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DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: Evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide

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ABSTRACT

Sperm DNA fragmentation (SDF) is not a static seminal parameter, since the longevity of sperm DNA decreases progressively with time following ejaculation or thawing. While the dynamics of SDF is a species-specific characteristic, in the case of humans, there is still significant variation within patients. To evaluate the suitability of the dynamic SDF assay to assess the adverse effects of agents that cause genetic damage, fresh semen samples from different donors were exposed in vitro to (1) increasing acute doses of ionizing radiation, (2) elevated temperature (41 °C and 45 °C), (3) acidic pH (pH 4) and (4) the nitric oxide (NO) donor sodium nitroprusside (SNP). Sperm DNA fragmentation was analyzed after an incubation period of chronic (24 h), or acute (1 h) exposure to each treatment followed by incubation at 37 °C over a period of 24 h. SDF was assessed using the sperm chromatin dispersion (SCD) test. Dynamic SDF for each treatment was analyzed using Kaplan–Meier survival curves. All agents, except for ionizing radiation, accelerated SDF kinetics following chronic exposure over a 24 h period. Transient exposure to NO and heat but not acidic pH increased the basal (T0) level of SDF. Despite the removal of the three toxicants, the remaining sperm following acute exposure showed a decrease in their expected DNA longevity. It is concluded that the assessment of sperm DNA fragmentation dynamics is an effective methodological approach for revealing latent damage associated with toxicants that is not initially expressed following a single initial observation of SDF.

1. Introduction

Every human ejaculate contains a proportion of sperm with fragmented DNA but this fraction may be elevated in patients with idiopathic infertility, obstructive or secretory azoospermia, varicocele, infections or cancer [1]. High levels of sperm DNA fragmentation (SDF) have also been associated with decreased oocyte fecundation, embryo quality and pregnancy rate [1,2]. Intriguingly, an intracytoplasmatic sperm injection (ICSI) study of mouse oocytes, using spermatozoa with a high percentage of SDF, revealed that a significant proportion of adult offspring produced by this procedure, showed a significant increase in the incidence of abnormal behavioral tests, malformations, tumors and signs of premature aging [3]. SDF is also an informative biomarker in studies of reproductive toxicology, as exposure of the testis to reproductive toxicants, such as thermal stress, ionizing radiation, chemotherapy, pesticides, air pollution and smoking have all resulted in a higher incidence of sperm DNA damage [4–6].

Given the recognized instability of the sperm cell post ejaculation, standard seminal parameters such as motility and vitality are typically evaluated at different times following collection or during semen processing. To the contrary, the frequency of sperm with fragmented DNA continues to be reported by andrology laboratories as a static number, without appropriate acknowledgment that sperm DNA is equally as susceptible to iatrogenic damage. Recent studies of human spermatozoa have indicated that SDF is in fact not stable, and that the frequency of sperm cells with fragmented

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DNA tends to increase with time after sample collection or following thawing of cryopreserved spermatozoa [7–10]; these studies have clearly shown that when examined in terms of fragmentation dynamics, human sperm DNA degrades progressively. A general standard pattern and velocity for human sperm DNA degradation has recently been established and appears to be distinctive from that of other mammals [7,11]. Nevertheless, apparent variation in the kinetics of SDF among individual donors or patients means that this phenomenon can potentially be used as a diagnostic or as a predictive measure of fertility.

The SDF dynamics evaluated by in vitro incubation provides important information about the evolution of DNA integrity in the mature spermatozoon that is typically not revealed on basal assessment of SDF [12]. In this paper, we examine the effect of known toxicants on the SDF rate of donors, in order to validate the dynamic procedure as a reliable assay within the context of reproductive toxicology. Semen samples from different donors were exposed in vitro to increasing doses of ionizing radiation, elevated temperature, an acidic pH environment and the nitric oxide (NO) donor sodium nitroprusside (SNP). Spermatozoa were incubated to mimic both chronic (24 h incubation at 37 °C) and acute (1 h) exposure of each toxicant and thereby improve the clinical relevance of the procedure. The overarching hypothesis of these investigations was that dynamic assessment of sperm DNA following exposure of sperm to known toxicants will reveal cryptic damage that is not normally revealed by a single initial baseline assessment of the SDF.

2. Material and methods

Semen samples were obtained from normozoospermic fertile donors, after informed, written consent.

