Preparation and Characterization of Chitosan-stabilized Selenium Nanoparticles for Ameliorating Experimentally Induced Diabetic Nephropathy in Rats

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Chitosan-stabilized selenium nanoparticles (CTS-SeNPs) were prepared by the reduction technique. Single phase structure of the SeNPs was confirmed using X-ray diffraction (XRD). Transmission Electron Microscope (TEM) showed that the particle size of the samples is in the range of 15 nm. This experiment aimed to study the potentialeffect of (CTS-SeNPs) as a therapeutic factor in diabetic nephropathy in rats. Streptozotocin (STZ) was used to induce diabetes in rats; thirty-six Wistar rats were divided into three groups; control, STZ-induced diabetic, and STZ-induced diabetic rats treated with CTS-SeNPs groups at a dose (2 mg Se/kg/d). All groups were given respective treatment orally via a stomach tube for 2 months. The obtained data showed that, the diabetic group revealed the presence of Microalbuminurea, the indicator of diabetic nephropathy and showed a significant (p < 0.05) increase in fasting bloodglucose, urea, creatinine and MDA, it is significant that high expression level of TGF-β1 and aldose reductase while the group treated with CTS-SeNPs revealed significant decline in all values compared to the diabetic group. The diabetic group showed a significant decrease in insulin level, total antioxidant capacity (TAC), GPx and SOD activity while, the treated group showed a significant (p < 0.05) increase in these values compared to the diabetic group. Kidney tissue showed normal histological picture except for mild vascular and glomerular congestion in treated group when compared with the diabetic one. The present investigation suggests that CTS-SeNPs can moderate diabetic nephropathy in streptozotocin-induced diabetic rats.

Keywords: Chitosan selenium nanoparticles, Diabetic, Nephropathy, oxidativestress

Introduction

Diabetic Nephropathy (DN), one of the most complications of diabetes mellitus supposed to be a risk factor for vascular disease, and is usually between type 2 diabetes’s peoples and is a main reason for the advancement of end stage renal disease (ESRD) in type 1 diabetes [1,2]. Approximately 20% to 40% of patients with type 1 or type 2 diabetes mellitus develop diabetic nephropathy [3]. SeNPs are considered a source of selenium that provides optimum in vivo bioavailability with a decrease of the risk of selenium toxicity. The biocompatibility and degradability of SeNPs in vivo are significantly preferable than noble metals as silver, gold and platinum. In addition, when compared to organic and inorganic forms of selenium, Se in many nanoforms has appeared lower toxicity and superior antioxidant and anti-tumor activity [4]. Besides, the anti-hyperglycemic activity of SeNPs has also been confirmed due to its antioxidant properties that can scavengen the free radicals generates due to hyperglycemia [5]. Chitosan (CTS), a natural polysaccharide is known to exhibit great bioactivities, such as antitumor, antibacterial, hypocholesterolemic, and antihypertensive activities, and the chitosan-
based nanoparticles exhibit high potency for treatment and prevention of diabetes and its complications, that is may be due to That CTS is a good stabilizer for nanoparticles.[6]. Due to the high antioxidant activity of chitosan (CS), the in-vitro antioxidant properties of selenite-loaded CS/TPP nanoparticles were significantly enhanced, compared with pure selenite [7].The reason for conducting this study was to examine the possible application of CTS-SeNPs as a therapeutic agent in diabetic nephropathy in rats through determination of: serum blood glucose level, serum insulin level, kidney function test (Urea and Creatinine), serum total antioxidant capacity (TAC), Antioxidant activities in Kidney homogenate; superoxide dismutase (SOD) and glutathione peroxidase (GPx), Malondialdehyde (MDA), Micro-albuminuria in urine sample & studying the gene expression of transforming growth factor-β1 (TGF-β1) & Aldose reductase in the kidney tissue by using Real time polymerase chain reaction, and finally histopathological examination of kidney tissue.

