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Streptozotocin - A Diabetogenic Agent in Animal Models

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ABSTRACT

Streptozotocin is a permanent diabetogenic compound, produced by the gram positive soil bacterium *Streptomyces achromogenes* that exhibits broad spectrum of antibacterial properties.STZ induces diabetes mellitus in laboratory animals by killing insulin-producing pancreatic β-cells.Streptozotocin is a toxic glucoseanalogue that preferentially accumulate in pancreatic beta cells via the low affinity glucose transporter GLUT2.The toxic effector mechanism of STZ starts with its decomposed products and the free radicals generated, which destroy the pancreatic β-cells by alkylating DNA, impairing mitochondrial system and inhibiting O-GlcNAcase.

INTRODUCTION

Streptozotocin is a permanent diabetes inducing drug. It is synthesized by a strain of the soil microbe *Streptomyces achromogenes* (gram positive bacterium) with broad spectrum of antibacterial properties (4). Streptozotocin is an unusual aminoglycoside containing a nitrosoamino group discovered in 1959 as an antibiotic, now marketed as a generic drug. The nitrosoamino group enables the metabolite to act as a nitric oxide (NO) donor. NO is an important messenger molecule involved in many physiological and pathological processes in the body. Streptozotocin is widely used to induce diabetes in rodent models by inhibition of β-cell O-GlcNAcase (5-10).

Streptozotocin features four important biological properties as evidenced by its antibiotic, β-cell (beta)-cytotoxic, oncolytic, as well as oncogenic effects (1-3). This product is an antineoplastic antibiotic and is used mainly in the treatment of pancreatic (islet cell) tumors (10). It is used for the treatment of malignant insulinoma (11). Current use of STZ is mostly as an investigational drug for diabetes research due to its specific toxicity associated with pancreatic β-cells (7). Low affinity glucose transporter- GLUT2 of βcells transports STZ into the cell and causes alkylation of DNA and irreversible necrosis of β cells (23). DNA synthesis in mammalian and bacterial cells is inhibited by action of STZ (12). STZ is widely used to induce both insulin-dependent (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) (8, 9). STZ is an antibiotic and antitumor agent, induces diabetes mellitus via reduction of nicotinamide adenine dinucleotide in pancreatic β-cells *in vivo* (9). This review will summarize the chemistry of STZ and its β-cell toxicity through the link between STZ and free radicals. In addition, dosage, route of administration and metabolism of STZ in experimental animal models to study diabetes will be addressed.

STRUCTURAL FEATURES OF STZ

Streptozotocin (2-deoxy-2-[3-methyl-3-nitrosourea] 1-D-glucopyranose) occurs in two anomeric forms, α and β (Figure1a), which can be separated by Chromatographic technique (HPLC) (6). It appears as pale yellow or off-white crystalline powder. Streptozotocin has a molecular weight of 265 g/mol, with molecular formula $C_8H_{15}N_3O_7$ (49). STZ molecular structure is similar to that of 2-deoxy-D-glucose with a replacement at C_2 with an N-methyl-N-nitrosourea group, which is the

cytotoxic moiety of STZ in damaging beta cells. Streptozotocin is a glucosamine nitrosourea compound with a methyl group attached at one end and a glucose molecule at the other end (4, 6) (Figure 1b).

