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INCREASED APOPTOSIS AND DNA FRAGMENTATION DURING WOUND HEALING OF STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Apoptosis or programmed cell death play a very significant role in the developmental biology and cellular homeostasis in an organism. Most disturbances of apoptotic cell death have been known to contribute in many diseases, which are related with proliferation and replacement of older cell population. However, in metabolic disorders like diabetes mellitus these studies are not very well conducted considering many variables which are likely affect the metabolic milieu. Apoptosis is involved in the regulation of inflammation and play an important role in the inflammatory phase of wound healing but in diabetic wound, apoptosis appearance is somehow increased corresponding with delay in granulation tissue formation and healing in relation to glycemic control. To determine the level of apoptosis in association with diabetes, DNA fragmentation and agarose gel electrophoresis which is biochemical hallmark was performed in wound of both control and diabetic rats. It was found that there was a significant increase in number of apoptotic cells and DNA fragmentation in wound of rats with diabetes compared with rats without diabetes. This premature cell death or apoptosis during healing process of wound in diabetes may inhibit the repair process as well as the cells responsible for carrying out their normal functions are lost.

KEYWORDS

Diabetes, Streptozotocin, Wound Healing, Apoptosis and DNA Fragmentation.

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INTRODUCTON

Apoptosis is important physiological death processes that play a significant and crucial role in development, tissue homeostasis and the pathogenesis of various diseases[1-2]. There are many diseases like cancer, neurodegenerative disorders, and tissue destructions after ischemic episode or autoimmune disorder where apoptotic phenomena are altered. It is important and inevitable event in the remodeling of tissues during development and aging[3-4]. Since the past three decades

which have been extensively used to unravel the mystery of apoptosis and its application in the clinical research and biotechnology. One area of particular importance is wound healing process in which apoptosis is responsible for the removal of inflammatory cells and granulation tissue[5]. While extensive literature in apoptosis have been published in many fields, very few have focused on role of apoptosis in wound healing specially in diabetic wound and the significance of apoptosis in relation to glycemic control.

Impaired wound healing in diabetes mellitus is a common well known complication. The underlying basis and mechanism of impaired healing in diabetes is poorly understood. It is presumed that diabetic complications results from periods of poor glycemic control. Wound repair in healthy individual depends on several processes, including migration of inflammatory cells into wound space to colonize provisional matrix, proliferation of fibroblast and vascular cells and apoptosis[6]. In normal wound healing process, apoptosis is responsible for events such as removal of inflammatory cells and evolution of granulation tissue into scar, which occur late in wound healing. Early apoptosis can lead to abnormal wound healing by removing granulation tissue including fibroblast and small vessels[7]. Apoptosis or programmed cell death is characterized by DNA fragmentation and other molecular features which is a biochemical hallmark of apoptosis. The present study involves the study of apoptosis and its impact on wound healing processes in experimental diabetes. To determine the occurrence and phenomena of apoptosis in association with hyperglycemia, morphological criteria, DNA fragmentation and agarose gel electrophoresis as marker of apoptosis was studied in wounds tissue of non-diabetic and streptozotocin induced diabetic rats.

MATERIAL AND METHODS

Experimental Diabetes

Experiment was conducted using laboratory bred male Wistar albino rats. All animal procedures were performed with the approval of the Animal Ethical Use and Care Committee at the North Eastern Hill University, School of Life sciences, Shillong India. Diabetes mellitus was induced in male wistar rats (n=10), 100-150g with an injection of 70mg/kg body weight streptozotocin intra-peritoneally dissolved in citrate buffer (pH=4.6). Citrate buffer-treated rat's (n=10) were used as a controls.

Plasma glucose measurement

Four days after streptozotocin injection, the rats were tested for glucose in blood by using commercially available kit to confirm the diabetes. Rats having the blood sugar level greater than 250 mg/dl were included in this study.

