International Journal of
Clinical Images and Medical Reviews

Original Article

The Effect of Gestational Diabetes on the α7 and α4 Subtypes of nAChRs in Human Placenta

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Abstract

Background: Gestational diabetes mellitus (GDM), as a teratogenic agent, is one of the most prevalent and critical metabolic disorders during pregnancy, which has irreversible effects on fetal development. This study aimed to evaluate the alterations of α7 and α4 subtypes of the nAChRs in the placenta of diabetic.

Materials and Methods: The present research was a case-control study performed on women who were referred to the cesarean section of the Allameh Bohlool Gonabadi Hospital from January 2021 to April 2021. These women were divided into the following two study groups: healthy pregnancy (n=10) and GDM (n=10). Placental samples were obtained from these mothers. Afterward, Immunohistochemistry (IHC) technique was performed for detecting α7 and α4 subtypes. Thereafter, the image quantification was done using ImageJ software. SPSS20 software was also used to perform statistical analyses and lastly, P≤0.05 was considered as statistically significant.

Results: Maternal and infant demographics data revealed no significant differences between the two groups (P<0.05). However, maternal fasting glucose level was significantly higher in the GDM group compared to the healthy group (P=0.002). Additionally, the α7 and α4 protein expression significantly differed between the two study groups, so that OD of the α7 and α4 was more in the GDM group compared to the healthy group (P=0.001; for each).

Conclusion: This study revealed that gestational diabetes could strongly affect the expressions of α7 and α4 subtypes in the Human placenta. This issue may reflect a compensatory protective physiological response, which may be responsible for fetal complications occurring during GDM.

Keywords: Gestational Diabetes, Nicotinic Acetylcholine Receptors, α7 Subtypes, α4 Subtypes, Human Placenta.

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Received: March 03, 2022 Accepted: May 14, 2022 Published: May 22, 2022

Introduction

Gestational diabetes mellitus (GDM) is developed among those pregnant women with inadequate pancreas function that cannot overcome resistance to insulin [1]. GDM increases blood glucose level that can be initiated or diagnosed for the first time in pregnancy. It was estimated that diabetes affects up to 15 % of pregnant women worldwide [2]. Moreover, its prevalence is increasing in all parts of the world [3]. According to previous studies, the congenital abnormalities in the newborns of diabetic mothers is 3 to 10 times higher than those of normal pups [4]. The basic reason of the impact of GDM on the embryo may possibly be due to maternal hyperglycemia that is associated with epigenetic changes in children [5]. Maternal hyperglycemia could induce immune activation, oxidative stress, hyperinsulinemia, chronic tissue hypoxia, and iron deficiency in the fetus [6], which consequently cause abnormalities in the structure and function of various tissues like placenta [7]. These abnormalities can impair the placental function, which can be considered as one of the main reasons for the increased fetal complications in diabetic pregnancies [7].

The human placenta is the most important fetal accessory structure, which establishes a close relationship between the fetal and maternal circulation. Accordingly, it has some functions such as the exchange of nutrients, respiratory gases, and metabolic wastes. In addition, Placenta is considered as a special endocrine organ that can produce protein, peptide, and steroid hormones. It also plays a critical role in the maintenance of the normal status of the fetus, while allowing anchorage to the maternal uterine wall [8]. It has been proved that both placental development and function are essential for retaining pregnancy and developing fetus health; hence, anything that disrupts placental development and function can disturb fetal growth and health [9]. Suter et al. in their study in 2020 believed that the placenta reflects a footprint of the in-utero experience [9]. Due to various exposures during pregnancy including GDM; several histological, pathological, and functional changes have been reported in the placenta [10-13].

Nicotinic acetylcholine receptors (nAChRs) belong to a family of pentameric ionotropic receptors, comprising of twelve identical subtypes

Citation: Sogol Arjmand[1], Raheleh Baradaran[2], Hasan Ahmadi Gharaei [3], Abdoljavad Khajavi[3], Azamsadat Mahmoudian*[4] . The Effect of Gestational Diabetes on the α7 and α4 Subtypes of nAChRs in Human Placenta . Int Clin Img and Med Rew. 2022; 2(2): 1069.

