

Role of Sperm Chromatin Damage in Abnormal Reproductive Outcomes

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Reproductive Toxicology Overview

The potential for reproductive ill effects from environmental, occupational, accidental, and therapeutic exposure to noxious agents is a major, growing public concern. Decades of animal and human research have solidly documented that a host of risk factors such as ionizing radiation and various chemical toxicants can reduce or eliminate male fertility. Adult exposure to chemicals is capable of decreasing sperm numbers produced by the mammalian testis, as well as reducing the motility, normal morphology, fertilizing capacity and genetic integrity of the remaining sperm.

Solid evidence also exists that exposure of the male to germinal mutagens before fertilization can have detrimental effects on embryo viability and health of offspring that can be manifested at birth or later in their life. Thus, there is increased interest in clinical matters such as fertility assessment and genetic counseling, and in human and veterinary assisted reproductive techniques. This contribution here will emphasize the use of flow cytometry (FCM) to measure end points of toxicology and fertility measures. Particular emphasis will be made on the use of acridine orange (AO) for the measurement of DNA and RNA content in testicular cells reflecting normal/abnormal testicular kinetics and the normality of sperm chromatin as a measure of toxicology damage and fertility potential.

Testicular Cell Kinetics And Toxicology

A suspension of testicular biopsy cells stained with AO and measured by FCM provides statistically robust measures on the relative percent of eight different testicular cell types. Figure 1 shows examples of cell type distributions from a normal bull testis and from a testis obtained from a bull that had been implanted with Zeranor. Note the loss of normal testicular cell kinetics as a result of the implant. This pattern is consistent with many potential reproductive toxicants. A significant increase in the percent of epididymal sperm with poor DNA integrity was observed in the Zeranor implanted bulls.

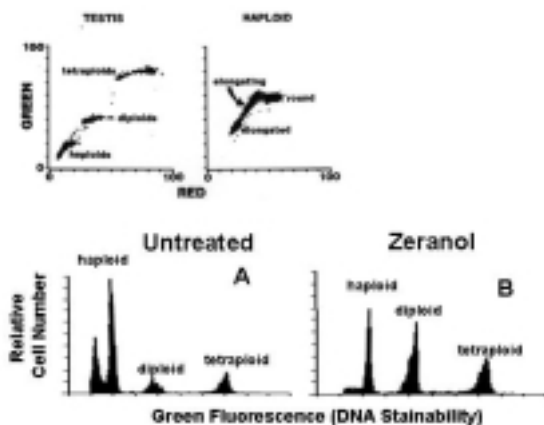


Figure 1. Effects of preweaning Zeranor growth implants (resorcylic acid lactone) on reproductive function in Simmental-Angus bulls. Normal distribution is shown in the cytograms at the top and in the untreated example (A). The testes of implanted bulls had an altered testicular cell distribution as seen in the frequency histogram on the right (B).

Sperm DNA Fragmentation and Toxicology

SCSA[®] protocol – Our laboratory is the originator of the Sperm Chromatin Structure Assay (SCSA[®]), a very rapid, highly repeatable flow cytometric assay [7,13]. The SCSA[®] defines abnormal chromatin structure as the susceptibility of DNA to low pH-induced denaturation *in situ*. DNA in normal sperm does not denature under the SCSA[®] conditions. In the SCSA[®], whole spermatozoa or sonication released sperm nuclei are treated for 30 sec with pH 1.2 buffer for 30 sec to potentially denature DNA *in situ*, stained with AO, a metachromatic dye, and then measured by FCM. AO intercalated into native, double stranded DNA fluoresces green following excitation with blue light, whereas AO associated with single stranded DNA fluoresces red. Thus, DNA denaturation results in a shift from green to red fluorescence [16]. The extent of DNA denaturation is quantitated by the expression, DNA Fragmentation Index, (DFI; [13]) which is red fluorescence / (red + green) fluorescence. SCSA[®] data from stallion semen are shown in Fig. 2.

