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Evaluation of the zebrafish embryo as an alternative model for hepatotoxicity testing

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Nederlandse Samenvatting

Korte introductie

Mensen worden gedurende hun hele leven blootgesteld aan een veelheid van schadelijke stoffen, via zowel natuurlijke als artificiële wegen. Soms vindt blootstelling aan deze schadelijke stoffen onbewust plaats, zoals blootstelling via voedsel, door inademing of door opname via de huid, of bewust wanneer men bijvoorbeeld een medicijn inneemt. Het gebruik van medicijnen kan leiden tot schade aan het lichaam, zeker wanneer er een te hoge dosering wordt ingenomen. Door middel van toxicologisch onderzoek probeert men de risico's in te schatten van deze schadelijke stoffen.

Bij toxicologisch onderzoek is de lever een belangrijk orgaan. Dit komt onder andere door de rol die de lever in het menselijk lichaam heeft. De lever komt als eerste in aanraking met giftige stoffen, nadat deze door de darm zijn opgenomen. Verder speelt de lever een rol in het omzetten van giftige stoffen naar mindere giftige stoffen die gemakkelijk door het lichaam zijn uit te scheiden. Dit proces verloopt normaal gesproken zonder problemen, maar soms kan deze omzetting van stoffen juist tot een giftiger product leiden waardoor de lever schade oploopt.

Leverschade kan resulteren in een verscheidenheid aan histopathologische veranderingen, waarbij cholestase, steatose en necrose de drie belangrijkste afwijkingen zijn. Cholestase is te zien in de levercel als geel-oranje druppeltjes. Steatose wordt gekenmerkt door ophoping van vet in de levercel en wordt zichtbaar door middel van histopathologische kleuringen. Bij necrose is er sprake van versterf van levercellen, wat afhankelijk van het stadium zichtbaar is als verkleuring of verval van levercellen. Alle vormen van leverschade kunnen uiteindelijk resulteren in leverfalen.

Om te kunnen voorspellen welke giftige stoffen tot leverschade leiden, wordt er gebruik gemaakt van zowel *in vitro* als *in vivo* modellen. Bij *in vitro* modellen analyseert men de effecten van schadelijke stoffen op cellijnen, schijfjes lever en primaire lever cellen. *In vivo* modellen richten zich op het testen van stoffen in dieren, en daarbij worden er voornamelijk muizen en ratten gebruikt. Aan deze *in vivo* dierstudies kleven een aantal ethische, economische en wetenschappelijke bezwaren. Er worden veel dieren gebruikt die voor langere tijd aan hoge doseringen van de giftige stoffen worden blootgesteld. Het belangrijkste bezwaar is echter dat de resultaten die met deze dierstudies worden behaald, lang niet altijd de situatie in de mens goed voorspellen. Dit wordt ook wel aangeduid als vals positieve of vals negatieve resultaten. Daardoor heeft het onderzoek de afgelopen jaren zich voornamelijk gefocust op de ontwikkeling van alternatieve testmethoden die de situatie in de mens beter kunnen voorspellen.

Een mogelijk goed alternatief model is het zebravis embryo model. Dit model combineert verschillende voordelen, waarvan er één is dat deze embryo's nog zo'n primitief zenuwstelsel hebben dat niet wordt verwacht dat ze pijn of ongerief kunnen ervaren. Zebravis embryo's worden daarom niet gezien als proefdier en het model draagt op die manier bij

aan de drie V strategie (Vermindering, Vervanging en Verfijning van proefdiergebruik).
Normaal gesproken word het effect van de giftige stoffen bepaald door het bekijken van histopathologische veranderingen en meten van leverenzymen. Deze methoden geven echter geen informatie over het mechanisme dat leidt tot leverschade, terwijl juist dit moleculaire mechanisme mogelijkheden geeft om leverschade goed te voorspellen. Om dit onderliggende mechanisme te kunnen begrijpen, wordt er gebruik gemaakt van verschillende moleculaire technieken, gericht op analyse van met name gen- en eiwitexpressie.
Dit proefschrift beschrijft hoe het zebravis embryo model in combinatie met gen-expressie analyse-technieken het beste gebruikt kan worden om leverschade in de mens te kunnen voorspellen.