2.1. Effect of ionizing radiation

Semen samples from three donors (A–C) were collected by masturbation and distributed into aliquots in insulin sterile syringes (Becton Dickinson, Franklin Lakes, NJ). Immediately after collection and dilution, the spermatozoa were exposed in a Clinac 2100 C/D radiotherapy unit (Varian Medical Systems, Palo Alto, CA) to different doses of X-rays 6 MeV: 0, 0.5, 1, 2, 5, 10 Gy, at 6.6 Gy/min. Following acute X-ray exposure, the spermatozoa were incubated at 37 °C for 0, 2, 4, 6, 8 and 24 h. After each incubation time period, samples were removed and processed to determine the percentage of sperm cells with fragmented DNA (%SFD) using the sperm chromatin dispersion (SCD) test, according to the instructions supplied with the kit (Dyn-Halosperm® kit, Halotech DNA SL, Madrid, Spain [13]). Using this technique, sperm cells immersed in an agarose microgel are treated with an acid solution followed by a lysing solution. Sperm nuclei with DNA fragmentation show very small halos or no halos of DNA dispersion, whereas those sperm nuclei without DNA fragmentation release their DNA loops forming large halos emerging from the core. Three hundred spermatozoa were scored per experimental point.

2.2. Effect of hyperthermia

Fresh semen samples from three donors (D–F) were incubated at 37 °C, 41 °C and 45 °C in a water bath over a 24 h period (chronic treatment) and aliquots of spermatozoa collected at 0, 0.5, 1, 2, 4, 6, 8, 24 h to determine %SDF. In order to induce a transient heat stress exposure, aliquots of the original sample were also incubated at 41 °C and at 45 °C for 1 h (acute treatment) and then subsequently cooled to 37 °C and incubated. Aliquots of spermatozoa were subsequently examined at 0, 0.5, 1, 2, 4, 6, 8, 24 h to determine %SDF; T0 represented the period immediately after 1 h incubation at the elevated temperature. SDF was determined following the method described above.

2.3. Effect of acidic pH

Fresh semen samples from two donors (G and H) were collected by masturbation. The pH of one aliquot was changed to 4 by adding a citric acid solution (1.5 M; pH 1.34); another aliquot of these semen samples was also processed as the control sample (pH 8). Spermatozoa were then incubated at 37 °C at the respective pH for 24 h (chronic treatment). In order to mimic a transient exposure to an acidic environment (pH 4), aliquots of the original semen sample were also exposed to pH values of 4 and 8 (control samples) for 1 h (acute treatment), centrifuged and re-suspended in PBS (pH 7) and then further incubated at 37 °C for 24 h. Aliquots of spermatozoa were collected at 0, 0.5, 1, 2, 4, 6, 8, 24 h to determine %SDF; T0 represented the period immediately after 1 h incubation at the designated pH. SDF was determined following the method described above.

2.4. Effect of NO

Semen samples from three donors (I–K) were collected by masturbation and immediately incubated at 37 °C for 24 h (chronic treatment) with 0, 5, 10, 25 and 50 mM SNP (Sigma, Madrid). Aliquots of each sample were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 24 h to determine %SDF. In order to mimic a transient exposure to NO, aliquots of the original semen sample were also exposed to SNP for a period of 5 min (acute treatment), then centrifuged, re-suspended in PBS (pH 7) and incubated at 37 °C for a 24 h period. Aliquots of spermatozoa were collected at 0, 0.5, 1, 2, 4, 6, 8, 24 h to determine %SDF, where T0 represented the period immediately after the acute 5 min incubation with SNP. Incubations in all experiments were performed in darkness, as SNP is light sensitive. SDF was determined following the method described above.

2.5. Statistical analysis

All data were analyzed using the SPSS 19 software package for Windows (SPSS Inc., Chicago). Pearson chi-square test was performed to assess if the acute treatment affected the %SDF immediately after the treatment (T0). To determine if treatments modified the dynamics of SDF over time, survival curves using a Kaplan–Meier analysis were determined; this approach reported the newly fragmented sperm DNA cells that appeared after each incubation time. The curves were compared using a log-rank test. During the analysis it was revealed that some sperm samples had similar dynamics but exhibited different basal %SDF values, so the test may have indicated significant differences in kinetics that were not real. This problem was evident when comparing the dynamics from the different donors and the dynamics that resulted after acute treatments, which affected the background of SDF. To avoid this complication, the SDF dynamics were standardized by subtracting the background SDF from each incubation time, thus producing a common starting survival value of 100% survival. Significance was defined as $p < 0.05$.

3. Results

3.1. Effect of ionizing radiation

The effect of X-ray dose on SDF dynamics of the three donors is shown in Fig. 1. While 3 donors showed DNA fragmentation dynamics within the normative range, X-ray dose, even as high as 10 Gy/min, had no apparent effect on the SDF dynamics of any of the donors.

3.2. Effect of hyperthermia

The effect of 24 h incubation at 37 °C, 41 °C and 45 °C of the 3 donors (D–F) is shown in Fig. 2. Significant differences in SDF were found between the three incubation temperatures ($p < 0.001$); the higher the incubation temperature, the higher the rate of SDF. In the case of acute heat stress for only 1 h, SDF increased immediately after the hyperthermic exposure and similar to that which occurred in the chronic heat stress, the higher incubation temperature was associated with a higher rate of increase in SDF. In fact, after 1 h at 45 °C, the T0 level of SDF was nearly double that of spermatozoa maintained at 37 °C (Fig. 2). The SDF rate following the acute heat stress period was accelerated, but less than that during chronic hyperthermia. In donor D, no significant difference was found between the 41 °C and 45 °C rates of SDF but both were higher when compared to the 37 °C rate. For donor E, the survival curves between 37 °C and 41 °C were statistically significant. For individual F, the higher temperatures of incubation resulted in rates of SDF that were significantly higher than the 37 °C rate ($p < 0.001$).

