

# Effect of Endogenous and Exogenous Nitric Oxide Induced Alterations in Pancreatic Architecture via Oxidative Stress in Female Mouse, *Mus musculus*

Rashmi Richa<sup>1</sup>, Chandra Mohini Chaturvedi<sup>2</sup>Research Scholar, Department of Zoology, Banaras Hindu University, Varanasi, India<sup>1</sup>Professor, Department of Zoology, Banaras Hindu University, Varanasi, India<sup>2</sup>

**ABSTRACT:** While STZ has been considered as endogenous NO donor and ameliorating pancreatic  $\beta$ -cell functions, SNP, a conventional NO donor associated with nitrosative stress and its effects on pancreatic architecture are still not clear. In view of the above background information, present study was undertaken with the objective to investigate the effects of endogenous (STZ) and exogenous (SNP) NO donor induced nitrosative stress on pancreatic architecture and  $\beta$ -cell physiology. Swiss strain female mice, *Mus musculus* were divided into 3 groups- control group, STZ group (an endogenous NO donor, administered with single dose of 16 mg/100 gm body weight) and SNP group (an exogenous NO donor, administered with SNP 0.25 mg/100 gm body weight) for 7 days intraperitoneally. Results showed that NO level was significantly increased in both STZ and SNP treated mice accompanied with increased AGE, MDA and AOPP level in plasma as well as in pancreatic tissue. Increased intracellular ROS and  $Ca^{2+}$  displayed by isolated pancreatic islets. Intense immunoreactivity for iNOS was seen in both treated groups as compared to control. However, strong immunoreactivity for insulin in the  $\beta$ -cells of pancreatic islets was observed in SNP treated mice which was lacking in STZ treated mice due to selective destruction of  $\beta$ -cells by the diabetogenic drug STZ. Morphometric results demonstrated that pancreatic architecture as well as  $\beta$ -cell physiology was altered in both endogenous (STZ) and exogenous (SNP) NO donor groups. Our findings suggest that both NO donors executed their effects by causing oxidative stress (increased ROS) and islet inflammation (increased *ir-iNOS*). Further, exogenous NO donor provokes hyperinsulemia like condition.

**KEYWORDS:** Nitric oxide, Hyperglycemia, Insulin,  $\beta$ -cell mass, iNOS, Inflammation.

## I. INTRODUCTION

Diabetes mellitus is a metabolic disease associated with hyperglycemia and involves accelerated non-enzymatic glycation reactions [17] [33], oxidative stress (imbalance between reactive oxygen and nitrogen species, with antioxidants enzymes), and carbonyl stress (increase of reactive carbonyl compounds caused by their enhanced formation or decreased degradation) [19] [2]. These processes aggravate the formation of advanced glycation end-products (AGEs) and advanced oxidation protein products (AOPP) and damaged the biologically important compounds [34][19]. Accumulation of AGEs has several toxic effects; it modifies proteins, directly damage the structure and metabolism of extracellular matrix or act *via* their specific receptors (e.g. RAGE, receptor for AGE). AGEs and AOPP accumulate in tissues and cause damage to biological membranes and thus take part in the development of diabetic complications [12].

Nitric oxide (NO) a signalling molecule synthesized endogenously from L-arginine has been shown to play a critical role in a wide variety of physiological functions including neurotransmission, vascular tone, platelet aggregation, immunological reactions, penile erection, endocrine and paracrine function [20]. NO has also been

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implicated as the effector molecule responsible for the selective destruction of beta cells of the pancreatic islet [13]. Excessive NO production is an important contributing factor which augmented increased expression of glucose-stimulated insulin release in the diabetic condition [27]. Numerous studies report the occurrence of a constitutive NO synthase (cNOS) activity in both endocrine cells and nerves of the pancreatic islet tissue [24]. Effects of NO on insulin secretion from the  $\beta$ -cells of pancreatic islets are not conclusive due to contradictory reports that NO may increase, [16], [30] or decrease insulin secretion [10].

Further, a lot of studies have addressed the effects of hyperglycemia on pancreatic  $\beta$ -cell function but less information is known on the effects of endogenous and exogenous NO on metabolic functions and pancreatic islet architecture. In view of the above background information and to correlate hyperglycemia and nitrosative stress present study was undertaken to study the effect of endogenous donor (STZ) and exogenous NO donor (SNP) on pancreatic architecture and  $\beta$ -cells with reference to hyperglycemia.