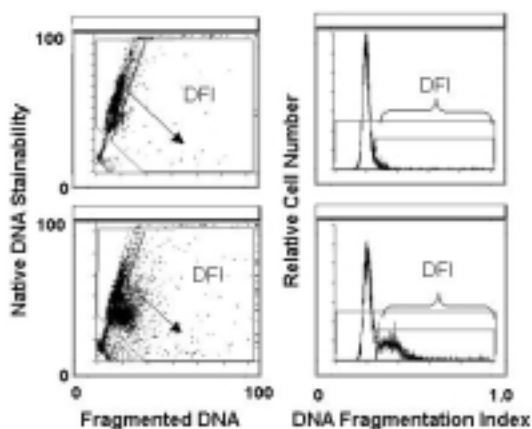


Figure 2. Native DNA Stainability (green fluorescence) vs Fragmented DNA (red fluorescence) cytograms are shown on the left; corresponding DNA Fragmentation Index histograms are shown on the right. The top row of FCM data is from a fertile stallion and the bottom from a stallion having poor fertility status. Note the increased % DFI in the bottom sample.

The percent sperm outside the main population (DFI) is determined on 5000 cells per sample. Sonicated sperm samples (cytoplasmic components separated from nuclei) produce the same results as whole sperm. Since this biomarker has been shown to be highly related to fertility and embryo development [2,3,5-7,12-15,16,19,24,25] it has been concluded that data are reliable and repeatable ($r \geq 0.98$). Measurements of sperm chromatin status on thousands of semen samples from rodents, small animals, bulls, stallions, boars and humans with potentially abnormal sperm chromatin have shown a strong relationship between SCSA[®] data, fertility and toxicant induced damage. Biomarkers for chromatin abnormalities, either independent or related to DNA denaturation are abnormal protein content [4] and specific DNA strand breaks [22]. The primary power of the SCSA[®] is to determine the percent of sperm in an ejaculate (or epididymal) with DNA that is highly susceptible to denaturation *in situ* following a low pH treatment and staining with AO. A variety of studies provide evidence that the amount of DNA denaturation per sperm is relative to the extent of DNA fragmentation, likely expressed as DNA strand breaks [13]. We have found, primarily from studies on humans [12,19 & unpublished], that when >30% of the sperm in an ejaculate have SCSA[®] - defined DNA damage, the statistical probability of producing a successful pregnancy is dramatically reduced (various studies show a reduction anywhere from <1 to 50%). Data from bulls and stallions suggest about the same threshold level.

SCSA and Uncompensable Fertility Factors — It is now clear that there are semen quality differences among males that are compensable in that fertility differences among such males can be minimized or eliminated by adjusting the quantity of sperm in the dosage [23]. On the other hand, there are subfertile males that cannot be brought to normal fertility by increasing the inseminate dosage, thus rendering the semen traits or deficiencies of such males, uncompensable [6,17,23]. Collaborative research between the laboratories of Drs. Saacke and Evenson, involved the effect of a mild thermal stress on bull sperm [17] and revealed that semen with specific abnormalities can be produced following a 48-hour scrotal insulation [28].

Specifically, experiments compared ejaculates obtained prior to thermal insulation with that obtained up to 21 days after insulation (+21d). By +21d high numbers of sperm with nuclear vacuoles were observed. Concomitantly, the sperm from +21d resulted in a significant increase in degenerate and fair to poor embryos at the expense of the excellent to good embryos when compared with the control sperm. Thus, nuclear vacuoles and diadem defects would appear to constitute an uncompensable factor in sperm. Of significance, the SCSA[®] detected chromatin abnormalities in samples +3d (Figure 3), while the light microscope did not detect abnormalities (e.g., nuclear vacuoles) until +7d to +11d. Thus, the SCSA[®] is very sensitive in detecting molecular abnormalities that result in more gross abnormalities later. This same phenomenon was also observed with mouse thermal testis insult [26], Figure 4.

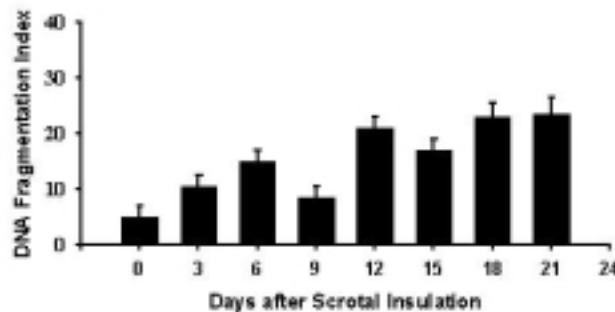


Figure 3. Effects of 48 hr scrotal insulation on sperm nuclear chromatin resistance to denaturation. Sperm collected on days 3, 6 and 9 were presumably in the epididymis or rete testis during the insulation period. (Reproduced with permission of Journal of Andrology, Karabinus et al, 1997, figure 2).