***DOI:** 10.55920/IJCIMR.2022.02.001069*

in placenta, including α2-10, β1, β2, and δ of which the expression levels differ according to the cell kind [14]. In addition, the α4 and α7 nAChR subtypes were abundantly found in the syncytiotrophoblast and endothelial cells of the placenta [14]. Of note, the properties of receptors are determined by the composition of the subtypes; for example, α subtypes (whether homomers or heteromers) have a high Ca+2 permeability [15]. The placental cholinergic system develops during the first half of gestation, and following that it is peaked at the 22nd week of gestation [16]. The syncytiotrophoblast produces Ach, which is known as the main placental signaling molecule. Accordingly, accompanied by nAchRs, it performs some important activities, including controlling the nutrient's uptake, blood flow and fluid volume of the placental vessels, the vascularization during the placental development, and contractile of placental myofibroblasts [16]. In previous studies, it has been shown that smoking and preeclampsia can induce some changes in placental nAChRs [14, 17, 18].

Therefore, due to the important role of these receptors in the development of the placenta, the present study aimed to investigate the effect of GDM on the densities of α7 and α4 subtypes in term women's placenta.

Materials and methods

Patient

The present research was a case-control study performed on units selected in the form of available sampling. After obtaining approval from the Ethics Committee of the Gonabad University of Medical Sciences (IR.GMU.REC.1399.103) and written informed consent, the included women were divided into the following two study groups: those with healthy pregnancy with normoglycemic (n=10) and those who had a GDM with fasting glucose more than 95 mg/dL (n= 10). Placental samples were then obtained within 5 minutes after cesarean from the women referred to Allameh Bohlool Gonabadi Hospital from January 2021 to April 2021.

Placental tissue collection & tissue process

In this study, to eliminate the bias, the obtained samples (2×2×2 cm3) were removed from the fetal disc of the placenta, close to the umbilical cord (as seen in figure 1). Thereafter, the samples were washed in normal saline solution (0.9% NaCl), and then fixed for 48 h in 10% formalin. Next, they were processed for paraffin embedding and then sectioned (5-µm-thickness) using a microtome (Leitz, Germany). Finally, after mounting the sections on poly-L-lysine coated slides, the immunohistochemistry (IHC) technique was done.

Immunohistochemistry

For detecting α7 and α4 nAChRs using immunohistochemical method, the slides paraffin were firstly removed by xylene (Sigma-Aldrich, USA), rehydrated by ethanol, and washed by phosphate-buffered saline (PBS). Secondly, thermal antigen retrieval was done by boiling these slides within PBS during 15 min, and then the samples were incubated by bovine serum albumin (BSA) dilution (0.01 mg BSA, 10 µl Triton X and 1000 µl PBS) at environment temperature. Thirdly, the specimens were incubated in 3% hydrogen peroxide for 15 min, in order to block the internal peroxides, washed three periods in PBS, and then incubated in 10 % normal goat serum for 20 min. Subsequently, the specimens were incubated by primary antibodies, rabbit anti- CHRNA7 antibody and rabbit anti- CHRNA4 antibody (Biorbyt, UK; Cat. No. Orb11096 and Cat. No. Orb11095, respectively) diluted 1:100, for an overnight at 4 °C, and then washing within PBS was performed in three periods. Afterward, the samples were incubated by secondary antibody, HRP-conjugated goat anti-rabbit IgG (Abcam, USA) diluted 1:1000 (0.02 g BSA, 20 µl Triton X and 2 cc PBS) at environment temperature for 60 min. Next, following three periods of washing in PBS, the studied receptors on the specimens were detected with 0.05 % diaminobenzidine (DAB; Sigma-Aldrich, USA) diluted with 0.01% hydrogen peroxide, and PBS for 15 min. Of note, for counterstaining, Harris hematoxylin was used. Finally, the specimens were dehydrated, cleared, and covered by coverslips [19].

Images analysis

To acquire photographs, a digital camera (DP12, Tokyo, Japan) connected with a light microscope (Olympus BX51, Tokyo, Japan) was used. Thereafter, image analysis was done using NIH Image/Image J software (version 1.48) and staining intensity in the placenta was measured as well. In the final step, the optical density was obtained using the following formula [20].

OD = log (225/Mean intensity).