## II. MATERIALS AND METHODS

### A. Animals

Twelve week old female ( $25\pm 3$ g) Swiss strain mice (*Mus musculus*) were procured from the central animal resource section of Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The animals were housed in 50cm x 30cm x 22cm polypropylene cages with maximum 10 animals per cage and acclimatized for a period of 10 days. Mice were housed under standard laboratory conditions of light: dark cycle of 12: 12, temperature ( $25\pm 3^{\circ}\text{C}$ ), relative humidity ( $55\pm 5\%$ ) and were provided standard rodent food pellets and tap water *ad libitum*.

### B. Experimental design

Female mice weighing  $25\pm 3$ g were randomly divided into three groups (n =10 in each group). 1) Control group, given normal saline 2) STZ group (treated with a single dose of STZ at a dose of 16 mg/100 gm body weight intraperitoneally 3) SNP group (treated with SNP at a dose of 0.25 mg/100 gm body weight for 7 days intraperitoneally). Animals were sacrificed by decapitation and blood was collected in microcentrifuge tubes and centrifuged at 5000 RPM for 20 minutes. After that, plasma was isolated and then it was stored in  $-20^{\circ}\text{C}$  for biochemical assay. All protocols used in this study were approved by the Institutional Animal Care and Use Committee and within the framework of the revised animals (Scientific procedures) Act of 2002 of the Govt. of India on the Animal Welfare.

### C. Pancreatic somatic index (PSI)

PSI is used for measuring the pancreatic weight of animals in relation to the body weight and is expressed as PSI (%) by using formula; Pancreas Weight / Body Weight x 100.

### Pancreatic islet isolation and preparation:

Pancreatic islets were freshly isolated from the splenic part of the pancreas of female mouse by a collagenase digestion technique. Islet isolation was performed by retrograde injection of a collagenase solution *via* the bile-pancreatic duct [25]. Briefly, islets were separated from pancreatic acinar tissue and picked up, using a finely drawn out with pipette in Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4.

### E. Fluorescent imaging of intracellular ROS

According to Ravier and Rutter [25] freshly isolated pancreatic islets were incubated in Krebs's Ringer bicarbonate (KRB) buffered solution containing DCF-DA (Dichlorofluorescein-Diacetate) at a final concentration of 5.0  $\mu\text{M}$  for 20 min at RT, then washed twice with KRB buffer. Islets were placed on glass slide and imaged under fluorescent microscope Carl Zeiss Axioskop plus 2. ROS generation was evaluated as fluorescence intensity  $E_x$  488 nm,  $E_m$  515 nm of DCF.

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### F. *Fluorescent imaging of intracellular Ca<sup>2+</sup>*

According to Ravier and Rutter [25] Freshly isolated pancreatic islets were incubated in calcium free KRB buffer containing 1 $\mu$ M Fura-2 AM working solution for 10 min at RT. Islet were placed on glass slide and images were taken under fluorescent microscope Carl Zeiss Axioskop plus 2. Intracellular Ca<sup>2+</sup> was evaluated as fluorescence intensity E<sub>x</sub> 340 nm to 380 nm in response to calcium binding and E<sub>m</sub> 505 nm.

### G. *Estimation of nitric oxide (NO<sub>x</sub>) level*

Total nitrate-nitrite concentration was measured in pancreatic tissue according to Sastry *et al.* [29]. Briefly, 10 % tissue homogenate (w/v) was prepared in 0.01M phosphate buffer pH 7.4. To 100  $\mu$ l of tissue sample or standard was added 400  $\mu$ l of carbonate buffer followed by a small amount (~0.15 g) of activated copper-cadmium alloy fillings and incubated at room temperature with thorough shaking. The reaction was stopped by the addition of 100  $\mu$ l of 0.35M NaOH followed by 120mM ZnSO<sub>4</sub> solution, vortexed and allowed to stand for 15 min. Tubes were then centrifuged at 8000 rpm for 10 min. 100  $\mu$ l of aliquots of clear supernatant were transferred into the 96 well micro plate (in triplicate) and Griess reagent (50  $\mu$ l of 1% sulphonilamide prepared in 2.5% orthophosphoric acid and 50  $\mu$ l of 0.1% *N*-naphthyl ethylenediamine (NEDD) prepared in distilled H<sub>2</sub>O) was added to it. After 10 min, the absorbance was read at 545 nm in an ELISA Reader (ECIL, India). Results were expressed as nmoles/mg of protein.

