Opportunities and limits of *in vitro* cytotoxicity test methods exemplified by powder metallurgy titanium alloys

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INTRODUCTION: Testing for *in vitro* cytotoxicity aims at obtaining data for a test material from simplified *in vitro* systems that can be used to make a prognosis for a key step within the processes occurring after implantation in the human body. Depending on the application of the test specimens, one can choose from a number of different assays to assess its bioacceptance in terms of absence of cytotoxicity. While guidelines (e.g. ISO, ASTM) describe a number of mandatory test procedures for official approval, additional tests have to be performed to properly evaluate the *in vitro* effects of test specimen on cells. However, in most cases it is difficult to select the appropriate assay. In addition, the conclusions that can be made out of the results are often limited [1]. They give trends rather than enable clear-cut recommendations. In this work we evaluate for the bioacceptance of different Ti alloys by two different assays; the agar overlay test and the extraction test, both described by the ISO 10993-5 guidelines. Opportunities and limits of cytotoxicity testing based on these and other methods will be discussed.

METHODS: Various Ti alloy samples were prepared by powder injection moulding. Sintered parts were sterilized by autoclaving or hydrogen peroxide gas plasma. The agar overlay test as well as the extraction tests were performed according to the ISO 10993-5 guideline. In case of the agar overlay test cells were vitally labelled using neutral red before use. After 24, 48 and 72 h of treatment toxicity was assessed taking the size of the zone without cells as an index. In case of the extract test cytotoxicity was assessed after 24h of treatment using neutral red and MTT (or WST-8) assays. From the obtained extracts the ions were quantified by ICP-EOS.

RESULTS: Large differences in outcome were obtained by evaluating the titanium alloys using agar overlay and extract tests with the agar overlay test being most sensitive. Only in the case of the agar overlay, the test cells were strongly affected by the samples after 24h. Basically, no such toxic effect could be observed using the extract test after the same treatment period.

DISCUSSION & CONCLUSIONS: Titanium is a widely used FDA approved implant material and passed all officially required *in vitro* as well as *in vivo* tests. Nevertheless, controversial data are reported (and also found in our lab) regarding the cytotoxic effects of Ti (alloy) materials (from no to moderate cytotoxicity). This was seen not only for the different alloys but also for the different cell assays, which most likely resulted from differences in preparation methods of the specimens and cell assay set-ups used.

The ISO 10993-5 extract test norm is rather flexible regarding extract medium, extraction method, treatment period and method to quantify cytotoxicity. As a result the outcome of different labs cannot be compared. The indirect extract test (which the overlay test in principle represents) in contrast is less prone to variations in experimental set-up. Furthermore, it might mimic better the local concentrations of the released constituents occurring *in vivo* in the tissue being in direct contact with the implant. In our hands this test showed to be most sensitive.

Thus while generally *in vitro* cytotoxicity testing represents a great opportunity to pre-evaluate the influence on cells regarding the released constituents of the test material, a careful, well documented and broad evaluation of assays is required in order to get an overall clear-cut impression concerning this key issue of bioacceptance.

REFERENCES: [1] A. Bruinink and R. Luginbuehl (2011) Evaluation of Biocompatibility Using In Vitro Methods: Interpretation and Limitations Adv. Biochem Eng Biotechnol, 117-52

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