Figure 1: Chemical structure of STZ

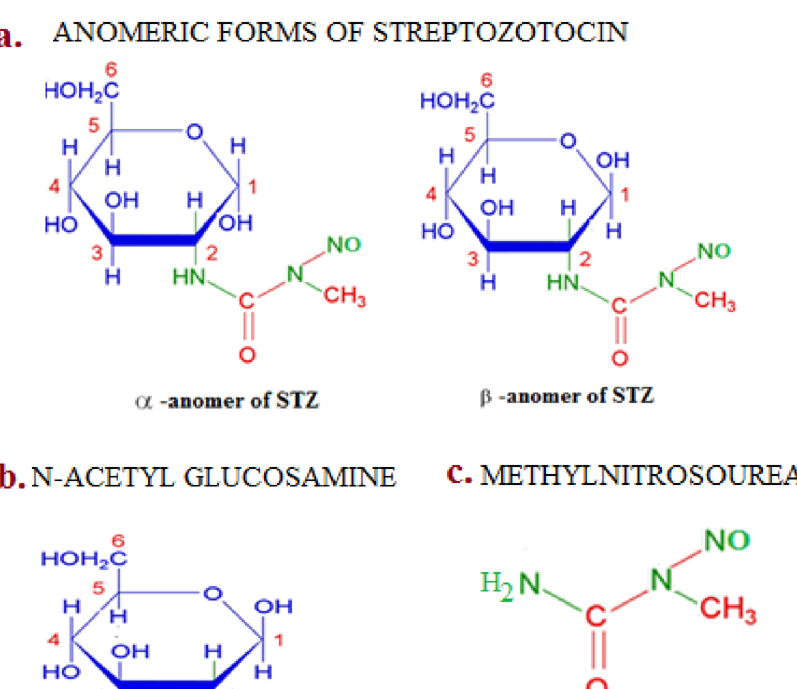

Figure 1: A schematic diagram representing - a) α and β Anomeric forms of STZb) structural analog of STZ - N-acetyl glucosamine c) cytotoxic moiety of STZ- N-methyl-N-nitrosourea

SOLUBILITY AND STABILITY

In case of solubility, STZ is highly soluble in water, ketones and lower alcohols, but slightly soluble in polar organic solvents (2). This product dissolves in water at 50 mg/mL to give a light yellow solution, from clear to slightly hazy. Aqueous solutions of STZ rapidly undergo mutarotation to an equilibrium mixture of alpha- and beta-anomers. Maximum stability of STZ solution is at pH 4, with stability decreasing rapidly at higher or lower pH. Freshly prepared solutions are clear and have a light straw color. On standing, they take on a yellow to brown color and effervescent, indicating decomposition (13). STZ can be stored at 4°C for short term, but long term storage needs -20 ^{o}C because it is stable at this temperature for at least 2 years.

The Streptozotocin solution (in citrate or acetate buffer, pH 4.5) should be administered "immediately" but not later than 15 to 20 min after dissolving, as recommended by different research consortia (15- 22). Solutions should be prepared just before use, since the product is unstable. Streptozotocin is dissolved in Phosphate buffer saline (PBS) instead of acidic citrate buffer, which allegedly rapidly inactivates the drug. Solutions of Streptozotocin will spontaneously give off NO gas at room temperature. This NO release is slowed, but not completely stopped even at -80°C, and the rate of NO release is also impacted by the solvent used (for example, dissolving Streptozotocin in buffers that contain sodium speeds up NO release). Since it does not seem to be possible to stop completely the NO release when Streptozotocin is in solution, and the NO donor function is critical to the majority of experimental applications, so it is recommended that to make STZ solutions only immediately before use (44 - 48). Streptozotocin is cytotoxic to pancreatic β-cells and its effects can be seen within seventy two hours after administration depending on the dose administered (54).

ROUTE OF ADMINISTRATION AND DOSE SCHEDULE OF STZ

A wide variety of dose schedules and routes of administration have been reported in inducing diabetes in rats with STZ. STZ is most commonly delivered by one of two routes, intraperitoneal (IP) or intravenous (IV) (9, 35), although other methods including subcutaneous, intracardiac, and intramuscular delivery have been used in rodents (36). Although IP offers a quick and easy method of administration, especially for studies involving multiple doses of the drug, accidental delivery into the bowel or sub-dermal space may result in increased moribundity or decrease in diabetogenic effect. Additionally, other studies have reported that IV administration of STZ produces a more stable and reproducible model of diabetes than IP administration (35).

The two most common protocols of dose schedule are intraperitoneal injection of a single high dose or multiple low doses (25). For diabetes induction through the single-high–dose regimen, reported doses vary from 100 mg/kg to 220 mg/kg. The low-dose protocol typically involves intraperitoneal administration of 5 consecutive daily doses of 40 mg/kg streptozotocin, but the use of 4 (35 mg/kg) and 6 daily administrations as well as of 2 separate courses of 5 injections of 40 mg/kg have been reported (16, 18, 19,21 and 26). The frequently used single intravenous dose of STZ in adult rats to induce IDDM is between 40 and 60 mg/kg b.w. (27), but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose,

but single dose below 40 mg/kg b.w. may be ineffective (28). Burcelin et. al. used intravenous injection of Streptozotocin in rats at a dose of 65 mg/kg body weight to induce diabetes (using cold 0.1 M citrate buffer pH 4.5) (13, 14).