Wound Preparations

After confirmation of diabetes, full thickness excisional wounds were made on the back of the 8-week-old rat in 8 mm diameter after giving the anesthesia injection of ketamine (80mg/kg body weight) through intra-peritoneally. Biopsy specimens were obtained at different days after wounding and divided into two parts. First part kept in phosphate buffer saline, stored at-20^{0}C for measurement of DNA fragmentation and second part was transferred to 10% neutral buffered formalin for histological study.

Biochemical Test for DNA Fragmentation

DNA fragmentation was studied by the methods described elsewhere[8]. It is based on the fragmentation of genomic DNA into larger and smaller fragments due to endo nuclease activity during apoptosis and could be separated by centrifugation. Briefly wound tissue was rinsed in PBS weighted and preminced with fine scissors and homogenized. The cells were washed with PBS, trypsinised and centrifuged at 110g for 5 minute to remove trypsin. The cell pellets were lysed with 0.4 ml of hypotonic lysis buffer (10mm Tris-HCl, 1mm EDTA PH 7.5) containing 0.5% triton x -100) and lysate were centrifuged at 13000g for 10 minute to separate intact chromatin from fragmented DNA. The supernatant, containing fragmented DNA were placed in separate microcertrifuge tube and both pellet and supernatant were precipitated overnight at 4^{0}C in 12.5% Trichloro acetic acid (TCA). The precipitates were sedimented at 13,000g for 4 minute. The DNA in pellets and supernatant was hydrolyzed by heating to 90^{0}C for 10 minute in 50ml of 5% TCA and quantified by modified method of Burton. In this method 100µl

of diphenylamine (DPA) reagent [150mg DPA, 150µl sulphuric acid, 50µl acetaldehyde (16mg/ml in glacial acetic acid)] was added to each tube. After overnight color development, 200µl of each sample was transferred to 96 wells of ELISA plates and an optical density was measured at 570nm in micro-plate reader. The percentage of DNA fragmentation was defined as a ratio of DNA in the 13,000g supernatant to the total DNA in the 13,000g supernatant and pellet.

DNA Agarose Gel Electrophoresis

Wound tissue was homogenized in digestion buffer consisting of (100mM NaCl, 10mM Tris-chloride, 25mM of EDTA, 0.5% SDS, and 0.1mg/ml of proteinase K) overnight at 50^0C. Nucleic acid in the homogenate was extracted with phenol/chloroform/isoamyl alcohol. After centrifugation, the top layer was transferred to another tube. Ammonium acetate (7.5M) (one -half volume of that top layer) and 100% ethanol (two fold volume) were added. After centrifugation again, the precipitate was washed with 70% ethanol. The samples were incubated with 100µg/ml ribonuclease A for 15 min at 37^0C to remove RNA. Resolving agarose gel electrophoresis was performed for 1.8% strength containing 10µg/ml ethidium bromide. Depending on the experiment, 20µg per well was loaded. DNA standards (0.5 µg per well) were included to identify the size of the fragments. Electrophoresis was performed for 2 hour at 70 V and DNA was visualized by ultraviolet florescence.

Statistical analysis

Data are presented as mean ± S.E. Differences were analyzed by using analysis of variance followed by an appropriate multiple comparison tests. A level of $p < 0.05$ was considered as statistically significant.

RESULTS

Blood glucose evaluation

The mean blood sugar level in control group rats on 5^{th}, 10^{th}, 15^{th}, 20^{th}, and 30^{th} day was 77.8±11.45, 89.5±9.56, 85.2±9.7, 80.54±8.5 and 93.6±9.44 mg /dl and in diabetic group rats it was 351±35.89, 381.56±39.76, 285.56±34.36, 365.24±33.54 and 378.54±35.87 mg/dl. The blood glucose level was maximum on 30^{th} day and it was minimum on 5^{th} day in control group. In diabetic group it was found maximum on 10^{th} day and minimum was on 15^{th} day. In both group the mean blood glucose were found statistically well significant ($p<0.01$) (Graph No.1).

Apoptotic morphology

Apoptotic cells in control and in diabetic wound group were identified on the basis of morphological features that induced central cell apoptotic bodies uniformly condensed chromatin and densely stained nucleolus or membrane bound apoptotic bodies containing one or more nuclear fragment (Figure No.1).

