UNIVERSITI PUTRA MALAYSIA

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

MOHD ROSNI BIN SULAIMAN

FBSB 2009 36

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

By

MOHD ROSNI BIN SULAIMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

2009

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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

By

MOHD ROSNI BIN SULAIMAN

Chairman : Nor Aripin Shamaan, PhD

Faculty : Biotechnology and Biomolecular Sciences

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 37.5ºC 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

MOHD ROSNI BIN SULAIMAN

Pengerusi : Nor Aripin Shamaan, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

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 37.5ºC 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-seolah 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.

ACKNOWLEDGEMENTS

First of all I would like to thank Allah S.W.T the Almighty for his blessings and wisdom to enable me to complete this thesis.

I would like to take this opportunity to express my deepest gratitude to my supervisor, Prof. Dr. Nor Aripin Shamaan for his invaluable criticisms, suggestions, guidance, motivation and patience throughout the research. I would also like to express my special gratitude to Assoc. Prof. Dr. Mohd Yunus Abdul Shukor, Assoc. Prof. Dr. Muhajir Hamid and Prof. Dr. Yasmin Anum Mohd Yusoff for their constant guidance, support, encouragement and constructive comments.

Furthermore, I would like to thank the Vice Chancellor of Universiti Malaysia Sabah (UMS), Kol. Prof. Datuk Dr. Kamaruzaman Ampon and the Dean of School of Food Science and Nutrition, UMS, Assoc. Prof. Dr. Ismail Abdullah, for extending my study leave that really helped in the completion of my research works and thesis write up, as well as for their constant encouragements. I would also like to thank the Ministry of Science, Technology and Innovation of Malaysia (MOSTI) for fully funding this research project (ScienceFund Grant No.: 02-01-04-SF0001), and also to Public Service Department/UMS for the SLAB scholarship.

Special thanks to all the staff of Faculty of Biotechnology and Biomolecular Sciences and Faculty of Medicine and Health Sciences at Universiti Putra Malaysia (UPM), especially staffs of Toxicology Laboratory, Enzymology Laboratory, Microbiology Laboratory, Haematology Laboratory, and Biotech 2 administration

office who had contributed directly or indirectly towards the success of this research. Thank you to Dr. Zainina Abu Seman, a Senior Lecturer from Pathology Department, for her kind assistance on haematological analysis. Also, thank you to Mr. Hussain Jirangon and Mdm. Sharipah Samah, Assistant Science Officers from Microbiology Department, for their assistance on microscopical observations and on the *in ovo* test substances administration technique to the chicken embryo. I would also like to thank Linggi Poultry Farm, Chengkau, Negeri Sembilan for supplying me the fertilized chicken eggs.

Special thanks to my friends, Nurul Ain, Hasliza Hassan, Natarajan, Tham Lik Gin, Ram Chandra Basnyat, Ahmad Razi, Nurlizah, Mohd Fadhil, Dr. Mohd Shukuri, Dr. Tengku Haziyamin, Chee Fah, Dr. Sharifudin Shaarani and Dr. Patricia Matanjun whose help, suggestions, comments and moral supports have helped in the improvement of this thesis. Also thank you to everyone who had contributed directly or indirectly to the success of this research.

Lastly but not least, my heartfelt gratitude goes to my mother, father, wife, children and family for their continuous encouragement, patience, understanding, support and love throughout the critical years of my study.

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.

Members of the Examination Committee were as follows:

Norhani Abdullah, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Noorjahan Banu Mohamed Alitheen, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Zalinah Ahmad, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

T. William Jordan, PhD
Reader
School of Biological Sciences
Faculty of Science
Victoria University of Wellington
New Zealand
(External Examiner)

_____

BUJANG KIM HUAT, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of Supervisory Committee were as follows:

Nor Aripin Shamaan, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Yunus Abd. Shukor, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Muhajir Hamid, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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:

TABLE OF CONTENTS

		Page
ABSTRACT		ii
ABSTRAK		v
ACKNOWLEDGEMENTS		viii
APPROVAL		x
DECLARATION		xii
LIST OF TABLES		xviii
LIST OF FIGURES		xix
LIST OF ABBREVIATIONS		xxiv
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	5
	2.1 N-Nitrosodimethylamine (NDMA)	5
	2.1.1 Basic physical and chemical properties	5
	2.1.2 Route of exposure to human and animal	5
	2.1.3 Detrimental effects	7
	2.1.4 Recent studies on NDMA	11
	2.2 Developmental toxicology	13
	2.2.1 History	13
	2.2.2 Mechanisms of developmental toxicity	15
	2.2.3 Interaction of toxicants with cellular molecules	17
	2.2.4 Animal model frequently used in developmental toxicity study	21
	2.3 Chicken embryo as a model in developmental toxicology	25
	2.3.1 Embryology of the chicken	25
	2.3.2 Advantages and disadvantages	26
3	**GENERAL MATERIALS AND METHODS**	30
	3.1 Sampling	30
	3.2 Experimental design	30
	3.3 Administration of NDMA	31
	3.4 Incubation of the eggs	32
4	**OPTIMIZATION OF METHODS FOR GROSS MORPHOLOGICAL, HAEMATOLOGICAL AND PROTEOMIC ANALYSES**	33
	4.1 Introduction	33

