DOCTORAL THESIS

Non-lethal Doses of Cadmium Exposure and Toxic Effects on Different Bone Cells by Using Mass Spectrometry-based Metabolomics

TIAN, Jinglin

Date of Award:
2021

ABSTRACT

Cadmium has been widely detected in the environment and various foods. The association between cadmium burden and osteoporosis has been studied in cohorts. However, the effects and mechanisms of environmental cadmium exposure on bone metabolism is poorly understood. This study aims at investigating the cadmium effects on bone cells and the associated mechanism by metabolomics analysis. Specifically, three types of bone cells (MG-63 cell from man, Saos-2 cell from woman and MC3T3-E1 cell from mouse) were exposed to a range of cadmium doses for 24 or 48 h, and the bone cell viability was evaluated. Then, the doses of cadmium that do not decrease the cell viability chosen to treat different types of bone cells for 24 h. The cell morphology was obtained by confocal imaging, and the nucleus and cytoskeleton were stained. Cell migration ability affected by such dose of cadmium was evaluated by wound-healing assay. ICP-MS was applied to quantify the cadmium in culture medium and cell precipitate. The cellular metabolites were extracted and analyzed by LC-MS. After the multivariate and univariate data analysis, the differential metabolic features were selected for further identification. The pathway analysis based on the identified differential metabolites showed that amino acid metabolism and energy metabolism were affected by cadmium exposure. It was observed that cadmium significantly affected malate-aspartate shuttle, alanine metabolism, glucose-alanine cycle, glutamate metabolism and citric acid cycle. Taken together, cadmium exposure could suppress the electrons transportation from the cytosol to mitochondrial matrix, and the impediment of the electron transport chain further inhibited downstream activities in citric acid cycle, which resulted in the accumulation of pyruvic acid and induced the energy associated metabolism abnormal. The levels of key enzymes involved in the shared affected pathways in different bone cells were also determined on transcriptional and translational levels. The results were consistent with and supported by the findings observed in metabolomics study. Dose-dependent effect of cadmium was observed in three types of cells although different cells displayed various susceptibility to cadmium. In general, this study unveiled the cadmium-induced metabolic perturbations in different bone cells and demonstrated the feasibility of the developed metabolomics pipeline applied to elucidate cadmium-induced effects and corresponding mechanism on bone cell at the nonlethal dose. The effective and efficient analytical methodology and risk assessment methods established in this thesis provide useful data and technological support for the investigation of metabolic markers in human samples exposed to cadmium. In addition, the study of metabolic disorders caused by cadmium stress provides a promising way for establishing protective strategies against cadmium-induced health risks.

Keywords: Cadmium; Bone cells; Metabolomics; LC-MS; Metabolic pathway

TABLE OF CONTENTS

DECLARATION ..... **i**

ABSTRACT ..... **ii**

ACKNOWLEDGEMENTS ..... **iii**

TABLE OF CONTENTS ..... **iv**

LIST OF TABLES ..... **x**

LIST OF FIGURES ..... **xii**

ABBREVIATIONS ..... **xviii**

Chapter 1 Literature review **1**

1.1 Overview of risk of being exposed to cadmium 1

1.2 Overview of cadmium toxicity studies 2

1.3 Bone health risk of being exposed to cadmium in cohort studies 3

1.4 Introduction of metabolomics 4

1.4.1 Instrumental analysis 7

1.4.2 Data analysis platforms 12

1.4.3 Overview of databases commonly used in metabolomics 18

1.5 Metabolomics studies of cadmium effect 18

1.6 Research gaps 19

1.6.1 Metabolomics analysis platform development 20

1.6.2 Appropriate models for mechanism investigation of cadmium effect 23

1.7 Objectives and significance 26

1.7.1 Analytical methods development for assessment of risk factors 27

1.7.2 Cadmium effects on bone cell lines 28

1.7.3 Development and validation of metabolomics methodology and pipelines 28

1.7.4 Investigation of mechanisms associated with cadmium effect on bone 28

1.7.5 Significance of this project 29

1.8 Research Design 29

1.8.1 Overview of the study 29

1.8.2 Method roadmap 30

Chapter 2 Cytotoxicity assessment of cadmium on three types of bone cell lines 36