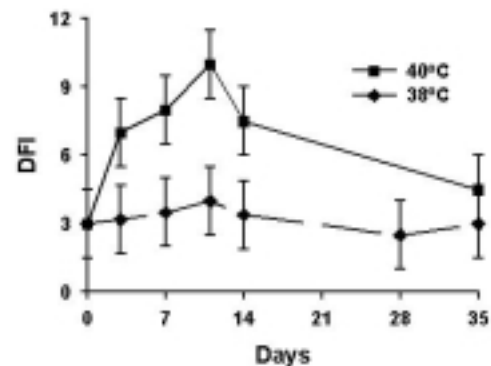
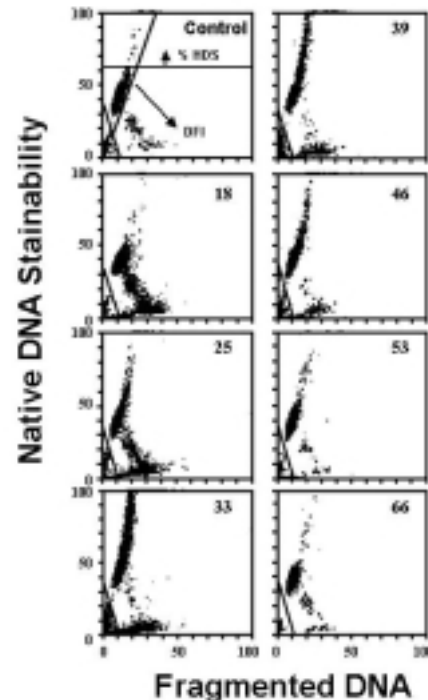


Figure 4. Effect of heat (38 and 40°F) applied to mouse testes and measured by the SCSA[®]. Graph shows DFI values at various time points after heat treatment. (Reproduced with permission of Journal of Andrology, Sailer et al, 1997, figure 6).

Whether external heat applied to bull or mouse testes has any similarity to human influenza induced fever of 104° F for a day is not known. However, the data are similar in that at 18 days post fever the SCSA[®] data showed a high DFI (Figure 5; [10]). Interestingly this phenomenon was then decreased and at 33 days post fever a high percent of the sperm showed increased DNA stainability. Dr. Rod Balhorn extracted from electrophoretic gels and partially sequenced an abnormally large protamine that was identified as the precursor to protamine 2. We have suggested that the fever affected the transcription or translation of the gene for the enzyme that

would normally cut the P2 protamine. The insertion of an abnormally long protamine into the paternal genome likely produced a less than normal chromatin condensation resulting in the higher accessibility of the AO into the DNA. The SCSA[®] pattern returned to normal after an approximate one-spermatogenic cycle thus showing that the fever affected a broad range of testicular cell types. Medical advice has for some time suggested that high fever is negative for male reproductive efficiency, and these data suggest a very significant alteration of sperm chromatin structure likely incompatible with good fertility potential. Similar patterns have been found in semen of bulls exposed to environmental heat stress [unpublished].

Figure 5. Native DNA Stainability (or double-stranded DNA quantitated by amount of green fluorescence) vs Fragmented DNA (single-stranded DNA quantitated by amount of red fluorescence) from sperm cells stained with AO and measured by FCM. Numbers in each cytogram indicate the number of days post fever. (Reproduced with permission of Journal of Andrology, Evenson et al, 2000, figure 1).



SCSA Data and Fertility

— **Boars.** In a previous heterospermic insemination trial, the SCSA[®] correctly identified the three highest and three lowest fertility ranking boars [15]. In some very exciting, current work [5], we evaluated 6 commercial boars with extensive fertility records. Of great interest, we “blindly” predicted the fertility ranking in the same order as the field trials. Of even greater interest, there was a high correlation between the SCSA[®] data and the number of pigs in the litters, i.e., with the best producing an average of 10 pigs and the poorer producing 8.5. This 1.5 pigs/litter difference represents a huge financial difference for hog producers.