IN VIVO TOXICITY AND STRENGTH

STZ has a reported LD_{50} of 240 mg/kg in mice. Streptozotocin has been used in research for many years to induce diabetes mellitus in rats and mice. Streptozotocin is hydrophilic in nature and does not cross the blood-brain barrier, but its metabolites are found in cerebral spinal fluid (10). Its biological half-life in cell culture medium was shown to be approximately 19 minutes (24). Streptozotocin degradation products resulting from incubation in pH 7.4 phosphate buffer or in plasma were not diabetogenic in rats (41). Studies indicate that as much as 77% of injected Streptozotocin is either broken down and eliminated or eliminated intact within 6 hours of injection. Of that amount 30% is eliminated within the first hour. The majority of the material/metabolites are eliminated (74%) in urine; the remainder (3%) is eliminated in feces. Most of the remaining material is found in the liver and kidney. Streptozotocin is cleaved into segments in the liver and degrades in plasma. Its cleavage/degradation products are not capable of causing diabetes but some remain biologically active and capable of crossing cell membranes (37-42). When administered intravenously, plasma levels of STZ rapidly decrease within 15 minutes and concentrate in the liver and kidneys. As much as twenty percent of the drug (or metabolites containing an N-nitrosourea group) is metabolized and/or excreted by the kidneys (43).

GENDER DIFFERENCE IN STZ SENSITIVITY

Rodents also show a substantial gender difference in STZ sensitivity. Male mice and rats tend to be more susceptible to STZ-induced diabetes (9, 29-34). This difference can be significant, with little or no response in female mice and severe hyperglycemia present in male mice receiving identical doses. In experiments with male and female mice, Leiter reported a highly significant difference ($P < 0.001$) of mean blood glucose values at all-time intervals after day 0, and a mean blood glucose > 200 mg/dL higher in males compared to females 35 days after STZ injection (34). This decreased sensitivity experienced by females may be attributed to estradiol's ability to protect pancreatic β cells from apoptosis induced by oxidative stress (33).

EFFICACY OF STZ OVER ALLOXAN

STZ and alloxan are two important diabetogenic drugs to create animal models of diabetes mellitus. STZ is the preferred agent to induce experimental type 2 diabetes, it has more advantages over alloxan over sustained hyperglycemia and the development of well characterized diabetic complications with a low incidence of ketosis and mortality (60). The range of the STZ dose is not as narrow as in the case of alloxan (9). Higher chemical stability and lower toxicity of STZ allow easier manipulation and more flexible dosing in comparison with alloxan. STZ administration is preferable also due to higher percentage of successful induction of diabetes and lower mortality of experimental animals when compared to alloxan treatment (55). STZ treatment induced diabetes in 95% of rats that is higher than alloxan which has caused diabetes only in 70% of rats (56-59).

DIABETOGENIC ACTION OF STZ

Despite its use to create animal models of diabetes for over several decades, the mode of action of STZ is not fully understood. Mode of action of STZ cellular toxicity includes its selective uptake by pancreatic β cells and its toxic effector mechanism in the animal body to create a diabetic model.

Selective transportation of Streptozotocin across β cell membranes:

Rodents (rats, mice, hamsters) and some other mammals (monkeys and rabbits) are susceptible to STZ induced diabetes. STZ toxic action involves its selective uptake into β cells *via* its low affinity glucose transporter GLUT2 present in the plasma membrane (6, 50). Unlike the general lipophilic nature of its group compounds- nitrosoureas, STZ is hydrophilic in nature due to glucose moiety substitution. Hydrophilic nature of STZ limits its free diffusion across phospholipid bilayered plasma membrane of cells because of their hydrophobicity (6). The 2-deoxy glucose moiety of STZ allows its selective uptake into β cells through glucose transporter (GLUT)-2 due to its structural analogy with glucose (Figure 2a). Hepatocytes and the renal tubular cells also express GLUT 2 transporter and are susceptible to STZ. This reasons the usual kidney and liver damages in STZ induced diabetic models (51). STZ is diabetogenic because it inhibits production of insulin and selectively destroys the insulin-producing beta cells by

inducing necrosis (Figure 2b). Non-β endocrine cells in pancreatic islets such as α- and δ-cell as well as the extra-pancreatic parenchyma remain intact after STZ challenge, indicating the beta cell selective properties of STZ (6,50,52). STZ also causes cardiac and adipose tissue damage and increases oxidative stress, inflammation, endothelial dysfunction with the concentrations of the drug or its metabolites in the liver, kidney, intestine and pancreas being consistently higher than those in the plasma (53). Once STZ enters into the cell, it inhibits the glucose metabolism and insulin secretion of beta cells and impairs the pancreas.

