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RESEARCH ARTICLE

ASTAXANTHIN AMELIORATES DIABETIC NEUROPATHY VIA MODULATING THE INFLAMMATORY CYTOKINES IN STZ INDUCED DIABETIC MICE

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Abstract

Background: Diabetic neuropathy (DN) is caused by hyperglycaemia which results in behavioral impairment, oxidative stress and inflammation.

Objective: The present study investigates the ameliorative effect of astaxanthin (AX) in brain tissue via modulating the inflammatory cytokines in diabetic neuropathic mice. This study was conducted by inducing diabetes in mice using STZ (40mg/kg i.p). To study ameliorative effect of AX on levels of TAS, TOS, OSI and expression of KLFs in brain tissue were analysed. Later, morphological impairments in neurons were studied in frontal cortex and levels of TNF-α, IL-1β, IL-6, inducible NO synthase (iNOS) and caspase-3 were analysed.

Results: AX administration attenuated STZ induced alternation in behavioural impairment, normalized the levels of TOS, OSI, TNF-α, IL-1β, IL-6, iNOS and caspase-3 level and significantly (p<0.001) increased the TAS.

Conclusions: This study revealed that AX optimised the levels of TOS, OSI, TAS and reducing the levels of TNF-α, IL-1β, IL-6, iNOS and caspase-3 in Diabetic mice. These suggest that AX could have a beneficial effect by ameliorates diabetic neuropathy via modulating the inflammatory cytokines.

Introduction:-

Diabetic neuropathy (DN) is one of the common dangerous complications of diabetes. It is caused by elevated glucose level which results in damage at cellular and molecular levels. According to the World Health Organization estimates, about five percent of the people are afflicted with diabetes and this number will double by 2025 (WHO). It is one of the chronic complications in which disease pathogenesis is often associated with pain, as confirmed by many neuropathic behavioural tests (Gaur et al., 2019; Kumawat et al., 2019). Increased glucose levels mediated glycation products formation, autoxidative glycosylation, and elevated polyol pathway activity, are some underlying mechanisms in DN. The major cause of DN is increased oxidative stress in neuronal tissues. Oxidative stress results from excess production of oxygen free-radicals like hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$) etc. which further result in reduced antioxidant enzyme's activity to eliminate them (Gaur et al., 2019).

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Astaxanthin (AX) is a reddish-orange xanthophyll carotenoid potent drug for the control of progression of different diseases including cardiovascular diseases, inflammatory diseases, nephropathy and nevertheless the neurodegenerative diseases. AX is also found in chlorophyta member of *Haematococcus pluvialisa* algae (Dhankhar et al., 2012; Gaur et al., 2022; Hussein et al., 2016). However, the effects of AX on KLFs and oxidative stress parameters in experimental DN have not been studied.

Therefore, in this study, we hypothesize that inflammatory cytokines level might be altered in diabetic neuropathic brain. Further we have studied whether the astaxanthin administration may normalize the levels of antioxidant status and behavioural impairments in diabetic neuropathic brain, employing the STZ induced mice model.

Material and Methods:-

Animals

Healthy male swiss albino mice (20-25g) were obtained from the Lala Lajpat Rai University of veterinary and animal science, Hisar (Haryana). We provided free access to standard feed and water to animals. All mice were housed in cages (three in each) at a temperature of 24 ± 1 °C and humidity of 55 ± 5%, with a 12-h light dark cycle. Chemicals like streptozotocin, astaxanthin were purchased from (Sigma, USA), glucose was estimated by the GOD-POD kit (Sigma, USA). All other chemicals were also analytical grade.

Diabetes Induction

Consecutive five doses of streptozotocin (STZ) (40 mg/kg i.p.) per day were injected to induce diabetes in mice. Control mice of the same age received the same amount of citrate buffer (vehicle). After 48 hrs since the last STZ injection, diabetes was confirmed. GOD/POD kit was used to estimate the plasma glucose levels and mice with glucose level >200 mg/dl were confirmed diabetic (Gaur et al., 2022).