**Experimental**

*Preparation of CTS-SeNPs*

For preparing Se NPS, an aqueous solution of chitosan (0.1%, 10 ml) was mixed with 80 ml (0.06 M) ascorbic acid under magnetic steering. 10 ml of 0.03 M sodium selenite was added slowly to the mixture. The mixture was left under sonification condition for 5 min [8]. The pH of solution controlled (5-6) by NaOH. The solution was left in refrigerator for 24 h. Se NPS were collected using centrifuge.

*Animals management*

Thirty six male adult Wistar rats 8 weeks age and weighing 200-220 gram were used in this study. The institutional animal Care and Use Committee of the Faculty of Veterinary Medicine, Zagazig University approved the present study (ZU-IACUC/2/F/91/2019). All animals were adapted for three weeks prior to the beginning of the study.

*Chemicals*

Streptozotocin (STZ) obtained from Sigma-Aldrich (Chemical Cp. St. Louis, Mo, USA). The STZ was mixed in freshly prepared sod.citrate buffer (pH of 4.5) then injected intraperitoneally for induction of T2DM [9].CTS-SeNPs were prepared by adjusted procedure [10].Nicotinamide was dissolved in normal saline and is usually administrated intraperitoneally [11].

**CTS-SeNPs characterization, particle size and morphology:**

The intended sample was characterized through X-ray diffractometer (XRD), X-lab Shimadzu X-6000), and identified with Cu-Kα radiation. The particle size was determined using a High-Resolution Transmission Electron Microscope (HR-TEM, Tecnai G20, FEI, Netherlands).

**Induction of diabetes**

Whole night-fasted adult Wistar rats (n=36) were outift type 2 diabetes via a single dose of Nicotinamide I/P (110 mg/kg BW) dissolved for 15 min in normal saline before injection of Streptozotocin I/P (65 mg/kg of BW) which dissolved in citrate buffer. Blood glucose levels were evaluated 2 days after STZ injection. Rats were evaluated diabetic when fasting blood glucose was 200 mg/dl or more [12].

**Animals grouping and dosing**

Thirty-six rats were obtained into three equal groups; control, STZ-induced diabetic and STZ-induced diabetic rats groups treated with CTS-SeNPs (2 mg Se/kg/d). for 8 weeks.

**Sampling**

The blood samples were obtained from orbital venous plexus then centrifuged and the top layer was used for determination of biochemical parameters[13].Immediately after scarifying, take kidney, weighted. Every sample was divided into 3 parts; one was wrapped in aluminum foil and put immediately in liquid nitrogen container to make snap-freezing for molecular investigation. The second part kept at -20℃ to be homogenized for antioxidants measurements. The last part kept in neutral buffered formalin 10% for histopathological analysis. The samples of urine were obtained from urinary bladder for evaluation of microalbuminuria.

**Biochemical determinations:**

Serum glucose concentrations were assayed enzymatically using glucose commercial kit according to Trinder [14].Serum insulin concentrations were analyzed according to Unger et al. [15].BUN was assayed by using Kit (Cat. No
5602-01) according to Marsh et al. [16]. Creatinine was assayed using Kit (Cat. No- ab65340) according to Husdan and Rapoport [17]. TAC was determined in serum using Total Antioxidant Capacity Assay Kit Cat. No- MAK187) according to Sies [18]. Determination of GPx activity conferring to the procedure was described by Paglia and Valentine [19]. Determination of SOD activity in kidney homogenate was evaluated according to the procedure granted by Misra and Fridovich [20]. Determination of MDA (Malondialdehyde) concentration was studied according to Esterbauer et al. [21].

**Molecular determinations:**
Determination of the levels of expression of (TGF-β1 and aldose reductase) was investigated using Real Time-PCR according to Ikeguchi et al. [22] and Maekawa et al. [23] using PureLink® RNA Mini Kit obtained from Ambion by life technologies by Thermo Scientific, Catalog numbers: 12183018A and using the manufacture instructions. The formation of cDNA was done by using High Capacity cDNA Reverse Transcription Kit obtained from Thermo Scientific, code4374966. Amplification was done using SYBR Green qPCR Master Mix (2X) kit obtained from Thermo scientific, catalog #K0251, to detect TGF-β1 and aldose reductase’s expression. The amount of target gene expression levels was estimated using the formula of 2^-∆∆Ct[24] and using internal control GAPDH. The primer sequences were designed by primer 3 program as fellow: aldose reductase primer, F-3` GGACCTCTACCTTATCCACTG-5’/R-3` TTGGCCCAGGCGCTTCAG-5’TGF-β1 primer, F-3` CTGAAACCAAGGAGACGGAT-5’/R-3`-GGTTCAATGTCATGGTGAG-5’ GAPDH primer, F3’-GGCACAGTCAGGCTGA-GAATG-5’/R-3ATGGGTGGTGAAGACGAGCCAGTA-5’

