



Ain Shams University.
Faculty of women for Arts,
Science and Education
Zoology Department.

Nanostructured therapy against bleomycin induced lung fibrosis in male C57BL/6

Submitted
By

Eman Adel Hassanin Sherif

Assistant lecturer, Zoology Department
Faculty of Women for Arts, Science and Education
Ain-Shams University
Thesis submitted for Partial Fulfillment Of
The Requirement for the Doctor of Philosophy Degree
In Science
(Physiology)

Supervisors

Prof. Dr. Nashwa Ahmed Fawzy El-Shinway

Prof. of Physiology - Zoology Department – Faculty of
Women for Arts, Science and Education- Ain Shams
University.

Prof. Dr. Waleed Khalid El-Zawawy

Prof. of Chemistry- Paper and Cellulose Department-
National Centre Research.

Assist. Prof. Dr. Afaf Hendawy Kamel

Assistant Prof. - Zoology Department – Faculty of Women for
Arts, Science and Education- Ain Shams University

2020

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ



صدق الله العظيم

سورة التوبة آية ١٠٥



Ain Shams University.
Faculty of Women for Arts,
Science and Education
Zoology Department.

APPROVAL SHEET

Title: Nanostructured therapy against bleomycin induced lung fibrosis in male C57BL/6.

Name: Eman Adel Hassanin Sherif

**Scientific Degree: Ph.D. In Zoology
(Physiology)**

Supervisors

Prof. Dr. Nashwa Ahmed Fawzy El-Shinway

Prof. of Physiology - Zoology Department – Faculty of
Women for Arts, Science and Education- Ain Shams
University.

Prof. Dr. Waleed Khalid El-Zawawy

Prof. of Chemistry- Paper and Cellulose Department- National Centre
Research.

Assist. Prof. Dr. Afaf Hendawy Kamel

Assistant Prof. - Zoology Department – Faculty of Women for
Arts, Science and Education- Ain Shams University

2020

QUALIFICATION

Name: Eman Adel Hassanin Sherif

Scientific Degree: M.Sc.

Department: Zoology

**College: Faculty of Women for Arts,
Science and Education.**

University: Ain Shams University

M.Sc. Graduation Year: 2015

Email: emanadel23@yahoo.com

eman.adel@women.asu.edu.eg

Acknowledgement

Thanks to GOD for helping and supporting me to do this work.

It is with my pleasure to acknowledge with sincere thanks and appreciation the help afforded to me by **Prof. Dr. Nashwa Ahmed El-Shinnawy**, professor of Physiology, Zoology Department, Faculty of Women, Ain Shams University, not only for her great efforts, suggestion the plan, cooperation in the manuscript but also for her continuous guidance, encouragement, excellent advice, enthusiastic help and valuable supervision. To her, I shall be forever grateful.

Any words would not be sufficient to express my deepest gratitude and appreciation to the great effort of **Prof. Dr. Waleed Khalid El-Zawawy**, Professor of chemistry, Cellulose and Paper Dept., National Research Center, for his supervision. I will be always thankful.

I would like to start by expressing my gratitude to my supervisor **Assistant Prof. Dr. Afaf Hendawy Kamel**, Zoology Department, Women`s College, Ain Shams University. Their expertise, understanding, and patience, added considerably to my graduate experience.

My work would not have been possible without the help of **Assistant Prof. Dr. Mona Ahmed**, Professor of Histology, for all of her assistance in reviewing all the histological results.

This thesis only becomes reality due to the kind support, of so many individuals whose names may not be mentioned, I would like to extend my sincere gratitude to them all.

I wish also to express my deepest thanks to all my colleagues and the staff members of the Zoology Department, Faculty of Women, Ain Shams University.

The word "DEDICATION" is written in a dark blue, serif font. It is surrounded by a decorative arrangement of various flowers, including pink roses, purple and yellow blossoms, and green foliage, which is centered behind the text.

DEDICATION

Glad to dedicate this work to my kind
family, special dedication to my parents,
husband and my children

CONTENTS

Title	Page
ABSTRACT	I
LIST OF ABBREVIATIONS	V
LIST OF TABLES	IX
LIST OF FIGURES	XII
INTRODUCTION	1
AIM OF THE PRESENT WORK	6
REVIEW OF LITERATURE	7
1- Pulmonary fibrosis.	7
2- Bleomycin	8
A- Effect of bleomycin on body weight and lung index.	9
B- Effect of bleomycin on total leukocytes and differential cell count	10
C- Effect of bleomycin on injury and oxidative stress markers.	13
D- Effect of bleomycin on collagen and inflammatory mediators.	15
E- Effect of bleomycin on apoptotic genes.	21
F- Effect of bleomycin on histological and histopathological changes in pulmonary fibrosis mice model.	26
3- Dexamethasone(Treatment and side effects)	28
A- Effect of dexamethasone on body weight and lung index.	30
B- Effect of dexamethasone on total and differential counts.	31