Experimental design

Animals were divided into two experimental groups of diabetic neuropathic mice and non-diabetic mice; each group was further divided into three groups containing 6 mice each. Diabetic group of mice was divided into 3 groups (1) diabetic control, (2) diabetic mice treated with (2mg/kg body weight/day) AX and (3) diabetic mice treated with (4mg/kg body weight/day). Non-diabetic group of mice were also divided into 3 groups (1) non-diabetic control, (2) non-diabetic mice treated with (2mg/kg) AX and (3) non-diabetic mice treated with (4mg/kg) AX. AX (commercial) was administered through intraperitoneal injections (2 and 4 mg/kg) for four weeks after confirmation of development of DN. Body weight of mice was monitored weekly which was already reported in our previous paper in detail (Gaur et al., 2021).

Behavioural Tests for confirmation the DN

Eddy's Hot Plate test

In this test, mice from the various groups were separately placed on the hot plate at a temperature of 55 ^{0}C. The first signal of jumping or paw licking to avoid the heat was considered as the reaction time; the cut off time was 10- 12 sec to avoid damage to the mice (Kuhad and Chopra 2009).

Randall Selitto test

In this test, pressure was applied at the paw of mice from various AX administered groups at a linear rate of 10g/s with cut off value < 50g. Paw pressure threshold was measured by the paw analgesia meter and expressed in mass unit (gram) (Kuhad and Chopra 2009).

Rota-rod treadmill

In this test, mice were placed on the rotating rod for two trials firstly to confirm the diabetic neuropathy and thereafter post 4 weeks treatment with AX to analyse the effect of AX on diabetic neuropathic brain. The mice were initially trained to adjust themselves according to the rotation of the rod for 3 minutes. Mice were scored for their latency to fall (seconds) in each trail (Kamboj et al., 2010).

Determination of Total Oxidant Status (TOS), Total Antioxidant Status (TAS) and Oxidative Stress Index (OSI)

Brain tissues from the various groups were extracted after ethically sacrificing mice. The tissues were subjected to homogenization followed by centrifugation at 5000 rpm for 10 minutes. The TAS of supernatant was estimated by using a colorimetric measurement method developed by Erel (Erel 2004). The TOS of supernatant was estimated by

using a colorimetric measurement method developed by Erel (Erel 2005). The TOS level to TAS level ratio was regarded as the OSI. The brain tissue TAS, TOS and OSI value was calculated through the method developed by Aycicek (Aycicek et al., 2005).

Determination of Protein

Protein concentration in the tissue supernatants were determined by incubating brain tissue samples after sacrificing the mice with Folin-Ciocalteau phenol in an alkaline medium for 30 minutes and measuring the OD at 750 nm. The amount of protein was quantified with bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

Estimation of levels of TNF-α, IL-1β, IL-6, iNOS, and caspase-3

Brain tissues from the various groups were subjected to homogenization followed by centrifugation at 5000 rpm for 10 minutes. The levels of TNF-α, IL-1β, IL-6, iNOS, and caspase-3 were detected using ELISA method according to the manufacturer's instructions. The results were recorded at 450 nm and the concentrations were calculated with the best fitting curve for data analysis (Huang et al., 2020; Kuhad and Chopra 2009).

Statistical Analysis

Results of each experimental group were represented as mean ± SD (n=6). The results were analysed by using one way ANOVA (analysis of variance) for multiple comparisons. Significance of difference between the groups was evaluated using Student's t-test by using Sigma stat 3.5, statistical software. The data was considered statistically significant at $p \leq 0.05$, $p \leq 0.01$and $p \leq 0.001$.

Results:-

Behavioural assessment

Eddy's Hot Plate test

Reaction time in the hot plate test in diabetic mice was found to be significantly ($p<0.001$) decreased (79%) as compared to nondiabetic mice. After administration of AX for four weeks with 2, 4 mg/kg doses exhibited significant ($p<0.001$) increase in reaction time as compared to controls (Fig. 1a).

Randall Selitto test

In the Randall Selitto test, paw withdrawal threshold values significantly ($p<0.001$) decreased (78%) in diabetic mice as compared to nondiabetic mice. After administration of AX with 2 and 4 mg/kg exhibited significant ($p<0.001$) increase in reaction time as compared to controls (Fig. 1b).

Rota-rod treadmill

Motor coordination was impaired due to decline in the retention time in diabetic mice. It was difficult for the diabetic mice to maintain themselves on the rotating rod. Retention time in diabetic mice was found to be significantly ($p<0.001$) reduced (46%) as compared to nondiabetic mice. But after administration with AX 2, 4 mg/kg exhibited a significant ($p<0.001$) increase in retention time as compared to controls (Fig. 1c).

