PROTECTIVE EFFECT AND ACTION MECHANISM OF GOLD AND ZINC-OXIDE NANO-PARTICLES ON EARLY DIABETIC NEPHROPATHY IN WISTAR RAT

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DEDICATION

This thesis is dedicated to my father and my mother, who always encourage me to go ahead and push the limits of my dreams. It is also dedicated to my beloved husband and sons for being there to support with their patient understanding, wishes, and love.

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ABSTRACT

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure and has become a global health issue. Recently, nanomedicines have attracted the attention of researchers; gold nanoparticles (AuNPs) and zinc oxide nanoparticles (ZnONPs) were demonstrated to have antihyperglycemic and antioxidative effects. This study aimed to explore the renoprotective effects of AuNPs and ZnONPs, and to identify the potential mechanisms of action in animal model of DN. Following induction of diabetes using a single dose of streptozotocin, 30 adult male rats were divided into three groups: 10 diabetic, 10 diabetic treated intraperitoneally for seven weeks with synthesised AuNP (51.8 ± 0.7 nm, 2.5 mg/kg/day), and 10 diabetic treated intraperitoneally with commercial ZnONP (<100 nm, 2.5 mg/kg/day). 10 non-diabetic rats were used as control. Pathohistological, biochemical, and molecular studies were performed to achieve the goals of this research. Diabetic animals treated with AuNPs exhibited significant reductions in blood glucose levels, urine albumin excretion, creatinine clearance, and blood urea nitrogen. The levels of oxidative stress markers: catalase, superoxide dismutase, and malondialdehyde were restored significantly evidence of antioxidant effect of AuNPs. Ultrastructural and histological findings highlighted the protective efficacy of AuNPs to reduce pathohistological hallmarks of DN, including glomerular basement membrane thickening, podocyte injury and glomerular sclerosis. Results from this study suggested that the protection against the development of DN was attributed to antioxidant and antihyperglycemic function of AuNPs. Both functions significantly reduced inflammation, fibrosis, and angiogenesis via the downregulation of tumor necrosis factor-α, transforming growth factor-β, and vascular endothelial growth factor-A. AuNP treatment also activated matrix metalloproteinase, which reduces the accumulation of extracellular matrix proteins, collagen IV, and fibronectin. Treatment also restored the expression of nephrin and podocin, which protects against podocyte injury. Equally, ZnONP treatment prevented the increase of blood glucose, urine albumin excretion, creatinine clearance, and blood urea nitrogen. Significant increases in oxidative stress markers (catalase and superoxide dismutase) and a reduction of malondialdehyde also indicated antioxidative effects of ZnONPs. Early histological hallmarks of DN (e.g., glomerular basement membrane thickening, podocyte injury, and glomerular sclerosis) were also reduced by ZnONP treatment. ZnONP treatment also significantly downregulated tumor necrosis factor-α, transforming growth factor-β, and vascular endothelial growth factor-A, but upregulated matrix metalloproteinase. The factors described above attenuated the hallmarks of DN while reducing the accumulation of extracellular matrix proteins, collagen IV, and fibronectin, thus preventing the progress of DN. Moreover, the expression of nephrin and podocin proteins, which protect against podocyte injury and albumin leakage in urine, was restored to normal levels. These results showed that AuNP and ZnONP have renoprotective effects against the development of DN via their antihyperglycemic, antioxidative, antiinflammatory, antifibrotic, and antiangiogenic effects.