C- Effect of dexamethasone on collagen profiles.	32
D- Effect of dexamethasone on injury and oxidative stress markers.	33
E- Effect of dexamethasone on inflammatory mediators.	33
F- Effect of dexamethasone on apoptotic genes.	35
G- Effect of dexamethasone on histological changes.	36
4- Nanotechnology in drug delivery system	38
- Chitosan	40
- Nano chitosan particle as apolymeric drug carrier for DEX	41
5- Curcumin treatment in pulmonary fibrosis.	42
A- Effects of curcumin on body weight and lung index.	44
B- Effects of curcumin on total and differential cell counts.	44
C- Effects of curcumin on collagen profiles.	45
D- Effects of curcumin on injury and oxidative markers.	46
E- Effects of curcumin on inflammatory mediators.	47
F- Effects of curcumin on apoptosis genes.	48
G- Effects of curcumin on histological changes.	49
MATERIAL AND METHODS	51
MATERIAL	51
1. Experimental Animals	
2. Experimental Chemicals and Drugs	51
a- Bleomycin	52
b- Dexamethasone	52
c- Nano Curcumin	53
d-Nano chitosan particle	54
3. Experimental design and animal grouping	
4. Chemical characterization.	57
a. Scanning electron microscope (SEM)	57
b. Transmition electron microscope (TEM)	57
c. Energy dispersive X-ray (EDX)	57
d. Fourier transform infra red (FT-IR)	58

METHODS	58
1. Housing of Experimental Animals.	58
2. Morphological Investigation.	59
a. Initial and final body weights.	59
b. Lung weight and Lung Indices.	59
3. Blood Sampling	60
a- Determination of (LDH) enzyme in serum	60
b- Determination of Malondialdehyde (MDA) as biomarker of oxidative stress in serum.	61
4- Tissue Sampling	
a- Collagen profile tests.	
1- Hydroxyproline ELISA Kit in lung tissue.	62
2- Collagen 1 alpha in lung tissue.	64
b. Inflammatory mediators.	
i. TNF-alpha ELISA Kit in lung tissue.	66
ii. Transforming Growth Factor β (TGF- β) in lung tissue.	68
iii. IFN-gamma ELISA Kit in lung tissue.	70
iv. Nuclear Factor (NF κ B) Elisa kit in lung tissue.	71
v. MMP-2 ELISA Kit in lung tissue.	73
d. Gene expression of Caspase, BCL2 and Muc5ac by Quantitative real time PCR	75
• RNA extraction.	76
• c DNA synthesis.	77
• Real-time qPCR using SYBR Green I.	78
5. Bronchoalveolar leavage fluid (BALF) technique	81
a. Total and differential cell counts	81

6- Histological Methods

Fixation	81
Processing	82
Staining	82
a. Hematoxylin and Eosin Stain.	82
b. Masson's trichrome stain.	83
c. Grades of pulmonary fibrosis	84

7- Statistical Analysis. 85

Results 87

1- Chemical characterization

1.1. Morphological structure.	87
1.2. Energy dispersive X-ray analysis	90
1.3. Foriour Transform Infra Red	92

2- Morphological investigation

a. Growth rate.	94
b. Lung weight.	97
c. Lung indices.	100

3- Markers of lung injury and oxidative stress.

1- Lactate dehydrogenase (LDH) content in serum.	103
2- Malondialdehyde (MDA) content in serum.	104

4- Collagen profiles

1- Hydroxyproline content in lung tissue.	110
2- Collagen 1- α content in lung tissue.	111

5- Inflammatory mediators 117

i. Tumor Necrosis Factor α (TNF- α) levels in lung tissue.	117
ii. Transforming Growth Factor- β (TGF- β) levels in lung tissue.	118
iii. Tissue Interferon Gamma (INF- γ) in lung tissue.	119
iv. Nuclear Factor Kappa B (NF kB) levels in lung tissue.	120
v. Matrix Metalloprotenise enzyme (MMP2) levels in lung tissue.	121
6- Genes expression by RT-PCR	
• Expression of Caspase gene activity in lung tissue.	133
• Expression of BCL2 gene activity in lung tissue.	134
• Expression of Muc5ac gene in lung tissue.	135
7- Total leukocytes and differential cell count in BALF.	
• Total cells count.	143
• Macrophage cells count.	144
• Lymphocyte cells count.	145
• Neutrophil cells count.	146
8- Histological investigation	
• Lung stain by Hematoxyline-Eosin.	165
• Grades of lung fibrosis stained by Masson's trichrome.	184
DISCUSSION	202
SUMMARY AND CONCLUSION	226
BIBLIOGRAPHY	233
ARABIC SUMMARY	

ABSTRACT

Pulmonary fibrosis is a chronic, progressive inflammatory lung disorder with well-established histopathological and pulmonary architectural changes. It is usually associated with significant impairment of respiratory functions. Indeed, inflammatory lung disorders are eventually associated with loss of alveolar architecture, accumulation of myofibroblasts, extensive extracellular matrix deposition and remodeling of lung parenchyma.