Statistical analysis

SPSS 20 software (IBM Corporation, USA) was used to perform all statistical analyses. The patients' characteristics and receptors' OD of the normal and GDM groups were compared using one- way analysis of variance (ANOVA). The obtained data were presented as mean± standard deviation (SD). A P-value of ≤0.05 was considered statistically significant.

Results

Maternal and infant demographics

A total number of 20 women participated in this study, n=10 healthy normal pregnancies and n=10 diabetic. The outcomes showed that maternal age, gestational age, birth weight, and Apgar scores at minutes 1 and 5 were not significant between the GDM and normal groups (P=0.434, P=0.695, P=0.647, P=0.556 and P=0.556; respectively). The maternal fasting glucose level was significantly more in the GDM women compared to that of the non-diabetic women (P=0.002) (Table 1).

Protein expressions of the α7 and α4 nAChRs

Protein expressions of the α7 and α4 nAChR subtypes were revealed at the cellular level by IHC (Figure 1). Image J software was used to quantify the staining intensity of both the α7 and α4 nAChR subtypes. The outcomes of One-way ANOVA showed a significant difference between the diabetic and normal women. As well, T-test as post hoc analysis revealed the maximum OD of both the α7 and α4 subtypes in the GDM group compared to the normal group (P=0.001; for each) (Figure 2).

Discussion

The present study found some significant differences in a7 and a4 subtypes of nAChRs between placentae of women with diabetic and normal pregnancies. In the diabetic placentae, an increase was observed in the levels of a7 and a4 subtypes compared to the normal placentae. It was reported that non-neuronal nAChRs play critical roles in the

Table 1: *The characteristics of the patients participating in the present study.*

Characteristic	Normal	Diabetic	P-value
No. of cases (n)	10	10	-
Maternal age (years)	30.80±4.16	29.20±4.76	0.434
Maternal blood glucose (mg/dL)	Fasting glucose: 74.5 ± 9.7	Fasting glucose: 120.38 ± 13.4	0.002**
Therapeutics	-	Diet (N = 7) and Metformin (N = 3)	-
Newborn gender	5 females / 5 males	4 females / 6 males	-
Gestational age (weeks)	39.10±.99	38.80±1.32	0.695
Birth weight (g)	3197.00±499.66	3098.00±450.85	0.647
Apgar 1 min	9	9	0.556
Apgar 5 min	10	10	0.556

The results are expressed as the mean± SD.
*** means P≤ 0.01.*
–: Not applicable.

