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# Non-Invasive Approaches to Epigenetic-Based Sperm Selection

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Since sperm size and form do not necessarily provide information on internal sperm structures, novel sperm markers need to be found in order to conduct assisted reproductive therapies (ART) successfully. Currently, the priority of andrologists is not only to select those sperm able to fertilize the oocyte, but also a high quality of sperm that will guarantee a healthy embryo. Evidence of this shows us the importance of studying sperm intensively on genetic and epigenetic levels, because these could probably be the cause of a percentage of infertility diagnosed as idiopathic. Thus, more attention is being paid to posttranslational modifications as the key for better understanding of the fertilization process and its impact on embryo and offspring. Advances in the discovery of new sperm markers should go hand in hand with finding appropriate techniques for selecting the healthiest sperm, guaranteeing its non-invasiveness. To date, most sperm selection techniques can be harmful to sperm due to centrifugation or staining procedures. Some methods, such as microfluidic techniques, sperm nanopurifications, and Raman spectroscopy, have the potential to make selection gentle to sperm, tracking small abnormalities undetected by methods currently used. The fact that live cells could be analyzed without harmful effects creates the expectation of using them routinely in ART. In this review, we focus on the combination of sperm epigenetic status (modifications) as quality markers, with non-invasive sperm selection methods as novel approaches to improve ART outcomes.

**MeSH Keywords:** **Andrology • Epigenomics • Microfluidics • Nanoparticles • Reproductive Techniques, Assisted • Spectrum Analysis, Raman**

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## Background

Widespread Assisted Reproductive Therapies (ART), such as *in vitro* fertilization (IVF) and mainly intracytoplasmic sperm injection (ICSI), have opened up a new perspective on infertility treatment. The successful outcomes of ART are based on selecting the most suitable gametes with which to conduct the process. And, despite the selection of oocytes in the female being limited due to the small number of oocytes with each individual feature, in the case of males, it is possible to perform strict selection to choose the finest spermatozoa with which to improve the outcome of ART.

Sperm heterogeneity in ejaculate is widely recognized. This diversity can be found at different levels and characteristics such as shape, motility, DNA content, or membrane composition [1,2]. Recently it has been shown that sperm are also epigenetically heterogeneous, although few reports have addressed this approach [3–5]. Epigenetic alterations of the genome and associated posttranslational modifications of DNA-binding histones equally affect gamete development, maturation, and embryogenesis. Therefore, posttranslational modification (methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation) and noncoding RNAs have been revealing such key markers of fertilization and embryo development abilities in sperm [6–9]. Their evaluation is paramount to provide relevant information for ART. Furthermore, the study of sperm epigenetic markers is also crucial because it has been suggested that they are involved in the etiology of male idiopathic infertility [10].

On the other hand, it might be advisable to follow up sperm changes derived from the use of ART and its consequences. The application of ART includes stages outside the male and female reproductive tracts, during which spermatozoa are subjected to procedures aimed at maximizing reproductive success [11]. This *in vitro* environment can affect sperm characteristics and function. It is currently also known that epigenetic changes are more frequent than DNA mutations, and their contributions during the performance of human ART is not well defined. Although many researchers have reported a higher incidence of imprinting disorders in ART offspring [12–14], a clear relationship has not been demonstrated. Recently, it has been reported that epigenetic alterations could rather arise from the process of gametogenesis rather than ART itself [15,16]. Thus, it can therefore be detected in the semen of the father. However, we should not overlook the effect of our manipulation with gametes on the stability of the epigenetic process. Based on these facts, sperm handling should be sensitive and invasiveness should be the last resort.

As mentioned above, the selection of viable sperm is an essential procedure to carry out ART. Thus far, centrifugation over

discontinuous density gradient (DG) has remained the most popular method, together with swim-up [17–21]. Both methods select morphologically normal and motile sperm, and, although these spermatozoa are expected to be epigenetically stable, few studies have been carried out in this direction [22]. Furthermore, there is evidence to support the fact that sperm morphology does not always reflect DNA status [23,24]. It is therefore crucial to find alternative sperm selection methods which allow us to isolate sperm based on novel parameters to better monitor sperm function.

