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Male reproduction in the polluted environment – review

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ABSTRACT

Endocrine Disrupting Chemicals (EDC-s) present in the environment can cause disturbance in the reproductive biology of animals and humans. While most of the studies focus on the adverse effects on the hormonal system, recent findings indicate a direct effect on germ cells. The present review introduces laboratory assays used to study several structural and physiological aspects of spermatozoa exposed to EDC-s.
(Keywords: environment, endocrine disrupting chemicals, reproduction, sperm, flow cytometry)

ENDOCRINE DISRUPTORS IN THE ENVIRONMENT

There is a growing interest in the presence of Endrocrine Disrupting Chemicals (EDC-s) in the environment (*Mills and Chichester*, 2005). EDC-s can disturb the normal reproductive physiology of animals (and humans as well), including abnormalities in sexual development (like hermaphroditism), changes in the normal sexual behavior, etc. Well known examples of EDC-s are polychlorinated biphenyls (PCB), alkyl-phenols or ethinyl-estradiol, a compound in human contraceptives (*Larsson et al.*, 1999; *Waring and Harris*, 2005).

Several countries initiated research projects to study the presence of EDC-s in the environment as well as to reveal their biological effects (*Hutchinson et al.*, 2000; *Vethaak et al.*, 2002). Most of the studies focused on the adverse effect of EDC-s on the endocrine homeostasis, however, a recent study clearly demonstrated effects at a different level: EDC-s can directly disturb the normal function of spermatozoa at physiologically relevant levels in the body fluids (*Schiffer et al.*, 2014).

Spermatology assays, originally developed for the routine semen quality controls at artificial insemination centers or human infertility clinics can be directly applied to the study of the effects of EDC-s on sperm structure and function. Such up-to-date automatized laboratory methods like flow cytometry (*Spano and Evenson*, 1991; *Hossain et al.*, 2011) or Computer-Assisted Semen Analysis (CASA – *Vetter et al.*, 1998) are precise and accurate tools of studying the disturbing effects of EDC-s at the cellular or subcellular level.

At the cell analysis laboratory of the Georgikon Faculty of the University of Pannonia we are currently testing the applicability of several sperm function assays based on flow cytometry to study the effect of EDC-s, heavy metals and adverse environmental conditions on spermatozoa, with a special focus on fish sperm since the gametes of externally fertilizing species theoretically may suffer more damage than the cells of internally fertilizing species (currently we use boar sperm as model to test the effects of EDC-s on mammals, too). We have chosen several cytotoxic and genotoxic

end points to measure, including mitochondrial trans membrane potential, phospholipid asymmetry and intactness of the plasma membrane, oxidative DNA damage and DNA fragmentation. Cytotoxic effects may trigger an intracellular cascade of events: defective mitochondria are the main source of intracellular Reactive Oxygen Species (ROS) which induce plasma membrane lipid peroxidation as well as DNA damage (first oxidative damage then consequently the development of DNA strand breaks leading to DNA fragmentation (*Aitken et al.,* 2012).

FLOW CYTOMETRIC ASSAYS TO STUDY SPERM STRUCTURE AND FUNCTION

Plasma membrane integrity, mitochondrial activity

Spermatozoa can be divided into the following domains: head, midpiece and tail, and into subdomains within these regions. The different subdomains of the sperm head plasma membrane are involved in separate gamete interaction events (zona binding, acrosome reaction, zona penetration, fusion with the oolemma, etc.). The acrosome, a large vesicle on the apical part of the sperm head, contains the hydrolytic enzymes necessary for zona pellucida penetration. The midpiece contains mitochondria and is involved in energy production. The tail is involved in motility. Several flow cytometric assays have been developed for assessing the plasma membrane integrity of the head, the integrity of the acrosome or for evaluating mitochondrial function. However, there is no flow cytometric assay for the evaluation of the plasma membrane integrity of the tail domain. Earlier we showed with a light microscopic staining method that spermatozoa with intact head membrane but with disrupted tail membrane are not motile therefore they are functionally dead (*Nagy et al.,* 1999). To be able to measure the same attribute by flow cytometry, we currently develop and test a fluorescent staining method specific for the integrity of the sperm tail plasma membrane. In order to distinguish spermatozoa from non-sperm particles (especially in extended semen) we apply the LIVE/DEAD® Fixable Viability Kits from Molecular Probes (Eugene, OR, USA) which label the viable and dead cells with the same color but different intensity (*Nagy,* 2007). This would be advantageous in multicolor flow cytometry as only one detector is needed to detect live/dead status. Moreover as our preliminary experiments indicated, these kits are able to indicate membrane rupture on every sperm subdomain, including the flagellum, not only the head. As a logical extension of the multiparameter approach, a fluorescent staining for the assessment of the mitochondrial activity could be added.