— **Bulls.** Previous work has repeatedly shown strong negative correlations between the susceptibility of sperm nuclear DNA to denaturation as measured by the SCSA[®] and measures of bull fertility, namely, nonreturn rate ($r=-0.77$, $P<0.001$ [3]) and competitive index ($r=-0.94$, $P<0.01$; Figure 6) based upon heterospermic performance. [2].

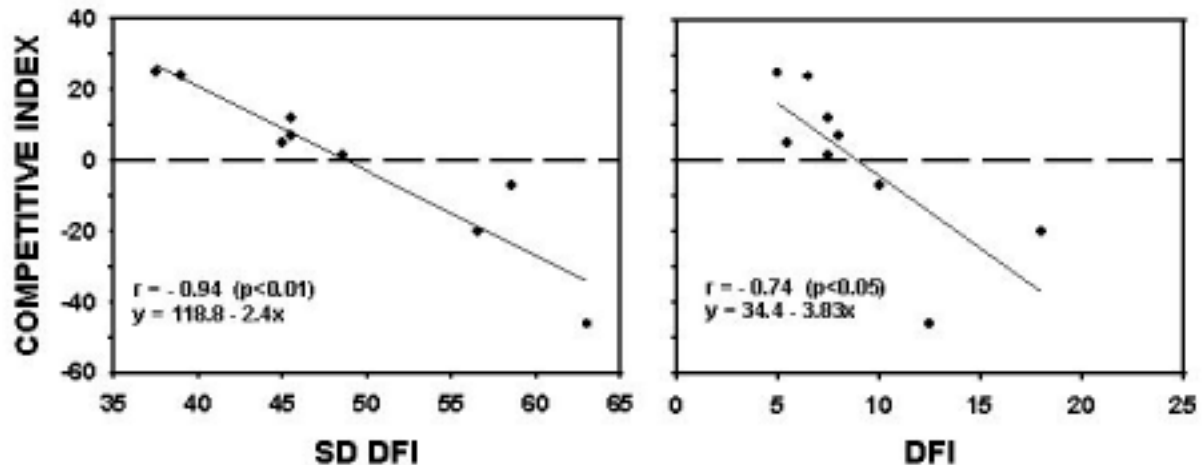


Figure 6. The SCSA[®] variable SD of DFI is correlated ($r=-0.94$, $P<0.01$), with the competitive index of a heterospermic insemination bull study [2]. Semen from 9 bulls with different phenotypes were mixed in various combinations and the % offspring of these provided a bull semen competitive index. (Reproduced with permission of Journal of Andrology, Ballachey et al, 1988, figure 2).

— **Stallions.** The table below shows a 60% decrease in seasonal pregnancy rate (SPR) when the mean DFI doubled from 16 to 32%.

Table 1.

Sperm chromatin structure and sperm parameters of fertile and subfertile stallions (mean $\pm$ sd)

Stallions	SPR*	Mean DFI*	SD DFI*	DFI (%)*	% Progressive motility*	% Morphologically normal*
Fertile	86 $\pm$ 8	220 $\pm$ 40	81 $\pm$ 31	16 $\pm$ 17	58 $\pm$ 17	56 $\pm$ 21
(n)	(60)	(48)	(59)	(54)	(32)	(24)
Subfertile	34 $\pm$ 23	262 $\pm$ 55	90 $\pm$ 32	32 $\pm$ 23	44 $\pm$ 22	42 $\pm$ 21
(n)	(21)	(16)	(33)	(33)	(23)	(26)

* Values within columns are different, $p<0.05$

(Reprinted with permission Biol Reprod Mono, Equine Reprod VI, Kenney et al, 1995, Table 1).