ABSTRAK

Nefropati diabetes (DN) merupakan punca utama kegagalan buah pinggang peringkat akhir yang menjadi isu kesihatan di seluruh dunia. Baru-baru ini, bidang nanoperubatan telah mendapat perhatian di kalangan para penyelidik, dimana nanopartikel emas (AuNPs) dan nanopartikel zink oksida (ZnONPs) telah ditunjukkan mempunyai kesan antihiperglisemia dan antioksidatif. Tujuan kajian ini adalah untuk meneroka kesan perlindungan renal nanopartikel AuNPs dan ZnONPs, dan mengenalpasti mekanisma tindakan yang berkemungkinan dalam haiwan model DN. Susulan pengaruhan diabetes menggunakan satu dos streptozotosin, 30 tikus jantan dewasa telah dibahagikan kepada tiga kumpulan: 10 diabetik, 10 diabetik yang dirawat selama tujuh minggu secara intraperitoneal dengan AuNP (51.8 ± 0.7 nm, 2.5 mg/kg/hari), dan 10 diabetik yang dirawat dengan ZnONPs komersial (<100 nm, 2.5 mg/kg/hari). 10 tikus bukan diabetik telah dijadikan kawalan. Kajian histopatologi, biokimia, dan molekul telah dilakukan untuk mencapai matlamat penyelidikan ini. Tikus diabetes yang dirawat dengan AuNPs telah menunjukkan pengurangan ketara paras glukosa darah, pengumuhan albumin air kencing, pembersihan kreatinin, dan urea nitrogen darah. Pemulihan ketara ke paras asal penanda tekanan oksidatif: katalase, superoksida dismutase, dan malondialdehid bukti kesan antioksidan AuNPs. Cerapan struktur ultra dan histologi menunjukkan keberkesanan perlindungan AuNPs mengurangkan ciri-ciri histopatologi DN termasuk penebalan membran tapak glomerulus, kecederaan podosit dan sklerosis glomerulus. Dapatan kajian ini mencadangkan perlindungan daripada kejadian ND disifatkan ke atas fungsi antioksida dan antihiperglisemia AuNP. Fungsi ini dengan ketara telah mengurangan keradangan, fibrosis, dan genesis-angio melalui penurunan faktor nekrosis tumor-α, faktor pengubah pertumbuhan-β, dan faktor pertumbuhan endotelia vaskular-A. Rawatan AuNPs juga mengaktifan metaloproteinase matriks yang mengurangkan pengumpulan protein matriks ekstraselular, kolagen IV, dan fibronektin. Rawatan juga memulihkan pengekspresan nefrin dan podosin yang melindungi daripada kecederaan podosit. Begitu juga, rawatan ZnONPs menghalang peningkatan paras glukosa darah, pengumuhan albumin urin, pembersihan kreatinin, dan urea nitrogen darah. Peningkatan ketara penanda tekanan oksidatif (katalase dan superoksida dismutase) serta pengurangan malondialdehid juga membuktikan kesan antioksida ZnONPs. Ciri-ciri histologi awal DN (seperti penebalan membran dasar glomerulus, kecederaan podosit, dan sklerosis glomerulus) dikurangkan oleh rawatan ZnONPs. Rawatan ZnONPs juga dengan ketara telah menurunkan faktor nekrosis tumor-α, faktor pengubah pertumbuhan-β, dan faktor pertumbuhan endotelia vaskular-A, tetapi meningkatkan metalloproteinase matriks. Semua faktor ini mengatenuasi ciri-ciri DN dan mengurangkan pengumpulan protein matriks ekstraselular, kolagen IV, dan fibronektin, lalu merencat kejadian penyakit DN. Tambahan pula, pengekpersan protein nefrin dan podosin, yang melindungi daripada kecederaan podosit dan kebocoran albumin dalam air kencing, telah dipulihkan ke paras normal. Secara keseluruhannya, penemuan kajian ini menunjukkan bahawa AuNPs dan ZnONPs mempunyai kesan perlindungan renal daripada kejadian DN melalui kesan antihiperglisemia, antioksidatif, anti-radang, antifibrotik dan antiangiogenik

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LIST OF ABBREVIATIONS

ACE	-	Angiotensin-Converting-Enzyme
AGE	-	Advanced Glycation End Products
ARB	-	Angiotensin Receptor Blocker
AuNPs	-	Gold Nanoparticles
BCA	-	Bicinchoninic Acid
BG	-	Blood Glucose
BSA	-	Bovine Serum Albumin
BUN	-	Blood Urea Nitrogen
CAT	-	Catalase
cDNA	-	Complementary DNA
CrC	-	Creatinine Clearance
COX	-	Cyclooxygenase
DLS	-	Dynamic Light Scattering
DM	-	Diabetes Mellitus
DN	-	Diabetic Nephropathy
ECM	-	Extracellular Matrix
ELISA	-	Enzyme Linked Immunosorbent Assay
ESRD	-	End Stage Renal Disease
DF	-	Degree of Freedom
FEG	-	field emission gun
FPW	-	Foot Process Width
GAPDH	-	Glyceraldehyde-3-Phosphate Dehydrogenase
GBM	-	Glomerular Basement Membrane
GFB	-	Glomerular Filtration Barrier
GFR	-	Glomerular Filtration Rate
GPx	-	Glutathione Peroxidase
GssG	-	Oxidized Glutathione
GOD	-	Glucose Oxidase
GOD-PAP	-	Glucose Oxidase Peroxidase Aminophenazone
GTJA	-	Glomerular-Tubular Junction