3.3. Effect of acidic pH

Continuous chronic exposure to pH 4 increased the SDF rate over time when compared to pH 8 for both donors ($p < 0.001$) (Fig. 3); there was no difference in rate of SDF between donors ($p = 0.83$). Both pH 4 sperm samples reached 100% SDF at 8 h incubation at 37 °C. Immediately following an acute exposure to pH 4 for 1 h, the

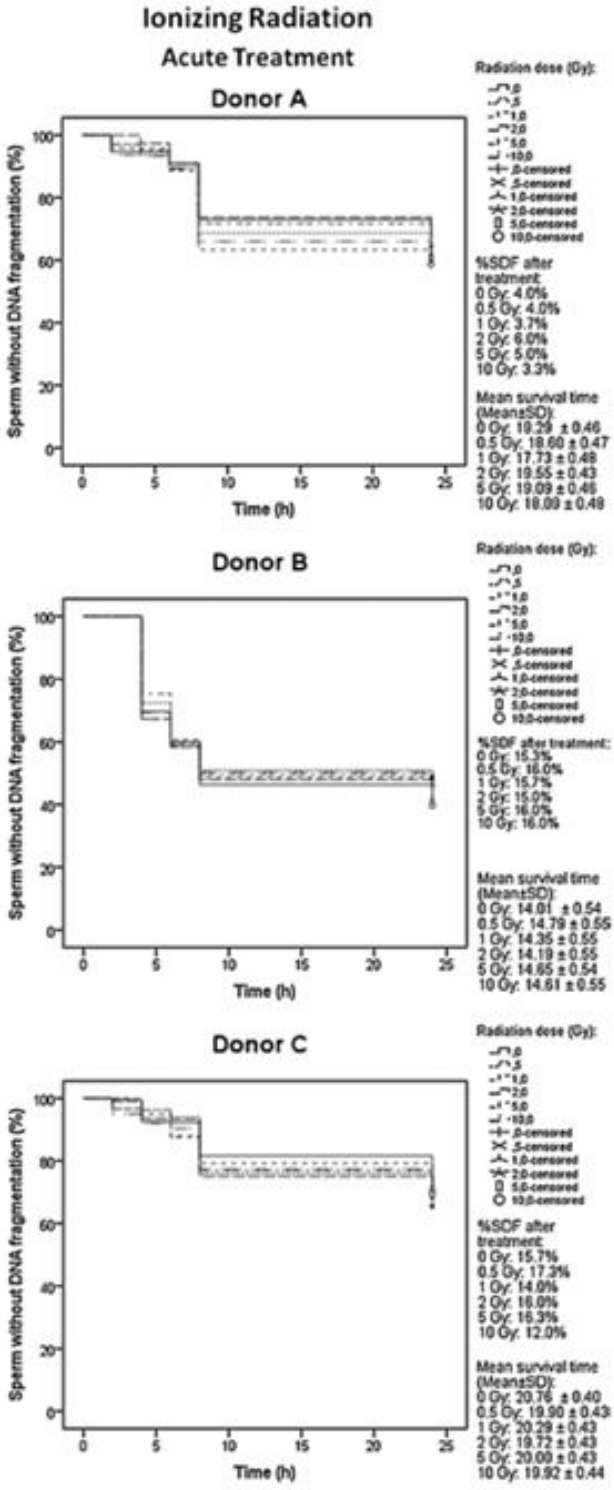

Fig. 1. Kaplan–Meier survival curves after exposure of sperm samples from donors A–C to increasing doses of X-rays, and incubated at 37 °C and at 45 °C for 24 h.

background SDF (T0) did not significantly increase compared to the control (pH 8). Nevertheless, the SDF rate from the remaining spermatozoa was significantly affected in both subjects ($p < 0.001$), although less strongly than in the samples chronically exposed to acidic pH. The rate of SDF of individual G was significantly greater than that from subject H, after acute exposure to pH 4 ($p < 0.001$).

3.4. Effect of NO

Chronic incubation with SNP for 24 h resulted in an extreme degradation of sperm DNA; most sperm were fragmented within a few hours, the intensity of the effect being similar irrespective of the SNP dose (Fig. 4). After subtracting the background SDF, the rate of SDF of all doses was found to vary significantly between individuals ($p < 0.001$). Immediately following only 5 min incubation with SNP, the %SDF increased in a dose dependent manner in all 3 donors. After subtracting this initially induced %SDF, the SDF dynamics of the remaining sperm was revealed to be clearly influenced by the previous acute SNP treatment, although it did not cause as a severe rate of SDF as that found in chronic treatment and it was found to be variable between donors; donor I showed a partial dose-effect, donor J only showed a significant alteration after the highest dose of SNP was used, whereas spermatozoa from donor K showed a similar rate of increase in SDF irrespective of the dose (Fig. 4).

