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Professional paper

Assessment of Sperm Nucleus Integrity in Infertile Men: A Novel Research Field for Anthropology in the Molecular Era

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A B S T R A C T

Anthropology has always been particularly interested in the origin of human life and the development towards adulthood. Although originally working with skeletal measurements and bio-morphological markers in modern populations, it has now entered the growing field of applied molecular biology. This relatively recent advance allows the detailed study of major events in human development and senescence. For instance, sperm DNA integrity and chromatin re-organization are crucial factors for fertilization and embryo development. Clinical researchers have developed improved methods for the evaluation of DNA integrity and protaminosis in sperm nuclei, such as the TUNEL and the CMA3 assays. DNA damage in spermatozoal nuclei is detected using the TUNEL assay which depends on the specific enzymatic reaction of TdT with the end strand breaks of DNA. Protaminosis in spermatozoal nucleus is evaluated using CMA3 assay, which is based on the in situ competition between CMA3 and protamines. Such measurements may provide useful data on human reproductive health, aiding the explanation of demographic differences across the world.

Key words*: male infertility, DNA damage, chromatin packaging, TUNEL, CMA3, demographics, population anthropology*

Introduction

Anthropology is a vast scientific field, interested in the study of the species *homo* in all its aspects and by all available means. Thus, it is only natural that as science and technology grow and more advanced tools become available, anthropology also faces new challenges, extending its applied research in further dimensions of human structure, function and activity. This is indeed a never-ending process, since every answer is bound to raise more questions. However, it is also a very intriguing field, because it explains the very way in which we have evolved, survived so far and achieved the culture observed today. At the same time, anthropology may provide information useful to solve challenges and risks, such as diseases and survival threats from our ever changing environment. A good example of this potential is the study of human reproductive failure, its causes and its reversibility, since a solution to this problem would be most helpful in the on-going attempt to solve the international demographic inequalities.

Globally, there is an increasing number of couples that confront infertility problems. Interestingly, in nearly 40% of all infertility cases, the cause is exclusively or partially attributed to the »male factor«, a fact not always publicly declared due to regional philosophical or religious barriers. Male infertility can be expressed in different ways, one of which is the production of low quality sperm (i.e. spermatozoa unfit to achieve fertilization). Standard or basic semen analysis constitutes an important diagnostic tool for the evaluation of descriptive parameters of ejaculates obtained by masturbation. The World Health Organization (WHO) has suggested a range of largely arbitary threshold values for the human semen parameters, such as concentration, motility and morphology[1]. According to the results of semen analysis, men are classified as fertile or sub-fertile and infertile. However, fertility depends not only on the absolute number of motile, morphologically normal spermatozoa, but also on their specific functional capabilities. These func-

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tional capabilities are affected by the integrity of DNA and the level of protaminosis in the spermatozoal nucleus.

The sperm nucleus is characterized by the fascinatingly high level of chromatin architecture organization that is responsible for the protection and transmission of the paternal genome to the oocyte, during fertilization. Sperm DNA integrity and chromatin organization have been postulated to be necessary prerequisites for the completion of fertilization and subsequent embryo development[2,3]. It has been proven that the integrity of DNA in the nucleus of spermatozoa is deficient in infertile men, as the spermatozoa of infertile men exhibit more DNA damage compared to the spermatozoa of fertile men[4]. DNA fragmentation in the sperm nucleus may be attributed to oxidative stress, abnormal chromatin packaging and poor DNA integrity[5]. DNA damage may emerge from excessive production of reactive oxygen species[6–8] (ROS), defective apoptosis before ejaculation[9–11] and abnormal chromatin packaging during spermiogenesis[12,13]. Regardless of the cause, it is directly associated with poor reproductive potential and, thus, remains a useful marker in reproductive health evaluation, at both individual and community level.

Evaluation of DNA Damage

DNA damage can be detected by various assays, directly or indirectly. The direct methods include: Single-cell gel electrophoresis (Comet Assay), *In situ* nick translation and terminal deoxynucleotidyl transferase-mediated dUTP-nick end labelling (TUNEL). Briefly, the comet assay is used for detection of nicks in both single and double-stranded DNA[14–17], while the *in situ* nick translation assay detects only single stranded DNA[18,19]. Due to the lower cost and more extensive experience of researchers, most available studies have been performed based on the TUNEL assay, although the trend has been reversed lately. This technique was originally described by Gavrieli et al. in 1992 and is still widely used to identify DNA strand breaks in spermatozoa[20].