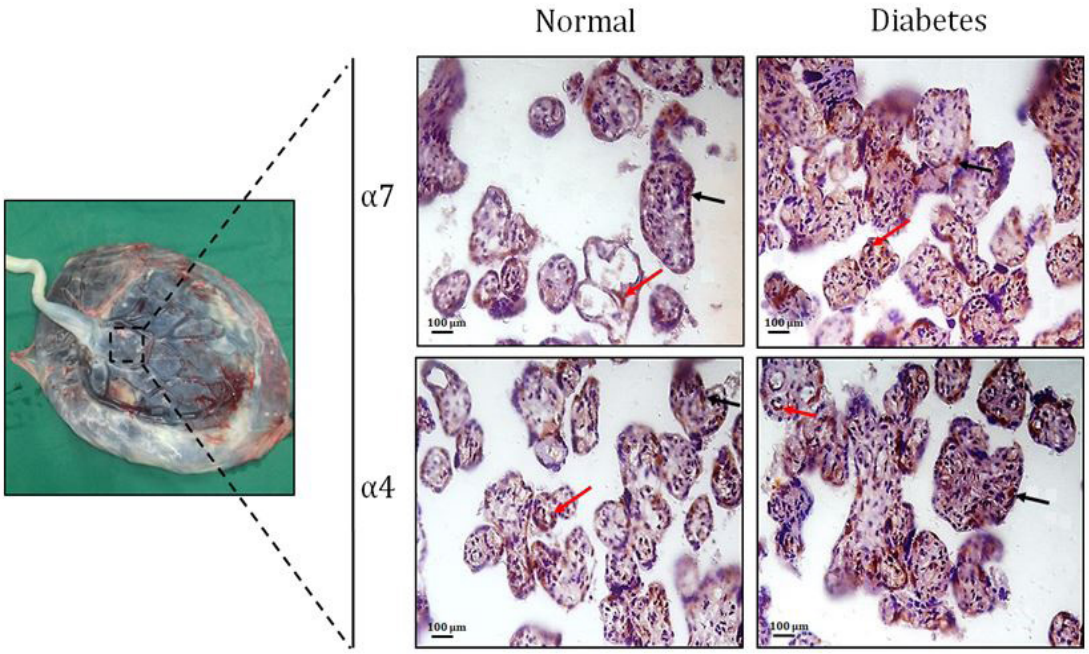

Figure 1: *nAChR IHC Staining of villous cells in normal and diabetic human placenta. Immunostaining in the human placenta showing positive staining for α7 and α4 nAChR subunits in the villous (Outer layer of connective cells that indicated with black arrow) and endothelial cells (within the villi that indicated with red arrow).*

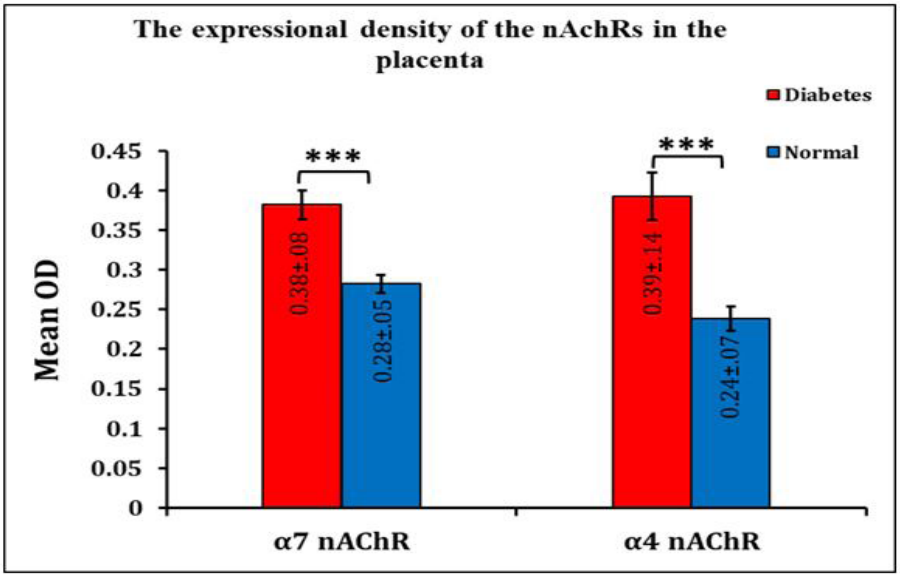

Figure 2: *Optical density of α7 and α4 nicotinic acetylcholine receptor immunoreactive cells in human placenta. The results are expressed as the mean± SD. *** means P≤ 0.001.*

vital cell functions, including mitosis, differentiation, organizing of the cytoskeleton, cell-cell connections, locomotion, and migration [21]. Additionally, acetylcholine is considered as a local hormone that in collaboration with its receptors, could modulate cell functions and also adapt the cell to new conditions [22]. Moreover, nAChRs control the nutrient's uptake, blood flow, and fluid volume of the placental vessels, the vascularization during the placental development and contractile of placental myofibroblasts [21]. During GDM, maternal glucose can cross the placenta unlike insulin, and consequently cause both fetal hyperglycemia and hyperinsulinemia. As a result, fetal hyperinsulinism causes macrosomia and organomegaly. Therefore, in order to provide nutrients and oxygen for the embryo, more blood supplies to the fetus are needed. In this case, the placenta may increase nAChRs to be adapted to these new conditions. In addition, it is proven that GDM leads to increases in volume and length of villous and capillaries [10, 11]. Therefore, the role of nAChRs can be considered as the reason for their increase during GDM.

GDM is associated with premature "aging" of the placenta. Under this condition, there is villous immaturity presenting with increases in villous diameter, hypercellular stroma, the proliferation of Hofbauer cells (resident macrophages), stromal fibroblasts, cytotrophoblasts, thickness of the basal membrane, and unusual capillary in villosities [11]. Considering that nAChRs are found in the cytotrophoblasts, syncytiotrophoblast, Hofbauer cells, pericytes, and endothelial cells of the placenta, it is expected that nAChRs increase in the placenta during GDM.