We have developed and tested a more objective method of evaluating sperm quality than the current subjective motility evaluations by testing the applicability of a novel fluorescent probe, Mitotracker Deep Red 633 (M-22426), for measuring the mitochondrial activity of spermatozoa both after freezing/thawing and after swim-up selection, using flow cytometry. The proportion of spermatozoa with high mitochondrial activity as determined by Mitotracker Deep Red 633 showed a high correlation with sperm motility (*Hallap et al.,* 2005).

Capacitation, early membrane changes

The capacitation process of spermatozoa involves complex changes in the composition and orientation of molecules at the surface of the sperm cell. Capacitation is defined as a preparative step; a sperm cell must undergo a priming process before it can bind to the zona pellucida and initiate the acrosome reaction. Flow cytometric assays like the merocyanine 540 staining (*Hallap et al.,* 2006) or the Annexin V-FITC assay

(conventionally used in apoptosis studies) allow discrimination between sperm subpopulations that undergo the capacitation induced transitions and cells that do not respond to the induction. Cryopreservation induces similar membrane changes in the surviving, intact sperm cell population. Such spermatozoa have shortened lifespan, therefore it would be interesting to see individually different responses to the cryopreservation process and relate them to fertility differences. This physiological process is extremely important area to study from the point of view of actions of EDC-s on sperm, as recent findings indicate that several EDC-s induce capacitation-like events via activation the CATSPER membrane channels and affecting intracellular calcium levels (*Schiffer et al.*, 2014).

DNA damage, chromatin status

The structure of the sperm chromatin is unique among other cell types, as histones are replaced by transition proteins and finally by protamines during spermatogenesis, resulting in an extremely condensed DNA (*Dadoune*, 1995). Proper condensation stabilizes the DNA and makes it less sensitive to oxidative damage, however, mature spermatozoa are not able to repair DNA damage as they are transcriptionally inactive. Abnormalities of the sperm chromatin structure can cause reduced fertility, abnormal pronuclear formation or early embryo quality and pregnancy outcome (*Evenson*, 1999).

Studies on domestic mammal species indicated that the early embryonic death is often caused by the nuclear defects (DNA fragmentation) of the fertilizing spermatozoa (*Evenson*, 1999). DNA strand breaks in spermatozoa can be caused by oxidative stress, heat stress, radiation or protamine deficiency (*Varner*, 2008). Due to incomplete protamination spermatozoa will be less compact and consequently more sensitive to attack by endogenous or exogenous agents, like nucleases, free radicals or mutagens (*Oliva*, 2006). Spermatozoa carrying damaged DNA look normal with conventional laboratory tests, but may induce failure in embryonic development. As mature spermatozoa are transcriptionally inactive, DNA damage may not be expressed until mitosis at the time of spermatozoon-oocyte fusion (*Varner*, 2008). Environmental factors like chemicals, excessive temperature, air pollution can cause abnormal sperm chromatin integrity (*Evenson and Wixon*, 2005).

The “gold standard” method to measure the fragmentation rate of sperm DNA is the so-called Sperm Chromatin Structure Assay (*Evenson*, 1999; *Spano and Evenson*, 1991). The test measures the ratio of the intact, double-stranded DNA and the fragmented, single-stranded DNA using acridine orange staining and flow cytometry. Earlier stage of DNA damage due to ROS attack can be detected with the OxyDNA assay which identifies 8-oxoguanine, a DNA adduct (*Hoornsta et al.*, 2003).

CONCLUSIONS, FURTHER DIRECTIONS

We have tested the adverse effect of several EDC-s, including irgasan, a bactericide agent and cadmium, a heavy metal with endocrine disrupting capabilities on silver carp and boar spermatozoa. Our initial results indicate negative effects on mitochondrial membrane potential, plasma membrane phospholipid asymmetry, moreover, oxydative DNA damage was also observed. Based on these findings we believe that flow cytometry offers a unique tool to study subcellular effects of EDC-s on spermatozoa. We plan to extend the array of tests with measuring intracellular calcium and ROS levels as well as applying dynamic flow cytometry with time parameter analysis to reveal the kinetic changes in sperm cells exposed to EDC-s.