HCl	-	Hydrochloric Acid
H & E	-	Haematoxylin and Eosin
HMC-1	-	Human Mast Cell Line
HUVECs	-	Human Umbilical Vascular Endothelial Cells
HSA	-	Human Serum Albumin
IL-1	-	Interleukin-1
IL-6	-	Interleukin-6
i.p	-	Intraperitoneal
KWI	-	Kidney Body Weight Index
MDA	-	Malondialdehyde
mRNA	-	Messenger RNA
MMP-9	-	Matrix Metalloprotenease-9
NCBI	-	National Centre for Biotechnology Information
NF-kB	-	Nuclear Transcription Factors
iNOS	-	Nitric Oxide Synthases
NPs	-	Nanoparticles
OD	-	Optical Density
PAS	-	Periodic Acid Shiff
PCCs	-	Pancreatic Cancer Cells
PKC	-	Protein Kinase C
PSCs	-	Pancreatic Stellate Cells
Q-RT-PCR	-	Quantitative Real Time Polymerase Chain Reaction
RAS	-	Renin Angiotension System
ROS	-	Reactive Oxygen Species
RT	-	Reverse Transcription
RT-PCR	-	Real Time Polymerase Chain Reaction
rpm	-	Rotate Per Minute
SD	-	Slit Diaphragms
SEM	-	Scanning Electron Microscope
SEM	-	Standard Error Mean
siRNA	-	small interfering RNA
Smad2	-	Drosophila Mothers Against Decapentaplegic 2
SOD	-	Superoxide Dismutase

STZ	-	Streptozotocin
TBA	-	Thiobarbituric Acid
TBM	-	Tubular Basement Membrane
TCA	-	Trichloroacetic Acid
TGF-β	-	Transforming Growth Factor-beta
TNF-α	-	Tumor Necrosis Factor- alpha
VEGF-A	-	Vascular Endothelial Growth Factor-A
ZnONPs	-	Zinc Oxide Nanoparticles
ZP	-	Zeta Potential

LIST OF SYMBOLS

g	-	gravity
M	-	Molar
N	-	Normality
Σ	-	Sigma
π	-	Pi
α	-	Alpha
β	-	Beta
Δ	-	Delta
IV	-	four

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

According to the World Health Organization and the International Diabetes Federation, 451 million people have diabetes. This number is expected to rise to 693 million by 2045 (Cho *et al.*, 2018). It was reported that approximately 20-40% of patients with either type I diabetes or type II diabetes develop diabetic nephropathy (DN) several years after diagnosis (Karthick *et al.*, 2014; Tonolo and Cherchi, 2014). DN is a widespread diabetes-related microvascular complication that is related to extreme morbidity and mortality (Umanath and Lewis, 2018). It is considered as the leading cause of end-stage renal disease (ESRD) in the world. In addition, this disease is a major cause of kidney dialysis and transplantation, with about 44% of ESRD cases in the United States caused by DN (Pourghasem *et al.*, 2015).

Different types of kidney cells, such as mesangial cells, podocytes, and tubulointerstitial cells, are susceptible to being affected by DN. Related complications lead to the early morphological lesion of DN, glomerular basement membrane (GBM) thickening, and mesangial expansion (Pourghasem *et al.*, 2015). Mesangial expansion and GBM thickening are consequences of increased deposition and/or decreased degradation of extracellular matrix (ECM) proteins, such as collagens and fibronectin (Fioretto and Mauer, 2007; Jefferson *et al.*, 2008). Moreover, it has been well-established that podocyte apoptosis, necrosis, or the loss of their adhesive interaction lead to the detachment of podocyte from the GBM. These cellular pathologies underlie the progression of DN and cause albumin to leak into the urine (Fioretto and Mauer, 2007; Jefferson *et al.*, 2008).

Various pathological mechanisms are involved in the development of DN. The major cause for DN pathogenesis is chronic hyperglycemia. Hyperglycemia is the

primary initiator of increased oxidative stress and is a major cause of the activation of several growth factors, such as transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor (VEGF-A) (Kitada *et al.*, 2010). The activation of these growth factors lead to changes in cellular functions and induce renal injury (Kumar *et al.*, 2014). Thus, affecting the pathways that lead to kidney damage early in the onset of diabetes is crucial to altering the development and progression of DN disease (Alicic *et al.*, 2017).