4. Discussion

The results of this study have shown that the dynamic assessment of SDF is a useful tool in the evaluation and diagnosis of the effect of a range of reproductive toxicants on sperm DNA. Conventional assessment of SDF is usually limited to an initial static assessment but this approach may not elucidate the possible underlying or cryptic damage, that is only expressed when the sperm is dynamically evaluated with time [12]; this may be particularly the case when exposure to the toxicant is only transient or temporary or if the effects of the toxic agent are subtle.

4.1. Effect of ionizing radiation

Ionizing radiation interacts randomly with matter, ejecting electrons and producing reactive free radicals. After X-ray exposure, most of the damage to the biomolecule is indirect, mainly mediated by the hydroxyl radicals produced by the radiolysis of the water molecules close to the biomolecule. DNA damage induced by ionizing radiation results in single- and double-DNA strand breaks, a variety of damage to base, base-free sites, inter-strand DNA cross-links and DNA–protein cross-links [14,15]. This damage is usually detected, signalized and repaired through several interconnected and finely regulated pathways but if this damage remains unrepaired or misrepaired, it may result in mutations, chromosomal aberrations or even cell death through the activation of apoptosis [16,17]. Of course, if the DNA damage occurs in the sperm, it cannot be repaired before fertilization of the oocyte. Radiation exposure of the testis leads to cell death of radiosensitive subpopulations, mainly associated with intermediate spermatogonia. Despite this, the mature sperm appears to be a radioresistant cell, as demonstrated by irradiation in vitro [18] and in the mouse in vivo [19–21]; these studies did not detect significant DNA breakage even after exposure to 4 Gy X-rays when they were assessed with the sperm chromatin structure assay (SCSA) or comet assay. The sensitive DNA breakage detection-fluorescence in situ hybridization (DBD-FISH) procedure was only able to detect significant DNA breakage after using extremely high doses [22]. Our results confirm the high degree of radioresistance of mature spermatozoa, even when the SDF of spermatozoa are dynamically assessed. The extreme and