— **Humans.** SCSA[®] data were predictive of time to pregnancy for 200 couples from the general population in a comprehensive fertility study at Georgetown University [12]. The SCSA[®] data were significantly different between the couples that conceived during the first three months of the study, vs. those who conceived over the next 9 months, vs those that were not pregnant by 12 months ($r=0.86$, $P<0.001$). In sharp contrast to the generally good quality of the semen samples in the Georgetown study (couples selected with no known reproductive problems), another

SCSA[®] evaluation of 140 consecutive patients at an infertility clinic showed much poorer quality SCSA[®] values [12]. These data showed differences in sperm chromatin quality for two human populations (the general population containing fertile and infertile individuals versus patients specifically presenting for infertility). Current ongoing studies of ~ 1000 IVF fertilizations, clearly show that SCSA data are statistically predictive of significantly reduced success of pregnancy when the percent of sperm with fragmented DNA in an ejaculate is over 30%. These and other animal data [1] show that sperm with damaged nuclear DNA can fertilize oocytes equally as well as sperm with normal nuclear DNA but with resulting embryonic loss.

SCSA[®] and Effect of Age on DNA Fragmentation — Sperm DNA integrity is age dependent. A current study on humans [Wyrobek, Evenson & Eskenazi, in preparation] clearly shows a decrease in sperm DNA integrity in men ranging in age from the 20's to 70's. Figure 7 shows the results of a healthy bull with semen samples taken over a four-year span. The first sample (A,D) is of excellent SCSA[®] quality and semen parameters. The second sample (B,E) also had excellent semen quality parameters but the SCSA[®] showed loss of DNA integrity corresponding to a loss of breeding efficiency. The final sample (C,F) from the same bull had nearly 100% DFI and the bull was functionally sterile.

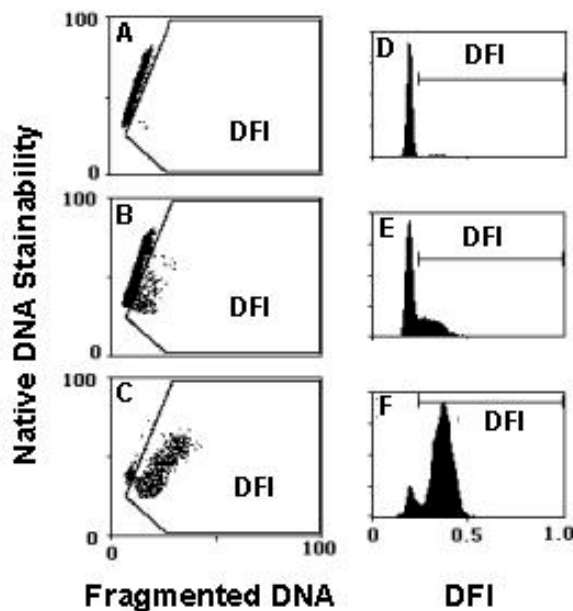


Figure 7. Flow cytometric data from acid-stressed AO-stained bull sperm. Cytograms on the left depict Native DNA Stainability vs Fragmented DNA. Corresponding DNA Fragmentation Index histograms are in the right column. (Reproduced with permission of Journal of Andrology, Sailer et al, 1996, figure 1).

SCSA[®] and Toxicology — An overview of the testicular differentiation pathway, reproductive tract events and potential modifiers of those events that result in loss of reproductive efficiency is seen in Figure 8. Since millions of spermatozoa per testis are produced daily that undergo very extensive differentiation and very unique macromolecular and cellular events, many modifiers can often target specific events [8] that result in loss of reproductive efficiency (see outline in Figure 9).

ENVIRONMENTAL DOSIMETRY		
DIFFERENTIATION PATHWAY	REPRODUCTIVE TRACT EVENT	MODIFIERS
SPERMATOGONIA	Macromolecular Synthesis	Chemicals
SPERMATOCYTES		
	Meiosis Exchange of Nuclear Histones for Protamines	Radiation
	S-S Bonding	Drugs
SPERMATIDS		Fever Heat Diet Air Pollutants
SPERM	Sperm Specific Morphology	

Figure 8. An overview of the testicular differentiation pathway, reproductive tract events and potential modifiers of those events.