Figure 2. Selective uptake of stz by β cells of pancreas

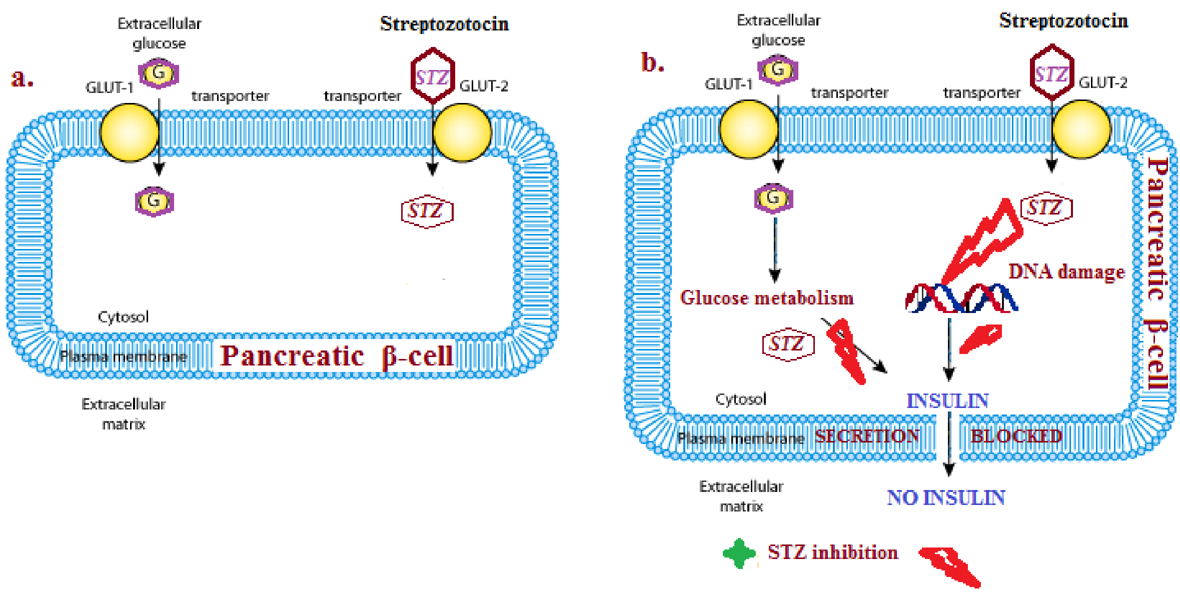

Figure 2: A schematic diagram representing - a)Model of STZ and glucose uptakeby pancreatic beta cells through selective transporters. b) Inhibition of glucose metabolism and inhibition of insulin secretion by STZ in pancreatic beta cells. Red marks represent the inactivation of the metabolic steps of insulin production by STZ.

EFFECTOR MECHANISM OF STZ TOXICITY

Proposed mechanism of β cellular toxicity of STZ includes –

1. Carbamoylation and alkylation of cellular components.
2. Release of nitric oxide (NO).
3. Free radical generation and Oxidative stress.
4. Inhibition of O-GlcNAcase.

1. Carbamoylation and alkylation of cellular components

Antibiotic STZ is a highly genotoxic alkylating agent, which can cause cellular damage including DNA strand breaks and will eventually lead to cell death (6, 62). Once STZ enters inside the cell, it is able to decompose spontaneously to form an isocyanate molecule and a methyldiazohydroxide molecule (62, 63) (Figure3). The isocyanate can undergo intramolecular carbamolyation or can carbamoylate other cellular components like proteins. The methyldiazohydroxide can decompose to form a highly reactive carbonium ion ($CH3^+$) which is believed to be a key player in STZ-induced DNA alkylation causing interstrand DNA cross-links (62,63) (Figure 3).