2.1 Introduction 36

2.2 Materials and methodology 37

2.2.1 Reagents and materials 37

2.2.2 Cell culture 37

2.2.3 Cell viability assessment 38

2.2.4 Bone cells morphology analysis 39

2.2.5 Detection of cadmium accumulated in cells and medium 39

2.2.6 Wound-healing assay 40

2.2.7 Statistical Analysis 41

2.3 Results and discussion 41

2.3.1 Cell growth curves 41

2.3.2 Cadmium effect on bone cell viability 43

2.3.3 Cadmium evaluation in cells and medium 47

2.3.4 Cell morphology affected by cadmium 50

2.3.5 Cell migration ability affected by cadmium 53

2.4 Summary 58

Chapter 3 Methods development of untargeted metabolomics 59

3.1 Introduction 59

3.2 Materials and methodology 61

3.2.1 Reagents and materials 61

3.2.2 Cell culture and cadmium exposure 61

3.2.3 Cellular metabolites preparation 62

3.2.4 Instrumental analysis 63

3.2.5 Data pre-processing 63

3.2.6 Multivariate and univariate statistical analysis 64

3.2.7 Identification of differential metabolites 65

3.3 Results and discussion 66

3.3.1 Metabolic features obtained by LC-MS/MS 66

3.3.2 Reproducibility and accuracy assessment of the analytical methods 69

3.3.3 Raw data quality assessment of nontargeted metabolomics data 70

3.3.4 Signal drift correction based on QC-RFSC 71

3.3.5 Sample similarity analysis 72

3.3.6 Differential metabolic features analysis using OPLS-DA model 75

3.3.7 Differential metabolic features analysis using PLS-DA model 78

3.3.8 Univariate statistical analysis for selection 83

3.3.9 Volcano plots of the differential metabolic features 86

3.3.10 Identification of Differential Metabolites 89

3.4 Summary 99

Chapter 4 Metabolomics analysis of cadmium effects on bone cells based on the developed methods 101

4.1 Introduction 101

4.2 Methods 102

4.2.1 Reagents and materials 103

4.2.2 Cell culture and cadmium exposure 103

4.2.3 Cellular metabolites preparation 103

4.2.4 Instrumental analysis 103

4.2.5 Metabolomics data analysis 104

4.2.6 Differential metabolic features and identification 105

4.2.7 Enrichment analysis of the differential metabolites 106

4.2.8 Topology analysis of the altered metabolites 106

4.3 Results and discussion 106

4.3.1 Precision and reproducibility assessment of the instrumental analysis 106

4.3.2 Signal drift correction based on the QC-RFSC 111

4.3.3 Multivariate analysis of the cadmium effect on bone cells 121

4.3.4 Metabolic differential features altered by cadmium 134

4.3.5 Perturbed metabolites identification 138

4.3.6 Perturbed metabolic pathways by cadmium 171

4.3.7 Shared perturbed pathways of bone cells 177

4.4 Summary 181

Chapter 5 The verification study of untargeted metabolomics study 183

5.1 Introduction 183

5.2 Materials and methods 184

5.2.1 Reagents and materials 184

5.2.2 RNA extraction 184

5.2.3 cDNA synthesis 185

5.2.4 RT-PCR for gene expression evaluation 185

5.2.5 Enzyme concentration determination 191

5.3 Results and discussion 192

5.3.1 Quality assessment of the RT-PCR products 192

5.3.2 Quantification of target genes on transcriptional level 195

5.3.3 Standard curves establishment of the targeted enzymes 203

5.3.4 Quantification of the targeted enzymes in cells 204

5.4 Summary 207

Chapter 6 Conclusions 208

6.1 Cadmium displays different toxic effects on different bone cells 208

6.2 Metabolomics analysis unveiled the metabolism perturbation induced by cadmium without change in cell viability 209

6.3 Non-lethal level cadmium disturbs the metabolism of energy and amino acid in different bone cells 209

6.4 Limitations and perspectives 212

REFERENCES 214

PULICATIONS 236

CURRICULUM VITAE 237

LIST OF TABLES

Table 1. Detection platforms applied in nontargeted metabolomics analysis 10

Table 2. Methods of raw data pre-processing and statistical analysis applied in nontargeted metabolomics 16

Table 3. Three types of bone cell lines 25

Table 4. The number of features with high VIP value in PLS-DA mode..83

Table 5. The number of features with eligible FC of peak intensity.....84

Table 6. The number of features in every comparative group with p-value <0.05 84

Table 7. The list of identified differential metabolites in MG-63 between 1 μM cadmium and control (in ascending order of molecular weight) 90

Table 8. The list of identified differential metabolites in MG-63 between 10 μM cadmium and control (in ascending order of molecular weight) 93

Table 9. The list of identified differential metabolites in Saos-2 between 1 μM cadmium and control (in ascending order of molecular weight) 139

Table 10. The list of identified differential metabolites in Saos-2 between 10 μM cadmium and control (in ascending order of molecular weight) 143

Table 11. The list of identified differential metabolites in MC3T3-E1 detected in positive ion mode. 156

Table 12. The list of identified differential metabolites in MC3T3-E1 detected in negative ion mode. 161

Table 13. Primer sequences for target genes in *Homo sapiens* (human). ..187

Table 14. Primer sequences for target genes in *Mus musculus* (house mouse). 189

LIST OF FIGURES

Figure 1. Outline of the metabolomics platform development and mechanisms investigation.....30

Figure 2. Detailed methods and experiments of the metabolomics platform development.....32

Figure 3. Cell growth curves of bone cells. 42

Figure 4. Cell viability of three bone cell lines exposed to cadmium evaluated by MTS assay. 44

Figure 5. Dose-time–response curves of cadmium on different bone cell lines. 46

Figure 6. The standard curve of cadmium established in ICP-MS analysis. 48

Figure 7. Cadmium in bone cells and medium evaluated by ICP-MS analysis. 49

Figure 8. The morphology of cell nucleus and cytoskeleton of MG-63 exposed to cadmium. 51

