9 Summary

Optimization and standardization of the rat whole embryo culture (WEC) as an *in vitro* embryotoxicity test through the development of a new culture medium

International guidelines of the Organisation of Economic Co-operation and Development (OECD) recommend the use of animal testing in reproductive toxicology to estimate the embryotoxic potential of chemicals. According to the REACH (registration, evaluation and authorisation of chemicals) Program of the European Union 30 000 of the "existing substances" must be investigated to determine their toxic potential within the next years. For this purpose, alternative methods to animal testing should be preferred, if they are validated according to the international validation guidelines and are accepted by regulatory agencies. Following the 3R-principle of Russel and Burch [1] many alternative methods were developed over the past decades, the WEC being one of them.

Today, the WEC is internationally validated to classify the embryotoxic potential of chemicals or drugs. However, in order to be fully accepted by regulatory agencies it is necessary to further develop the WEC method to achieve general standardization. The recommended culture medium (100 % rat serum) has two disadvantages for the WEC embryotoxicity test. First, the rats have to be sacrificed to gain blood for the culture medium. Second, the charge variability of the rat serum biases the comparability of the obtained results. Therefore, not all given evaluation parameters of the embryos such as the growth could be incorporated into the prediction model during the validation study. At that time there was no alternative culture medium to rat serum which could have enabled a standardization of the culture system. Therefore, it was the aim of this thesis to establish a culture medium which would be superior to rat serum.

A direct comparison of the commonly used culture media of the WEC (human serum, human serum supplemented with 10 % rat serum and rat serum) and a bovine serum produced according to the protocol of Klug et al. [72] revealed that all heterologous sera have comparable development-promoting potencies for rat embryos in culture. The use of commercially available bovine serum did not lead to sufficient results. A financially supported joint project was initiated to analyze this problem. In cooperation with a producer of culture media and animal sera the development-promoting potencies of commercial bovine sera (fetal bovine serum [FBS], donor bovine serum [DBS] and adult bovine serum [ABS] were investigated. As a first partial success, a combination of FBS and DBS supplemented with 10 % rat serum was identified which supported the development of rat embryos comparable to rat serum.

Continuing investigations of possible influencing variables in the serum production led to a successful transfer of a modified protocol to produce bovine serum (suitable for the WEC [72]) under industrial standards.

A new subtype of industrially produced DBS, donor pregnant bovine serum (DPBS) was established. We achieved the highest development-promoting potency of all commercial DBS for rat embryos ever tested before. A second partial success was that DPBS supplemented with 10 % rat serum supported the development of rat embryos comparable to rat serum.

Furthermore, we investigated the significance of the growth factors (insulin-like growth factor [IGF-1], epidermal growth factor [EGF] and vascular endothelial growth factor [VEGF]) as an alternative to the 10 % rat serum supplementation to improve the development-promoting potencies of heterologous sera for rat embryos. RT-PCR analyses of the growth factor receptors revealed differences in there mRNA levels of IGF-1 and VEGF (Flt-1) receptors in the whole embryos in comparison between *in vivo* and *in vitro*, but not for the EGF receptor. An increase of embryonic development could be caused by the supplementation of IGF-1 and even more profound by VEGF. These observations indicated that both growth factors are involved in the effect observable after a supplementation of 10 % rat serum to heterologous sera.

To exclude the possibility of changed sensitivity and sensibility of the embryos to test substances caused by the new culture media, five reference substances were investigated. The observed effects using the new established culture media: 1. Serum mixture of ABS : FBS : RaS in the ratio 4.5 : 4.5 : 1 or 2. DPBS supplemented with 10 % rat serum were compared to the effects observed previously in WEC studies or *in vivo*. Both, the concentration-effect relationships and the specific adverse effects of penicillin G, ethanol, valproic acid, all-trans retinoic acid and 5-fluorouracil were comparable.

The new culture media of the WEC provide the possibility to reduce the needed number of animals to produce the culture medium for the WEC by 90 % and to increase the batch size of the culture medium from 50-80 ml up to 20 000 to 50 000 ml. To ensure the availability of the new culture media they were incorporated into the commercially available product assortment of the cooperating company (Biochrom AG, Berlin). A German pharmaceutical company as well as the head of the WEC Validation Study were able to reproduce the development-promoting potencies of these culture media for rat embryos.

Two new culture media could be established for the WEC, enable the demanded widespread standardization of the WEC, ensure the reproducibility of all evaluation parameters, are easily available world-wide, and above all, will dramatically reduce the sacrificing of animals for the WEC.