Effect of AX on TOS, TAS and OSI

The TAS, TOS and OSI levels in mice brain, are presented in Fig. 2. There was a significant ($p<0.001$) decline (40%) in the TAS levels in the brain tissues of the diabetic mice as compared to the control groups. However, AX treated diabetic mice groups (2mg/kg and 4mg/kg) significantly ($p<0.001$) increase TAS levels as compared with nondiabetic groups. The levels of TOS and OSI in the brain tissue found to be significantly ($p<0.001$) elevated (79% and 62.7%) in diabetic mice as compared to nondiabetic mice groups. However, TOS and OSI levels were ($p<0.001$) significantly declined in AX (2mg/kg and 4mg/kg) administered diabetic groups compared with nondiabetic groups.

AX decreases levels of TNF-α, IL-6, iNOS, and caspase-3

Levels of TNF-α, IL-1β, IL-6, iNOS, and caspase-3 were significantly ($p<0.001$) increased (306%, 154%, 153%, 187% and 522.3%) in diabetic groups as compared to non diabetic mice. The serum levels of TNF-α, IL-1β, IL-6, iNOS and caspase-3 were significantly decreased in AX (2 mg/kg and 4mg/kg) ($p<0.001$) administered groups of mice as compared to controls. ANOVA showed that the detected changes were significant after treatment in the TNF-α, IL-1β, IL-6, iNOS, and caspase-3 Fig. 3.

Discussion:-

DN generally affects the central and peripheral nervous system causing pain and numbness in legs and feet. Development of DN in mice is traditionally confirmed by evaluating the sensory and motor coordination of animals by performing various behavioural tests. In case of DN, nerve blood flow is decreased due to imbalanced oxidative stress, which often results in cerebral dysfunction, decrease in cognitive functions leading to various neurological diseases. In our study AX administration ameliorates the behavioural impairment caused in diabetic neuropathic mice.

In this study, the measurement of TAS, TOS and OSI provided an accurate and definitive index of oxidative stress in DN. TAS levels were found to be decreased in brain tissue in different neuropathic rats (Alp et al., 2012). In our study also, in consistency with existing literature, diabetic neuropathic groups of mice have shown elevated TOS, OSI and decreased TAS levels. Treatment with AX (2mg/kg and 4mg/kg) helped in significantly decreasing the levels of reactive oxygen species. This is probably because AX significantly stabilizes or reduces the levels of ROS/RNS, due to the presence of the hydroxyl (OH) group on each ionone ring of AX. This rationalizes some of distinctive features of AX, like, the ability to be esterified, a higher antioxidant activity and a more polar nature than other carotenoids. It can quench ROS and free radicals in both the inner and outer layers of the cellular membrane (Gaur et al., 2021).

The role of inflammatory cytokines like TNF-α, IL-1β and IL-6 in diabetic has been well known (Huang et al., 2020; Kuhad and Chopra 2009). Our study shows that levels of TNF-α are increased in diabetic neuropathic mice brain. AX administration reversed the increased TNF-α, IL-1β and IL-6 level in diabetic groups. This is possibly because of anti-inflammatory role of AX (Wu et al., 2015). This result supports anti-inflammatory potentials of AX via reduction in TNF-α mediated pathway. The generation of increased ROS and inducible NOS (iNOS) causes oxidative damage to the cellular proteins, membrane lipids and even DNA, resulting in unbalanced homeostasis and cellular apoptosis of neurons (El-Akabawy and El-Kholy 2014; Fukudome et al., 2008). In our study the frontal cortex of the diabetic mice showed an increase in expression of iNOS, as a marker of oxidative stress. AX administration for four weeks in diabetic mice decreased the iNOS levels in our study.

Conclusions:-

This study concludes that AX helps in amelioration of the behavioural impairments and normalizing TOS, TAS, OSI, TNF-α, IL-1β, IL-6, iNOS and caspase 3 levels that might lead to decrease in adverse effect of DN in mice.

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Author contributions

Author SG has performed the experiments and SG, SS, RM and SB has analyzed and interpreted the data.SG and SB has written the manuscript. All the authors have read and approved the final manuscript.

Conflict of interest:

Author Sonal Gaur, Shreshtha Gaur, Sonu Singhal, Rakesh Mishra, and Surabhi Bajpai, declare no conflict of interest.