4.2 Materials and Methods 36
4.2.1 Measurement method for the development of chicken embryo 36
4.2.2 Total soluble protein (TSP) content measurement 39
4.2.3 Two dimensional electrophoresis (2DE): Modification, development and optimization of a cut long pipette tip with syringe as a practical gel solution filling technique 42
4.2.4 Two dimensional electrophoresis (2DE): Modification, development and optimization of new tube gel labeling technique using a cut blue pipette tip connected to syringe 44
4.2.5 Two dimensional electrophoresis (2DE): Development and optimization of a simple method to minimize black region effect at the bottom part of 2D gel 45

4.3 Results and Discussion 47
4.3.1. A newly developed and optimized virtual measurement method using Adobe Photoshop measuring tool 47
4.3.2 An optimized modified Bradford microassay method for total soluble protein content measurement 49
4.3.3 A new gel solution filling technique using a cut long pipette tip connected to a syringe 51
4.3.4 A newly developed technique for labeling the tube gel using a cut blue pipette tip connected to a syringe 54
4.3.5 A simple method to minimize black region effect on the 2D gel 57

4.4 Conclusion 60

5 GROSS MORPHOLOGICAL ANALYSIS OF THE EFFECT OF NDMA TO THE CHICKEN EMBRYO 62

5.1 Introduction 62

5.2 Materials and Methods 64
5.2.1 Preparation and handling the egg yolk with embryo 64
5.2.2 Direct photography technique 64
5.2.3 Image processing in Adobe Photoshop 64
5.2.4 Measurement of growth parameters using Adobe Photoshop measuring tool 65
5.2.5 Comparison between the observed groups 66
5.2.6 Statistical analysis 66

5.3 Results and Discussion 66
5.3.1 General visual observation 66

5.3.2 Comparison of blastoderm diameter 74
5.3.3 Comparison of embryonic width and length 77
5.3.4 Comparison of vitelline vasculature width and length 82

5.4 Conclusion 86

6 GROSS MORPHOLOGICAL AND HAEMATOLOGICAL ANALYSES OF THE EFFECT OF NDMA ON THE CHICKEN EMBRYO DURING MID EMBRYOGENESIS 88

6.1 Introduction 88

6.2 Materials and Methods 90
6.2.1 Preparation and handling of the embryonated egg yolk 90
6.2.2 Direct photography technique 90
6.2.3 Blood withdrawal from embryo 90
6.2.4 May-Grunwald Giemsa staining of the blood smear 91
6.2.5 Blood cells scoring and differential count 91
6.2.6 Blood analysis 92
6.2.7 Image processing 93
6.2.8 Measurement of growth parameters using Adobe Photoshop 93
6.2.9 Statistical analysis 93

6.3 Results and Discussion 94
6.3.1 General observation of embryonic growth related to haematological analysis 94
6.3.2 Comparison of blood cells score and count 100
6.3.3 Comparison of blood parameter 106

6.4 Conclusion 111

7 PROTEOMIC ANALYSIS OF THE EFFECT OF NDMA ON THE CHICKEN EMBRYO DURING EARLY EMBRYOGENESIS 112

7.1 Introduction 112

7.2 Materials and Methods 114
7.2.1 Protein extraction and solubilization 114
7.2.2 Protein dialysis 115
7.2.3 Protein standard curve development 116
7.2.4 Total soluble protein determination 116
7.2.5 Native- and SDS-PAGE 117
7.2.6 Two dimensional electrophoresis 119
7.2.7 Staining the gel 130

7.2.8 Image analysis of 2D gels using Melanie version 7.0 software 135
7.2.9 Protein spots for MALDI-TOF peptide mass fingerprinting and their identifications 137
7.2.10 Peptide masses application in databases mining for protein identifications 138