DNA Fragmentation

The mean DNA fragmentation on 5^{th}, 10^{th}, 15^{th}, 20^{th}, and 30^{th} day were 42.32±5.95, 43.25±5.88, 46.23±5.6, 45.21±5.38 and 41.56±5.65 respectively ($p<0.01$) in control group. In diabetic rats wound, the mean DNA fragmentation were 62.56±10.45, 66.47±8.67, 62.33±6.21, 67.58±8.47 and 63.25±8.26. The DNA fragmentation was maximum on 15^{th} day and minimum on the 30^{th} day in control group. The DNA fragmentation in diabetic group was maximum on 30^{th} day and minimum on 10^{th} day (Graph No.2).

Agarose Gel Electrophoresis

Cells undergoing programmed cell death or apoptosis exhibited chromatin fragmentation in a characteristic internucleosomal pattern thought to represent endogenous endonuclease activation, the amount of DNA in each fragment being a multiple of 180-200bp in diabetic group of rats at different days. Sometimes smearing pattern of DNA molecules was noticed (Figure No.2).

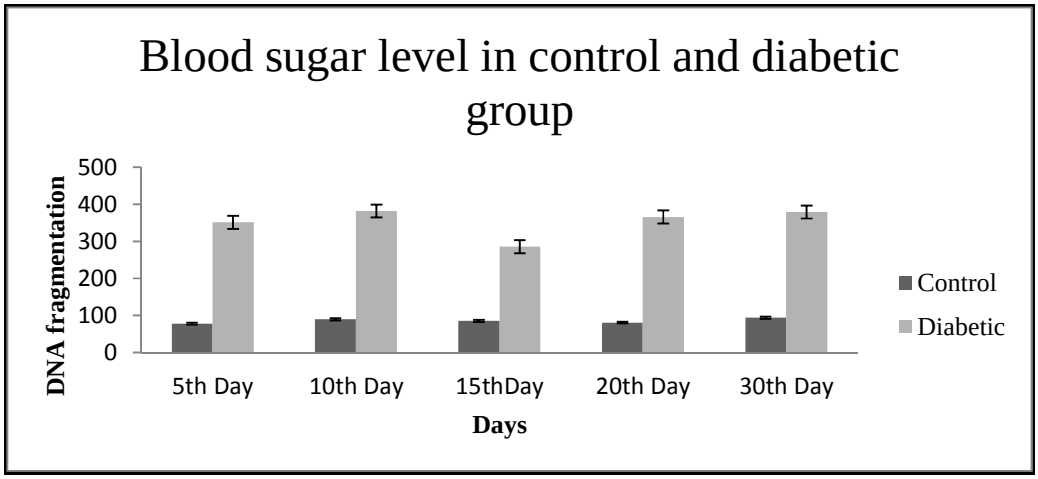
DISCUSSION

The present study demonstrates that the apoptosis was increased in diabetic wounds granulation tissue with respect to the advancement of time during wound healing process. It has been shown previously that apoptosis is involved in the loss of cells which occurs as the highly cellular granulation tissue involves into a less cellular scar[5]. In diabetic wound we found the subsequent increased apoptosis in comparison with non-diabetic wound. We also noticed that there was not consistent increased apoptosis in both the group during healing process. This premature cell death during healing may inhibit the repair process as well as