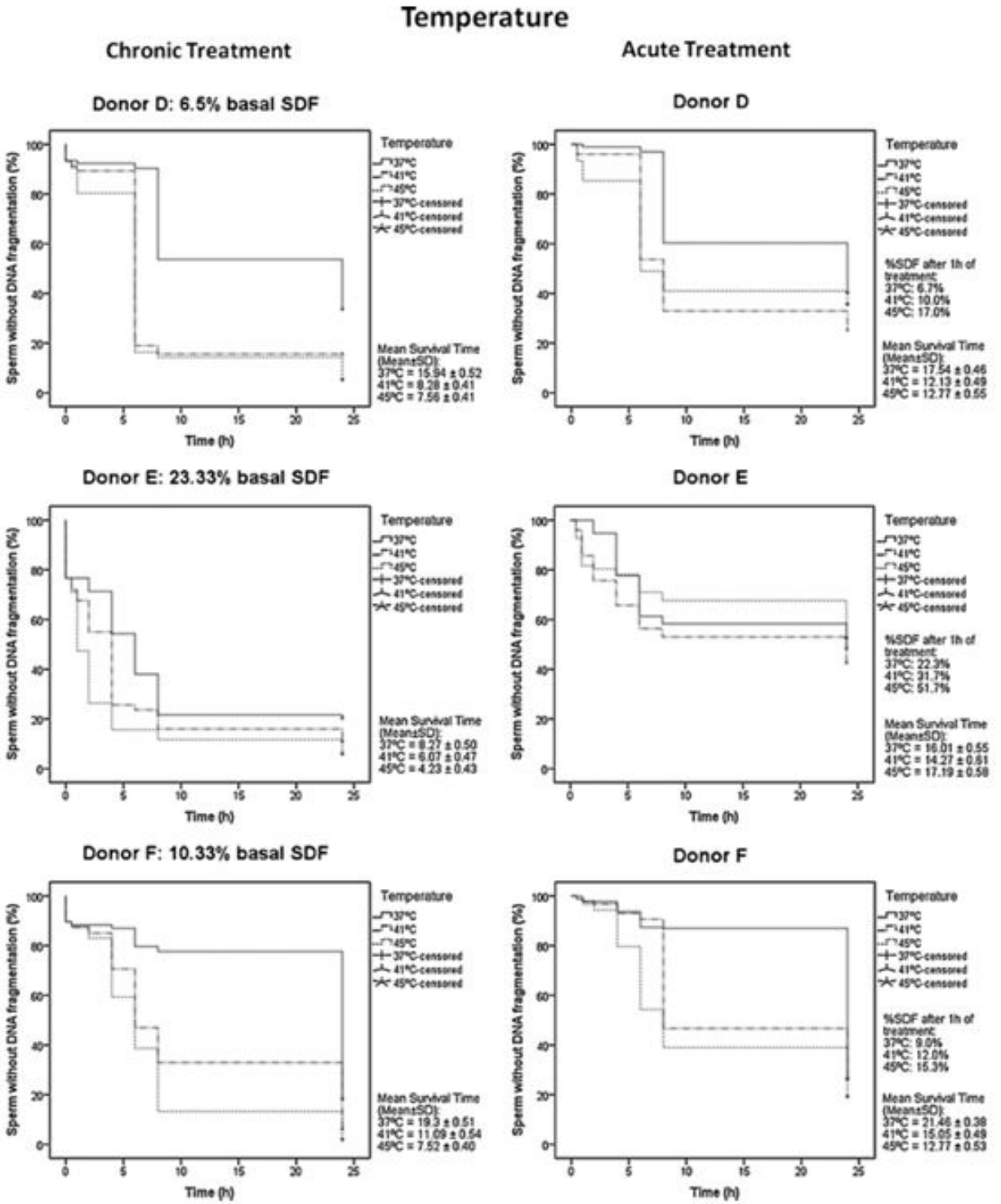

Fig. 2. Kaplan–Meier survival curves after exposure of sperm samples from donors D–F to 37 °C, 41 °C and 45 °C, chronically for 24 h, or at 41 °C and 45 °C acutely for 1 h and then 24 h at 37 °C.