In recent years, there has been great developments related to nanotechnology in the field of science and technology. Nanomaterials have been used in several biomedical applications due to their unique properties (Zhao and Castranova, 2011). For example, gold (Au) and zinc oxide (ZnO) nanoparticles (NPs) were found to have antioxidant and antihyperglycemic effects in streptozotocin (STZ)-induced diabetic animal models (Barathmanikanth *et al.*, 2010; Umrani and Paknikar, 2014).

However, the effects of AuNPs and ZnONPs on DN have not been thoroughly established, particularly in relation to structural, ultrastructural, biochemical, and molecular changes. Therefore, the current study investigates the protective potential of AuNPs and ZnONPs. The secondary aim of this work is to provide insights into the mechanisms by which NP-based treatments might exert effects in kidneys with DN in a rat model with STZ-induced diabetes.

1.2 Problem Statement

DN is a common cause of end-stage renal failure and a major indicator of dialysis and transplantation. Around one-third of all diabetic patients develop DN. DN is often associated with substantial financial and social problems (Donate-Correa *et al.*, 2015; Tuttle *et al.*, 2014). Despite the large number of experiments that have been conducted on human and experimental animal models, few therapeutic medications are available, and effective therapy does not yet exist (Keri *et al.*, 2018; Kim and Park, 2017; Quiroga *et al.*, 2015). Most therapies for DN are adapted to reduce hyperglycaemia and hypertension and, thus, slowdown the development of glomerular

damage. Such therapies include the use of steroids, immunosuppressive agents, ACE-inhibitors, angiotensin receptor blockers (ARBs), and aldosterone antagonists (Colombo *et al.*, 2017; Lim, 2014). However, these therapies have side effects that range from relatively minor issues, such as muscle contractions and coughing, to critical problems, including hypotension, hyperkalemia, acute renal failure, heart failure, cardiovascular mortality, and strokes (Kim and Park, 2017; Lim, 2014).

The field of nanomedicine holds unlimited prospects for the imaging, diagnosis, and treatment of different diseases, including cancer. However, there are technological gaps in the assessment of nanomedicine for kidney diseases diagnoses and cures (Williams *et al.*, 2016). NP-based drug delivery strategies have been investigated, and it has been suggested that they can treat different kidney diseases, including acute kidney injury (Chen *et al.*, 2015), chronic kidney disease (Zuckerman *et al.*, 2015), glomerular diseases (Shimizu *et al.*, 2010), and kidney cancer (Ko *et al.*, 2013). A few studies have used AuNP-based technology to test for the early recognition and development of DN by testing the volatile organic compounds from breath samples (Marom *et al.*, 2012). However, very few studies have investigated the use of AuNPs or ZnONPs as treatments for DN.

Consequently, the search for substances that protect the kidney from the effects of diabetes has high priority in biomedical research. Recent studies have reported that AuNPs and ZnONPs exhibit antioxidant and antihyperglycemic effects on an STZ-induced diabetic animal model (Barathmanikanth *et al.*, 2010; Karthick *et al.*, 2014; Umrani and Paknikar, 2014; Wahba *et al.*, 2016). The pathogenesis and progression of DN result from hyperglycemia, which raises oxidative stress and enhances the expression of transforming growth factor-beta (TGF-β) and angiogenic proteins (Soldatos and Cooper, 2008). However, no studies to date have addressed the effects of AuNPs and ZnONPs on the oxidative status and the complex system controlling the extracellular matrix (ECM), TGF-β, and angiogenic proteins expression in DN status.

In this study, ultrastructural and histological evaluations were performed, and the potential biochemical and molecular mechanisms by which AuNPs and ZnONPs might exert their actions in the diabetic kidney were examined.

1.3 Objectives of the Study

The primary aim of the current study was to evaluate the protective effects of AuNPs and ZnONPs on early DN. The second aim was to investigate the possible mechanisms by which these NPs exert their action in the diabetic kidney from the onset of diabetes in an STZ-induced rat model with diabetic renal disease. To achieve these goals, the following specific objectives were considered:

(i) To determine the effects of AuNPs and ZnONPs on blood glucose (BG) and renal function parameters, blood urea nitrogen (BUN), creatinine clearance (CrC), and urine albumin excretion rate (UAE), which is the clinical hallmark of DN.

(ii) To evaluate the histological and ultrastructural changes in GBM, mesangial cells, and podocytes in diabetic rats treated with AuNPs and ZnONPs.

(iii) To detect the antioxidant effects of NPs by measuring catalase (CAT) and superoxide dismutase (SOD) activity, as well as the concentration of malondialdehyde (MDA).