GDM-induced hypoxia increases the fetal need for blood supply. Furthermore, the placenta may probably increase nAChRs as a defensive reaction, in order to control blood flow, fluid volume of the placental vessels, and vascularization during the placental development. Heeschen et al. in their study in 2002 showed that hypoxia and ischemia can increase α7 nAChRs during vessel proliferation. As well, nAChR-induced angiogenesis was found to be done via phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways [23]. On the other hand, the placenta increases capillary and villous branching to compensate hypoxia. Accordingly, these events are regulated by some genes, including vascular endothelial growth factor (VEGF), angiopoietins [24], and nitric oxide [25]. It was previously proved that the nAChRs stimulation can significantly release VEGF and NO [23]. Additionally, GDM was observed to be associated with the upregulation of placental nutrient transporters [12]. It should be mentioned that nAChRs-mediated entrance of Ca2+ ions into cells could be considered as one of the main reasons for such gene expression changes, since it can activate the cAMP response element-binding protein (CREB) [26]. As well, GDM-induced hypoxia leads to the thickening of the lining of blood vessels in the placenta that can justify more expressions of the studied receptors in endothelial cells [27]. In another study, Machaalani et al. in 2015 showed that nAChR subtypes increase in the pre-eclamptic placenta. Correspondingly, they stated that pre-eclampsia-induced hypoxic environment can affect endogenous ACh levels and lead to compensatory increases in some nAChR subtypes [18].

In another study, Machaalani et al. in 2014 demonstrated that the increased α7 nAChRs can be known as a possible mechanism for the increase in calcification and apoptosis in the placentas of smoking mothers [17]. Moreover, apoptotic changes in diabetic rats' placenta were reported in a study by Gul et al. in 2015 [28]. In this study, another proposed mechanism for a7 and a4 nAChRs increase was high Ca2+ influx into cells that induced apoptosis.

A literature review highlighted the placenta's role as a source of inflammatory mediators, an inflammation place, and an adaptive mediator. It was found that placental hypoxia can raise the secretion of inflammatory mediators into the maternal circulation. Hypoxia can also activate leukocytes in the intervillous space, and amplify the production of pro-inflammatory cytokines by the placenta. An increase in IL-8, and leptin was reported in the placentas with GDM, as well [29]. Lappas et al. in their study in 2010 reported that GDM- induced oxidative stress leads to the release of some cytokines such as IL-1b and TNF-a [13]. On the other hand, α7 subtypes were indicated on the Hofbauer cells of the human placenta, highlighting their role in the anti-inflammatory cholinergic pathway. Moreover, the α7 subtypes modulate cytokine/chemokine release; for example, it can prevent TNFa release from Hofbauer cells [30]. Thus, it is possible that α7 expression increases to dampen the inflammatory response caused by GDM- induced hypoxia and oxidative stress.

Conclusion

In brief, our results indicate that the α7 and α4 nAChRs expressions were significantly higher in placenta of the diabetic mothers compared to the healthy mothers. Together, hypoxia, apoptosis, inflammation, and oxidative/nitrative stress induced by GDM led to α7 and α4 nAChRs increase. We suggest that the increased a7 and α4 in GDM may reflect a compensatory protective physiological response and may be responsible for developing fetal complications during GDM. However, further studies in the future are needed to evaluate the effects of maternal diabetes on the expression of other nAChRs subtypes and molecules involved in signaling pathways in the placenta.

Acknowledgments

We would like to thank from Deputy Research of Gonabad University of medical science (GMU) for financially supported this research by grant No. A-10-1998-1.

Financial support and sponsorship

This research was sponsored by Gonabad University of medical science (GMU) (A-10-1998-1).

Disclosure of conflicts of interest

The authors have no conflicts of interest to declare.

Authors' contribution

This study was designed by AS Mahmoudian, R Baradaran, H Ahmadi Gharaei, AJ Khajavi, and S Arjmand. AS Mahmoudian, R Baradaran, and S Arjmand have made substantial contributions to acquisition of data. H Ahmadi Gharaei and AJ Khajavi analysis and interpretation of data. AS Mahmoudian, R Baradaran, H Ahmadi Gharaei, AJ Khajavi, and S Arjmand have been involved in drafting the manuscript and revising it critically for important intellectual content.

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