Assessing the toxicity of engineered nanomaterials in the male reproductive system: Developing improved methods and strategies for hazard assessment

Bryony L Ross[1,2], Eva M Malone[2], Mick T Rae[2], Hans Bouwmeester[3], Meike van der Zande[3] & Gary R Hutchison[2]

1. Blue Frog Scientific Ltd. Edinburgh | 2. School of Applied Sciences, Edinburgh Napier University | 3. RIKILT, Waginingen UR

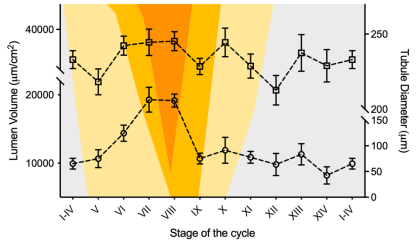
CONTACT:
Dr Bryony Ross
bryony.ross@bluefrogscientific.com
https://www.linkedin.com/in/bryonyross/

INTRODUCTION: Male fertility rates are declining; in areas they are now below replacement level[1]. Developing a better understanding of the multiple factors which can affect the male reproductive system is paramount, particularly in light of intensified attention from regulators on reproductive & endocrine endpoints. One relevant novel field requiring greater understanding of potential risk is engineered nanomaterials (ENM).

Our lab previously reported the impact of engineered nanomaterials on key cells of the testes; the Sertoli and Leydig cell[2]. In an attempt to assess whether *in vitro* toxicity tests using murine Leydig and Sertoli cells could be used to predict changes in testicular function, we examined relatable endpoints in animals exposed to a similar panel of ENM. The aim was to ascertain if cellular changes *in vivo* could be observed and potentially be related back to changes observed *in vitro,* and in turn develop an improved testing strategy for hazard assessment of DART. This study highlights data collated in relation to silver ENMs and ions.

Importantly, accurate examination of male reproductive tissue requires staging; a skill that takes years of training and experience to undertake. As part of this study, we developed a new simplified method by which to stage testicular tubules for histopathological analysis.

METHODS: Using a panel of highly representative ENM, assays to screen for toxicity in male testicular cell lines were established & optimised *in vitro*. Findings were validated for reliability & reproducibility against those generated using similar test systems with cells from alternative organs of the body. Assessment of cell and endocrine function also provided a deeper understanding of cellular responses following acute sub-lethal ENM exposure. Comparison of outcomes *in vitro* to *in vivo* was enabled by appraisal of tissues from animals exposed orally to the same ENM. Through this, a new method by which to stage tubules for histopathological analysis was developed, and for the first time a truly thorough morphological and stereological examination of tissues for markers of effect was provided.



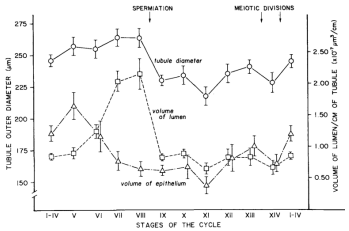


Fig 1: Comparison of hybrid Dazl-enabled technique to published staging data. (Lefr) Diameter of the seminiferous tubule and lumen volume at various stages of the spermatogenic cycle, superimposed onto the Dazl immunostaining intensity scores recorded. (Right) Wing & Christensen, 1982. NB. lumen volume is expressed per unit length of the seminiferous tubule in order to try and correct for shape differences between tubule.

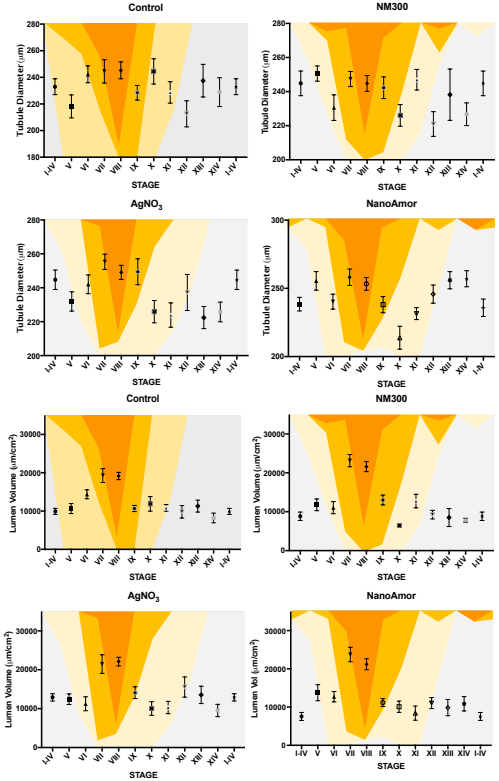


Fig. 2: Average (A) Seminiferous Tubule diameter (B) Lumen Volume (+/- SEM) overlaid on Dazl staining intensity scores, both according to stage of the cycle. Dazl staining intensity scores followed the same pattern regardless of whether animals were exposed to engineered nanomaterials or not. Tubule diameters recorded showed some alteration from the pattern recorded in control animals.

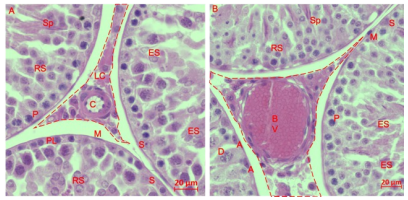


Fig. 3: Generalised morphology of healthy rat testis with focus on interstitial cell populations. (A) Adult Leydig cell populations surrounding a capillary. (B) Adult Leydig cells lying in close association to a larger blood vessel containing red blood cells. Scale bar represents 20µm.