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REFERENCES

Aitken, R. J., De Iuliis, G. N., Gibb, Z., Baker, M. A., (2012) The Simmet lecture: New horizons on an old landscape--oxidative stress, DNA damage and apoptosis in the male germ line. Reproduction in domestic animals = Zuchthygiene, 47. 7-14.

Dadoune, J.P. (1995). The nuclear status of human sperm cells. Micron, 26 (4), 323-345.

Evenson, D.P. (1999). Loss of livestock breeding efficiency due to uncompensable sperm nuclear defects. Reprod Fertil Dev. 11. 1-15.

Evenson, D.P., Wixon, R. (2005). Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Structure Assay (SCSA®). Toxicology and Applied Pharmacology, 207. 532-537.

Hallap, T., Nagy, Sz., Håård, M., Jaakma, Ü., Johannisson, A., Rodriguez-Martinez, H. (2005). Mitochondrial activity of frozen-thawed bull spermatozoa assessed by MitoTracker Deep Red 633. Theriogenology, 6.: 2311-2322.

Hallap, T., Nagy, Sz., Jaakma, Ü., Johannisson, A., Rodriguez-Martinez, H. (2006). Usefulness of a triple fluorochrome combination Merocyanine 540/Yo-Pro 1/Hoechst 33342 in assessing membrane stability of viable frozen-thawed spermatozoa from Estonian Holstein AI bulls. Theriogenology, 65. 1122-1136.

Hossain, Md. S., Johannisson, A., Wallgren, M., Nagy, Sz., Siqueira, A.P., Rodriguez-Martinez, H. (2011) Flow cytometry for the assessment of animal sperm integrity and functionality: State of the art. Asian Journal of Andrology, 13. 406-419.

Hoornstra, D., Andersson, M.A., Mikkola, R., Salkinoja-Salonen, M. , (2003) A new method for for in vitro detection of microbially produced mitochondrial toxins. Toxicology In Vitro, 17, 745-751.

Hutchinson, T.H., Brown, R., Brugger, K.E., Campbell, P.M., Holt, M., Lange, R., McCahon, P., Tattersfield, L.J., van Egmond, R. (2000). Ecological risk assessment of endocrine disruptors. Environ Health Perspect, 108. 1007-1014.

Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P-E., Forlin, L. (1999). Ethinyloestradiol - an undesired fish contraceptive? Aquatic Toxicology, 45. 91-97.

Mills, L.J., Chichester, C. (2005). Review of evidence: are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? Sci Total Environ, 1. 1-34.

Nagy, Sz., Házas, G., Bali Papp, Á., Iváncsics, J., Szász, F., Szász Jr. , F., Kovács A., Foote, R.H. (1999). Evaluation of sperm tail membrane integrity by light microscopy – Theriogenology, 52. 1153-1159.

Nagy, Sz. (2007). Bikaspermiumok vizsgálata doménspecifikus fluoreszcens jelöléssel, Magyar Állatorvosok Lapja, 129. 464-468.

Oliva, R. (2006). Protamines and male infertility. Hum. Reprod. Update. 12. 417-435.

Schiffer, C., Müller, A., Egeberg, D.L., Brenker, C., Rehfeld, A., Frederiksen, H., Wäschle, B., Kaupp, U.B., Balbach, M., Wachten, D., Skakkebaek, N.E., Almstrup,

K., Strünker, T. (2014). Direct action of endocrine disrupting chemicals on human sperm. EMBO Reports, 15. 758-765.

Spano, M., Evenson, D.P. (1991). Flow cytometric studies in reproductive toxicology. Prog Clin Biol Res, 37. 497-511.

Varner, D.D. (2008). Developments in stallion semen evaluation. Theriogenology, 70. 448-462.

Vethaak, A.D., Lahr, J., Kuiper, R.V., Grinwis, G.C., Rankouhi, T.R., Giesy, J.P., Gerritsen, A. (2002). Estrogenic effects in fish in The Netherlands: some preliminary results. Toxicology, 27. 147-150.

Vetter, C.M., Miller, J.E., Crawford, L.M., Armstrong, M.J., Clair, J.H., Conner, M.W., Wise, L.D., Skopek, T.R. (1998). Comparison of motility and membrane integrity to assess rat sperm viability. Reprod Toxicol, 12. 105-114.

Waring, R.H., Harris, R.M. (2005). Endocrine disrupters: a human risk? Mol Cell Endocr,1. 2-9.

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