Compliance with ethical standards

All the experiments were conducted strictly in accordance with guidelines of the Institute Ethical Committee regulated by the Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA). Handling and experimentation of animals was strictly according to the standard guidelines of the Institutional Ethics Committee. The experimental protocol was approved by institutional animal ethical committee (BV/IAEC/4063/2020).

Figures with legends

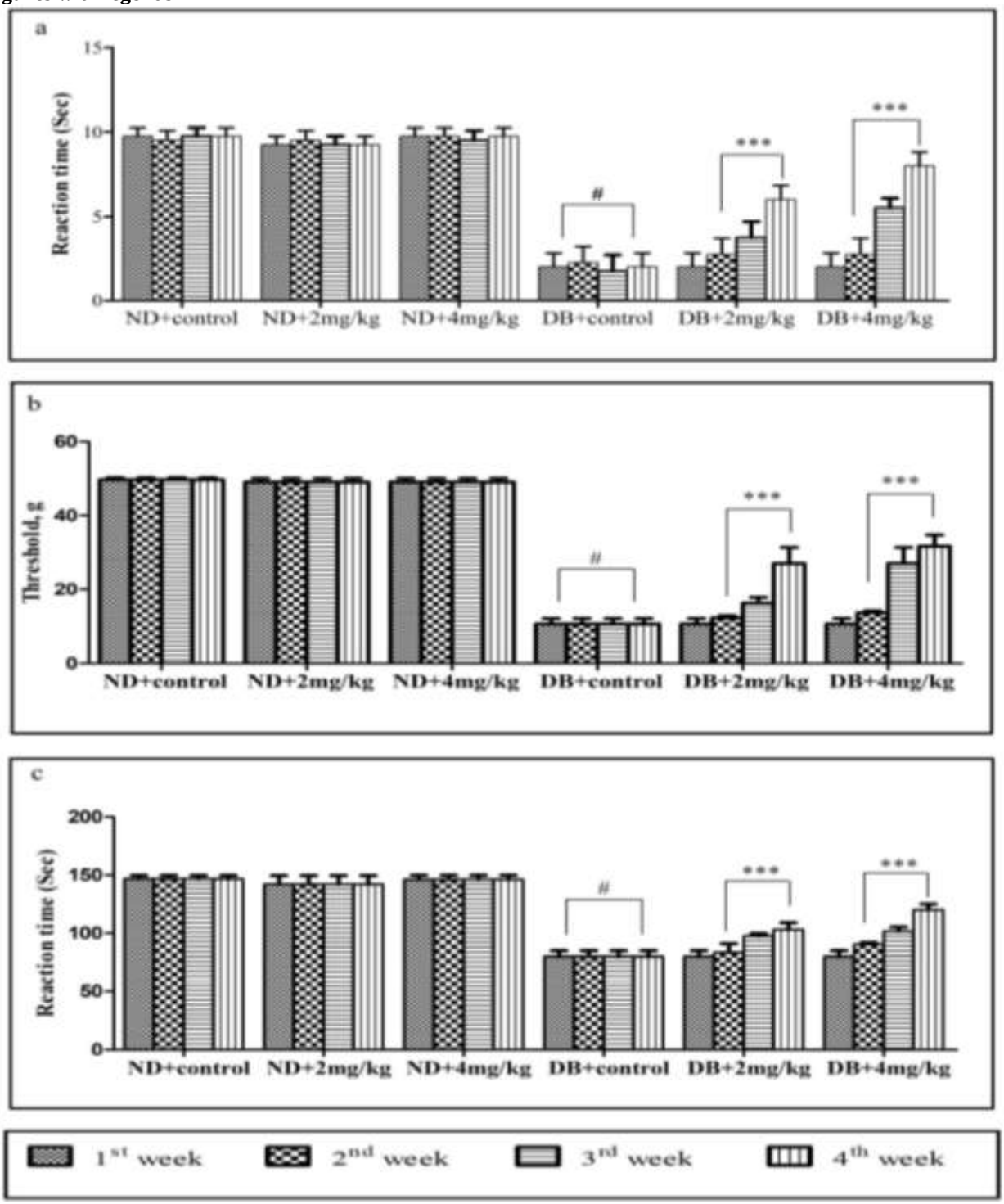

Fig.1:- Effect of AX (2 and 4 mg/kg. i.p) on Eddy hot plate test (reaction time, sec) (a), Randall &Selitto test (Threshold, g) (b) and Rota rod test (reaction time, sec) (c) in diabetic and nondiabetic mice. Data are expressed as mean ± SD. ***p<0.001 vs diabetic mice, #p<0.001 vs control mice.