**Histopathological examination of Kidney:**
Sections from kidney were cut and stained by Hematoxylin& Eosin and examined microscopically according to Suvarna et al. [25].

**Statistical analysis:**
The results were done using mean and standard error (Mean ± SEM). ANOVA test has been done to test the significant changes among different groups. Duncan multiple range test was considered as a post hoc test. The statistical analysis was done using IBM SPSS version 25.

**RESULTS**

**CTS-SeNPs Characterization**
For the X-ray diffraction analysis, the containing Se-NPs was dried as a powder and analyzed using a powder X-ray diffractometer. The peaks were noted to confirm the presence of Se-NPs. The instrument used for the analysis was X-lab Shimadzu X-6000), and identified with Cu-Kα radiation.

**Particle size and Morphology**
The particle size was observed using a High Resolution Transmission Electron Microscope (HR-TEM, Tecnai G20, FEI, Netherlands).

**Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on serum glucose and insulin levels**
The diabetic group revealed a significant (p < 0.05) increase in blood glucose level compared with control group, while, the group treated with CTS-SeNPs showed a significant (p < 0.05) decrease in glucose level when compared to the diabetic one. The diabetic group showed a significant (p < 0.05) decrease in serum insulin level and the group treated with CTS-SeNPs showed a significant (p < 0.05) increase in insulin level compared to the diabetic one. (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120.40± 3.33</td>
<td>25.50± 0.87</td>
</tr>
<tr>
<td>Diabetic</td>
<td>237.47± 10.84</td>
<td>5.17± 0.44</td>
</tr>
<tr>
<td>Diabetic with CTS-SeNPs</td>
<td>179.29± 1.90</td>
<td>15.83± 1.17</td>
</tr>
</tbody>
</table>

Values are represented as the mean ± SE . The means within the same column carrying different superscripts (a, b, c) are significant at p < 0.05.
Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on renal injury biomarkers

In Table (2), showed that the diabetic group revealed a significant (p < 0.05) elevation in the levels of kidney injury biomarkers (urea, creatinine and Microalbuminurea), while the treated group showed a significant (p < 0.05) decrease in kidney injury biomarkers when compared to the diabetic group.

Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on kidney oxidant/antioxidant status

Compared with the control group, the diabetic group revealed a significant (p < 0.05) decrease in TAC, GPX & SOD level, while the treated group showed a significant (p < 0.05) increase when compared to the diabetic group. Meanwhile, the treatment with CTS-SeNPs for 2 month resulted in a significant (p < 0.05) decrease in lipid peroxidation marker MDA level when compared with the diabetic group. (table 3).

Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on TGF-β1 and Aldose reductase gene expression level

Table (4) showed that the diabetic group is significantly (p < 0.05) increased in the level of expression of TGF-β1 & Aldose reductase compared with control group. While, the treated group showed a significant (p < 0.05) decrease in TGF-β1 & Aldose reductase gene expression level when compared to the diabetic group.

Table (2): Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on urea, creatinine and microalbuminurea levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urea (mg/dl)</th>
<th>Creatinine (mg/dl)</th>
<th>Microalbuminurea (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.15 ± 2.56</td>
<td>0.56 ± 0.0</td>
<td>44.77 ± 1.14</td>
</tr>
<tr>
<td>Diabetic</td>
<td>63.51 ± 3.90</td>
<td>1.35 ± 0.26</td>
<td>100.93 ± 1.39</td>
</tr>
<tr>
<td>Diabetic with CTS-SeNPs</td>
<td>45.78 ± 1.57</td>
<td>0.62 ± 0.03</td>
<td>77.33 ± 1.41</td>
</tr>
</tbody>
</table>

Values are represented as the mean ± SE. The means within the same column carrying different superscripts (a, b, c) are significant at p < 0.05.