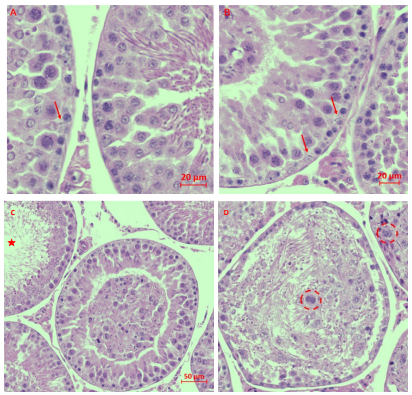


Fig. 4: Illustrative images captured from treated animals. (A+B) NM300 treatment, retention of mature step 19 spermatids in stage IX (A) and X (B) tubules (red arrows). Retention of mature spermatids after spermiation at stage VIII is a sensitive indicator of toxicity. Scale bar represents 20µm. (C) NanoAmor: Sloughing of tubular contents into central lumen, observed alongside tubules which are healthy in appearance (star). Scale bar represents 50µm. (D) +NM300: Potential development of multinucleated giant cells within morphologically degenerative tubules (red circles). Scale bar represents 50µm.

RESULTS:

- Detailed examination of morphological markers in tissues from animals exposed orally over 28-days to silver ENM and ions panel was undertaken following development of a new methodology for staging of testicular tissue in toxicity studies. The hybrid technique combined Dazl immunostaining (to quickly allow grouping of seminiferous tubule stage) with a decision key (incorporating key cell populations, morphological and stereological identifiers) to assign individual stage. This then allowed meaningful stereological and histopathological analysis, by stage (Fig. 1 & 2).
- Stereological analysis showed no alteration in tubule diameter with stage following exposure to ENM, indicating that at a structural level there was no link between tubule diameter and effect observed. However, it was possible to identify some very specific changes in lumen volume. These included increases compared to control for stage VII tubules in Nano Amor treated animals, and an increase in volume for stage IX tubules in treated animals, both of which indicate potential disruption of tubules associated with spermiation (stage VIII).
- Effects common to all animals included generalised tubular dysmorphism, presence of pyknotic nuclei, degeneration of germ cell populations and sloughing of tubular contents into the central lumen, all of which are widely accepted indicators of testicular toxicity, and relatively sensitive indicators of a disturbance in spermatogenesis, either via direct injury or disturbances in the HPG axis (Fig. 3 & 4).
- The Johnsen score provided evidence of increased apoptotic cells in particle treatments with both particles showing greater levels of impaired spermatogenesis when compared to the negative and ionic control (Table 1).

CONCLUSIONS:

- It was possible to confirm that all animals treated with silver suffered adverse changes within their testicular tissue following exposure, which were maintained even after a 6- week period of wash out, a finding which aligns completely with both that of van der Zande *et. al.*[3], who confirmed that silver ENM accumulated in the testis and were not excreted following this recovery period, and our own findings *in vitro* .
- New methods developed to stage seminiferous tubules to compare across treatment groups helped assess impact of ENM exposure & identify sensitive markers of reproductive toxicity.
- Observed histological changes and slight impaired spermatogenesis would suggest that nanosilver could have implications for men close to subfertility.

1. Makarow M, Hojgaard L. (2010) Male reproductive health. Its impacts in relation to general wellbeing and low European fertility rates. European Science Foundation. Science policy briefing 40. http://www.esf.org
2. Zhu, Donghui, Farcal, Lucian, Torres Andón, Fernando, Di Cristo, Luisana, Rotoli, Bianca Maria, Bussolati, Ovidio, Bergamaschi, Enrico, Mech, Agnieszka, Hartmann, Nanna B., Rasmussen, Kirsten, Riego-Sintes, Juan, Ponti, Jessica, Kinsner-Ovaskainen, Agnieszka, Rossi, François, Oomen, Agnes, Bos, Peter, Chen, Rui, Bai, Ru, Chen, Chunying, Rocks, Louise, Fulton, Norma, Ross, Bryony, Hutchison, Gary, Tran, Lang, Mues, Sarah, Ossig, Rainer, Schnekenburger, Jürgen, Campagnolo, Luisa, Vecchione, Lucia, Pietroiusti, Antonio and Fadeel, Bengt (2015) *Comprehensive In Vitro Toxicity Testing of a Panel of Representative Oxide Nanomaterials: First Steps towards an Intelligent Testing Strategy. PLOS ONE*, 10 (5). e0127174. ISSN 1932-6203
3. Van der Zande et al. (2012) Distribution, elimination, and toxicity of silver nanoparticles and silver ions in rats after 28-day oral exposure, ACS Nano, 28;6(8)

Table 1: Summary of morphological changes observed within tissue samples from treated with two types of silver ENM, silver ions and vehicle control. Results of Johnsen scores (as % of total tubules count) on spermatogenesis (scores are unpublished data received via personal communication from Bouwmeester and van der Zande, 2015).

	Dysmorphism	Germ Cell degeneration / atrophy	Pyknotic germ cell Nuclei (apoptosis)	Giant Cells	Interstitial effects	Germ Cell Sloughing	Germ Cell layer atrophy / 1-2 cells / tubule	Spermatid retention	Johnsin score (n) 10	Johnsin score (n) 9	% of apoptotic cells
Control	-	-	-	-	-	-	-	-	100		0
Silver Nano Amor	Y (widespread)	Y	Y	-	-	Y	Y	Y	25	75	27.6
Silver NM-300	Y (focal)	Y	Y	Y	Inflammation-like changes	Y	-	Y	36.6	63.4	8.2
Silver $AgNO_3$	Y (focal)	Y	Y	-	Leydig Cell Hyperplasia	Y	Y	-	92.2	7.8	7.6