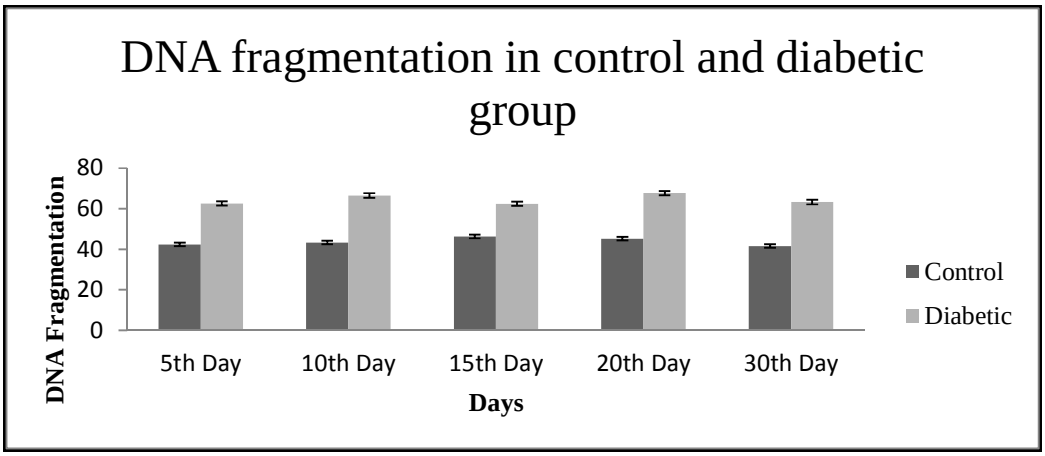
cells are lost before carrying their normal functions and finally leads the delay in healing process during the diabetes.

The mechanism by which apoptosis is increased in diabetic wounds remains unclear but various mechanism has been proposed for hyperglycemia induced increased apoptosis. *In vitro* studies have shown induction of apoptosis in endothelial cells exposed to high glucose in cultured medium[9-10]. Exposure of human endothelial cells to high glucose results in increased p42/44 and p38 MAPK activity causing increases apoptosis[11]. This effect is independent of the increases in osmolarity associated with high glucose. It has been demonstrated that protein kinase B (also called AKT) activation via insulin receptor plays an important role in apoptosis. The exact mechanism by which apoptosis is increased in diabetic wounds remains unknown. There are some evidence on animal models that hyperglycemia can induce apoptosis of endothelial cells [12]. Furthermore, *in vitro* studies have shown induction of apoptosis in endothelial cell exposed to high glucose in culture medium[9-13]. Thus hyperglycemia may be responsible for increased level of apoptosis seen in diabetic wounds. In addition it has been shown that interruption of cell matrix adhesion as it may occur in case of excessive matrix degradation may lead to apoptosis by inhibition of integrin binding to matrix proteins[14-15].

DNA fragmentation is considered the most characteristic features of apoptosis or programmed cell death[16]. Thus the appearance of DNA fragments become the hallmark of apoptosis, so increased DNA fragmentation showing the fragmentation of DNA in to oligonucleo some by activation of endogenous endonuclease common to all processes of apoptosis[17]. In our study we noticed during measuring of DNA fragmentation and agarose gel electrophoresis it shows the least DNA fragmentation and appeared as smear in control group of rats wound. The reason might be due to very low level of glycemic control which causes the least apoptosis bia different signaling cascade system and production of reactive oxygen species. In this study DNA fragmentation (Biochemical marker) and agarose gel electrophoresis were taken as a determinant of apoptosis. The biochemical techniques are easier for detection of apoptosis but some time do not distinguish between apoptotic and necrotic pathways. When DNA fragmentation and agarose gel electrophoresis are used together, it is consider as a very reliable method to detect apoptosis [18]. As it was mentioned earlier the activity of endogenous endonuclease leads to fragmentation of DNA into 180-200bp. These changes even occur before any appreciable change in the cellular morphology. The percentage of DNA fragment in control and diabetic rats groups will be directly proportional to the extent of apoptosis in that groups[18]. In our study we observed the mean DNA fragmentation in control group were 20.12±1.6, 35.61±2.4, and 38.5±3.8 respectively and in diabetic group it was 40.05±2.6, 45.61±4.8, and 68.2±3.2 ($p<0.01$). All the above results were statistically well significant.