### H. *Malondialdehyde (MDA) assay*

Malondialdehyde (MDA), an end product of unsaturated fatty acid peroxidation, can react with thiobarbituric acid (TBA) to form a colored complex called thiobarbituric acid-reactive substance (TBARS). For each sample to be assayed, the reaction mixture contained 8% SDS, 20% acetic acid, BHT, TBA, 0.2% Triton-X were incubated at 95<sup>o</sup>C for 1 hr. Samples were centrifuged at 3000 rpm for 10 minutes. 200  $\mu$ l of supernatant was transferred to 96 well microplate and absorbance was measured at 532 nm using a microplate ELISA reader (ECIL, India). Concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10<sup>5</sup> and was expressed in  $\mu$ mol MDA/ mg of protein.

### I. *Advanced glycation end products (AGE) assay*

AGE products can be measured spectrofluorometrically according to the method of Kalousova *et al.* [12] with slight modifications. For AGE- related fluorescence, plasma was diluted 1:50 with PBS. Fluorescence was determined in a Perkin Elmer LS-45 Luminescence Spectrometer at the wavelengths 498 nm/ 426 nm (excitation/emission). Results were expressed in arbitrary units (AU)/ mg of protein.

### J. *Advanced oxidant protein products (AOPP) assay*

AOPP can be biochemically assayed according to Kalousova *et al.* [12]. Reaction mixture contains 1.16mol/L of potassium iodide (KI), glacial acetic acid (100 % v/v) and PBS (20mmol/L). Chloramine-T stock solution (10 mmol) was diluted 100 times with PBS to make working solution of 100  $\mu$ mol/L. Chloramine-T standards for PBS calibration curve were prepared by diluting this solution with PBS to concentrations of 12.5, 25, 50, 75, 100  $\mu$ mol/L. The samples were processed at room temperature. 160  $\mu$ L of PBS reactive was added on 40 $\mu$ L plasma and incubated for 25 seconds. The absorbance of the mixture was measured spectrophotometrically at 340 nm. Subsequently 20 $\mu$ L of acetic acid was added and incubated for 25 seconds the absorbance was re-measured. Finally 10 $\mu$ L of KI solution was added and absorbance was re-measured after another 25 sec of incubation. Concentration of AOPP was determined in chloramine units ( $\mu$ mol/L) and values adjusted for protein levels were calculated as ( $\mu$ mol/L/g protein).

### K. *Enzymatic assay of aldose reductase (AR)*

Aldose reductase activity was assayed according to the method of Saraswatet *al.* [28]. The assay mixture in 1ml contained 50 mM potassium phosphate buffer pH 6.2, 0.4 M lithium sulphate, 5 mM 2- mercaptoethanol, 10 mM DL-glyceraldehyde, 0.1 mM NADPH and brain samples. Appropriate blanks were employed for corrections. The assay mixture was incubated at 37<sup>o</sup>C and the reaction was initiated by the addition of NADPH at 37<sup>o</sup>C. The change in the absorbance at 340 nm due to NADPH oxidation was followed in a Lamda35 spectrophotometer (Perkin-Elmer, Shelton, U.S.A.). Results were expressed in U/mg of protein.

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## L. *Histological procedures*

Pancreatic tissues were excised from the mice sacrificed by decapitation weighed, and fixed in paraformaldehyde (PFA) for 24 h. Fixed tissue was sequentially embedded in paraffin wax blocks according to standard protocol and 6  $\mu$ m thick sections were cut using rotary microtome. These sections attached on gelatin coated glass slides were deparaffinised with xylene, and processed for routine histology with Haematoxylin and Eosin. Slides were examined using a light microscope (Carl Zeiss Axioskope plus 2).