It is demonstrated that the methylnitrosourea metabolite has four times alkylating activity than intact STZ, suggesting that the presence of the glucose moiety reduces the alkylating action of STZ (50, 64). The carbonium ions (carbocation-$CH3^+$) are highly reactive and can react with unshared pairs of electrons of the nitrogen and oxygen molecules located within the nucleophilic center of DNA (63). Structure activity studies have provided evidence suggesting that the most predominant site for DNA alkylation is at the O^6 position of guanine (6, 63) (Figure 4a).
The methylation at O^6 may interfere with hydrogen bonding and allow guanine to mispair with thymine, thus causing a point mutation (6, 63). In addition to the bases, STZ-mediated DNA alkylation can be targeted to the phosphate backbone resulting in the formation of phosphotriesters, which can cause conformational changes in DNA (6, 50, 63). STZ could also react at other sites of DNA such as the ring nitrogen and exocyclic oxygen atoms of DNA bases, predominantly producing 7-methylguanine, 3-methyladenine which leads to DNA breaks activates poly-ADP-ribose polymerase (PARP) and subsequently depletes NAD+ (6, 50, 61). Here activated PARP catalyzes covalent addition of ADP-ribose groups to many nuclear proteins, causing rapid depletion of NAD^+.

It is hypothesized that the diabetogenic action of STZ in animals is mediated through a reduction of nicotinamide adenine dinucleotide (NAD^+) in pancreatic cells (61).The DNA damage caused by STZ-mediated alkylation is repaired by an excision repair process, which requires the activation of the NAD-dependent enzyme poly (ADP-ribose) synthetase (PARP) (50,63). It is postulated that overstimulation of DNA repair mechanisms in the beta cell this enzyme is

continuously activated, thus depleting the cell of NAD^+ and consequently, ATP stores (Figure 5-1). Cellular ATP stores become depleted in an effort to replenish NAD^+, resulting in the inhibition of insulin synthesis and induces diabetes. The critical loss of NAD^+ leads to a cessation of cellular function and eventually beta cell death (6, 63) (Figure 2b), (Figure 5-1).

Inhibitors of poly ADP-ribosylation suppress the process of DNA methylation. Thus, injection of nicotinamide and other PARP inhibitors in parallel with, or prior to the administration of streptozotocin is well known to protect beta cells against the toxic action of streptozotocin and to prevent the development of a diabetic state (50, 67). Also, mice deficient in PARP are resistant to beta cell death mediated by streptozotocin, in spite of DNA fragmentation. The absence of PARP prevents the depletion of the cofactor NAD+ and the subsequent loss of ATP (65, 66) and thus cell death.