7.3 Results and Discussion 140
7.3.1 Comparison of the total soluble protein 140
7.3.2 Native protein bands pattern 144
7.3.3 Denatured protein bands pattern 147
7.3.4 Reproducibility of 2D gels 152
7.3.5 General 2D gel spots pattern of embryonated egg yolk 161
7.3.6 Spot detection and matching in same group of 2D gels 167
7.3.7 Spot detection and matching in different group of 2D gels 171
7.3.8 Protein spots identification by peptide mass fingerprinting (PMF) 175
7.3.9 Remarkable proteins expression throughout the incubation hours in all groups 185
7.3.10 The selected most remarkable proteins involved in normal and abnormal chicken embryogenesis 191
7.3.11 The *in ovo* administration method effect on the chicken embryo early embryogenesis at proteome level 212

7.4 Conclusion 213

8 OVERALL FINDINGS 216

8.1 General development and growth effect on early and mid embryogenesis of the chicken treated with NDMA 216

8.2 Angiogenesis inhibition and/or hematoxic effect of NDMA on early and mid embryogenesis of the chicken 217

8.3 The *in ovo* NDMA administration method effect on early and mid embryogenesis of the chicken 220

8.4 The suitability of chicken embryo as a model for developmental toxicity test 221

9 SUMMARY, GENERAL CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH 223

9.1 Summary 223

9.2 General conclusion 228

9.3 Recommendation for future research 228

REFERENCES 230
APPENDICES 251
BIODATA OF STUDENT 257
LIST OF PUBLICATIONS 259

LIST OF TABLES

Table		Page
2.1	Detrimental effects of NDMA and their common manifestations in various laboratory animals	8
4.1	Comparison of measurement methods for embryonic size parameters in control 48 hours chicken embryo	48
4.2	The accuracies of the modified Bradford microassay method and its original Bradford microassay method in the BSA blind samples determinations	51
6.1	Scoring for different types of blood cells at day 10 and day 11 chicken embryos	104
6.2	Blood parameters analyses at day 10 and day 11 chicken embryos	108
7.1	Solution mixtures for stacking and resolving gels of the Native- and SDS-PAGE	118
7.2	Matching percentage for overall reproducibility of 2DE gels	156
7.3	Percentage volumes variation for selected protein spots in duplicate control 0 hour 2D gels	158
7.4	Estimated molecular masses and pI values of protein spots on a representative 2D gel	162
7.5	List of identified protein spots after databases searching and matching	176

LIST OF FIGURES

Figure		Page
2.1	Chemical structure of NDMA	5
2.2	An example of Hamburger and Hamilton stages (stage 3 to stage 21) of chicken embryo development	27
2.3	An example of labeled Hamburger and Hamilton stages (stage 4, 11, 25 and 26) of chicken embryo development	28
3.1	Experimental design applied throughout the whole process of laboratory work	31
4.1	An Adobe Photoshop screenshot of a raw calibration image of yolk with ruler	37
4.2	An Adobe Photoshop screenshot of the cropped and reduced size calibration image of yolk with a 1 cm black straight line marked on the ruler	37
4.3	An Adobe Photoshop screenshot of the adjusted calibration image of yolk with a 1 cm black straight line marked on the ruler which was ready to be visually measured	38
4.4	An Adobe Photoshop screenshot of the measuring tool selected and used in visual calibration and measurement of chicken embryo size parameters	38
4.5	Photo of 20 glass tubes fixed in the casting stand that were ready to be filled with IEF gel solution	43
4.6	Photo of a long beveled needle used in the original gel solution filling technique	43
4.7	Photo of a long beveled needle attached to a 3 ml syringe used in the original gel solution filling technique	43

4.8	An Adobe Photoshop screenshot of the calibration process by measuring the 1 cm black straight line mark using Adobe Photoshop virtual measuring tool (as shown by white straight line)	48
4.9	Microplate used in BSA protein quantification for development of standard curve	50
4.10	Standard curve of BSA protein for total soluble protein quantification	50
4.11	The photo of components used (A) and a diagram with the exact scales (B) for the new modified gel solution filling technique	52
4.12	The application of this new modified gel solution filling technique for casting of IEF tube gels in the laboratory	53
4.13	Photo of an original blue tip and its cut that was used in a newly developed IEF tube gel labeling technique	55
4.14	Photo showing the application of this newly developed IEF tube gel labeling technique in the laboratory	55
4.15	Photo of a successfully labeled IEF tube gel using a cut blue tip labeling technique which was newly developed in the laboratory	56
4.16	Modification of running condition in second dimensional electrophoresis to minimize the black region effect	58
5.1	Gross morphological comparisons of the control chicken embryos development over different incubation hours	67
5.2	Gross morphological comparisons of the control vehicle chicken embryos development over different incubation hours	68
5.3	Gross morphological comparisons of the NDMA-treated chicken embryos development over different incubation hours	69
5.4	Trends of blastoderm development in control, control vehicle and NDMA-treated groups of fertilized egg yolks for 0 up to 48 hours of incubation	75
5.5	Trends of embryonic width development in control, control vehicle and NDMA-treated groups of fertilized egg yolks for 36 up to 120 hours of incubation	79
5.6	Trends of embryonic length development in control, control vehicle and NDMA-treated groups of fertilized egg yolks for 8 up to 120 hours of incubation	81