## M. *Immunohistochemistry of Insulin and i-NOS*

Pancreatic tissues were excised and fixed in 4% PFA (paraformaldehyde) for 24 hrs. Tissue were dehydrated and embedded in regular paraffin wax. Tissue blocks were prepared and serial sections of pancreas were cut (6  $\mu$ m thickness) on a microtome (LEICA) and collected on gelatin-coated slides. Tissue sections were deparaffinised rinsed in PBS and endogenous peroxidase activity was blocked by immersion in 0.03% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature. Sections were preincubated in 10% normal goat serum for 1 hr. and subsequently incubated in rabbit anti-insulin antibody (1:250, Santa Cruz) and anti-iNOS antibody (1:500, Sigma, USA) for overnight at 4°C. The second incubation with Vectastain Avidin-Biotin complexes Kit (Vector Laboratories, Burlingame, CA, USA) with some modifications as described by Singh and Chaturvedi, [31]. Pancreatic sections were visualized with 0.06% 3, 3-diaminobenzidine (DAB) in PBS containing 0.3% H<sub>2</sub>O<sub>2</sub>. Counter-staining was performed with haematoxylin and were rehydrated in different grades of alcohol, cleared in xylene and mounted in DPX. Haematoxylin was used as the counterstain. Immunopositive areas were measured using analysis software (Image J System, USA). The image analysis software NIH Image J was used to calculate the total pancreatic tissue area, pancreatic islet area, and insulin-positive cells in the pancreatic islets to determine the % pancreatic islet area (relative to the pancreatic area) and the % cell count (relative to the pancreatic islets).

## N. *Morphometric assessment of the pancreatic islets*

For determination of the  $\beta$ -cell areas, entire pancreatic sections were immunoperoxidase stained for insulin were imaged at 10X magnification (10X objective) using a Carl Zeiss Axioskope microscope equipped with a motorized stage. The fractional areas of the pancreas, stained positive for insulin were digitally quantified using a color-based threshold using Image J analysis software (USA) to measure insulin+ve surface areas, and to determine the number of nuclei in the insulin +ve areas..

The following equations were used to calculate morphometric parameters of islet architecture:

$$\text{Pancreatic somatic index (PSI, mg/100g body weight)} = \text{Pancreas weight} / \text{Body weight} \times 100$$
$$\beta\text{-cell mass} = \beta\text{-cell relative volume} \times \text{pancreas weight}$$

## K. *Statistical Analysis*

Data is expressed as means ( $\pm$  SEM). Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by the Dunnett test for post hoc analysis.  $p < 0.05$  was considered statistically significant.

## III. RESULTS

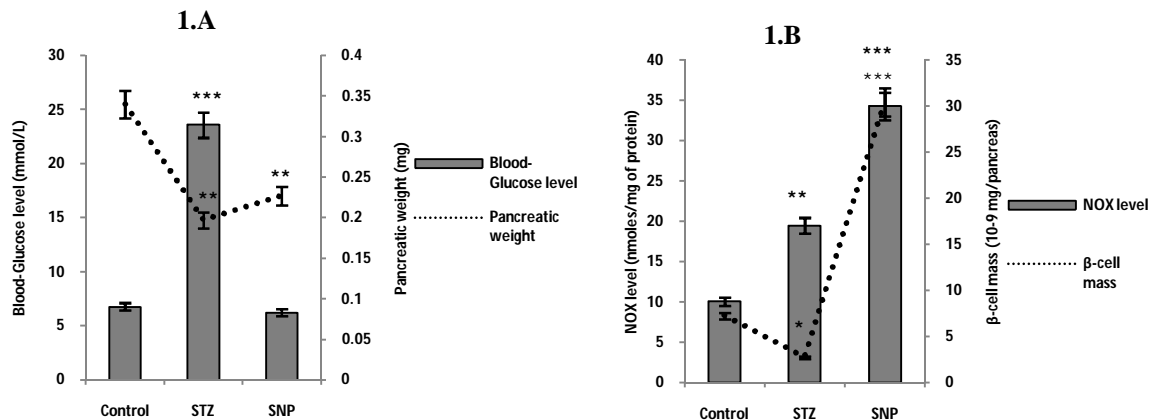
### Endogenous and exogenous NO donors alter islet parameters via intracellular ROS and Ca<sup>2+</sup> production

Significant increase in blood glucose level was noted in STZ treated mice although no change in blood glucose level was noted in SNP treated mice as compared to control (Fig. 1A). Significant decrease in pancreatic weight was noted in STZ and SNP treated mice as compared to control (Fig. 1A). On the other hand, increased NO<sub>x</sub> level accompanied with increased  $\beta$ -cell mass in both the treated groups compared to control (Fig. 1B). However, pancreatic somatic index (PSI) were significantly declined in STZ and SNP treated mice with significantly increase in islet area of both groups compared to control (Fig. 2A and B).