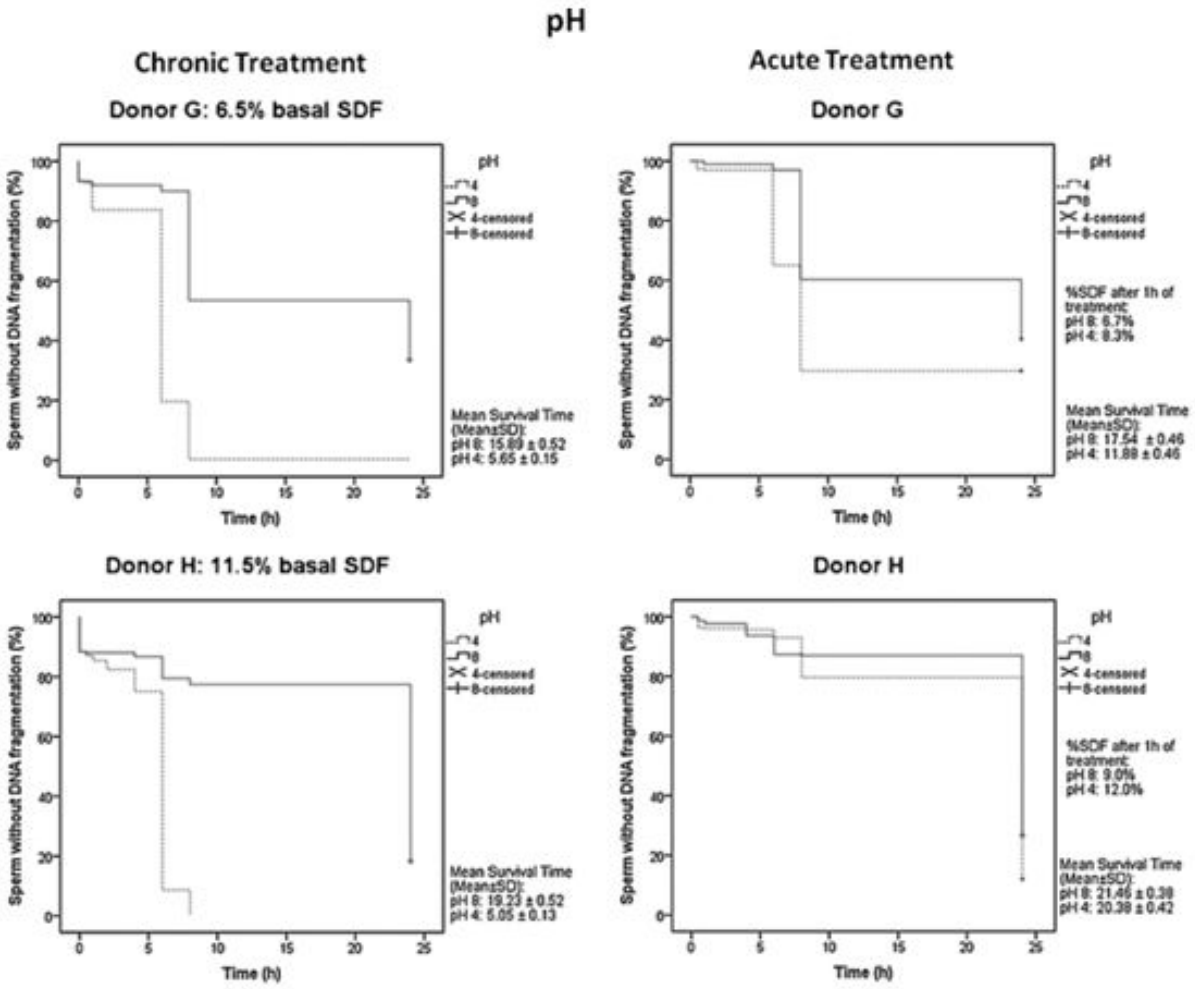

Fig. 3. Kaplan–Meier survival curves after exposure of sperm samples from donors G and H to pH 8 (original sample) and pH 4, chronically for 24 h, or acutely for 1 h and then 24 h in PBS at pH 7 at 37 °C.

tightly packed nature of the chromatin of the mature spermatozoon may reduce the water solvation layer surrounding the DNA strands, so that hydroxyl radical attack on DNA would be reduced; in fact, it is possible that this form of DNA packaging in the sperm nucleus may have evolved to provide a protective function.

4.2. Effect of hyperthermia

Heat is a pleiotropic damaging physical agent. It can alter the structure of certain heat-labile proteins that may aggregate after denaturation. Although it is unlikely to directly induce DNA breaks, it may interfere with the repair of DNA damage so that the resulting chromatin structure may be affected. Membranes can also be modified following hyperthermia, with a possible role of reactive oxygen/nitrogen species (ROS/NOS). These may induce sphingomyelin hydrolysis and ceramide formation, which in turn induces the JNK/SAPK cascade that is involved in heat-induced apoptotic cell death [23,24]. Specifically in the testis, high temperature is associated with an increase of ROS, which reacts quickly with substrates such as lipids, causing lipid peroxidation that affects sperm membranes [25]. In addition to these effects, the mitochondrial membrane can also be disrupted in spermatozoa and in germ cells leading to the activation of pathways of programmed cell death [26,27].

The effect of hyperthermia on DNA integrity from sperm has been mostly studied in vivo, in animals, after testes–epididymal exposure. This was due to the well-known adverse influence of heat stress on spermatogenesis as spermatids are more sensitive to heat stress, resulting in increased levels of apoptosis and cell loss. Nevertheless, reports about a direct influence of heat stress on the DNA integrity specifically in the mature spermatozoa are difficult to find, especially in human. A study of heat stress in the stallion for which the testes were insulated for 48 h, detected damage to sperm chromatin starting only 10 days after heating, so that DNA damage was apparently not associated with epidydimal transit [28]. In contrast, scrotal heating of the mouse to 40 °C for only 1 h resulted in a slight increase of sperm with fragmented DNA only 3 days after exposure [29]; these results were interpreted as an alteration of chromatin packaging of spermatozoa that were traversing the cauda and corpus epididymides, possibly mediated by alterations in epididymal function, as indicated by Paul et al. [30]. Elevated scrotal temperature for 48 h in bulls affected both epididymal and testicular sperm seen by an increased doubling of the %SDF (4–8%) within 3 days and a 5-fold increase after 12 days [31]. Banks et al. [32] exposed the mouse scrotum to mild heat stress (42 °C for 30 min) and showed