Table (3): Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on kidney oxidant/antioxidant status

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GPx (ng/mg)</th>
<th>SOD (U/mg)</th>
<th>MDA (nmol/ml)</th>
<th>TAC (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.75 ± 1.01</td>
<td>36.33 ± 1.86</td>
<td>29.55 ± 1.62</td>
<td>16.83 ± 0.60</td>
</tr>
<tr>
<td>Diabetic</td>
<td>13.00 ± 0.38</td>
<td>8.42 ± 1.86</td>
<td>124.67 ± 3.18</td>
<td>3.83 ± 0.60</td>
</tr>
<tr>
<td>Diabetic with CTS-SeNPs</td>
<td>24.58 ± 0.96</td>
<td>26.33 ± 0.93</td>
<td>64.00 ± 3.21</td>
<td>12.17 ± 0.94</td>
</tr>
</tbody>
</table>

Values are represented as the mean ± SE. The means within the same column carrying different superscripts (a, b, c) are significant at p < 0.05.
**Histopathological results**

The control rat showed normal histological structure (Fig. 1A). The STZ-induced diabetic rats, kidney showed marked epithelial necrosis, vascular congestion, cast formation, severe hemorrhages and vascular congestion (Fig 1B). The STZ-induced diabetic rats administered CTS-SeNPs kidney showed mild cast formation and regenerative epithelium, Fig (1C).

**Discussion**

Fig (2) shows the XRD pattern for Se NPs. The XRD pattern for the Se-NPs shows diffraction peaks at 2θ (degrees) of 23.68°, 29.84°, 41.4°, 43.74°, 45.56°, 51.88°, 56.34°, 61.78°, 65.34° and 71.68° which correspond to the (100), (101), (110), (102), (111), (201), (112), (202), (210) and (113) planes of the Se-NPs. All the diffraction peaks in the 2θ range correspond to the hexagonal structure of selenium. The lattice parameters \((a\) and \(c)\) were calculated according to the equation:

\[
\frac{1}{a^2} = \frac{4}{3} \left( \frac{h^2 + h k + k^2}{a^2} \right) + \frac{l^2}{c^2},
\]

where \(h\), \(l\), and \(k\) are miller indices. The calculated lattice parameters values \(a = 4.337\) Å and \(c = 5.631\) Å and have an agreement with the results were obtained by [25]. The crystallite size \(D\) of the Se-NPs has been calculated using Debye-Scherrer's equation:

\[
D = \frac{0.94 \lambda}{\beta \cos \theta}
\]

where \(\lambda\) is the wavelength of the X-ray radiation, \(\beta\) is the full width at half maximum and \(\theta\) is the angle of diffraction. The crystallite size of the Se-NPs recorded 21.47 nm. There is a deviation from the results from the Debye-Scherrer's Eq. from the results from the TEM images. This is because of Debye-Scherrer's constant 0.89 or 0.9 for \(\beta\) was taken as spherical crystals with cubic unit cells. [26].

**Table (4): Effect of CTS-SeNPs (2 mg/kg/b.wt) in STZ induced diabetic rats on TGF-β1 and Aldose reductase gene expression level**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TGF-β1</th>
<th>Aldose reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.27</td>
<td>1.00 ± 0.22</td>
</tr>
<tr>
<td>Diabetic</td>
<td>3.48 ± 0.23</td>
<td>3.66 ± 0.24</td>
</tr>
<tr>
<td>Diabetic with CTS-SeNPs</td>
<td>1.86 ± 0.38</td>
<td>2.20 ± 0.24</td>
</tr>
</tbody>
</table>

Values are represented as the mean ± SE. The means within the same column carrying different superscripts (a, b, c) are significant at \(p < 0.05\).