Graph No.1: Mean blood glucose level and DNA fragmentation in control rats



Graph No.2: Mean blood glucose level and DNA fragmentation in diabetic rats

Control rats (A) Diabetic rats (B)

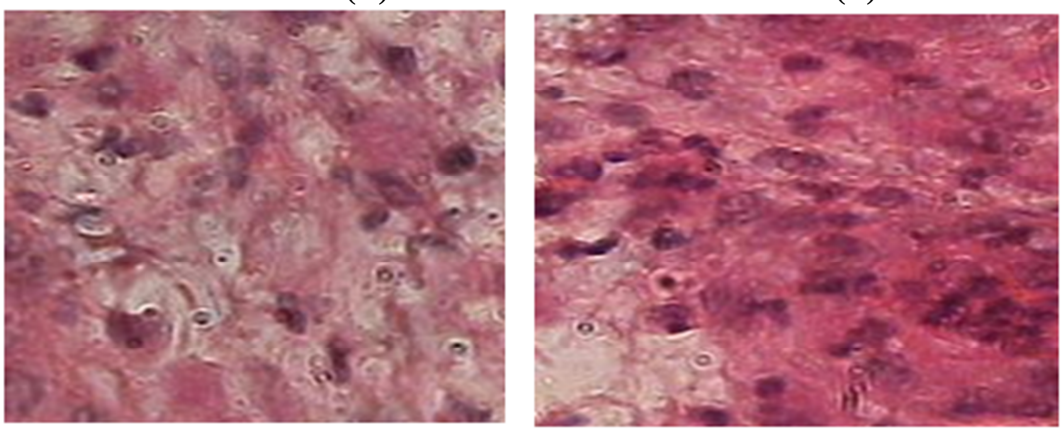


Figure No.1: Apoptotic cells were found in both groups (the apoptotic cells characterized by nuclear condensation and fragmentation) but more number of apoptotic cells observed in diabetic rats wound group (B) compared with control group (A)

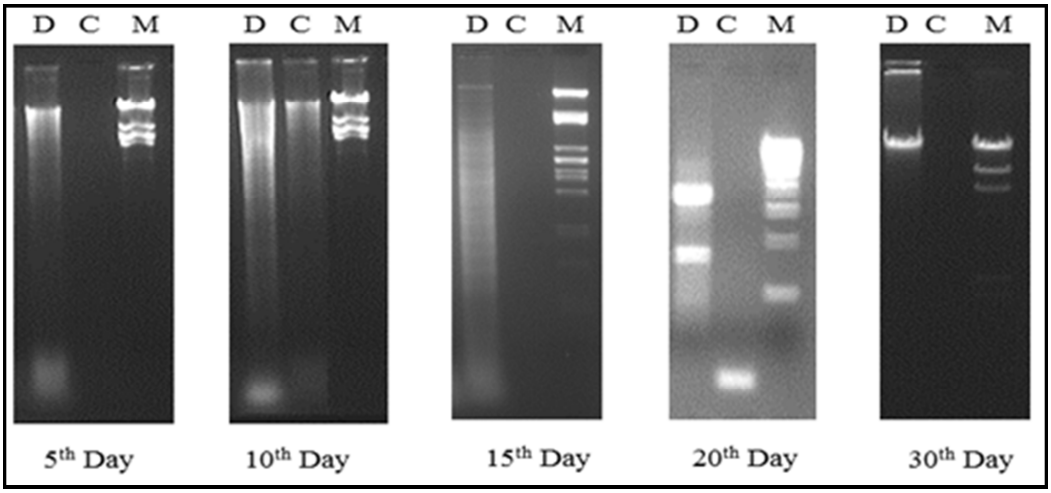


Figure No.2: Agarose gel electrophoresis was performed on different days in diabetic and control group of rat's wound. The DNA molecular weight marker (lane 1), the DNA fragmentation resolved as smear in control group (lane 2) and characteristic DNA fragmentation on different days (lane 3, 4 and 5)

CONCLUSION

In the present study, we observed that apoptosis and DNA fragmentation was increased in the diabetic rats wound with poor glycemic control. However the control group has shown least DNA fragmentation and minimum number of apoptotic cells explains definitely that hyperglycemia plays significant role in increased apoptosis. The further study is required to explore the mechanism of increased apoptosis in diabetic rats wound which helps to the clinicians and researchers for better management of wound healing during diabetes. In summary, we can say that high glucose concentration mediates an increased in the apoptosis involving DNA fragmentation and a reduction in granulation tissue survival.

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CONFLICT OF INTEREST

The authors hereby certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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