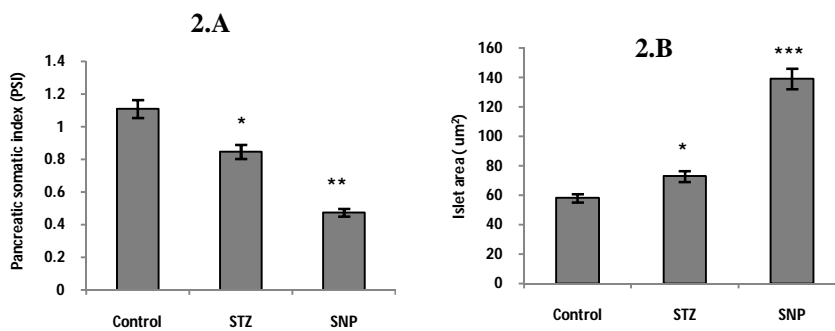
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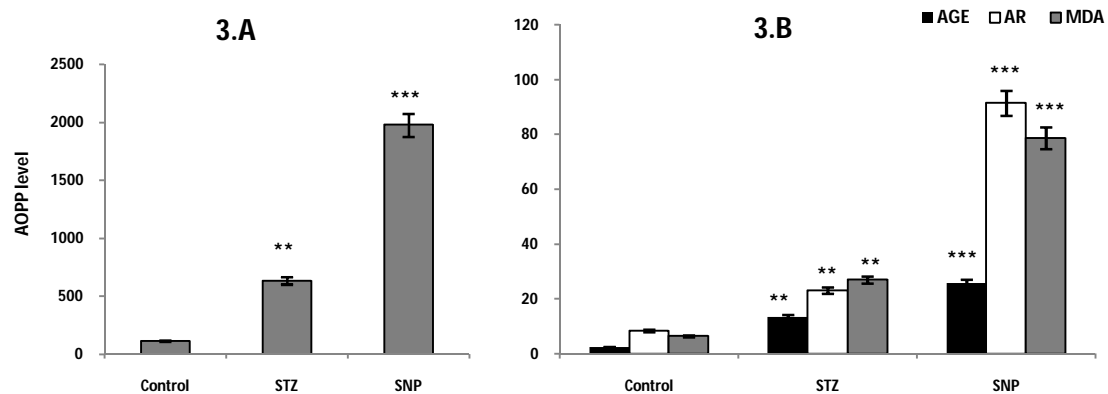
**Figure 1** Fig. 1.A shows blood-glucose level and pancreatic weight in control, STZ and SNP treated mice. Fig. 1.B shows total nitrite- nitrate concentration (Nitric oxide level) and β-cell mass in control, STZ and SNP treated mice. Data are presented as mean ± S.E., \*\*p<0.01, \*\*\*p<0.001 significance of difference from control group.



**Figure 2** Fig. 2.A shows pancreatic somatic index (PSI) in control, STZ and SNP treated mice. Fig. 2.B shows islet area in control, STZ and SNP treated mice. Data are presented as mean ± S.E., \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 significance of difference from control group.

## Nitrosative stress affects metabolic parameters

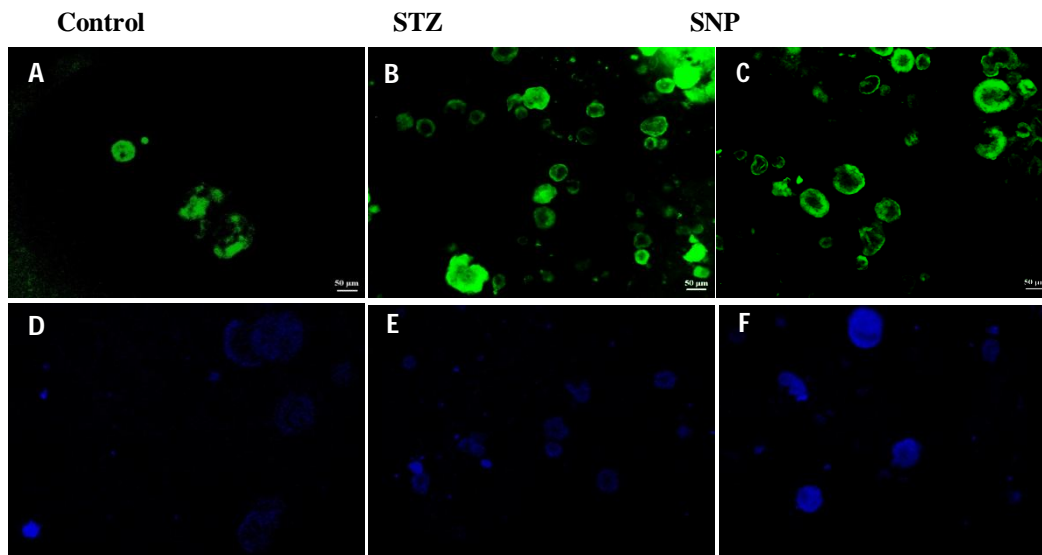
Plasma levels of AGE and AOPP were significantly increased in STZ and SNP group (Fig. 3A). Same results were obtained in MDA content accompanied with increased activity of AR in pancreatic tissue of both the treated groups as compared to control (Fig. 3B).



