

70. Ollero M, E. Gil-Guzman, M.C. Lopez, R.K. Sharma, A. Agarwal, K. Larson, D. Evenson, A.J. Jr Thomas, J.G. Alvarez: Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod* 16, 1912–1921 (2001)

71. Gergely A, E. Kovanci, L. Senturk, E. Cosmi, L. Vigue, G. Huszar: Morphometric assessment of mature and diminished-maturity human spermatozoa: sperm regions

that reflect differences in maturity. *Hum Reprod* 14, 2007–2014 (1999)

72. Lachaud C, J. Tesarik, M.L. Canadas, C. Mendoza: Apoptosis and necrosis in human ejaculated spermatozoa. *Hum Reprod* 19, 607-10 (2004)

73. Sotolongo B, T.T. Huang, E. Isenberger, W.S. Ward: An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. *J Androl* 26, 272-80 (2005)

74. Cayli S, D. Sakkas, L. Vigue, R. Demir, G. Huszar: Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. *Mol Hum Reprod* 10, 365-72 (2004)

75. Blanco-Rodriguez J and C. Martinez-Garcia: Apoptosis is physiologically restricted to a specialized cytoplasmic compartment in rat spermatids. *Biol Reprod* 61, 1541-7 (1999)

76. Grunewald S, U. Paasch, T.M. Said, R.K. Sharma, H.J. Glander, A. Agarwal: Caspase activation in human spermatozoa in response to physiological and pathological stimuli. *Fertil Steril* 1, 1106-12 (2005)

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