Fig. (1): Histopathological results on kidney revealed that A) Kidney of control rat showing normal histological structure, H&E, X 400. B) Kidney of rat showing severe hemorrhages and vascular congestion, H&E, X 400. C) Kidney of rat showing mild cast formation and regenerative epithelium, H&E, X 400.
PREPARATION AND CHARACTERIZATION OF CHITOSAN—...
Excretion of ≥300 mg in a 24 hour collection or macroalbuminuria and abnormal renal function [32].

The generation of ROS due to hyperglycemia leads to apoptosis and renal damage, further leading to diabetic microvascular complications. So, it is clear that necessary to explore clinical therapies against oxidative stress as additional standard treatments for diabetic patients [29].

Anser et al. [33] investigated that Se pretreatment increase TAC levels significantly compared to the AgNP group. The Se and SeNPs administration effects lead to antioxidative and protective effects evidenced by the increase of TAC and reduction in MDA contents. and this findings comes beside our results.

Eidet et al. [34] investigated that nano-Se supplementation upregulated activities of SOD, GPx, GSH in serum and liver tissue of growing rabbits, as well as liver TAC. It also down regulated (P<0.01) serum GSSG, NO levels and MDA and 8-OHdG in liver tissue. In one line, nano-Se induced plasma GPx, SOD, TAC and declined MDA of non-stressed rats more than in control. GPX catalyzes the reduction of lipid peroxides into corresponding alcohols and free hydrogen peroxide into water via reduced glutathione. This function of GPX is significantly important in antioxidant defense and in maintenance of the health of cells and organisms.

Selenium can improve diabetes viacollaborationin GPx1. GPx-1 is effective in the protection of pancreatic beta cells against the damage which caused by STZ. The study of selenium effects on diabetic-induced rats has shown that the treatment with selenium causes recovery and restoration of endothelial dysfunction and vascular disorders through regulating antioxidant enzymes and releasing nitric oxide [35]. SeNPs is known to have antioxidative effects. This leads to increase activities of both GPX and glutathione S-transfers leading to less oxidative stress [36]. Nafiu and Rahman, [37] documented the impact of Se²⁺ or Zn²⁺ added papaya extracts in vivo excision wound model. Se²⁺ added to papaya extract induces expression of TGF-β and VEGFA for wound repair. Fu et al. [38] explained that VEGFA and TGF-β play a part in the natural cutaneous wound healing by attracting inflammatory cells, cellular proliferation, neovascularization and epithelial migration.

Another study explained that Se deficiency causes increased oxidative stress via TGF-1 in normal and STZ diabetic rats. Results indicated that Se deficiency led to renal oxidative stress and renal injury via TGF-1 [39] and these findings agreed with our study.

Inhibition of aldose reductase (AR), the main enzyme in polyol pathway lead to decline in the expression of some inflammatory markers in the heart and aorta, and IL-1β in the serum of STZ induced diabetic mice [40].

In silico studies of eleven ligands explained that the glycoalkaloids (vicine) possessed good glide score against the receptor aldose reductase comparable with that of a standard drug metformin for Type-2 diabetes mellitus' treatment [41]. Asadi et al. [42] documented the induction of diabetes induced glomerular hyperfiltration and to the structural changes as cell growth, mesangial expansion, and glomerular basement membrane thickening [423]. This finding in agreement with previous researches since the presence of glomerular hyperfiltration in incipient DN having previously been reported in both clinical trials and animal models [44]. Sections from untreated diabetic kidney showed several glomerular and tubuleinterstitial changes as compared to control. Observed lesions as dilated tubules with thickening of the basement membrane of glomeruli [45] and this agreed with our histopathological finding.

**Conclusion**

These findings suggest that CTS-SeNPs possess a potential effect in preventing diabetic nephropathy via improving oxidative stress and kidney functions. Future investigations regarding CTS-SeNPs in the prevention of development and progression of diabetic nephropathy introduce the hope for use of lower doses or in combination with other hypoglycemics which add to the safety and efficacy of both drugs. However, future studies are needed to understand the hypoglycemic activity of SeNPs and/or the possible toxicity increases after SeNPs are administered for a long time before starting any clinical trials.
Disclosure statement
The authors declare no conflicts of interest, financial or otherwise.

References
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