Figure 3. Carbamoylation and alkylation

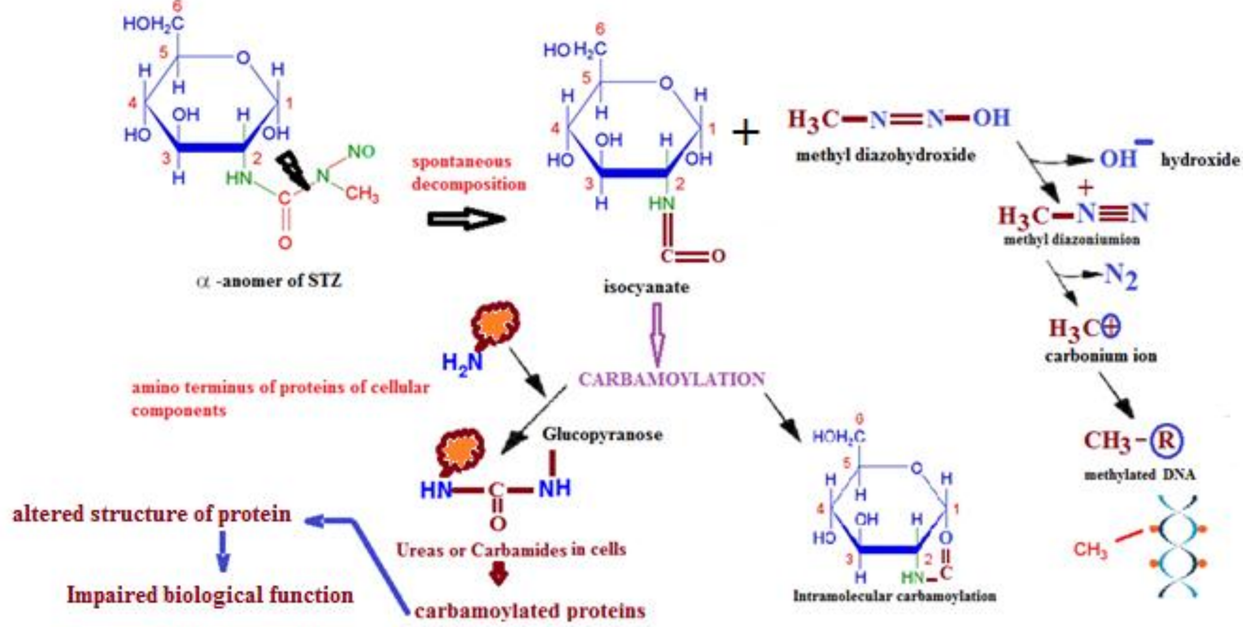

Figure 3: A schematic diagram representing – Spontaneous decomposition of STZ into an isocyanate and methyldiazohydroxide. The isocyanate either carbamoylate proteins or can undergo intermolecular carbamolyation. The methyldiazohydroxide decomposes to form a carbonium ion ($CH3^+$) that alkylate DNA, causing interstrand DNA cross-links and abnormal base pairing.

Figure 4. Methylation and abnormal base pairing

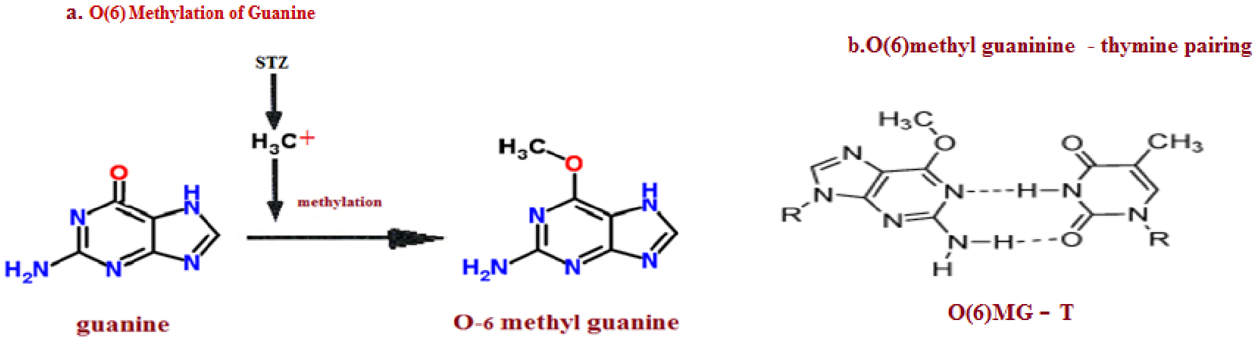

Figure 4: A schematic diagram representing – a) carbonium ion ($CH3^+$) generation bySTZ that alkylate DNA by methylation of guanine at its O^6 position to form O^6 methyl guanine. b) O^6 methylation of guanine leading to abnormal base pairing, where guanine mispairs with thymine.

2. Release of nitric oxide (NO)

Like other nitrosoureas STZ is a potential NO radical donor in *in vivo* conditions, that mediates the destruction of pancreatic β islet cells through DNA damage. After two hours of STZ injection NO release is observed in pancreatic β cells of rats (9, 68). In order to confirm that the NO formation was not attributed to nitric oxide synthtase (NOS), inhibitors of NOS used in the experiment did not block the NO formation generated in the presence of STZ (44). NO release after STZ injection is evidenced by increased activity of guanyl cyclase and the formation of cGMP, which are the characteristic features of NO in execution of its biological function (6, 46).

Variable low levels of free radical scavenging enzymes in pancreatic β-cells makes them more sensitive to cytotoxic action of NO radical (69). It still remains to be determined which are the intracellular targets for NO-induced damage. But available data supports that NO radical produced during STZ metabolism have the following possible intracellular targets, which makes the β-cell dysfunction and death. NO radical inactivates the mitochondrial enzyme aconitase, impairing substrate oxidation and ATP production (6,9,70-72) (Figure 5-2).

Ionic channels and complexes I and II of the mitochondrial electron transport chain are two other possible targets for NO effects which may impair insulin secretion. NO also leads to nuclear DNA damage in both rat and human pancreatic beta-cells, as evaluated by the 'comet assay' (75).

The effects of NO at the DNA level are complex, and involve formation of N-nitrosoamines. NO damages DNA in two ways that is by nitration of nucleic acids and deamination of purines and pyrimidines, or damage induced by peroxynitrite formation by reacting with super oxide free radical (73, 74). Besides inducing over DNA damage, NO may also inactivate DNA repair/replication enzymes. The outcome of NO-induced beta-cell DNA damage can be cell death by apoptosis or, in some cases, necrosis (71-75).