This study aims to evaluate the therapeutic effectiveness of loading dexamethasone on Nano chitosan particles as a novel Nano-targeted treatment for bleomycin-induced pulmonary fibrosis in male C57BL/6 mice model. This is achieved with minimal systemic and cellular side effects and effective drug release of dexamethasone to the lung tissue. Dexamethasone loaded on Nano chitosan treatment was further compared with the widely used treatment dexamethasone either alone or combined with the improved form of curcumin which is nano curcumin.

Nano chitosan (NCH) particles were chemically analyzed by scanning electron microscope (SEM) Transmission electron microscope (TEM), Energy dispersive X-ray analysis (EDXA) and Fourier transform infrared spectroscopy (FTIR). These chemical analysis were carried out for Nano-chitosan particles before and after loading with dexamethasone. Chemical characterization of the novel synthesized nano particle

confirmed that dexamethasone was effectively loaded onto NCH-particles.

In vivo experiment was achieved to attain this goal on a total number of 128 male C57BL/6 mice with average weight 18-33 gm. They were divided into eight groups each of 16 mice. The first group served as normal control group which received distilled water, the second group represented the dexamethasone group which was injected intraperitoneally with a dose of 0.45 mg/kg body weight/ day for 4 weeks. The third group represented the Nano-curcumin group which was given a dose of 100 mg/kg body weight of nanocurcumin /day for 4 weeks. The fourth group represented the bleomycin group which was given a single intra-tracheal dose of BLM 1 mg/ kg body weight. The fifth group represented the bleomycin + dexamethasone. The sixth group represented the bleomycin + dexamethasone loaded on Nano chitosan particles. The seventh group represented the bleomycin + Nano-curcumin and the eighth group represented the bleomycin + dexamethasone + Nano-curcumin. Mice were dissected after two durations of 14 days and 28 days. Eight animals of experimental study period from each group were dissected in both durations. After every duration serum, Broncho alveolar lavage fluid and lung tissue were collected from each animal to evaluate physiological, immunological, molecular genetics, histological and histopathological alterations.

All results were compared with the corresponding normal control group then after, bleomycin treated groups were further compared with bleomycin group. The present findings were then discussed in view of the relevant literature available in similar fields of study.

The present study elucidated a significant decrease in body weight in C57BL/6 after intratracheal BLM instillation for 28 days. This was accompanied with a significant elevation in lung weight, lung index, serum MDA and LDH either after 14 or 28 days of study period. In addition, there was an elevation in lung tissue collagen profile, inflammatory mediators. Moreover, Caspase-3 and Muc5ac gene expressions showed a significant increase while the gene expression of BCL2 was declined. Furthermore, a significant increase of total leukocytes thus increased inflammatory cell counts in BALF of neutrophils, lymphocytes and macrophages were also established after both 14 and 28 days of bleomycin instillation. Besides, recognized histopathological alterations in mice lung sections following were seen.

Nano chitosan nanoparticles as a novel nano targeted treatment for bleomycin-induced pulmonary fibrosis in male C57BL/6 mice model were analyzed by SEM, TEM, EDXA and FTIR. These analyses were carried out for Nanochitosan before and after loading with dexamethasone. Also SEM and TEM confirmed that dexamethasone was adsorbed on the surface and the absorbed inside Nano-chitosan. While EDX analysis confirmed the elements content in each component, DEX, NCH and DEX-NCH particles. Furthermore, FTIR proved all functional groups presented in DEX, NCH particles and DEX-NCH particles. The treatment with DEX alone or DEX loaded on NCH particles or under DEX and NanoCURC produced a significant decline in lung weight, lung indices, serum LDH and MDA. This was also accompanied by a decrease in the inflammatory mediators including $TNF\alpha$, $TGF\beta$, $INF\gamma$, $NFkB$ and lung tissue inflammatory marker MMP2. In addition, microscopical investigation for total leucocytic count and differential cell counts of macrophages, lymphocytes and neutrophils in BALF revealed likewise a