Samenvatting van de studies beschreven in dit proefschrift

Hoofdstuk 2 beschrijft twee technieken die gebruikt zijn om te bepalen of het zebravis embryo als alternatief model gebruikt kan worden bij de voorspelling van leverschade. Hierbij hebben we veranderingen in extracten van het gehele embryo vergeleken met veranderingen in de lever van de volwassen zebravis, om de vraag te beantwoorden of effecten op de lever wel in het zebravis embryo gemeten kunnen worden. Allereerst hebben we met behulp van histologie gekeken of er daadwerkelijk morfologische veranderingen in de lever van zowel de volwassen zebravis als de embryo waargenomen kunnen worden. Hieruit kwam naar voren dat er leeftijd- en stofspecifieke veranderingen optraden in beide modellen. Daarna hebben we met behulp van next generation sequencing onderzocht of we in staat waren om genexpressie veranderingen in het embryo te kunnen waarnemen, omdat de lever maar in geringe plaats inneemt in het embryo. Na analyse kwam naar voren dat we in staat zijn om leverspecifieke veranderingen waar te nemen in het gehele embryo.
Omdat we in staat waren lever- geassocieerde genexpressie te kunnen bepalen in het gehele embryo, hebben we in **hoofdstuk 3** geanalyseeerd of het mogelijk is om op basis van genexpressie profielen (transcriptomics) onderscheid te maken tussen de verschillende typen leverschade. Voor deze studie hebben we gebruik gemaakt van dezelfde stoffen als in **hoofdstuk 2**, maar zijn de genexpressie profielen op een andere manier geanalyseerd, namelijk met behulp van microarrays. Het bleek dat in het zebravis embryo model op die manier de verschillende klassen van leverschade niet te onderscheiden zijn, omdat de genexpressie profielen van alle klassen veel overlappen. Wel bleek het mogelijk om genexpressie markers te identificeren die leverschade zouden kunnen voorspellen in de mens.
Omdat met genexpressie geen onderscheid gemaakt kon worden tussen de verschillende typen leverschade hebben we in **hoofdstuk 4** gebruik gemaakt van een andere techniek, namelijk het meten van eiwitexpressie veranderingen (proteomics). Maar ook met deze techniek bleek het niet mogelijk om in het zebravis embryo model onderscheid te maken tussen de verschillende typen klassen van leverschade. Daarentegen waren we wel in staat om een kleine lijst met markers te selecteren die leverschade in de mens zouden kunnen voorspellen.
Om echt de voorspellende waarde van het zebravis embryo model te kunnen definiëren, hebben we in **hoofdstuk 5** een overkoepelende analyse uitgevoerd die de genexpressie profielen van de verschillende testmodellen voor leverschade vergelijkt. In plaats van alleen te kijken naar veranderingen in expressie van alleen genen, hebben we bestudeerd of groepen van genen, de zogenaamde pathways, veranderde na blootstelling. Uit deze vergelijking bleek dat het zebravis embryo model met betrekking tot gereguleerde pathways overlap heeft met zowel de *in vivo* als de *in vitro* modellen. Wanneer we alle modellen met elkaar vergelijken, zowel *in vivo* als *in vitro,* komt er één pathway naar voren die in alle modellen gereguleerd is.
In de afsluitende discussie (**hoofdstuk 6**) van het proefschrift worden de voor- en nadelen van de gebruikte technieken beschouwd, en besproken welke aanvullende studies nodig zijn om het zebravisembryo model daadwerkelijk in te zetten voor het voorspellen van leverschade door chemische stoffen in de mens. Als slotconclusie wordt geconcludeerd dat het zebravis embryo model gebruikt kan worden als alternatief voor dierproeven bij het voorspellen van leverschade in de mens.

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Curriculum Vitae

Marja Driessen was born on January 23, 1985 in Breda, The Netherlands. After graduating from secondary school at Stedelijk Lyceum in Roermond in 2003, she started her study in Health Sciences at the University of Maastricht with a major in Bioregulation & Health and a minor in Movement Sciences. During her studies, she completed several internships. Her Bachelor internship was conducted at the Academic Hospital Pharmacy in Maastricht under the supervision of Dr. Leo Stolk and Dr. Joyce Pullen. Her Master internship was performed at the Department of Tumor Immunology, Maastricht University under the supervision of Dr. Michel van Gelder and Peter Frings. After obtaining her Master degree in 2007, she applied for a second Master in Bio-Informatics at the Catholic University of Leuven, Belgium. There she conducted her internship in the Department of Electrical Engineering (ESAT) under the supervision of Prof. Dr. Bart de Moor and Tunde Adeshoyla Adefoyie. After graduating in 2009, she started her PhD study on the applicability of the zebrafish embryo as a screening model for hepatotoxicity under the direct scientific supervision of Dr. Leo T.M. van der Ven and Dr. Anne. S. Kienhuis (RIVM, Laboratory for Health Protection Research). Since February 2014, Marja has a position as a postdoctoral fellow in the Structural Computational Biology Department of the European Molecular Biology Laboratory (EMBL) Heidelberg, Germany.

List of publications

Articles

Driessen M, Duijvesteijn – van der Plas S, Vitins AP, Kienhuis AS, Pennings JL, van den Brandhof EJ, Roodbergen M, van de Water B, Spaink HP, Palmblad NM, van der Ven LTM. *Toxicogenomics approach in the zebrafish embryo to assess hepatotoxicity: combination of protein and gene expression changes*. In preparation

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Book chapter

Driessen M and van der Ven, LTM. Chapter in Toxicogenomics-Based Cellular Methods, *Alternatives to Animal Testing for Safety Assessment*, 1st Edition. *Academic Press*, 2014 March 7 ISBN: 9780123978622

Patent

Driessen, M and van der Ven LTM. *Hepatotoxicity testing in zebrafish embryo*. Patent application number EP13165649.8 (European)