3. Free radical generation and oxidative stress

Reports say that there is a tissue-specific increase in oxidative stress with persistent increase in the generation of free radicals. Free radical production in the early stages of streptozotocin induced diabetic rats includes reactive oxygen and nitrogen species (ROS and RNS) like superoxide radical ($O_2^{\circ-}$), hydroxyle radical ($OH^{\circ-}$), hydrogen peroxide (H_2O_2) and Peroxynitrite ($ONOO^-$), that cause oxidative stress (76,78,80,81) (Figure 5-3). Several studies have demonstrated that hyperglycemia in STZ induced diabetes has been associated with increased formation of reactive oxygen species (ROS) and oxidative damage to tissue compounds (77). Oxidative stress in STZ induced diabetic animals is due to glucose auto-oxidation, protein glycation, formation of advanced glycation products and the polyol pathway that generates free radicals (83, 84).

In STZ induced diabetic rats, there is a significant increase in the levels of plasma glucose, total lipids, triglyceride, cholesterol, lipid peroxides, nitric oxide and uric acid. There is a significant decrease in the levels of antioxidants ceruloplasmin, protein albumin and total thiols found in the plasma of diabetic rats. Lipid peroxide levels are significantly increased in erythrocyte lysate and in homogenates of pancreas, liver and kidney, while catalase; glutathione peroxidase and superoxide dismutase (SOD) activities are decreased in tissue homogenates of pancreas, liver and kidney (6, 70, 79). Superoxide($O_2^{\circ-}$), is generated by mitochondrial respiratory chain, NADPH Oxidase, Xanthine oxidase, respiratory burst, auto oxidation of several biomolecules.Superoxide dismutase (SOD) catalyses the disproportion of weak Superoxide ($O_2^{\circ-}$) to H_2O_2.The hydrogen peroxide (H_2O_2) subsequently generates free radicals such as O_2^- and OH^- (6, 85, and 87). These reactive compounds can cause peroxidation of lipids, resulting in the formation of hydroperoxy fatty acids and endoperoxides.

Superoxide ($O_2^{\circ-}$) reacts NO at 37^0C to form Peroxynitrite ($ONOO^-$) (6,89). Peroxynitrite (PXN) is a short lived free radical and more reactive than its precursors $O_2^{\circ-}$ and NO. PXN is a two electron oxidizing agent, mediates its cytotoxicity through NO and ($O_2^{\circ-}$) (85,86) (Figure 5-3). Some reports have demonstrated its cytotoxicity through NF-κB activation, cell signaling and protein phosphorylation. PXN can initiate self-sustained peroxidation reaction in lipids through its free radicals generated by single electron transfer reactions (6,9,50,85-90). The mitochondria appear to be a highly sensitive target for STZ toxicity. The mitochondrial membrane potential and enzyme activities are altered in STZ treated cells resulting in the inhibition of ATP synthesis (76, 82). Aconitase, a ROS sensitive enzyme present in mitochondrial matrix, that protects mitochondrial DNA (mtDNA) from degradation, markedly inhibited in the diabetic rat tissues (6, 70, and 76). The activities of mitochondrial respiratory enzymes ubiquinol: cytochrome c oxidoreductase (Complex III) and cytochrome *c* oxidase (Complex IV) are also decreased significantly (76). But NADH: ubiquinone oxidoreductase (Complex I) and succinate: ubiquinone oxidoreductase (Complex II) increases moderately in diabetic rats, which are confirmed by the increased expression of the 70 kDa Complex II sub-unit. Increased expression of oxidative stress marker proteins Hsp-70 and HO-1 indicate the susceptibility of pancreas to STZ's induction of oxidative stress (6, 76).

4. Inhibition of O-GlcNAcase

STZ specifically kills islet cells by inhibiting O-GlcNAcase (OGA). O-GlcNAcase is a glycoside hydrolase that cleave the beta-O-linked GlcNAc (N-acetyl glucosamine (O-GlcNAc)) frommodified proteins in the cytosol of β-cell during protein posttranslational modification for the generation of good and safer functional proteins (6, 90-96). Inhibition of OGA (O-GlcNAcase) by STZ results in the hyper-O-GlcNAcylation (due to irreversible O- glycosylation), which cause the accumulation of harmful proteins and activation of stress pathways leading to apoptosis (6, 9, 50, 91, 92) (Figure 5-4). The mode of inhibition involves covalent modification of the enzyme or the enzyme-catalyzed formation of a tight binding inhibitor (93-96).