5.7	Trends of vitelline vasculature width development in control, control vehicle and NDMA-treated groups of fertilized egg yolks for 48 up to 120 hours of incubation.	83
5.8	Trends of vitelline vasculature length development in control, control vehicle and NDMA-treated groups of fertilized egg yolks for 48 up to 120 hours of incubation.	84
6.1	Photographs of day 10 (D10) and day 11 (D11) chicken embryo experimental groups showing their growth and peripheral blood vessels formation (labeled with arrow head).	95
6.2	A representative photomicrograph (×400) of chicken embryo blood cells (control at day 11)	101
6.3	Representative photomicrographs (×400) of chicken blood cells for control and control vehicle at day 10 and day 11 respectively.	102
7.1	A screenshot of paramaters set up for database mining using MS-Fit software	139
7.2	Total soluble protein (TSP) content in the egg yolks of all groups throughout different incubation hours	141
7.3	Native-PAGE gels for control, control vehicle and NDMA-treated egg yolk samples	145
7.4	SDS-PAGE gels for control, control vehicle and NDMA-treated egg yolk samples	148
7.5	Denatured protein bands intensities in control, control vehicle and NDMA-treated groups at 0, 8, 16, 24, 36, 48, 72, 96 and 120 hours of incubation	149
7.6	Screenshot for the 2D gels reproducibility test of the 3 different pairs of control samples	153
7.7	Screenshot for the 2D gels reproducibility test of the 3 different pairs of control vehicle samples	154
7.8	Screenshot for the 2D gels reproducibility test of the 3 different pairs of NDMA-treated samples	155
7.9	Scatter plot of percentage volumes correlation analysis between selected spots of duplicate control 0 hour 2D gels	160
7.10	A representative 2D gel of fertilized chick egg yolk (with embryo) stained with silver staining	161

7.11	Screenshot of control gels after spots matching step	168
7.12	Screenshot of control vehicle gels after spots matching step	168
7.13	Screenshot of NDMA-treated gels after spots matching step	169
7.14	Spot counts in control, control vehicle and NDMA-treated groups at nine different hours of incubation	169
7.15	Spot matching percentages in control, control vehicle and NDMA-treated groups at nine different hours of incubation	170
7.16	Spot detection and matching for group comparison at 0, 8 and 16 hours of incubation	172
7.17	Spot detection and matching for group comparison at 24, 36 and 48 hours of incubation	173
7.18	Spot detection and matching for group comparison at 72, 96 and 120 hours of incubation	174
7.19	Spot matching percentages comparison among control, control vehicle and NDMA-treated groups at nine different hours of incubation	175
7.20	Venn diagram representing remarkably expressed proteins in each of the chicken embryos group	186
7.21	Triangular diagram representing remarkably expressed proteins in all groups of the chicken embryos	187
7.22	Venn diagram representing most remarkably expressed proteins among the remarkable proteins in both individual groups and between all groups of the chicken embryos	188
7.23	Mass spectra from peptide mass fingerprinting for the identification of PIT54 isoforms	192
7.24	PIT 54 protein expression in individual group and all groups of the chicken embryos	193
7.25	Mass spectra from peptide mass fingerprinting for the identification of VEGF-D	196
7.26	VEGF-D protein expression in individual group and all groups of the chicken embryos	198

.

7.27	Mass spectra from peptide mass fingerprinting for the identification of ApoA1	199
7.28	ApoA1 protein expression in individual group and all groups of the chicken embryos	201
7.29	Mass spectra from peptide mass fingerprinting for the identification of unnamed protein product of IgY	204
7.30	Unnamed protein product of IgY (spot no. 24), TBP-like protein 1 (spot no. 23) and Kelch-like protein 7 (spot no. 22) expressions in control group	205
7.31	Unnamed protein product of IgY (spot no. 24), TBP-like protein 1 (spot no. 23) and Kelch-like protein 7 (spot no. 22) expressions in all groups at 72 and 96 hours of incubation	206
7.32	Mass spectra from peptide mass fingerprinting for the identification of TBP-like protein 1	209
7.33	Mass spectra from peptide mass fingerprinting for the identification of Kelch-like protein 7	211

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 substraction angiography
DTT	Dithiothreitol
E1	Primitive normochromatid erythrocyte (NCE)
EDTA	Ethylene diamine tetraacetic acid
EG&K	Eyal-Giladi and Kochav
FAS	Fetal alcohol syndrome
H_2O_2	Hydrogen peroxide