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Diabetes during pregnancy enhanced neuronal death in the hippocampus of rat offspring

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ABSTRACT

Background: Diabetes in pregnancy has a detrimental effect on central nervous system (CNS) development and is associated with an increased risk of short- and long-term neurocognitive impairment in the offspring. This study aimed to investigate the effect of maternal diabetes and also insulin treatment on the numerical density of apoptotic cells in rat neonate's hippocampi during the first two postnatal weeks.
Methods: Wistar female rats were maintained diabetic from a week before gestation through parturition and their male pup's brains were collected at postnatal days (P); P0, P7 and P14, equivalent to the third trimester in human. Numerical density of total neurons and percentage of apoptotic (TUNEL-positive) cells in different subfields of hippocampus (CA1, CA2, CA3, and DG) was calculated by stereological methods.
Results: Immediately after birth, we found a significantly decline in the total neuronal density only in hippocampal CA3 area in neonates born to diabetic animals ($p < 0.0001$). Moreover, the number of neurons was significantly decreased in all hippocampal sub-regions of diabetic group pups when compared to control and insulin treated diabetic pups at both P7 and P14 ($p < 0.0001$ each). Nevertheless, in diabetic group, the percentage of apoptotic cells in different subfields of hippocampus were higher in all studied time-points compared to control or insulin treated diabetic groups ($p < 0.0001$ each). There were no significant differences either in the total number or apoptotic cells in the different hippocampal sub-fields between the insulin-treated diabetic group and controls ($p > 0.05$).
Conclusion: Our data indicate that diabetes in pregnancy induce the neuronal cell apoptosis in offspring hippocampus. Furthermore, the maternal glycaemia control by insulin treatment in the most cases normalized these effects.

1. Introduction

Diabetes mellitus is the most seriously metabolic condition in pregnancy period and is associated with a high rate of congenital anomalies, disturbances of intrauterine growth and often short- and long-term neurocognitive and neurobehavioral abnormalities in the offspring (Zhao and Reece, 2013; Anderson et al., 2005; Georgieff, 2006; Magaton et al., 2007; Persaud, 2007; Ornoy, 2005). multiple lines of evidence clearly indicate a lower IQ (Ornoy et al., 1999; Rizzo et al., 1991), a greater inattention and hyperactivity (Ornoy, 2005; Ornoy et al., 2001), and a poorer general cognitive function (Veena et al., 2010; Schwartz and Teramo, 2000) in the offspring born to diabetic mothers. The hippocampus in human and rodents brain plays a crucial role in memory/learning and processing information about spatial locations (Burgess et al., 2002). The results from the majority of experimental studies also indicate that diabetes negatively impacts hippocampal cell proliferation and survival (Ho et al., 2013; Chen et al., 2012; Foghi and Ahmadpour, 2013; Li et al., 2002a,b; Jafari Anarkooli et al., 2008). On the other hands, some of experimental studies revealed a reduction in the numerical densities of neurons in some portions of fetal CNS,

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especially in the hippocampus due to maternal diabetes which reflects in lower memory and learning skills and defect in memory storage (Ghafari et al., 2015; Razi et al., 2014; Golalipour et al., 2012; Khaksar et al., 2011; Tehranipour and Khakzad, 2008). Meanwhile, there is no study that evaluated the impact of diabetes during pregnancy period on neuronal cell apoptosis in different sub-regions of rat hippocampus during brain development.

Apoptosis, or programmed cell death, is an active, tightly regulated, and genetically encoded form of cell death (Elmore, 2007; Haanen and Vermes, 1996; Brill et al., 1999; Voiculescu et al., 2000; Lichnovsky et al., 1999; Hardy, 1999; Hung and Chow, 1997). This suicidal pathway is characterized by several morphological and biochemical aspects including membrane blebbing, mitochondrial depolarization, condensation of the nucleus and cytoplasm, and degradation of nuclear DNA (Elmore, 2007; Hung and Chow, 1997; Umanskii, 1996; Goldar et al., 2015; Wang, 2015; Wu et al., 2015). Preclinical literature consistently report that the hippocampal environment of hyperglycemic animals favors apoptosis, as evidenced by significant elevations in apoptotic markers (Chen et al., 2012; Foghi and Ahmadpour, 2013; Li et al., 2002a,b; Revsin et al., 2005). There are also several *in vitro* and *in vivo* studies indicating that hippocampal apoptotic neuronal loss occurs in diabetic encephalopathy in animals and that this may be a major contributing mechanism to memory and learning impairments (Foghi and Ahmadpour, 2013; Li et al., 2002a; Jafari Anarkooli et al., 2008; Sima et al., 2004).