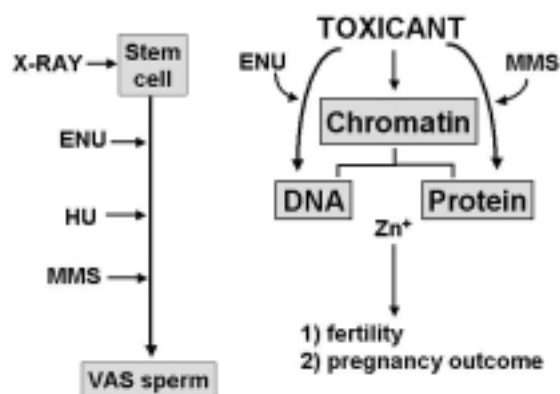


Figure 9. Many modifiers can target specific reproductive tract events that result in loss of reproductive efficiency.

Data on epididymal sperm obtained from mice 40 days after their testes were exposed to X-radiation are shown in Figure 10. Thus the damage incurred by the stem cells remained during passage through the reproductive tract and was measured as DNA damage by the SCSA[®] in mature sperm. The SD of the DNA fragmentation index showed a statistically significant level of damage following exposure to 25 rads. Only 3 mice were used per dosage level and it is likely that if more had been used the data would have been significant at the 12.5 rad level making the SCSA[®] the most sensitive assay for non-invasive detection of radiation induced damage in mature sperm.

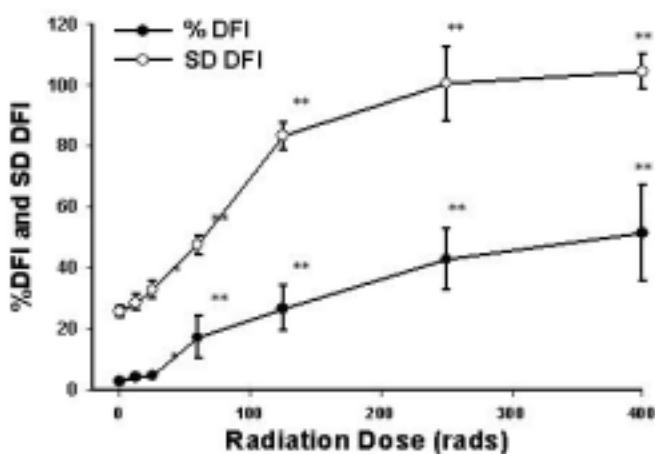


Figure 10. Relationships between DFI measures in epididymal sperm 40 days after testicular X- radiation exposures from 0 to 400 rads. (Reproduced with permission of Environmental & Molecular Mutagenesis, Sailer et al, 1995, figure 5).

An example of an agent targeting spermatozoa in the narrow window of epididymal passage was the use of methyl methanesulfonate (MMS), a known alkylating agent of free -SH groups present on cysteine residues of sperm protamines [9]. This alkylation breaks the DNA strands [27]. Following exposure of the MMS, the first epididymal sperm sample was measured by the SCSA[®] at 3 days post exposure. Already, 80% of the sperm showed significant levels of DNA

fragmentation (Figure 11a). Using the exact same protocol, Sega found that sperm obtained from MMS treated mice 0-4 days after exposure produced normal fertilization and embryo development. However, animals bred 5-8 days after MMS exposure produced a significant loss of the resulting embryos, most likely due to DNA strand breaks (as shown to exist) that made the paternal genome incompetent for embryo development [25]. SCSA[®] data indicated the early molecular events that led to more dramatic and lethal effects at a later time point (Figure 11b).

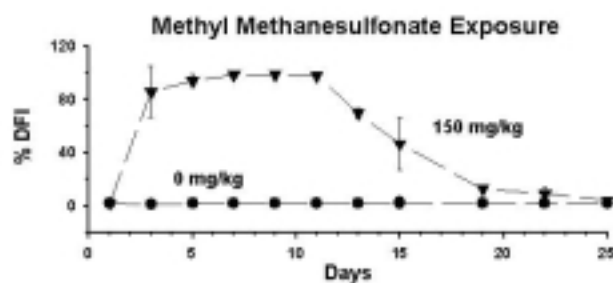


Figure 11a. Effects of 0 or 150 mg/kg MMS exposure on % DFI as measured by the SCSA[®]. (Reproduced with permission of Environmental & Molecular Mutagenesis, Evenson et al, 1993, figure 6).