Figure 9. The morphology of cell nucleus and cytoskeleton of Saos-2 exposed to cadmium. 52

Figure 10. The morphology of cell nucleus and cytoskeleton of MC3T3-E1 exposed to cadmium. 53

Figure 11. The migration ability evaluation of MG-63 cells exposed to cadmium. 55

Figure 12. The migration ability evaluation of Saos-2 cells exposed to cadmium. 56

Figure 13. The migration ability evaluation of MC3T3-E1 cells exposed to cadmium. 57

Figure 14. The positive ion mode TICs of the substance extracted from MG-63 cells. 67

Figure 15. The negative ion mode TICs of the substance extracted from MG-63. 68

Figure 16. The overlapped ion chromatograms of the internal standard.....69

Figure 17. Median of mass precision of internal standard. 70

Figure 18. The intensity RSD of all features in MG-63 cells. 71

Figure 19. The intensity RSD of features in MG-63 cells after signal and correction. 72

Figure 20. PCA score plots of MG-63 samples. 74

Figure 21. OPLS-DA score plots and the validation plots of positive ion mode. 76

Figure 22. OPLS-DA score plots and the validation plots of negative ion mode. 77

Figure 23. PLS-DA of MG-63 samples in positive ion mode. 80

Figure 24. PLS-DA of MG-63 samples in negative ion mode.....82

Figure 25. Volcano plot of metabolic features in positive ion mode.....87

Figure 26. Volcano plot of metabolic features in negative ion mode.....88

Figure 27. The detailed metabolomics data analysis diagram. 100

Figure 28. The overlapped ion chromatograms of the internal standard in Saos-2 cells. 108

Figure 29. Median of mass precision of internal standard in Saos-2 cells..109

Figure 30. Overlapped ion chromatograms of the internal standard 110

Figure 31. Median of mass precision of internal standard in MC3T3-E1 cells. 111

Figure 32. TICs of the Saos-2 cell samples in positive ion mode. 113

Figure 33. TICs of the Saos-2 cell samples in negative ion mode. 114

Figure 34. Violin plots of the RSD of metabolic features in Saos-2 cells. ...116

Figure 35. TIC of the MC3T3-E1 cell samples in positive ion mode.....118

Figure 36. TIC of the MC3T3-E1 cell samples in negative ion mode.....119

Figure 37. Violin plots of the RSD of metabolic features in MC3T3-E1 cells. 121

Figure 38. PCA plot of the metabolomics data of Saos-2 cells. 123

Figure 39. PLS-DA plot of the metabolomics data of Saos-2 cell. 125

Figure 40. Validation plot of PL-SDA model of Saos-2 cell in positive mode. 126

Figure 41. Validation plot of PLS-DA model of Saos-2 cell in negative mode. 127

Figure 42. PCA plot of the metabolomics data of MC3T3-E1 cells. 129

Figure 43. PLS-DA models of the metabolomics data acquired in MC3T3-E1 cells. 131

Figure 44. Validation plot of PLS-DA model of MC3T3-E1 cell in positive mode. 132

Figure 45. Validation plot of PLS-DA model of MC3T3-E1 in negative mode. 133

Figure 46. Volcano plots displayed the differential metabolic features in Saos-2 cells. 135

Figure 47. Volcano plots displayed the differential metabolic features in MC3T3-E1 cells. 137

Figure 48. Heatmap of the changed metabolites in Saos-2 obtained in positive ion mode. 153

Figure 49. Heatmap of the changed metabolites in Saos-2 obtained in negative ion mode. 154

Figure 50. Heatmap of the changed metabolites in MC3T3-E1 obtained in positive ion mode. 170

Figure 51. Heatmap of the changed metabolites in MC3T3-E1 obtained in negative ion mode. 171

Figure 52. Pathway analysis of Saos-2 cells exposed to low dose of cadmium. 173

Figure 53. Pathway analysis of Saos-2 cells exposed to high dose of cadmium. 174

Figure 54. Pathway analysis of MC3T3-E1 cells exposed to low dose of cadmium. 176

Figure 55. Pathway analysis of MC3T3-E1 cells exposed to low dose of cadmium. 177

Figure 56. The melting curves of PCR products of MG-63 cells by RT-PCR. 193

Figure 57. The melting curves of PCR products of Saos-2 cells by RT-PCR. 194

Figure 58. The melting curves of PCR products of MC3T3-E1 cells by RT-PCR. 195

Figure 59. Quantitative analysis of targeted genes expression related to cadmium exposure in MG-63 cells. 197

Figure 60. Quantitative analysis of targeted genes expression related to cadmium exposure in Saos-2 cells. 200

Figure 61. Quantitative analysis of targeted genes expression related to cadmium exposure in MC3T3-E1 cells. 202

Figure 62. Standard curves of the enzymes established by ELISA. 204

Figure 63. Evaluation of the target enzymes level in bone cell lines.....206

Figure 64. Proposed metabolic pathways perturbed by cadmium in bone cells. 211