**Figure 3** Effect of endogenous and exogenous NO donors on the plasma levels of AGE, AOPP and AR activity and MDA level in pancreatic tissue of control, STZ and SNP treated mice. Data are presented as mean  $\pm$  S.E., \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  significance of difference from control group.

#### Nitrosative stress contributes to intracellular ROS and $Ca^{2+}$ production

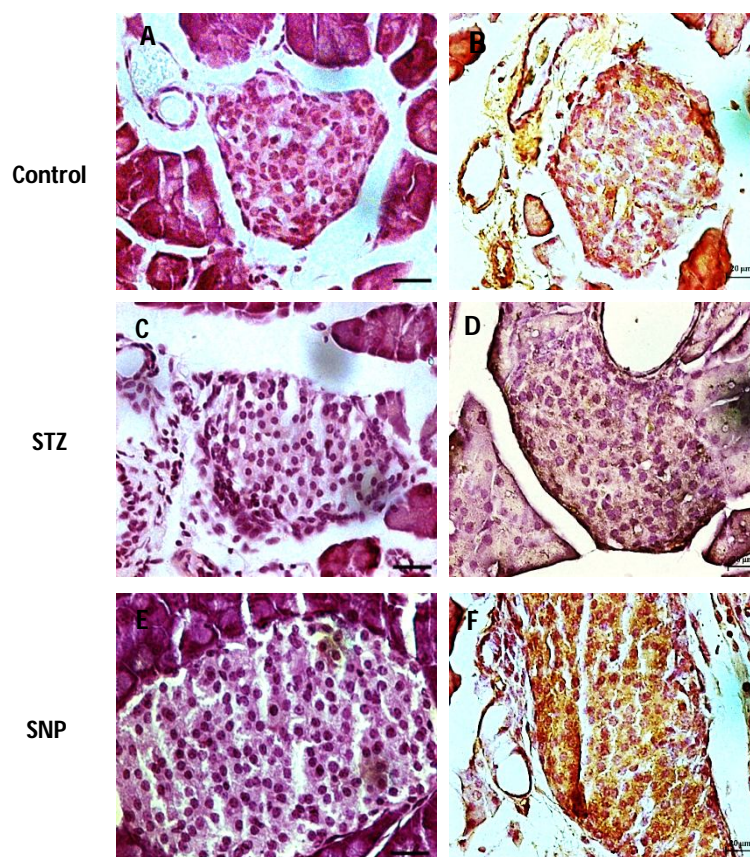
Isolated pancreata displayed intense staining of DCF-DA in the islet cells exhibiting increased intracellular ROS in both the treated groups accompanied with enhanced intracellular free  $Ca^{2+}$  stained with calcium binding dye FURA-2 AM (Fig.4).



**Figure 4** Photomicrographs of isolated pancreatic islet cells stained with DCF-DA fluorescent dye showing bound intracellular ROS and cytosolic free  $Ca^{2+}$  stained with FURA-2 AM dye in (A,D) control group, (B,E) STZ and (C,F) SNP treated mice. Note increased amount of ROS and free  $Ca^{2+}$  in both the treated mice groups compared to control. Results shown are representative images from different pancreas samples from each group.

### Histology of pancreatic islets

Pancreatic islet of (C) STZ treated diabetic mice pancreas displayed degranulated and reduced cell density with in distinct islet border whereas reduced cell density and hypertrophied islet size were observed in the pancreatic islets of (E) SNP treated mice compared to (A) control group (Fig. 5).