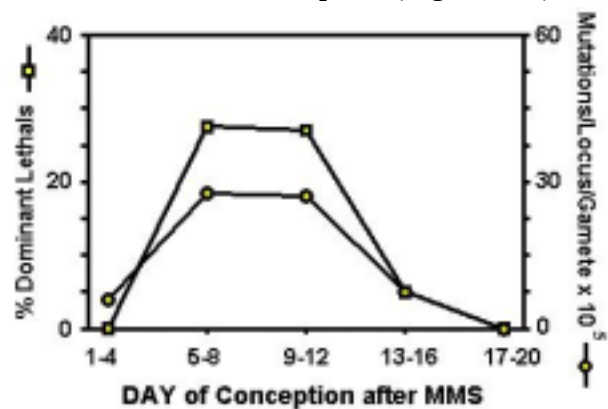


Figure 11b. % Dominant Lethals and Mutation levels in embryos at various days after conception following exposure to the male with MMS. (Reproduced with permission of Mutation Research, Sega et al, 1983).

The role of the nuclear intra- and intermolecular disulfide bonding may be important to produce a nuclear chromatin structure that is highly impervious to physical and chemical assault [21]. This is evident by the fact that sperm nuclei are unaffected by sonication at a level that destroys somatic cells as well as the cytoplasm of the sperm. Reports [18] have suggested that the DNA denaturation of the SCSA[®] is dependent on the level of the free disulfide bonds in the sperm. Our limited data as shown in Figure 12 [11] do not support this view.. In this case we have measured 28 samples of stallion sperm by the SCSA[®] and a -SH specific dye and showed no statistical significance between the level of stallion sperm DNA fragmentation and the extent of sperm nuclear S-S bonding.

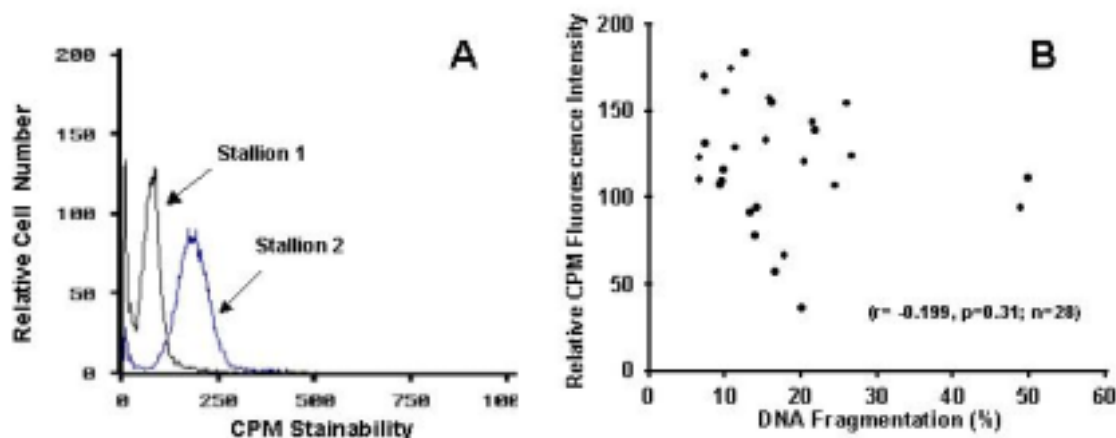


Figure 12a. Frequency histogram of CPM (7-diethylamino-3-(4'-malemidylphenyl)-4-methyl-coumarin) stainability of free –SH groups from 2 stallions. Stallion 2 has the greatest number of free –SH groups that bind to the CPM stain (Reproduced with permission of J Reprod and Fert Suppl, Evenson et al, 2000, figure 2).

Figure 12b. Scatter plot of the coordinates for each of 28 stallion semen samples related to the percentage of cells with fragmented DNA (% DFI) and mean blue fluorescence of free –SH groups measured by CPM stainability. Note the heterogeneity in values from both biomarkers and the lack of correlation between them. (Reproduced with permission of J Reprod and Fert Suppl, Evenson et al, 2000, figure 3).

Conclusion

The SCSA[®] is a very practical, statistically rigorous assay that can easily be used to detect semen samples containing sperm with damaged DNA leading to reproductive failure.

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