The TUNEL method is used in order to detect damage in the DNA within the sperm nucleus. In detail, the TUNEL method identifies nicks of cleaved DNA (either double-stranded or single-stranded) by using terminal deoxynucleotidyl transferase (TdT) to transfer biotinylated-dUTP to free 3'-OH terminal ends of the strand breaks of cleaved DNA. It should be pointed out that, the enzymatic reaction of TdT specifically depends on the presence of bivalent metal ions (Co^{2+}). The biotin-labeled cleavage sites are then detected by reaction with fluorescent Texas red conjugated streptavidin (Figure 1a). The polymer can be observed using an epifluorescence microscope. The evaluation of healthy normal sperm nuclei is done with the fluorescent probe DAPI (4´,6-diamidino-2-phenylindole) that is used as counterstain. DAPI associates with DNA in various binding modes[21] and the formed DAPI-DNA complexes can be visualized using an epifluorescence microscope, as the spermatozoal nucleus is stained blue. Hence, spermatozoal nuclei with broken DNA strands are stained red with Texas red (TUNEL-positive spermatozoa), contrary to the healthy normal spermatozoal nuclei, without DNA nicks, which are stained blue with DAPI, the latter being used as a counterstain (TUNEL-negative spermatozoa) (Figure 1b). At least 500 spermatozoa must be evaluated per patient and the so-called »DNA fragmentation index« (DFI) is yielded by the ratio of spermatozoal nuclei with DNA breaks (red) divided to the total number of spermatozoal nuclei (red and blue).

Many studies have shown that DNA fragmentation is highly observed in the spermatozoal nuclei of infertile men compared to those of men with proven fertility[22,23]. Moreover, DNA fragmentation is also highly observed in infertile men affected by various specific pathologies[24]. Moreover, there are indicative findings showing a high proportion of cells with DNA fragmentation in low motility sperm fractions from ejaculates of infertile men, suggestive of a possible association between DNA integrity and sperm kinetics[25]. Several studies have also reported the effects of age on sperm DNA damage[26,27]. In one of them, an age-related decrease in conventional semen parameters, an increase in DNA damage and poor chroma-

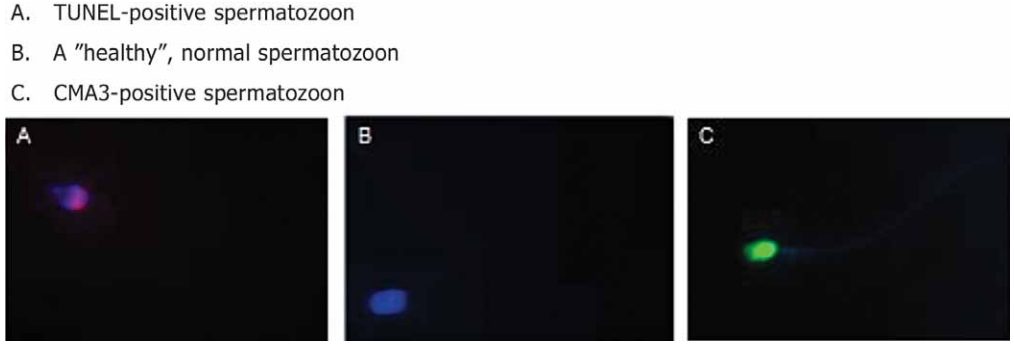

Fig. 1. a, b) A TUNEL-positive spermatozoal nucleus (i.e. one with fragmented DNA) is stained red with Texas red, while the TUNEL-negative spermatozoal nucleus (i.e. without fragmented DNA) is stained blue with DAPI. c) A CMA3-positive spermatozoal nucleus (i.e. one with abnormal chromatin condensation) is stained yellow with CMA3, while the CMA3-negative spermatozoal nucleus (i.e. with normal chromatin condensation) is stained blue with DAPI.

tin packaging were simultaneously demonstrated in infertile men, aging over 35 years[28].

Evaluation of Protaminosis

Many assays are applied for measuring chromatin packaging and protamine content in sperm nuclei, such as Acidic Aniline or Toluidine blue and Chromomycin A3 (CMA3). Briefly, Acidic Aniline and Toluidine blue detect lysine-rich histones, allowing the identification of chromatin abnormalities in the spermatozoal nuclei related to their nucleoprotein content[29–31]. On the other hand, CMA3 staining is used to evaluate the protamine levels in the sperm nucleus of the infertile men and appears in a consistently growing paste in reproductive endocrinology-andrology literature.