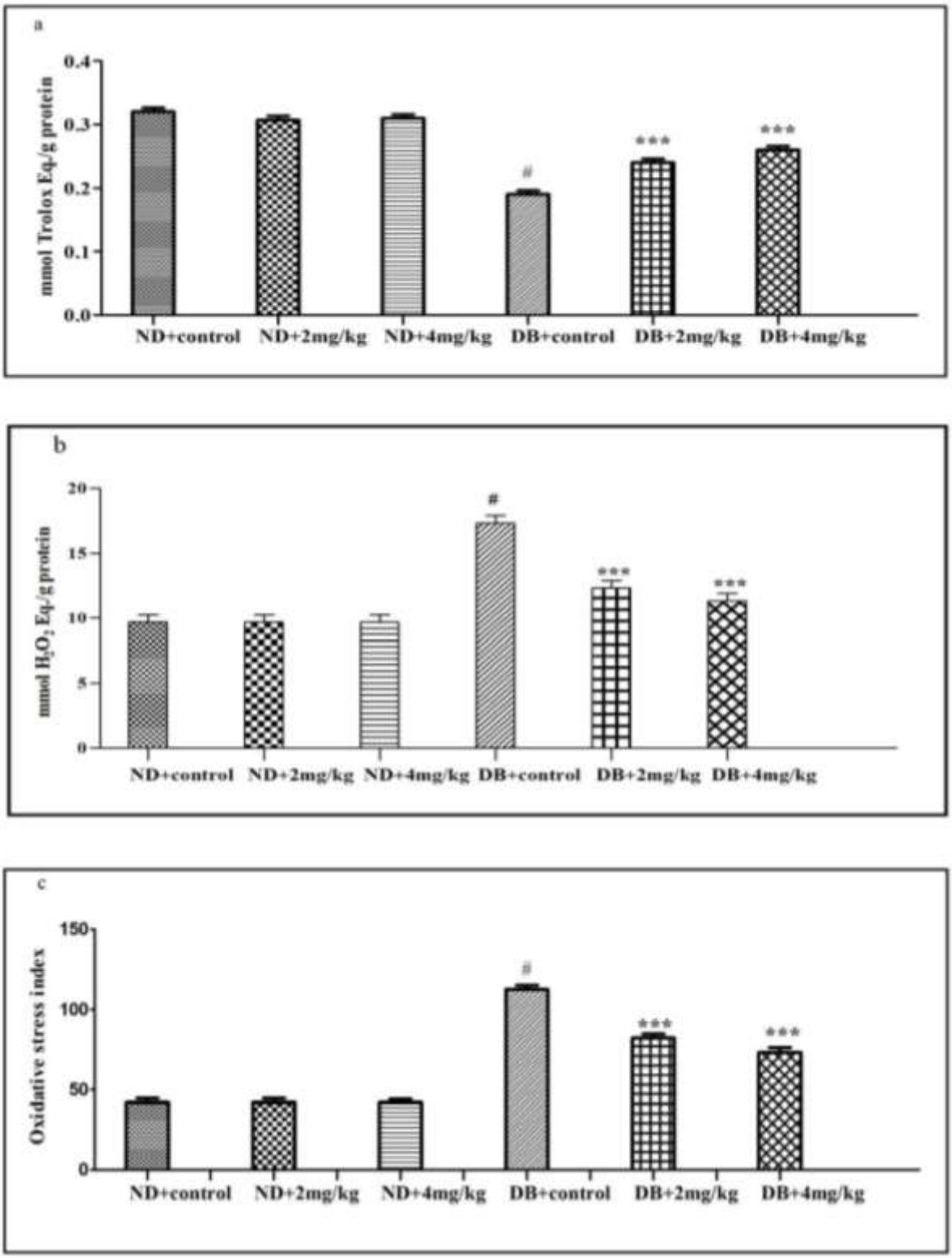

Fig.2:- Effect of AX (2 and 4 mg/kg. i.p) on TAS (a), TOS (b) and OSI (c) in diabetic and non-diabetic mice. Data are expressed as mean ± SD. ***p<0.001 vs diabetic mice, #p<0.001 vs control mice.

