



**UNIVERSITI PUTRA MALAYSIA**

**CHICKEN EMBRYO AS MODEL FOR EFFECTS OF N-NITROSODIMETHYLAMINE USING MORPHOLOGY, HAEMATOLOGY AND PROTEOMIC ANALYSES DURING EMBRYOGENESIS**

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EMBRYOGENESIS**

**By**

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This study was carried out to investigate the developmental toxicity effects of an established carcinogen namely N-Nitrosodimethylamine (NDMA) and its administration method on the early and mid embryogenesis of chicken. The study was also conducted to test the suitability of chicken embryo in its early and mid embryogenesis stages as a model for developmental toxicity test. Fertilized eggs were divided into three groups (control (untreated), control vehicle and NDMA-treated) with six eggs in each group and incubated at 37.5°C for 11 different incubation times. Several methods and techniques were modified, optimized and developed prior to be used in the analyses. The effect of NDMA was assessed based on gross morphological (early and mid embryogenesis), haematological (only in mid embryogenesis) and proteomic (early embryogenesis) analyses of the developing chicken embryos. The newly developed method and technique, i.e., Adobe Photoshop gross morphological measurement method and isoelectric focusing (IEF) tube gel labeling technique were optimized and applied throughout this study. The

normal development and growth of the chicken embryos in the early and mid embryogenesis were found to be severely affected by NDMA as indicated by gross morphological and haematological data. Malformations in the development of embryos and failure of peripheral blood vessels formation (angiogenesis) in their yolk sac were visibly apparent for NDMA-treated group. The administration method of NDMA did not affect the normal chicken embryos early and mid embryogenesis as there were no significant ( $p > 0.05$ ) difference between control and control vehicle groups in all of the gross morphological and haematological parameters tested. Around 100 to 180 protein spots were resolved on the 2DE gels in the control, control vehicle and NDMA-treated groups. A total of six most remarkably expressed protein out of 51 identified proteins were found to be directly or indirectly involved in NDMA possible angiogenesis inhibition and/or hematotoxic effect in the early chicken embryogenesis. These six proteins were identified as PIT54, VEGF-D, ApoA1, unnamed protein product of IgY, TBP-like protein 1 and Kelch-like protein 7, respectively. The PIT54, VEGF-D, and ApoA1 proteins seemed to be directly affected by the NDMA metabolite (s) or by its (their) angiogenesis inhibition effect. The unnamed protein product of IgY, TBP-like protein 1 and Kelch-like protein 7 which seemed to be indirectly affected by NDMA, were closely interrelated with each other and simultaneously upregulated only in the control group at 72 and 96 hours of incubation. At the proteome level, the *in ovo* administration method of NDMA seemed to affect the embryos by suppressing their normal responses to the possibly adverse IgY-antigens interaction. This as indicated by the downregulation of the unnamed protein product of IgY and its interrelated proteins (TBP-like protein 1 and Kelch-like protein 7) in the control vehicle group of embryos. It is uncertain whether this effect is harmful or not to the general chicken embryo development and

growth since the gross morphological and haematological results showed no observable effect of this administration method. However, any disturbance to the normal cellular response should be taken into a serious consideration. In conclusion, NDMA in its normal carcinogenic dosage could potentially cause developmental toxicity in the early and mid embryogenesis of chicken through its possible primary role as an angiogenesis inhibitor and/or secondary role as a hematotoxicant. The *in ovo* administration method of NDMA using sterile dionized water is evident not to adversely affect the normal physical development and growth of the embryos in their early and mid embryogenesis. However, it seemed to affect the expression of certain proteins at proteome level. Therefore, it is a must for any future study involving this *in ovo* administration method to also include the control vehicle in their experimental designs to avoid false positive results that might arise from the administration method itself. It is also evident from this study that chicken embryo in its early and mid embryogenesis stages is a suitable model for developmental toxicity test.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**EMBRIO AYAM SEBAGAI MODEL UNTUK KESAN N-NITROSODIMETILAMIN MENGGUNAKAN ANALISIS-ANALISIS MORFOLOGI, HEMATOLOGI DAN PROTEOMIK PADA PERINGKAT KEJADIAN EMBRIO**

Oleh

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Kajian ini dilakukan untuk melihat kesan ketoksikan perkembangan sejenis karsinogen iaitu NDMA dan juga kaedah pemberiannya ke atas ayam pada peringkat awal dan pertengahan pembentukan embrionya. Kajian ini juga bertujuan untuk menguji kesesuaian embrio ayam pada peringkat awal dan pertengahan pembentukan embrionya untuk digunakan sebagai model ujian ketoksikan perkembangan. Telur ayam tersenyawa telah dibahagikan kepada tiga kumpulan dengan enam biji telur setiap satu iaitu dinamakan sebagai kawalan, kawalan pembawa dan perlakuan-NDMA dan dieramkan pada 37.5°C dengan 11 masa yang berbeza. Beberapa kaedah dan teknik telah diubahsuai, dioptimumkan dan dibangunkan terlebih dahulu sebelum digunakan dalam analisis. Kesan NDMA kemudiannya dipantau dengan analisis- analisis morfologi kasar (awal dan pertengahan kejadian embrio), hematologi (pertengahan kejadian embrio) dan proteomik (awal kejadian embrio) ke atas embrio-embrio ayam yang sedang berkembang. Kaedah dan teknik baru yang dinamakan sebagai kaedah Adobe Photoshop untuk pengukuran morfologi kasar dan teknik pelabelan tiub gel pemfokusan isoelektrik (IEF) telah dibangunkan,