For detection of distinct epigenetic markers, staining of the cell is usually required, together with flow cytometry or immunocytochemistry [25]. These approaches are valid for identifying mistakes in posttranslational modifications, but the sperm cannot subsequently be used in ART. Estimation of markers based on the epigenome for evaluation of sperm quality, in combination with harmless procedures that are able to detect them, could be one of the solutions to help infertile couples. In this context, non-invasive and label-free methods, such as microfluidics, nanopurification, and Raman spectroscopy, are promising.

## Microfluidics

The female reproductive tract has many functions, including the facilitation of sperm migration to the site of fertilization, as well as the selection of healthy sperm [26]. The mechanisms by which the female genital tract selects spermatozoa still remain unknown, but it selects against spermatozoa containing damaged DNA, acting as a biological sensor to screen spermatozoa. This fact demonstrates the existence of unrecognized processes for detection and interpretation of markers on the sperm surface that link phenotypic and genotypic qualities of each individual sperm [27]. It has also been reported that the oviduct responds to sperm presence by modifying the oviductal environment [28].

It is known that many of these characteristics of the oviductal environment are impossible to reproduce during ART. However, some features, such as unidirectional, laminar or gradient flow, containment in a 3-D physical environment, and changing the chemical composition in the medium, can be obtained in a microfluidic environment [29].

These microfluidic devices use microchannels to sort sperm according to normal morphology, motility, and higher DNA integrity for IVF techniques [30,31]. Important advantages have been shown compared to traditional selection techniques, such as the potential to work with small sperm sample volumes, short processing times, and the ability to manipulate single cells in a non-invasive manner. In addition, the potential to be a versatile tool for selection applications or fundamental

studies on sperm has also been shown [24,32]. Furthermore, the yield of the selected sperm by microfluidic technique was estimated at 41%, what is comparable to the recovery rate of currently used conventional methods [33].

Another of the advantages of using a microfluidic device in ART is its one-step sorting protocol without the need to centrifuge. Eliminating the centrifugation step minimizes the exposure of sperm to reactive oxygen species (ROS), preserving the integrity of the chromatin [34]. DNA fragmentation is significantly decreased in treated sperm with the microfluidic sperm sorting system [35,36]. Wang et al. [37] compared the swim-up method with a microfluidic device, resulting in a significantly lower rate of DNA damage (16.4% swim-up vs. 8.4% microfluidic). A recent microfluidic study [30] utilized polycarbonate filter paper with different-sized pores to determine how the different pore sizes affect sperm retrieval, ROS production, and DNA integrity. The device was efficient in selecting motile and morphologically normal sperm. In addition, there was significantly less ROS production and improved sperm retrieval using the microfluidics versus the swim-up method [30]. Another research group created a device that uses a radial array of microchannels to select the most motile sperm, and they were able to obtain 80% improvement in sperm DNA integrity after sorting [38].

Decreasing levels of DNA fragmentation and ROS can prevent epigenetic changes, since it has been observed that, in sperm damaged by oxidative stress, impaired DNA sequence prevents the process of DNA methylation [39]. Other studies have suggested that oxidative stress due to smoking affects protamine protein levels and histone retention rates in mature sperm [40,41]. The fact that the epigenetically aberrant sperm populations were preferentially found in men with severe sperm abnormalities and that non-imprinted genes are also affected, suggests that there might be a link between phenotype and epigenotype [4]. However, sometimes normal morphology is not necessarily associated with DNA integrity [24]. Moreover, epigenetic markers had significant variation between samples from different men, as well as significant variation within the same semen sample. This reinforces that epigenetic patterns may be used as biomarkers for sperm quality [42].

Future studies should investigate creating sperm-sorting devices that can isolate spermatozoa to limit genetic diseases, while also maintaining sperm viability. New sperm sorting techniques have been shown to be able to improve DNA integrity, morphology, and motility, but there are still some conflicts as to whether these are significant improvements over the conventional centrifuged-based techniques [43].

In summary, microfluidic technology could offer a new non-invasive method for selecting sperm with good molecular characteristics to prepare sperm for IVF or ICSI, which would greatly improve

the current point-of-care ART. Although newer microfluidic-based sperm sorting methods showed promising results, these devices should be thoroughly analyzed for their clinical utility to continue progress in the fields of microfluidics and andrology.

## Sperm Nanopurification

Nanotechnologies and their application have undergone huge expansion in different disciplines, including the medical field. The suggestion exists for the use of nanoparticles in drug delivery, diagnostics, and, in the case of sperm, as a non-invasive sperm selection method [44,45]. While methods such as Raman spectroscopy, described below, can function with any sperm molecules, nanoparticles for sperm selection interact with external molecules of the sperm membrane or acrosome. This could seem to be a disadvantage, but as mentioned before, we are witness to sperm ability reflecting their inner condition on the sperm surface [46,47].

Nanotechnologies function with different nanoparticle materials, but some harmful side effects can be found [48,49]. To date, the most successful method for sperm selection using nanoparticles is magnetic-activated cell sorting (MACS), which separates apoptotic and non-apoptotic spermatozoa [50,51]. However, in recent years, a new method based on magnetic nanoseparation of abnormal sperm has been implemented and was applied in cattle, utilizing ferritin nanoparticles coated with specific antibodies [52]. The sperm nanopurification process is relatively easy to implement. First, a mixture of sperm with ferritin nanoparticles is created and coated with antibodies against specific molecules, especially those which are expressed on the surface of defective sperm [52]. Spermatozoa with these molecules interact with the coated nanoparticles, and a magnetic separator is placed at the bottom of a tube for final nanopurification. Thus, the pellet containing unhealthy sperm attached to nanoparticles is removed. Utilizing this technique for bull semen purification, 25–30% spermatozoa were eliminated and it was possible to obtain double the number of artificial insemination doses per semen collection [47,52]. Success in sperm nanopurification in cattle has been reported, increasing the number of pregnancies after insemination and IVF [52].

As the most suitable antibodies, those against the ligand of lectins PNA (peanut agglutinin from *Arachis hypogaea*) and PSA (*Pisum sativum agglutinin*) that are more abundant on the acrosome membrane of defective sperm [46,53] were selected, as well as ubiquitin, which is a small chaperone molecule known mainly from posttranslational modifications called ubiquitination. While monoubiquitination participates mainly in the regulation of gene transcription, cell signalling, and silencing of X chromosomes [54,55], polyubiquitination plays a

role in protein turnover by the ubiquitin proteasome system. Ubiquitin as a quality control marker is secreted by epididymal epithelium to eliminate defective spermatozoa by subsequent phagocytosis [56–58]. Nevertheless, some of the defective spermatozoa tagged by extracellular/cell surface ubiquitination are carried over into the ejaculate. This fact supports the utilization of ubiquitin as an appropriate sperm marker [58,59]. The estimation of ubiquitin for sperm selection was confirmed by sperm ubiquitin-tag immunoassay (SUTI). The intensity of ubiquitination in sperm correlated with the fertilization rate and success of ART, such as ICSI and IVF [59–61]. However, SUTI has been combined with flow cytometry or fluorescence microscope requiring sperm staining, which makes subsequent use of these sperm for fertilization in a harmless way unlikely.

The expectation of the application of sperm nanopurification in human reproductive medicine is based on the current information about its non-invasiveness. To date, no harmful side effects have been noted to be caused by using ferritin nanoparticles or the magnetic field, which is a crucial argument for its use in humans [52]. Furthermore, ubiquitin seems to be the proper marker for sperm selection, not only for its accessibility on the sperm membrane, but mainly for its ability to reflect the DNA status [60]. All this information supports the application of sperm nanopurification in connection with ubiquitin in human reproductive medicine and, due to its non-invasiveness, in the treatment of couples who have problems conceiving.

## Raman Spectroscopy

Sperm heterogeneity is widely accepted [4,5], and is based on several sperm features, including slight molecular differences which determine the sperm function. Changes in DNA packaging or epigenetic modifications can be important [62–64]. However, these small but important details are difficult to evaluate by standard methods, without disturbing or even destroying the sperm cell.

Raman spectroscopy (RS) is an optical laser-based technique which provides information on the vibrational energies of the biomolecular constituents of the sample [66]. Any changes in structure are translated into distinct Raman spectra from each molecule or tissue, without use of fixatives or labels [66–68]. However, a laser with suitable wavelengths and intensities to guarantee its invasiveness is required [68]. The combination of Raman spectroscopy with confocal microscopy, called Raman microspectroscopy, allows us to obtain 3D spatial resolution and makes possible investigations into single live cell tracking *in situ* changes in cell components [68,69].