(iv) To evaluate the effects of AuNP- and ZnONP-based treatments on inflammatory markers, angiogenic factors, ECM proteins, and podocyte proteins, both at the protein and mRNA expression levels.

1.4 Scope of the Study

The current study was designed to examine the renoprotective activity of AuNPs and ZnONPs. Different parameters were measured to assess DN status. BG, BUN, CrC, and UAE were measured using available assay kits. Furthermore, histological and ultrastructural evaluations of the glomerulopathy were done after the treatment with AuNPs and ZnONPs. Numerous methods were used to achieve accurate histological diagnoses. Routine stain (hematoxylin and eosin (H&E)) was used to estimate the NPs' pathohistological effects. To estimate glomerulosclerotic index, Periodic acid-Schiff (PAS) stain was used, while Masson trichrome was used to assess

tubulointerstitial fibrosis. Moreover, electron microscopy was used for the ultra-structural evaluation of GBM thickening and podocyte injury.

To explore the possible mechanism of action of AuNPs and ZnONPs to attenuate DN, the activities of the oxidative stress markers SOD and CAT and the concentration of MDA in renal tissue were measured using the available kits. In addition, qRT-PCR was used to examine the effects of AuNPs and ZnONPs on the mRNA expression levels of various intracellular proteins in kidney cells (fibronectin, nephrin, podocin, TGF-β1, VEGF-A, matrix metalloproteinase-9 (MMP-9), and tumour necrosis factor-alpha (TNF-α)). Moreover, immunohistochemistry was carried out using different antibodies to assess the effects of treatments on protein expression levels. The used antibodies were against collagen IV, fibronectin, TGF-β1, VEGF-A, nephrin, podocin, and MMP-9 proteins.

1.5 Significance of the Study

Recently, nanomedicines have become an important therapeutic strategy used in biomedical research. However, the development of medication for DN using nanotechnology has not yet been investigated. DN is the most common cause of ESRD, which is extremely related to morbidity and mortality. Despite the large number of experiments on human and experimental animal models, few therapeutic medications have been developed to stop or protect against the progression of DN. Therefore, effective therapy is still not available (Keri *et al*., 2018; Kim and Park, 2017).

This study explores the incidence and contribution of using specific nanoparticles (AuNPs and ZnONPs) to protect against the development of DN. This study adds important new information for researchers in the nanomedicine field to consider so that they can better understand the effects of AuNPs and ZnONPs on the histological, ultrastructural, and pathophysiological determinants of DN. This work evaluates the renoprotective capability of AuNPs and ZnONPs and illustrates the mechanism pathways through which this protection might be afforded. AuNPs and

ZnONPs demonstrate renoprotective effects against the development of DN through their anti-hyperglycaemic, anti-oxidative, anti-inflammatory, anti-fibrotic, and anti-angiogenic effects.

The search for new, efficient medication will help diabetes patients to protect their kidneys from the disastrous effects of diabetes. It will also help to minimise DN status and, thus, help in reducing ESRD, which causes many social and economic burdens. The results of the present study contribute knowledge to the field of nanomedicine regarding the protective effects of AuNPs and ZnONPs against the development of DN. This study is a potential pathway for other researchers to develop an efficient and cost-effective medication that protects against DN.

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LIST OF PUBLICATIONS

1- Alomari, G., Al-Trad, B., Hamdan, S., Aljabali, A., Al-Zoubi, M., Bataineh, N., *et al.* (2019). Gold nanoparticles attenuate albuminuria by inhibiting podocyte injury in a rat model of diabetic nephropathy. Drug Delivery and Translational Research, 1-11. DOI: 10.1007/s13346-019-00675-6. **[Q2: Impact factor 3.111]**.

2- Ghada Alomari, Salehhuddin Hamdan, Bahaa Al‑Trad. Gold nanoparticles as a promising treatment for diabetes and its complications: Current and future potentials. Brazilian journal of Pharmaceutical Sciences. (accepted for publication in 16-May-2019). **[Q4: Impact factor 0.512]**. **(Accepted for publication).**

3- Ghada Alomari, Bahaa Al Trad, Salehhuddin Hamdan, Alaa Aljabali, Mazhar Al-Zoubi, Gregory J Eaton, Almuthanna K Alkaraki, Walhan Alshaer, Saja Haifawi, Khairunadwa Jemon. Zinc Oxide nanoparticles alleviate diabetic nephropathy in a rat model of streptozotocin-induced type 1 diabetes. Journal of Experimental Nanoscience, **[Q2: Impact factor 2.482]**. **(Under review).**