Although, the exact mechanism by which diabetes during pregnancy period increases the risk of congenital abnormalities in developing brain is not completely understood, Multiple lines of evidence suggest that the high maternal glucose concentration is a major teratogenic factor that impairs the development of central nervous system (CNS) in the offspring (Hartling et al., 2014; Allen et al., 2007; Gabbay-Benziv et al., 2015). Isolated embryos in high concentrated glucose culture have been shown to develop malformations similar to those seen in human diabetic pregnancy (Eriksson et al., 2000; Gareskog et al., 2007; Zhao and Reece, 2005). The embryos from diabetic mice also showed a significant increase of apoptosis on the surface of neural folds during organogenesis as well as elevating of cell death in the neural tube (Phelan et al., 1997; Zabihi and Loeken, 2010).

Therefore, the objective of this study was to examine the effect of maternal diabetes on the numerical density of apoptotic neurons in various hippocampal subfields of rat neonates at postnatal days (P) 0, 7, and 14. In this regard, the neurotoxic effect of diabetes during pregnancy period on the rat offspring hippocampus was investigated by TUNEL staining for apoptosis examinations.

2. Materials and methods

2.1. Animals

Thirty virgin female Albino Wistar rats (200–250 g body weight, 4–6 weeks old) were purchased from Birjand University of Medical Sciences Experimental Animal House (Birjand, Iran). The rats were housed in individual polycarbonate cages under controlled environment (12 h light/dark cycles and 21–25 °C temperature), fed with standard laboratory animal's pelleted diet (Javanneh-Khorasan Co., Iran) and tap water, *ad libitum*. The experimental procedures used in the present study were approved by the Ethic Committee of Laboratory Animals of the Birjand University of Medical Sciences, Birjand, Iran.

2.2. Experimental design

The animals randomly divided into 3 groups as follow:

a Diabetic (STZ-D) group (n = 11)
b Diabetic treated with insulin (STZ-INS) group (n = 11)
c Controls (CON, n = 8)

These allocations were based on difference of fertility between diabetic and non-diabetic rats. Existing evidence suggest direct deleterious effects of hyperglycemia on female reproductive functions (Bestetti et al., 1985; Ballester et al., 2007; Hashimoto et al., 2010). Thus, we considered 11 animals for each diabetic group to achieve acceptable number of pregnant females per group.

2.3. Induction of diabetes

The overnight fasted female rats were made diabetic with a single intraperitoneal (i.p) injection of 50 mg/kg streptozotocin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in cold 0.9% saline. STZ is one of the most commonly used diabetogens, inducing insulin-dependent diabetes mellitus in rodents by causing a selective destruction of insulin-secreting beta cells in pancreatic islets (Moazzen et al., 2015; Dowling et al., 2014a). These experimental diabetic animal models have been widely used to study the molecular mechanisms of maternal diabetes induced malformations in various organs, including the neural tissues (Hami et al., 2015a,b,c, 2013a; Dowling et al., 2014b; Haghir et al., 2013; Delascio Lopes et al., 2011; Jawerbaum and White, 2010). Blood glucose was measured using a commercially available digital glucometer (BIONIME, Switzerland) and the rats with FBG levels above 350 mg/dl (on 3 days after injection and delivery day) were considered as diabetic and used in this study.

Treatment of diabetic animals was conducted after the verification of diabetes. The diabetic rats from STZ-INS group were treated with protamine-Zinc insulin (NPH) (EXIR Pharmaceutical Company, Iran) twice per day (6U/day, subcutaneously, 2U at 8:00 AM and 4U at 5:00 PM) as previously described (Alves-Wagner et al., 2015; Bahey et al., 2014; Freitas et al., 2005). The other groups (STZ-D and CON) received both subcutaneous saline injections at the same moment of insulin injection, in equal volume.

Animals were mated with non-diabetic males overnight starting a week after treatments. The presence of a vaginal plug at the following morning was designated as day 1 of pregnancy (GD1). At the end of pregnancy, animals were allowed to deliver naturally; the day of birth was defined as postnatal day (P0). Newborn rats born to diabetic and Insulin-treated diabetic mothers were fostered onto control mothers to exclude other effects by the milk of diabetic rats, and thus enabled to focus only on the environment of the fetal period. Male offspring were randomly assigned to three age groups, P0 (n = 8), P7 (n = 8), and P14 (n = 8).

2.4. Tissue preparation and TUNEL assay

In each group, 8 male pups from different mother animals/timepoint (at P0, P7, and P14) were anesthetized with diethyl ether and for P14 pups transcardially perfused with cold 0.09% saline, followed by a fixative solution containing 4% paraformaldehyde and 0.1 M glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was done. Then, their brains were rapidly removed and fixed/post fixed (P14) in the same fixative for 24 h at 4 °C. The tissues were processed by routine histological methods and embedded in paraffin blocks. The brain samples transversely were cut (10 μm thick) using rotary microtome (Leitz, Italy) and 6 sections from each animal with 150 μm intervals were collected. To estimate the total number of neurons in different hippocampal subfields, the sections were stained by Toluidine blue.

TUNEL assay was carried out using the in situ cell death detection kit (Roche, Germany) according to kit instruction, to label nicked DNA. Briefly, the brain sections were deparaffinized in