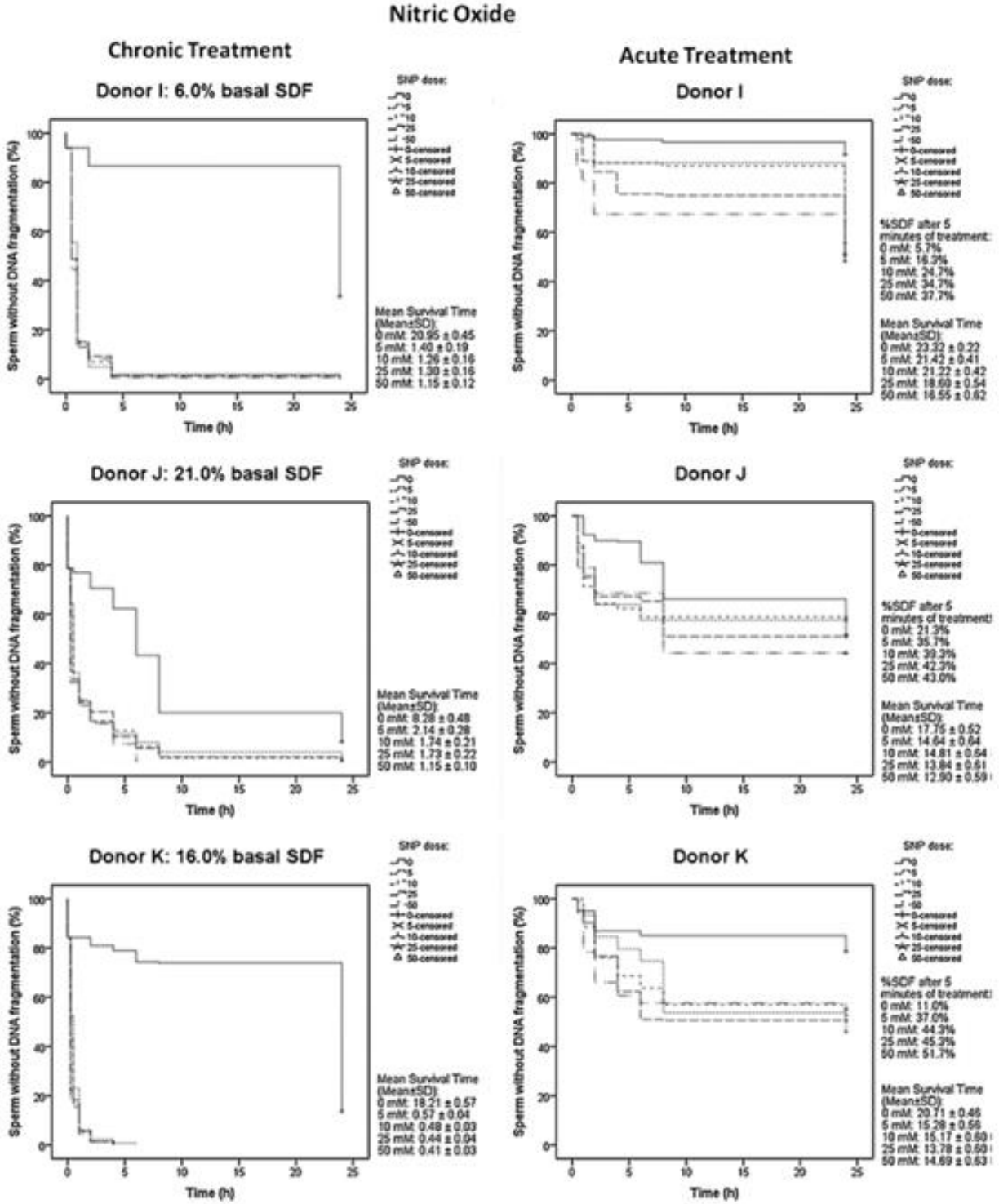

Fig. 4. Kaplan–Meier survival curves after exposure of sperm samples from donors I–K to increasing doses of SNP chronically for 24 h, or acutely for 5 min and then 24 h in PBS at 37 °C.

a significant increase in the SDF of mature spermatozoa from the epididymis. They showed that SDF increased from 5% to 30% after 1 h (the shortest time assayed) and then to 60% after 6 h and then decreasing followed by a decrease. In a similar model, Pérez-Crespo et al. [33] also obtained a similar increase in SDF, 6 h after scrotal heating which represented the minimum time following the application of the heat stress. It has been suggested in these reports that sperm DNA damage could be a result of an unfavorable epididymal environment, due to enhanced oxidative stress by the increase in epididymal temperature.

To our knowledge, there is only one report about the effect of heat stress on DNA in mature human sperm; exposure in vitro to 40 °C for 4 h only affected DNA integrity in the fraction of spermatozoa associated with poor capacitation [34]. Our results clearly detected a significant SDF increase after 1 h exposure to elevated temperature. Moreover, the dynamic assay was efficient in revealing damage in spermatozoa that initially showed no DNA fragmentation following hyperthermia, the intensity of the impact being variable among the individuals. Our observations also indicated that these spermatozoa were experiencing some kind of heat-induced damage, which was latently expressed as DNA fragmentation. The dynamic sperm DNA fragmentation for humans is likely to have a range of application when examining for the effect of hyperthermia, especially in those males exposed to (1) hot environments such as bakers and welders [35], (2) extended periods in a sedentary position, such us professional drivers, (3) males who use tight-fitting underwear and (4) high-fever or pyrexic episodes [36].

4.3. Effect of acidic pH

Most enzymatic processes are influenced by pH, so pH variations should therefore have a major global affect on metabolic activity. Acidic media are known to reduce the percentage of motile sperm and decrease the straight-line velocity of spermatozoa [37]. To our knowledge, no data exist about SDF induction by pH variations. In this study, we have demonstrated that exposure to acidic pH can result in SDF. Although no significant SDF increase was evident immediately after transient exposure for 1 h, prolonged incubation for 24 h revealed a significant increase in SDF. Only the dynamic SDF assay was capable of detecting the detrimental effect of the transient acidic pH, thereby, again proving the efficacy of the dynamic approach for the detection of latent sperm DNA damage. It is possible that the decrease in SDF found in this study is mediated via oxidative induced injury to the sperm membrane. From a practical point of view, it must be taken into account that spermatozoa are normally subjected to pH stress after ejaculation in the vagina. Vaginal pH in the woman varies between 3.8 and 4.5, except in the menstrual phase, where it is close to 7 [38,39]. Our results suggest that acidic vaginal pH could have an adverse effect on sperm DNA integrity, even after a short-term contact of 1 h. Although SDF may be not initially be apparent, transient exposure to acid pH could lead to accelerated SDF kinetics with increased time in the female tract. It is likely that the acidic pH of the vagina may be important as a natural selective mechanism against sperm with DNA that is potentially susceptible to fragmentation, as some of these spermatozoa are likely to be fragmented when they reach the site of fertilization and thereby not participate in oocyte fertilization.