dioptimumkan dan digunapakai dalam kajian ini. Perkembangan dan tumbesaran normal embrio-embrio ayam pada peringkat awal dan pertengahan kejadian embrio menerima kesan yang sangat teruk daripada NDMA seperti yang ditunjukkan oleh data morfologi kasar dan hematologi. Kecacatan bentuk dalam perkembangan embrio-embrio dan kegagalan pembentukan salur-salur darah sekitaran (angiogenesis) di atas kantung kuning telur kelihatan dengan jelas bagi kumpulan perlakuan-NDMA. Oleh kerana tiada perbezaan yang ketara ( $p > 0.05$ ) di antara kumpulan kawalan dan kawalan pembawa bagi kesemua parameter morfologi kasar dan hematologi yang diuji, maka kaedah pemberian NDMA didapati tidak meninggalkan kesan sampingan ke atas perkembangan normal embrio-embrio ayam pada peringkat awal dan pertengahan kejadian embrio. Sekitar 100 ke 180 titik-titik protein telah didapati muncul di atas gel-gel 2DE dalam kumpulan-kumpulan kawalan, kawalan pembawa dan perlakuan-NDMA. Sejumlah 6 protein terpilih yang paling menonjol diekspreskan daripada 51 bintik-bintik protein yang berjaya dikenalpasti identiti telah didapati terlibat secara langsung atau tidak langsung dalam kemungkinan kesan perencatan angiogenesis dan/atau kesan hematotoksik NDMA terhadap kejadian embrio ayam pada peringkat awal. Keenam-enam protein ini telah dikenalpasti identiti sebagai PIT54, VEGF-D, ApoA1, protein tidak bernama hasilan IgY, protein 1 mirip-TBP dan protein 7 mirip-Kelch. PIT54, VEGF-D, dan ApoA1 kelihatan seolah-olah menerima kesan langsung daripada metabolit NDMA ataupun kesan perencatan angiogenesisnya. Protein tidak bernama hasilan IgY, protein 1 mirip-TBP dan protein 7 mirip-Kelch yang kelihatan seolah-olah menerima kesan secara tidak langsung daripada NDMA adalah saling berkait rapat antara satu sama lain dan peningkatan pengekspresan mereka pula adalah secara serentak dalam hanya kumpulan kawalan iaitu pada 72 dan 96 jam. Pada peringkat proteom, kaedah

pemberian NDMA secara *in ovo* kelihatan seolah-olah memberi kesan ke atas embrio dengan menekan tindakbalas normal mereka terhadap kemungkinan kesan buruk interaksi IgY-antigen. Ini adalah seperti yang ditunjukkan oleh penurunan pengekspresan protein tidak bernama hasilan IgY dan juga protein-protein yang berkaitan dengannya (protein 1 mirip-TBP dan protein 7 mirip-Kelch) dalam embrio-embrio kumpulan kawalan pembawa. Ianya tidak pasti samada kesan ini adalah merbahaya atau tidak terhadap perkembangan dan tumbesaran normal embrio ayam kerana keputusan-keputusan morfologi kasar dan hematologi telah menunjukkan yang kaedah pemberian ini tidak mempunyai kesan yang boleh dilihat. Walau bagaimanapun, sebarang gangguan terhadap tindakbalas normal sel seharusnya diambil perhatian yang serius. Secara kesimpulannya, NDMA dalam dos karsinogenik biasa mungkin telah menyebabkan ketoksikan perkembangan ke atas ayam pada peringkat awal dan pertengahan kejadian embrio melalui peranan utamanya sebagai perencat angiogenesis dan/atau peranan keduanya sebagai hematotoksikan. Kaedah pemberian menggunakan air nyahion steril telah dibuktikan tidak memberikan sebarang kesan buruk ke atas perkembangan normal embrio-embrio pada peringkat awal dan pertengahan kejadian mereka. Walau bagaimanapun, ianya kelihatan seolah-olah memberikan kesan ke atas pengekspresan protein-protein tertentu pada peringkat proteom. Oleh itu, sebarang kajian yang menggunakan kaedah pemberian secara *in ovo* ini mestilah melibatkan juga kawalan pembawa dalam rekabentuk eksperimen mereka bagi mengelakkan keputusan-keputusan positif yang salah yang berkemungkinan disebabkan oleh kaedah pemberian bahan ujian itu sendiri. Embrio ayam pada peringkat awal dan pertengahan pembentukan juga adalah ditunjukkan di dalam kajian ini sebagai sesuai untuk digunakan sebagai model untuk ujian ketoksikan perkembangan.



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I certify that an Examination Committee has met on **10 December 2009** to conduct the final examination of **Mohd Rosni Bin Sulaiman** on his **Doctor of Philosophy** thesis entitled “**Chicken Embryo As A Model To Study The Effects Of N-Nitrosodimethylamine Using Morphology, Haematology and Proteomic Analyses During Early And Mid Embryogenesis**” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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Date: 17 March 2010

## **DECLARATION**

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

---

**MOHD ROSNI BIN SULAIMAN**

Date:

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## LIST OF ABBREVIATIONS

2DE	Two dimensional electrophoresis
ACE	Angiotensin converting enzyme
ADI	Acceptable daily intake
AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ApoA1	Apolipoprotein A1
APP	Acute phase protein
APR	Acute phase response
APS	Ammonium persulphate
BMPs	Bone morphogenetic proteins
BSA	Bovine serum albumin
Ca	Calcium
CAM	Chorioallantoic membrane
CNS	Central nervous system
CV	Coefficient of variation
DMEs	Drug-metabolizing enzymes
DNA	Deoxyribonucleic acid
DSA	Digital subtraction angiography
DTT	Dithiothreitol
E1	Primitive normochromatid erythrocyte (NCE)
EDTA	Ethylene diamine tetraacetic acid
EG&K	Eyal-Giladi and Kochav
FAS	Fetal alcohol syndrome
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide