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Evaluation of Zinc Oxide Nanoparticles for Insulin, Insulin Receptors and Insulin Receptors Substrates Gene Expression in Streptozotocin-Induced Diabetic Rats Hussein, S. A.¹, EL-Senosi, Y. A.¹, El-Dawy, K.² and Hind A. Baz.¹

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ABSTRACT

The use of nanoparticles in medicine is an attractive proposition. In the present study, evaluation of the anti-diabetic activity of zinc oxide nanoparticles (ZnONPs) on insulin, insulin receptors and insulin receptors substrates gene expression in streptozotocin-induced diabetic rats were investigated. One hundred and sixty male albino rats with weight 130 ± 30 and age 12-16 weeks were used. Animals were grouped as follows: control; did not receive any type of treatment, control positive received single daily oral dose of 5 mg/kg ZnONPs in suspension, diabetic rats; received a single intra peritoneal dose of streptozotocin (50 mg/kg), diabetic + insulin; received a single daily subcutaneous dose of insulin (2U/kg), diabetic + ZnONPs I, received single daily oral dose of 5mg/kg ZnONPs in suspension, diabetic + ZnONPs II, received single daily oral dose of 10mg/kg ZnONPs in suspension, diabetic+ ZnONPs + insulin I; received single daily oral dose of 5mg/kg ZnONPs in suspension and a single daily subcutaneous dose of insulin (2U/kg) and diabetic+ ZnONPs + insulin II; received single daily oral dose of 10mg/kg ZnONPs in suspension and a single daily subcutaneous dose of insulin (2U/kg). The pancreatic insulin gene expression, hepatic insulin receptor A (IR-A), hepatic insulin receptor substrate-2 (IRS-2) and muscular insulin receptor substrate-1 (IRS-1) mRNA levels were determined. The results indicated that the expression of insulin, IR-A, IRS-1 and IRS-2 were depressed in diabetic rats, while they are induced in rats that administrated ZnONPs and/or insulin in a dose dependant. In conclusion, zinc oxide nanoparticles act as potent inducer for insulin, insulin receptors and insulin substrates gene expression.

KEY WORDS: ZnONPs, insulin, insulin receptors, gene expression.

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1. INTRODUCTION

Diabetes mellitus is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. High blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Wild et al., 2004). A large number of people suffer from diabetes all over the world (Lin and Sun, 2010). These patients would require the development of several medications with multiple modes of actions. Many researches demonstrated the role of metals in glucose

metabolism and the association of their deficiency with diabetes as Vanadium (Thompson, et al., 2009), chromium (Wang and Cefalu, 2010) magnesium (Wells, 2008), and zinc (Chausmer, 1998) have been reported to play a role in blood sugar maintenance and have been included in diabetes therapy. Zinc, an essential metal, is an activator for more than three hundred enzymes in the body (Haase et al., 2008), and plays a key role in different metabolic pathways including glucose metabolism. Zinc promotes hepatic glycogenesis through its actions on the insulin pathways and thus improves glucose utilization

(Jansen *et al.*, 2009). Zinc is also known to keep the structure of insulin and has a role in insulin biosynthesis, storage and secretion (Chausmer, 1998). There are several zinc transporters in pancreatic β -cells (Smidt *et al.*, 2009); like zinc transporter 8 which has a potent role in insulin secretion (Rungby, 2010). In addition, zinc improve insulin signaling by increasing insulin receptor phosphorylation, enhancing PI3K activity and inhibition of glycogen synthase kinase-3 (Jansen *et al.*, 2009). Zinc deficiency is positively correlated with diabetes and may also affect the progress of Type 2 diabetes also, decreased zinc in the pancreas may reduce the ability of the islet β -cells to produce and secrete insulin (Meyer and Spence, 2009). Furthermore, knowing zinc's antioxidant role, reduced zinc may exacerbate the oxidative stress-mediated complications of diabetes. Thus, there exists a complex inter-relationship between zinc, diabetes and diabetic complications. Developing a zinc-based agent for treatment of both Type 1 and Type 2 diabetes and associated complications thus becomes an attractive proposition. The beneficial role of zinc in diabetes has been implicated by studies of the zinc supplies in diabetic rats (Ukperoro *et al.*, 2010), (Alkaladi *et al.*, 2014) reported the antidiabetic effects of ZnONPs through induction of insulin, IR and glucose metabolizing enzymes gene expression. In the same line Umrani and Paknikar (2014) proved the ability of ZnONPs in controlling of blood glucose in diabetic rats, these are only two studies that monitored the effect of ZnONPS on diabetic rats therefore this work was designed to investigate the ability of ZnONPs to modify the insulin mechanism of action in diabetic rats through measuring of its effects on the insulin, IR and IS mRNA levels in the different tissues of STZ-diabetic rats.

2. MATERIAL AND METHODS

2.1. Experimental animals

One hundred and sixty white male albino rats, 12-16 weeks old and average body weight 130 ± 30 gm were used in the experimental investigation of this study. Rats were obtained from laboratory animals research center, Faculty of Vet. Medicine Zagazig university, Egypt. Animals were housed in separate metal cages, fresh and clean drinking water was supplied ad-libitum through specific nipple. Rats were kept at constant environmental and nutritional conditions during the course of the experiment. Cleaning and changing water and food was done for all animals twice daily. The animals were left 7 days for acclimatization before the beginning of the experiment. The animals were fed on constant ration through the course of the experiment in the form of concentrated diet composed of carbohydrate 58%, protein 21%, lipid 3.4%, cellulose 2.6%, minerals 1.49% calcium 0.9% phosphorus 0.59% and moisture 12%.

2.2. Zinc Oxide Nanoparticles (ZnONPs)

ZnONPs was obtained in the form of dispersion (Sigma-Aldrich, Steinheim, Germany). of the following properties, concentration 50 wt.% in H₂O, the average nanoparticle size <35 nm, the particle size distribution (hydrodynamic diameter) <100 nm using dynamic light scattering (DLS) technique, pH 7 ± 0.1 (for aqueous systems) and density $1.7 \text{ g/mL} \pm 0.1 \text{ g/mL}$ at 25 °C

2.3. Diabetes Induction

One hundred and twenty Rat were fasted for 18 hours and allowed free access of water. Fasting prior to streptozotocin-injection is required in order to reduce the blood glucose level in animals blood to a level that circulating glucose does not out-compete streptozotocin for GLUT-2 binding and transport β -cells. The experimental induction of diabetes in male rats was induced by a single intraperitoneal (i.p) injected dose of 50 mg /kg body wt. of streptozotocin (STZ) (Sigma Chemical Co. P.O. St. Louis, U.S.A.) freshly dissolved in citrate buffer, PH 4.5. After STZ

injection the animals were allowed to drink glucose solution (5%) w/v overnight to avoid hypoglycemia which might be induced by streptozotocin (STZ). Control rats (n= 40) were received an equivalent amounts of vehicle (citrate buffer) alone .A week later, STZ–treated rats were fasted for 12 hours, and blood samples were collected from the orbital venous sinus for blood glucose determination. Rats in diabetic group with blood glucose levels higher than 250 mg /dl were considered diabetic and included for further studies (Ramanathan et al., 1999).

2.4. Animal grouping

After four weeks of diabetes induction the diabetic rats were randomly sub-divided into eight groups, 20 animals in each, placed in individual cages and classified as follow:-Group I (control non-treated group): didn't receive any type of treatment. Group II (control positive ZnONPs treated group) Rats were received ZnONPs (5 mg / kg body weight oral daily) served as positive control ZnONPs for all experimental groups. Group III (diabetic non-treated group): Rats were received no drugs and served as STZ-induced diabetic groups. Group IV (diabetic insulin treated group): Rats were injected with 2U/kg insulin (HumulinR U-100) S/C daily for one month. (Izbeki et al., 2008) Group V (diabetic ZnONPs treated group I): Rats were received 5 mg /kg bwt ZnONPs orally once daily for one month (Umrani and Paknikar , 2014). Group VI (diabetic ZnONPs treated group II): Rats were received 10 mg /kg bwt ZnONPs orally once daily for one month (Umrani and Paknikar, 2014).Group VII (diabetic ZnONPs with insulin treated group I): Rats were received 5 mg /kgbwt ZnONPs orally once daily for one month (Umrani and Paknikar , 2014).and injected with insulin at dose of 2 U/ Kg subcutaneously (Izbeki et al., 2008). Group VIII (diabetic ZnONPs with insulin treated group II): Rats were

received 10 mg /kg bwt ZnONPs orally once daily for one month (Umrani and Paknikar , 2014). and injected with insulin at dose of 2 U/ Kg subcutaneously (Izbeki et al., 2008). At the end of experiment rats were anesthetized, small parts of pancreas, liver and thigh muscle were desecrated and kept in liquid nitrogen for molecular investigations

2.5. Determination of insulin, IR-A , IRS-1 and IRS-2 gene expression

Using a semi-quantitative RT-PCR according to (Meadus ,2003).Total RNA will be extracted from liver tissues; using an RNA extraction Kit (AXYGEN, Biosciences, Central Avenue, Union city, CA, USA). First strand cDNA was synthesized using RevertAid™ H Minus (Fermentas, life science, Pittsburgh, PA, USA). Each RT reaction contained RNA template (3 µg/µL), 200 U/µL RevertAid™ H Minus M-MuL V Reverse Transcriptase (Fermentas, life science, Pittsburgh, PA, USA), 100 µM, 0.2 µg/µL random hexamer primers, 10 mM dNTPs mix, 20 U/µL RiboLock™ RNase inhibitor (Fermentas, Life Science, Pittsburgh, PA, USA), and 5× reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT). Primers for all examined genes were chosen according to the following parameters: primer length within 20 bases; GC content 55%. The PCR reaction was started by using (DreamTaq™ Green PCR Master Mix (2×), Fermentas, Life Science, Pittsburgh, PA, USA). The reaction was performed using thermal cycler (Biosystems, Carlsbad, CA, USA). The primer pairs for amplification were shown in table 1. Amplified PCR products were then electrophorised on 1.5% Agarose gel in 1× Tris acetate EDTA running buffer (1 × TAE) with condition of 100 V/40 min. Samples were visualized using UV transilluminator T2621BS, (BioRad, Berkeley, CA, USA).

Table 1. Oligonucleotide primers sequences.

Gene name	Size pb	5' primer	3' primer
Insulin	331	GCCCAGGCTTTTGTCAAACA	CTCCCCACACACCAGGTAGAG
IR-A	258	TTCATTCAGGAAGACCTTCGA	AGGCCAGAGATGACAAGTGAC
IRS-1	490	AGCACCTGGTGGCTCTCTACA	CAGCTGCAGAAGAGCCTGGTA
IRS-2	416	GCAGTTCAGGTCGCCTCTGC	GGAGCCACACCACATTCGCA
B- actine	230	ACGAGGCCAGAGCAAGA	TTGGTTACAATGCCGTGTTCA

2.6. Statistical analysis

Data analysis were expressed as mean \pm S.E. and were statistically analyzed by (Kempthorn, 1969).

3. RESULTS

The pancreatic insulin, IR-A, IRS-1 and IRS- 2 mRNA levels were depressed in

diabetic rats as compared with the other groups. When the diabetic rats treated with ZnONPs in doses 5 and 10 mg/kg b.w. for 30 days either alone or with insulin, the mRNA levels were induced as compared with the diabetic rats. The best results were observed in diabetic rats treated with ZnONPs in a dose of 10mg/kg b.w. with insulin. The induction of genes expression with ZnONPs is a dose dependant (table 2 and figure 1).

Table 2. mRNA levels of insulin, insulin receptor-A, insulin receptor substrate-1 and 2 in the different treated groups(Calib Vol ,ng).

Groups	insulin	IR-A	IRS-1	IRS-2	β -actine gene
G1	700.00	740.76	832.76	1093.83	811.06
G2	814.76	810.76	930.76	1583.09	810.96
G3	313.52	363.52	584.52	680.74	810.76
G4	480.91	580.91	792.91	993.83	810.81
G5	580.74	490.74	589.74	980.74	810.98
G6	687.19	677.19	789.19	1283.09	810.96
G7	901.64	924.46	974.46	998.83	811.08
G8	1024.46	961.64	991.64	1267.09	811.1

G1, Control group; **G2**,Control positive group; **G3**,Diabetic group; **G4**, Diabetic group treated by insulin; **G5**,Diabetic group treated by ZnONPs (5 mg/kg); **G6**,Diabetic group treated by ZnONPs (10 mg/kg); **G7**, Diabetic group treated with ZnONPs (5mg/kg) &insulin; **G8**,Diabetic group treated with ZnONPs (10mg/kg) &insulin.

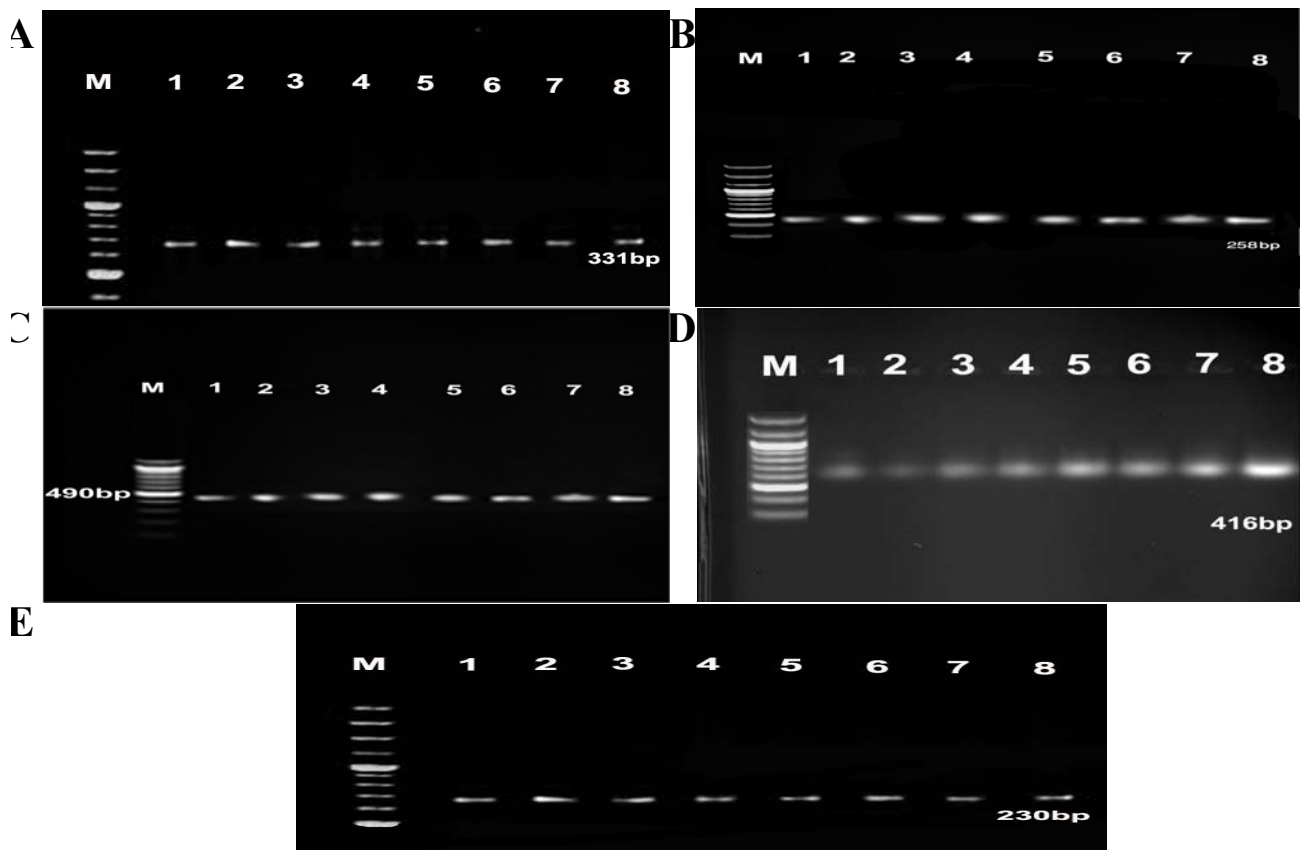


Figure 1. Gel picture of the examined genes bands after Agarose gel electrophoresis (A) Insulin gene; (B) Insulin receptor A gene; (C) Insulin receptor substrate-1; (D) Insulin receptor substrate-2; (E) β -actine; M, DNA-ladder; 1, Control group; 2, Control positive group; 3, Diabetic group; 4, Diabetic group treated by insulin; 5, Diabetic group treated by ZnONPs (5 mg/kg); 6, Diabetic group treated by ZnONPs (10 mg/kg); 7, Diabetic group treated with ZnONPs (5mg/kg) & insulin; 8, Diabetic group treated with ZnONPs (10mg/kg) & insulin.

5. DISCUSSION

Diabetes mellitus is a chronic metabolic disorder due to the relative deficiency of insulin secretion and varying degrees of insulin resistance and is characterized by high circulating glucose (Aylward ., 2005). In the present study we evaluated the possible therapeutic effect of zinc oxide nanoparticles on streptozotocin-induced diabetic rats as well as its compared effect to insulin treatment. Our results pointed out that mRNA expression level of insulin gene, IR-A IRS-1 sand IRS-2 appeared to increase in ZnONPs and insulin treated groups if compared with the diabetic non-treated group. In the same line (Akaladi et

al, 2014) reported the ability of ZnONPs for induction of insulin and IR-A gene expression in STZ-diabetic rats. The insulin receptor is a heterotetrameric transmembrane glycoprotein composed of 2 α and 2 β -subunit structures (Myers and White, 1993).

The extracellular α -subunits contain the insulin binding domain, while the β -subunits span the extracellular, membrane and intracellular domains .The adequate binding of insulin to its receptor is influenced by the concentration of circulating insulin, the concentration of

receptors and the affinity of the receptor for insulin (Kahn, 1994). In DM-2 and other insulin resistant states such as obesity, higher concentrations of circulating insulin will decrease insulin receptor concentration in a dose-dependent manner in a process known as 'down-regulation' (Gavin *et al.*, 1974). In a study by (Bathena *et al.*, 1986) rats fed a high-fat diet developed insulin resistance due to lower insulin receptor numbers and binding affinity, which were then restored to normal with a standard diet. (Gomot *et al.*, 1992) observed that a zinc deficient diet produced a significantly lower insulin receptor binding in rat adipocytes when compared to ad libitum controls. (Ezaki, 1989) found that Zn²⁺ ions also stimulated glucose transport activity in adipocytes when compared to buffer alone and it is well-known that all tissues that respond to insulin are able to express insulin receptors (IRs) with different levels and the ability of insulin to perform its function need to bind to α -subunits of its receptors has lead to the phosphorylation of β -subunits (White and Yenush, 1998). This supports our experimental results which showed high expression levels of IR-A gene in hepatic tissues in the groups treated with ZnONPs. Insulin receptor substrate molecules are key mediators in insulin signaling and play a central role in maintaining basic cellular functions such as growth, survival and metabolism. They act as docking proteins between the insulin receptor and a complex network of intracellular signaling molecules. Four members (IRS-1, IRS-2, IRS-3, IRS-4) of this family have been identified that differ according to tissue distribution, sub-cellular localization, developmental expression, binding to the insulin receptor, and interaction with SH2 domain containing proteins.

In fact, IRS-1 appears to have its major role in skeletal muscle whereas IRS-2 appears to regulate hepatic insulin action as well as pancreatic β -cell development and survival. The two major IRS isoforms IRS-1 and IRS-2, are highly expressed in livers of

diabetic animals and humans (Aytug *et al.*, 2003). The role of IRS molecules as mediators of insulin signaling through the insulin receptor is firmly established (White, 1998). Ablation of IRS-1 results in growth retardation and mild insulin resistance (Tamemoto *et al.*, 1994), whereas ablation of IRS-2 causes death due to a combination of insulin resistance and failure to develop compensatory response of β cells (Withers *et al.*, 1998). Ablation of IRS-3 and IRS-4 yields no apparent phenotype, raising the question of whether these 2 molecules play any role in insulin and IGF signaling (Lavan *et al.*, 1998). The different phenotypes caused by ablation of the 2 receptors or ablation of their substrates suggest that multiple substrates are required to mediate the actions of each receptor. Insulin receptors are indeed important for embryonic growth in late gestation (Louvi *et al.*, 1997). Likewise, it is striking that mice lacking insulin receptors, either as a result of total ablation or as a result of β cell-selective ablation, develop normal numbers of β cells whereas mice without IRS-2 fail to develop a sufficient number of β cells, and mice without IRS-1 show impaired insulin secretion (Kulkarni *et al.*, 1998). Recent study of a common amino acid variant of IRS-1 also suggest that IRS-1 may be implicated in insulin secretion (Porzio *et al.*, 1999). Based on recent evidence, it appears that IGF-1R is required for β -cell growth (Withers *et al.*, 1999). Thus, the emerging paradigm is β -cell signaling is that IR, acting through IRS-1, is important for secretion, whereas IGF-1R, acting through IRS-2, is important for growth. (Yoshiaki *et al.*, 2003), showed that the combined heterozygosity for null alleles of IR and IRS-1 results in a synergistic impairment of insulin action in multiple tissues, leading to β -cell hyperplasia and an increased incidence of diabetes (Bruning *et al.*, 1997). In these experiments, double heterozygosity for *ir* and *irs-1* resulted in an approximately 4-fold increase in the prevalence rate of diabetes, which is similar to the increased

recurrence risk of type 2 diabetes in first degree relatives of diabetic patients, suggesting that an oligogenic model with 2 predisposing alleles can indeed account for the entire genetic susceptibility to type 2 diabetes (Ghosh and Schork, 1996). Moreover, several sequence variants have been identified in the human IRS-1 gene, which may impair IRS-1 signaling and predispose to diabetes (Porzi et al., 1999). These data indicate that IRS-1 plays an important role in metabolic regulation.

6. Conclusion

ZnONPs were elucidated as antidiabetic agents. They lead to induction of insulin synthesis and activation of insulin mechanism of action through induction of insulin, insulin receptors and insulin receptors substrates gene expressions.

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تقييم جزيئات أكسيد الزنك النانوية على التعبير الجيني للأنسولين ومستقبلاته في الفئران المحدث بها مرض السكري

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الملخص العربي

ان استخدام الجزيئات متناهية الصغر في الطب أصبح موضع اهتمام و جذب. وفي هذه الدراسة تم استخدام الباحثين جزيئات أكسيد الزنك متناهية الصغر لتقييم مدي تأثيرها في مقاومة السكري. حيث استخدمت مائة وستين ذكرا من الجرذان البيضاء وزن 130 ± 30 و عمر 12-16 أسابيع وتم تقسيمهم الي المجموعات الاتية: المجموعة الضابطة والتي لم تتناول أي عقار والمجموعة الضابطة ايجابيا والتي تم تجريعها عن طريق الفم 5ملجم / كجم من محلول أكسيد الزنك متناهية الصغر جرعة واحدة يوميا والمجموعة الفئران المصابة بالسكري و حقنت مادة الاستربتوزوتزين مرة واحدة تحت الغشاء البريتوني (50 ملغ / كلغ) ومجموعة السكري المعالجة بالانسولين والتي حقنت تحت الجلد 2 وحدة دولية من الانسولين جرعة واحد يوميا ومجموعة السكري والمعالجة بجزيئات أكسيد الزنك متناهية الصغر بمعدل 5ملجم/كجم جرعة واحدة فمويه يوميا ومجموعة السكري والمعالجة بجزيئات أكسيد الزنك متناهية الصغر بمعدل 10ملجم/كجم جرعة واحدة فمويه يوميا ومجموعة السكري المعالجة بكلا من الانسولين 2وحدة دولية يوميا تحت الجلد وتطعم أيضا جرعة 5ملجم/كجم من محلول جزيئات أكسيد الزنك متناهية الصغر فمويا جرعة واحدة يوميا ومجموعة السكري المعالجة بكلا من الانسولين 2وحدة دولية يوميا تحت الجلد وتطعم أيضا جرعة 10ملجم/كجم من محلول أكسيد الزنك النانو فمويا جرعة واحدة يوميا. وتم قياس مستويات التعبير الجيني للانسولين بالبنكرياس ومستقبلات الانسولين بالكبد وكذلك المواد المستقبلية للانسولين 1,2 وأشارت النتائج الي انخفاض التعبير الجيني لهم في الفئران المصابة بالسكري بينما الفئران المعالجة بجزيئات أكسيد الزنك متناهية الصغر الانسولين اظهرت ارتفاع ملحوظ للتعبير الجيني في كلا من الانسولين ومستقبلاته وكذلك المواد المستقبلية للانسولين

الخلاصة تعمل جزيئات أكسيد الزنك متناهية الصغر كمحفز قوي لعمل هرمون الأنسولين من خلال تحفيز للتعبير الجيني لجينات للانسولين ومستقبلات الانسولين وكذلك المواد الركيه لمستقبلات الأنسولين.

(مجلة بنها للعلوم الطبية البيطرية: عدد 27(1):166-174, سبتمبر 2014)