4.4. Effect of NO

Incubation of spermatozoa in vitro with different sources of ROS/NOS has resulted in an increase in DNA fragmentation [40–43]. In this study, the effect of oxidative stress on the SDF dynamics was evaluated by incubating spermatozoa with the NO donor SNP. NO is a small non-polar molecule which freely diffuses through membranes, so that it may act at a relatively long distance from its point of production. It is a versatile molecule that physiologically acts as a cellular messenger mediating various functions, including the regulation of blood flow, thrombosis, neural activity and immune response [44]. Nitric oxide may be produced in the spermatozoon during capacitation and the acrosome reaction [45]. While NO is in itself typically unreactive toward DNA, it is nevertheless unstable in the presence of molecular oxygen and forms nitrous anhydride (N_2O_3), which causes nitrosative stress. Moreover, NO also reacts with the superoxide anion ($O_{2^-}{}^{\bullet}$), yielding the powerful oxidizing peroxynitrite ($ONOO^-$). These reagents may induce DNA breaks and abasic sites in DNA and cause lesions in guanine residues [46]. SNP in blood, incubated in vitro at 37 °C, decomposes by 50% at 26.6 min and more than 90% by 2 h [47]. Accordingly, in this experiment, incubation with SNP produced a significant increase in SDF primarily within the initial 2 h exposure period. The different doses of SDF yielded similar kinetic damage responses in the three donors, suggestive of a saturation effect with time and which was apparent even with the lowest dose of SNP. Interestingly, a brief SNP exposure of only 5 min, irrespective of dose, produced a significant increase in SDF. After removal of SNP, the remaining spermatozoa that did not initially display DNA fragmentation, showed an increased SDF rate when evaluated dynamically. As was the case for exposure to hyperthermia and acidic pH, not all the damage induced by SNP was apparent immediately. NO may not only attack DNA but also produce oxidative damage to proteins and lipids. In fact, spermatozoa are particularly susceptible to the damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids which readily experience lipid peroxidation by ROS, resulting in a loss of membrane integrity [48,49]; this could trigger a late DNA degradation apoptotic-like process. In fact, apart from induction of direct oxidative DNA damage, ROS have also been implicated in the regulation of specific cellular functions, acting as signaling molecules for apoptosis. Mitochondrial damage may also be implicated in sperm DNA fragmentation as these organelles are both a source and target of ROS, which mediate cytochrome *c* release resulting in the triggering of caspase activation [50]. It is clear that ROS overproduction may activate an apoptotic process, leading to DNA fragmentation [51] and it is important to reduce this damage during processing of sperm for manipulative procedures.

Many andrological pathologies involve oxidative stress in their pathophysiology, including inflammatory and infectious diseases. Moreover, excessive nitric oxide release within dilated spermatic veins has been identified in subfertile males with varicocele [52]. While an increase in the background SDF is evident in these patients immediately after ejaculation, we propose that the dynamic assay would be more sensitive at detecting further cryptic sperm damage that was not initially obvious by a static assessment. Our experiments also emphasize the importance with respect to the assessment of SDF following ejaculation, since different SDF values can be obtained at different times after DNA damage induction. We suggest that an abnormally accelerated SDF dynamic identified in a patient may be suspicious of cryptic sperm damage and may be completely independent of the background level of SDF initially recorded after the ejaculation.

5. Conclusions

Dynamic assessment of SDF after transient heat, pH stress and NO exposure has revealed the importance of incubating the sperm cell at 37 °C to allow the detection of cryptic damage that is not necessarily revealed by an initial static assessment; i.e. DNA longevity is affected and this may resemble a form of delayed apoptosis that occurs in somatic cells. While the X-ray doses used in this experiment were unable to induce SDF, there was a clear effect following hyperthermia, exposure to acidic pH and NO. The dynamic SDF assay may be of interest to reproductive genetic toxicologists in revealing not only immediate damage of sperm DNA but latent or cryptic damage that is only lately expressed. If dynamic assessment is used as the model to evaluate sperm DNA damage, the extent and incidence of DNA damage in patients associated with pyrexia, local heating, inflammation, infection and varicocele could be much higher than previously reported. We conclude that the sperm DNA fragmentation dynamic assay as demonstrated in this study is

ideally suited as a tool for those clinicians interested in elucidating the subtle and cryptic effects of reproductive toxicants and pathological conditions on sperm DNA integrity.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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