Although RS has been used in different biomedical fields [70,71], in reproductive medicine and mainly in andrology, it has started

to be utilized in recent years [71–73]. Using RS, researchers have characterized different sperm regions such as the head, acrosome, middle piece, and flagellum, as well as inner organelles such as the nucleus and mitochondria [71–73]. This success is a prerequisite for deeper sperm characterization on a molecular level.

Currently, Raman spectroscopy is used mainly in the detection of sperm nuclear damage [67], and also in sex sperm selection [71]. The PO<sub>4</sub> backbone of DNA is characterized by peak intensity from 1055 cm<sup>-1</sup> to 1095 cm<sup>-1</sup> [67,69,73]. Any changes in the intensity are signs of DNA damage, and it is possible to make a map of sites with DNA fragmentation. The results that were obtained by Raman microspectroscopy in the field of DNA evaluation are in correlation with the DNA fragmentation index (DFI), which is associated with infertility and spontaneous abortions [66,69,74]. For sex sperm selection, variations of Raman peaks from DNA content and sex membrane proteins were reported in bovine sperm [71,75]. These results showed, with more than 90% accuracy, that RS may be applied in the near future for sperm sexing in a label-free and non-invasive manner, compared with the sex sorting by flow cytometry used to date [72,76]. Recently, the damage induced by sperm sorting and freezing-thawing procedures have been quantified by laser tweezers Raman spectroscopy [72]. These authors reported a variation of DNA, lipid, carbohydrates, and protein contents in sperm during flow cytometry process. Liu et al. [77] also showed the ability of Raman microspectroscopy to distinguish zona pellucida-bound sperm from unbound sperm, identifying differences in the intensity of Raman spectra on the acrosome region.

Thus, Raman spectroscopy allows us to evaluate DNA and RNA, as well as histones and protamines [78–80]. Poplineau et al. [78] used laser tweezers Raman spectroscopy to isolate a living human cell and monitor epigenetic modifications. These researchers treated Jurkat cells with histone deacetylase inhibitors, which resulted in an increase of histone code acetylation and changes in chromatin. The aforementioned effect was analyzed by different methods (laser tweezers Raman spectroscopy, electrophoresis, and nuclear image cytometry). Laser tweezers Raman spectroscopy showed the best discrimination between treated and control cells.

Noticeably, according to recent studies, if epigenetic modifications of sperm could be evaluated by non-invasive Raman spectroscopy, similarly to Jurkat cells, they would be suitable biomarkers of sperm quality in routine ART, providing relevant information on male infertility.

## Conclusion and Perspectives

Correct sperm evaluation and selection of the most representative markers may be the key to resolving male infertility



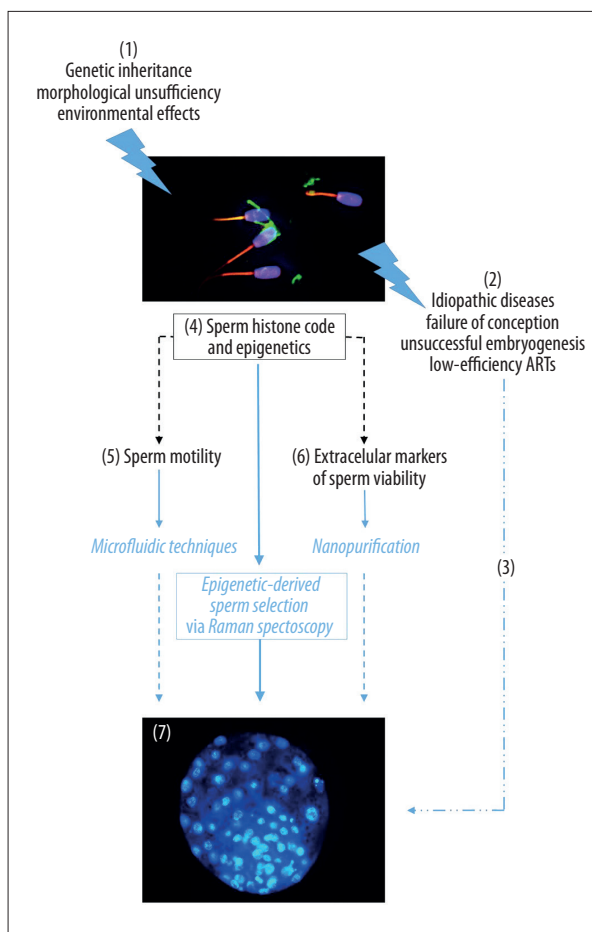
problems. The most feasible option for finding informative biomarkers that will be a guarantee of successful fertilization and proper embryo and offspring development could be found in the epigenome. Many researchers have suggested the possible usefulness to ART of analyzing epigenetic markers in routine sperm analysis [6,11,15,16]. However, further studies are required to confirm the relationship between sperm epigenetic features and the outcome of ART, as well as alterations in offspring generated by ART.