Figure 5.Mechanism of STZ toxicity

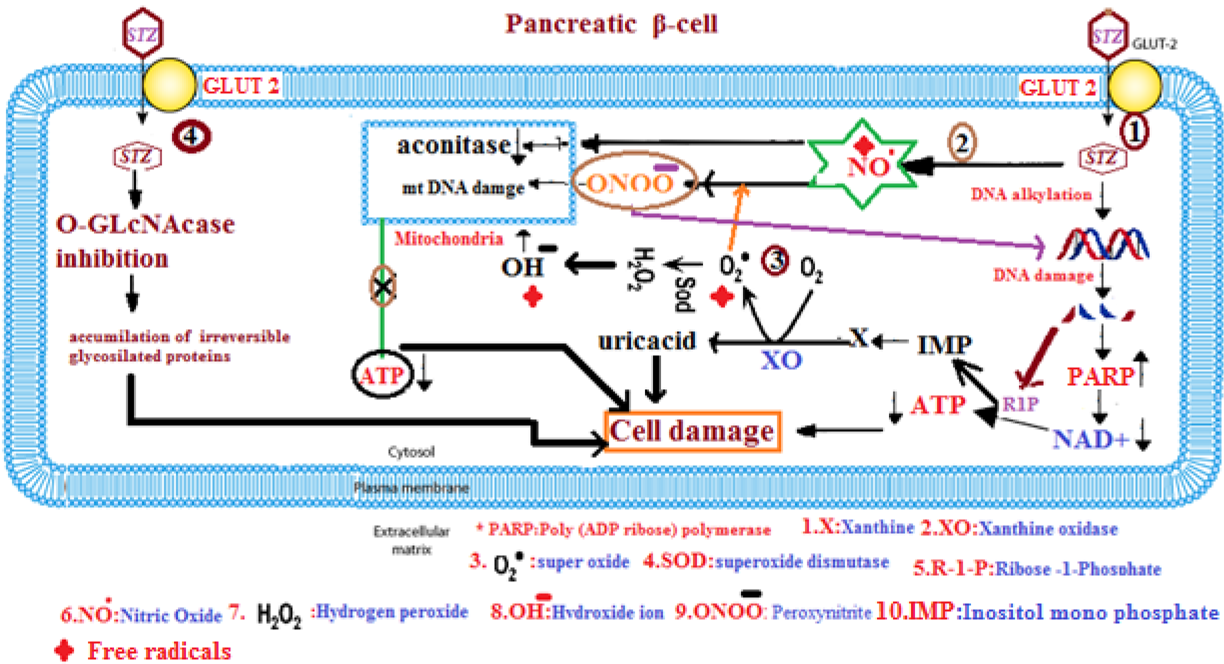

Figure 5: Schematic diagram representing – 1) alkylation of DNAby STZ causing DNA damage, PARP activation,leading to depletion of NAD$^+$and ATP stores that causes pancreatic beta cell death 2) Spontaneous release of NO by STZ, impairment of mitochondria by NO, inhibition of APT synthesis ultimately leading to beta cell death.3) Generation of free radicals like superoxide ($O_2^{\circ-}$), hydroxide ($OH^{\circ-}$), peroxynitrite ($ONOO^-$), causing beta cell damage. 4) Inhibition of O-GlcNAcase by STZ, formation of irreversible glycosylated proteins that damage pancreatic beta cell.

CONCLUSION

Streptozocin is a natural diabetogenic agent that induces permanent diabetes in animal models by damaging pancreatic β-cells that stops insulin production. Its β-cell toxicity is reasoned through Carbamoylation of proteins, alkylation of DNA, release of free radicals (ROS and RNS) and inhibition of O-GlcNAcse. β-Cell insulin production is impaired by methylation of DNA through formation of carbonium ion ($CH3^+$), resulting in the provocation of nuclear enzyme poly ADP-ribose synthetase (PARP) and therefore, depletion of NAD$^+$and ATP. Free radicals generated during decomposition and metabolism of STZ diminish the activities of mitochondrial enzymes and inhibit O-GlcNAcse a (glycoside hydrolase) causing totarnish energy levels of cell and

suppressing biological function of proteins of islet cells. The above mentioned harmful events induced by STZ are responsible for necrosis of pancreatic β-cells and induction of experimental diabetes mellitus in laboratory animal models.

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