The antitumor antibiotic CMA3 is isolated from *Streptomyces griseus*[32] and it was originally described in a series of sperm studies by Hayasaka T. and Inoue Y. in 1969[33]. CMA3 forms a dimer stabilized by a single divalent metal ion[34–35] such as Mg^{2+} and binds to the minor groove of DNA around G/C-rich sequences. The specificity of the chromomycin dimer is due to the particularly strong intermolecular CMA3-DNA hydrogen bonds between specific oxygen atoms of the chromophore and the 2-amino atoms of gouanine bases in DNA[36–38]. The CMA3 assay outcome is inversely correlated with the protamination state of spermatozoa (and thus, with sperm structural normality), as it is based on the *in situ* competition of CMA3 with protamine[39]. Spermatozoal nuclei with defective protaminosis are stained yellow with CMA3 (CMA3-positive spermatozoa), contrary to the healthy spermatozoal nuclei with normal protaminosis that are stained blue with DAPI used as counterstain (CMA3--negative spermatozoa) (Figure 1c). It must be pointed out that binding of CMA3 to the DNA can deter DNA polymerase I access to the DNA and since CMA3 is unable to accesss DNA in the presence of protamines and normally formed disulphide bonds[13,40,41], almost none of the CMA3-negative spermatozoa present broken DNA strands.

Several studies have supported a significant correlation of CMA3 staining with sperm morphology, fertilization and assisted reproduction outcome[42–45] in patients attending reproductive clinics. Furthermore, a strong correlation has been observed between DNA damage and low quality semen in infertile men[46]. These findings are particularly relevant in population anthropology, because they indicate a possible predisposing factor for differential reproductive potential among human populations. Reproductive survival is the key prerequisite for species survival and, therefore, such studies may prove useful to predict the trends for world human population kinetics for the forthcoming decades and detect intervention needs.

Conclusions

TUNEL and CMA3 assays constitute important tools, which allow clinicians to analyze defective protaminosis and fragmented DNA in spermatozoa[47]. Injecting defective spermatozoa into oocytes will possibly result in failure of sperm decondensation and subsequent fertilization, increasing the possibilities of unsuccessful conception and impaired pregnancy outcome. Therefore, the co-operative application of the TUNEL and CMA3 assays is important during evaluation of sperm and, thus, can assist in eliminating the risk of using defective spermatozoa during assisted reproductive technology. However, generalized clinical application cannot be supported at present, since baseline values still differ considerably among populations and among different laboratories, making interpretation of intermediate (»gray zone«) samples unsafe. Therefore, their application is currently focused on scientific research protocols and epidemiological-anthropological studies, rather than an individualized clinical application, although the latter remains a »hot« diagnostic challenge[48].

In modern population cohorts, TUNEL and CMA3 may be used to evaluate male fertility potential and predict demographic kinetics in biological anthropology. On the other hand, the application of DNA fragmentation techniques may be difficult in paleo-anthropological specimens, in which biological fluids are generally not preserved and extractable DNA quality is usually considerably impaired by environmental exposure over time. This may be less true for mitochondrial DNA, which tends to be better preserved and also retains the unique characteristic of exclusive mother – offspring transition, thus making it a genetic relic reflecting evolutionary history. In this context, the study of DNA damage remains a useful tool in the evaluation of the environmental agents involved in human physical anthropology and population dynamics during various crucial evolutionary periods.

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PROCJENA CJELOVITOSTI JEZGRE SPERMIJA U NEPLODNIH MUŠKARACA: NOVO ISTRAŽIVAČKO POLJE ZA ANTROPOLOGIJU U MOLEKULARNO DOBA

S A Ž E T A K

Antropologija je uvijek bilo posebno zainteresirana za porijeklo ljudskog života i razvoju prema odrasloj dobi. Iako se prvobitno radilo o skeletnim mjerama i biomorfoloških markera u modernim populacijama, sada se sve više primjenjuje molekularna biologija. Ovaj relativno nedavni napredak omogućuje detaljnu studiju o glavnim događajima u ljudskom razvoju i starenju. Na primjer, cjelovitost DNK spermija i reorganizacija kromatina ključni su faktori za oplodnju i razvoj embrija. Klinička znanstvenici razvili su poboljšane metode za procjenu cjelovitosti DNK i protaminoze u jezgrama spermija, kao što su TUNEL i CMA3 testovi. Oštećenja DNK u jezgrama spermiaj je otkriveno pomoću TUNEL testa, koja ovisi o specifičnoj enzimskoj reakciji TDT sa završecima dijelova lanaca DNK. Protaminoza u jezgri spermija se ocjenjuje CMA3 testom, koja se temelji na in situ odnosu između CMA3 i protamina. Takve mjere mogu pružiti korisne podatke o ljudskom reproduktivnom zdravlju, pomažući u objašnjavanju demografskih razlika diljem svijeta.