Correct chromatin structure involves the precise realization and timing of posttranslational modifications and protamination, essential for healthy progenitive sperm. Accordingly, deeper insight into DNA structure and epigenome generally seems to be promising in the clarification of processes for sperm fertilization and embryo development [6–8,10,81,82]. Posttranslational processes have an evident impact on embryo imprinting, and defects of imprinting participate in different offspring disorders, such as Angelman, Beckwith-Wiedemann, Prader-Willi, Silver-Russell, Goiter, Kabuki, and Claes-Jensen X-linked mental retardation syndrome [12,13,83,84]. For all these reasons, the development of adequate therapeutic options and sperm selection technologies based on epigenetic quality are crucial for improving ART outcomes [4].

Although the importance of epigenetics is obvious, it is currently not used in sperm selection methods or other ART techniques. The latest knowledge forces us to consider new perspectives for tracking these novel sperm parameters. The above-mentioned techniques (microfluidic device, sperm nanopurification, and Raman spectroscopy) could be options (Figure 1). Furthermore, a combination of microfluidic and Raman spectroscopy is possible and could increase sperm selection efficiency in some infertility cases [24,85].

These methods are not only non-invasive, they also have a huge potential for identification of epigenetic markers and use in successful ART. Actual studies of potential techniques show good results that are in correlation with the DNA fragmentation index (%DFI) and success of fertilization, ICSI, and others [32,36,52]. In addition, another use could be to mimic the natural sperm environment and natural sperm selection in the female genital tract, such as microfluidics [86,87].

However, although the sperm separation methods currently in use cannot use epigenetic markers for selecting undamaged sperm, Raman spectroscopy could be a promising method of identifying small differences in the chemical structure which are associated with changes in cell physiological properties (Figure 1). Furthermore, the most recent information on the application of Raman spectroscopy in evaluation of the histone acetylation state of cancer cells, suggests it could be used in sperm and expanding research to other posttranslational



**Figure 1.** Raman spectroscopy for sperm epigenetics as an innovative tool for sperm selection resulting in successful embryonic development. When various environmental pressures or genetic burden (1), showed as idiopathic infertility followed by conception failure (2), decrease a chance for successful conception (3). On the other hand, sperm population is predestined for strict selection more than oocytes. Therefore, qualified selection of markers of sperm fertilization ability is crucial for advanced approaches to live sperm selection. Based on current knowledge, sperm epigenetics (4) seems to be a general phenomenon regulating clinical obvious features (motility (5) and sperm viability (6)) as well as invisible sperm quality leading to correct embryonic development (7). Raman spectroscopy is a versatile tool for advanced epigenetic-derived sperm selection. Therefore, Raman spectroscopy-selected sperm can improve ART outcomes, even with low-quality ejaculate.

modifications, such as phosphorylation and DNA methylation. The level of histone phosphorylation detected by flow cytometry correlates with DNA damage and infertile male patients [88]. In addition, the DNA methylation pattern varies between fertile normospermic males and IVF patients, even if it seems to be a proper indicator of embryo quality after IVF [89]. However,

it would be better if Raman spectroscopy could be used for these objectives and this seems to be possible.

Progress in Raman spectroscopy suggests that in the near future we will be able to better observe the epigenome of gametes without disturbing or destroying them. The results obtained by these methods show that sperm selected in these ways, in comparison to standard methods, exhibit better quality. Nevertheless, although the results and application in ART seem to be promising, further observations are necessary to confirm their safety in the epigenetic context.

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## Competing interests

The authors declared no competing interest.

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