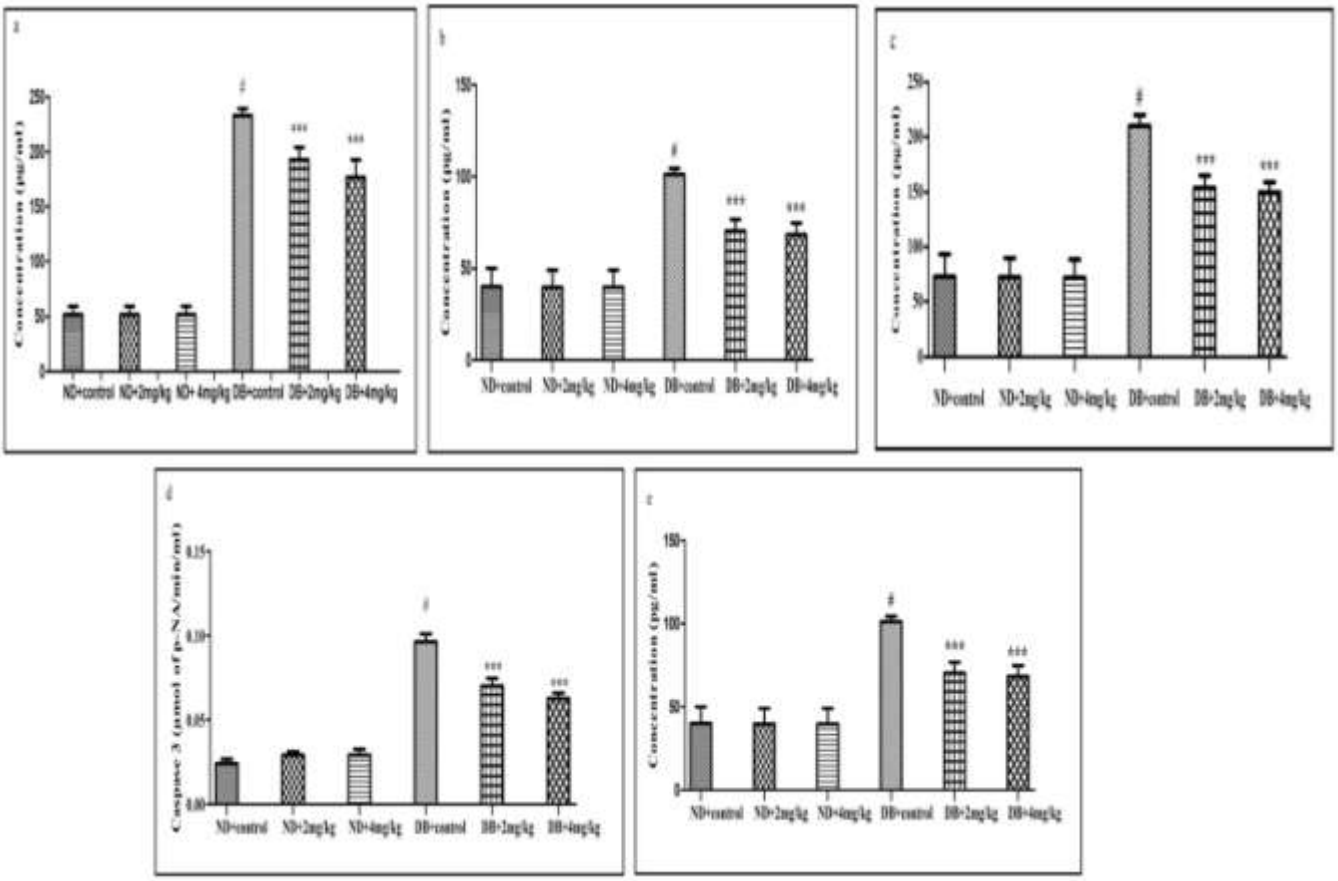

Fig.3:- Effect of AX (2 and 4 mg/kg. i.p) on TNF-α (a), iNOS (b) IL-6 (c) caspase-3 (d) and IL-1β (e) in diabetic and nondiabetic mice. Data are expressed as mean ± SD. ****p*<0.001 vs diabetic mice, #*p*<0.001 vs control mice.

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