**Figure 5 Immunohistochemical localization of insulin in pancreatic islets.** Note the absence of *ir*-insulin in (D) STZ treated mice pancreas and strong immunoreactivity for insulin in (F) SNP treated mice compared to (B) control. T.S of pancreas stained with Haematoxylin & Eosin showed degranulated and reduced cell density in the pancreatic islet of (C) STZ treated diabetic mice. Increased islet size with reduced cell density were observed in (E) SNP treated mice compared to (A) control. Results shown are representative

### Nitrosative stress modulates insulin secretion

Immunohistochemical staining of the pancreatic tissue in (B) control group displayed normal islet architecture with normal immunostaining of *ir*-insulin in  $\beta$ -cells. A single high dose of STZ (endogenous NO donor) provoked extensive  $\beta$ -cell degranulation, reduced cellular density and as a result, significantly decreased pancreatic insulin expression in (D) STZ group mice. In contrast, an exogenous NO donor, SNP exhibits an insulintrophic effect on pancreatic islets showed intense immunostaining of *ir*-insulin in pancreatic islet of (F) SNP treated group (Fig. 5).

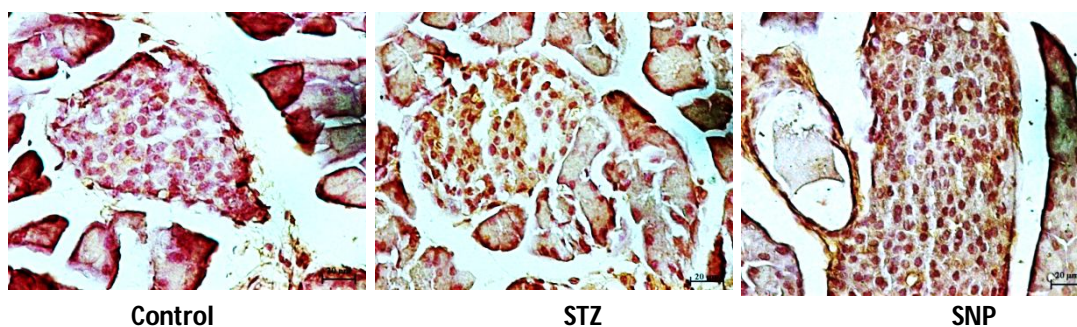
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### Nitrosative stress stimulates iNOS secretion from pancreatic islets

Intense immunoreactive *i*-NOS staining have been found in most of the cells of pancreatic islets and in exocrine parenchyma of STZ and SNP treated groups compared to control (Fig. 6).



**Figure 6 Immunohistochemical localization of *i*-NOS.** Note intense immunostaining of *i*-NOS in STZ and SNP treated groups as compared to control group. Results shown are representative images from different pancreas samples immunostained for *ir*-iNOS from each group.

### IV. DISCUSSION

Present study examined the effects of endogenous (STZ) and exogenous NO donor (SNP) on pancreatic architecture and pancreatic functions with special reference to  $\beta$ -cells. These findings indicate that STZ treatment not only induced an increase in blood glucose level leading to hyperglycemia but also decreases pancreatic weight of STZ and SNP treated groups (Fig.1A). Previous studies have indicated that NO has been proposed as a mechanism mediating the diabetogenic effects of STZ and there is an overproduction of NO when animals are injected with STZ and diabetes mellitus develops [13]. Precise level of NO is reported to be crucial in determining its resultant effect, higher levels of NO becoming cytotoxic [16] and causes cellular damage. However, in spite of the finding that STZ induced hyperglycemia was accompanied with elevated NOx level and decreased  $\beta$ -cell mass, whereas SNP administration induced NOx level results in increased  $\beta$ -cell mass, was not accompanied with hyperglycemia as compare to control (Fig.1.B).

Our results have shown that both STZ and SNP treatment resulted in high amount of NO production from the pancreatic islets resulted in nitrosative stress resulted in decreased pancreatic somatic index (PSI) accompanied with increased islet area observed only in SNP treated group but it was decreased in STZ treated group due to selective action of hyperglycemic drug causes islet destruction (Fig. 2A, 2B) as well as augmented the intracellular generation of reactive oxygen species (ROS) and  $Ca^{2+}$  (Fig. 4). NO is able to combine with reactive oxygen species (ROS) and thus induces oxidative damage [27]. Nitrosative stress increases oxidative load which damages the islet cells and created an inflammatory environment. In response to inflammatory stimuli, pancreatic  $\beta$ -cells produce high cytotoxic levels of NO production *via* *i*-NOS activity leading to  $\beta$ -cell damage and dysfunction (Fig. 6). Our results coincide with [13] reported that high levels of NO produced by *i*-NOS in a calcium-independent manner over prolonged periods of time, are implicated in the cytotoxicity of activated macrophages. Sustained elevation of islet *i*-NOS activity and excessive production of NO cause pancreatic inflammation in pancreatic islets of STZ and SNP treated group give rise to certain pathological conditions. Excess NO production by a cytokine inducible NOS (iNOS) participates in host defense and immunological reactions and may play a role as effector molecule in autoimmune diseases [32]. Increased  $\beta$ -cell mass in SNP treated mice correlated with increased insulin secretion, showed intense expressions of *fir*- insulin in pancreatic islets (Fig 1.B and 5).



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In the present study, morphometric analysis of islet of Langerhans was studied with special reference to  $\beta$ -cells under different experimental conditions. It was observed that STZ and SNP induced nitrosative stress affected these parameters variably. These responses indicate that as expected, STZ increased blood glucose level, (which were not affected by SNP). It was noted that STZ induced hyperglycemia was associated with an increase in NO level but SNP induced NO level was not accompanied by increased blood glucose level. In diabetes, elevated levels of AOPP and AGE products were reported. Our results showed that high levels of advanced oxidant protein products (AOPP) and AGE level was present in the pancreatic tissue of STZ group as well as in SNP treated group compared to control (Fig. 3A). It was also interesting to found that nitrosative stress accelerated the activity of aldose reductase (AR) in both STZ and SNP treated group (Fig. 3B). AR is a key enzyme in polyol pathway and converted the excess glucose into fructose which was further converted to sorbitol by sorbitol dehydrogenase (SD) enzyme. AGEs formed in the presence of both oxygen and sugar, namely glycoxidation products, but those derived from lipoxidation [23] or advanced lipoxidation end products (ALEs) in diabetic vascular complications [5]. Concentrations of AOPP were correlated with advanced glycation end (AGE) products, as the indices of oxidant-mediated protein damage in pancreatic tissue. Like hyperglycemic condition, nitrosative stress induces protein crosslinking too and alters pancreatic architecture through nitro-oxidative damage.

On the other hand in response to nitrosative stress, PSI and islet area were significantly increased in STZ and SNP treated group whereas pancreatic weight was decreased in both the treated groups compared to control. Further,  $\beta$ -cells mass as well as *ir*-insulin was decreased in STZ treated mice but significantly increased in SNP treated group compared to control. Studies done by Larsen *et al* [12] suggests that  $\beta$ -cells responded to combined glucose and arginine stimulation correlated with  $\beta$ -cell mass. In our results, we observed that in response to exogenous NO donor (SNP), increased *ir*-insulin expression was observed in most of the islets result in hypertrophy of  $\beta$ -cells due to accumulation of AOPP and AGE products. Increased  $\beta$ -cells mass and islet area were associated with  $\beta$ -cell hypertrophy and hyperplasia as studies done by Montanya *et al*, [22]. In this study exogenous NO (SNP) has been pointing towards a stimulatory effect of pancreatic NOS activity on  $\beta$ -cell function, hence insulin secretion. Anne *et al*. [1] colocalized pancreatic nNOS and the insulin and indicated that nNOS is associated with insulin secretory granules. This concept was strengthened by our morphometric analysis data in which  $\beta$ -cell mass was significantly increased suggesting the involvement of endogenous and exogenous NO in altered islet architecture.

## V. CONCLUSION

In conclusion, the current study has revealed that nitrosative stress induced by either endogenous (STZ) or exogenous source (SNP), plays an important role in the  $\beta$ -cell function and hence altered pancreatic architecture via oxidative stress and pancreatic inflammation.

## VI. ACKNOWLEDGEMENT

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## VII. DISCLOSURE

The authors have no competing interest.

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## BIOGRAPHY



**Rashmi Richa** is a Research Scholar in the Department of Zoology, Banaras Hindu University, Varanasi, UP, India



**Chandra Mohini Chaturvedi** is working as a Professor in the Department of Zoology, Banaras Hindu University, Varanasi, UP, India