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Original Article

The beneficial effect of gamma aminobutyric acid on diabetic nephropathy in type 2 diabetic rat model and their offspring

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Abstract

Background and purpose: Diabetic nephropathy (DN) in the first and second generations of diabetic rats and improving kidney function by gamma aminobutyric acid (GABA) were investigated.
Experimental approach: Male and female rats and their offspring were used. Diabetes was induced by a high-fat diet and a low dose of streptozotocin. Animals were divided into the diabetic positive control (D) group, the diabetic group receiving insulin (D + insulin), and the diabetic group receiving GABA (D + GABA). In addition, two groups of non-diabetic parents were assigned as negative control (NDC) groups. Each animal was monitored for 16 weeks, and offspring were fed with normal diet. The blood glucose level, urine volume, and water intake, as well as renal function, including the serum levels of blood urea nitrogen (BUN), creatinine (Cr), and glomerular filtration rate (GFR) were assessed. Also, the hyperinsulinemic-euglycemic clamp and gene expressions of *Nox4* and *Icam1* in the kidneys were measured for all subjects.
Findings/Results: GABA administration in parents and offspring decreased blood glucose level, insulin resistance, GFR, serum levels of BUN and Cr compared to the D groups. GABA reduced the urine Cr, BUN, and albumin loads in both parents and offspring in comparison to the D groups. GABA decreased *Nox4* and *Icam1* gene expression in both parents and offspring.
Conclusion and implications: GABA decreased the risk of DN, hyperglycemia, and insulin resistance in both diabetic parents and their offspring by improving kidney function, highlighting the potential therapeutic benefits of GABA in managing type 2 diabetes complications.

Keywords: Diabetes; GABA; Hyperinsulinemic-euglycemic clamp; Insulin resistance; Kidney function; Nephropathy.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by high blood glucose levels. One of the leading causes of type 2 DM (T2DM) is insulin resistance (1,2). The most prevalent form of diabetes, T2DM, affects vascular function in individuals (3,4). Organ difficulties, including kidney issues, are brought on by vascular issues. Twenty to fifty percent of those with diabetes have been diagnosed with diabetic nephropathy (DN). DN is characterized by persistent albuminuria and progressive decline in renal function (5,6). The pathological process in DN leads to most molecular events that one of which is oxidative stress, which links DN and T2DM (7). These molecular events affect the production of chronic inflammation, glomerular and tubular hypertrophy.

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The alteration of redox leads to albuminuria, proteinuria, glomerulosclerosis, and tubule-interstitial fibrosis. Oxidative stress in DN has the value to consider as a trigger, modulator, and link within the complex web of pathological events that occur in DN (8). The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) plays a significant role in the development of oxidative stress (9). The Nox family has 7 different isoforms, one of which, *Nox4*, is primarily expressed in the cortex of the kidney. According to earlier research on the kidney, diabetes increases the expression of *Nox4*, leading to renal pathologies such as interstitial fibrosis, mesangial enlargement, and renal structural degradation (9,10). Intercellular adhesion molecule-1 (Icam1) is considered to clarify the process of oxidative stress on the pathology of DN (11). *Icam1* expression has considerably risen in nephropathy, which affects the immune system, cytokines homeostasis, and reactive oxygen species (ROS) (12-14). Previous studies have shown that *Icam1* plays a crucial role in the development of diabetes (15,16).

Gamma-aminobutyric acid (GABA) is one of the compounds co-secreted with insulin by β cells (14)Its receptors are expressed in α and β cells, which provide membrane depolarization in β cells, increase insulin secretion, and suppress α cells to decrease glucagon secretion (17). In earlier research, type 1 and type 2 diabetic rat models were used to test the effects of GABA administration on insulin resistance, blood glucose levels, vascular tone, and blood pressure (18,19). In another study, the administration of GABA showed protective and beneficial effects on the liver and kidney associated with diabetes in an animal model, and GABA plays a role in protecting the tissues from lipid peroxidation and the remission of vascular function (20,21). In addition, the previous work indicated that GABA therapy in diabetic parents reduced the risk of insulin resistance in their offspring (22).

Since diabetes increases the plasma concentration of free fatty acids, which can pass through the placenta, disturb the insulin signaling pathway, and cause insulin resistance in children of diabetic mothers (23-25). So far, it was unclear the role of GABA in improving kidney function following the reduction of insulin resistance in both sexes and their offspring. Therefore, the current study examined the effect of GABA to prevent DN in the first and second generations of the diabetic rat model and improve kidney function *via* decreasing insulin resistance and the expression of *Nox4* and *Icam1*genes in the male and female diabetic rat model.

MATERIALS AND METHODS

Animals

Forty-eight male and female Wistar rats (4-week-old) were purchased from Razi Institute (Tehran, Iran) and kept in the animal house (Medical School, Isfahan University of Medical Sciences, Isfahan, Iran) at the temperature of 22 ± 2 °C, relative humidity of 50 ± 5%, 12/12 h light/dark cycle, and free access to water and pellet diet. All experimental procedures were approved by the Ethical Committee of Isfahan University of Medical Sciences (Ethical code: IR.MUI.MED.REC.1398.120).

Experimental design

Six rats in each sex were assigned as the non-diabetic control group (NDC), which received standard chow. The rest of the animals received a high-fat diet (HFD) containing 58% fat, 25% protein, and 17% carbohydrate for 3 months. Then, the animals received intraperitoneally (IP) a single dose (35 mg/kg) of streptozotocin (STZ, Sigma Aldrich, Hamburg, Germany) dissolved in saline (22). To confirm type 2 diabetes, one week after STZ injection, the blood glucose level and insulin tolerance test (ITT) were measured, and the animals with blood glucose levels above 250 mg/dL and impaired ITT were included in the study. The diabetic animals were randomly divided into the groups (n = 6) including non-treated diabetic animals as positive control group (D), the diabetic animals treated with insulin (D + insulin) at the dose of 2.5 U/kg, twice/day, IP (22) , and the diabetic animals treated with GABA (Sigma Aldrich, Hamburg, Germany) at the dose of 1.5 g/kg, IP (22) as group D + GABA. All animals were housed for 3 months in a clean cage in the animal room under the above-mentioned conditions. Male and female

animals from each group were permitted to mate after a 2-month treatment period (receiving insulin or GABA). In addition, the animals received insulin, GABA, and HFD during the periods of mating, pregnancy, and lactation. The females nursed their offspring for a month while the males were separated after parturition. Then, the male and female offspring of all groups were separated and kept in independent cages with labels similar to the parental groups. Finally, the offspring were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA (Fig. 1).

Urine collection

Every month each rat was individually placed in a metabolic cage with a collection container below. The floor was designed so that urine dropped through while feces remained behind. This manner allowed the collection of separate urine and feces for 24 h in all animals over a set time period.

Insulin tolerance test

ITT was performed monthly in all animals. To perform ITT, all animals received insulin (2.5 U/kg, IP). Then, the blood glucose of animals was measured with a glucometer through the tail at 0, 20, 30, 60, 90, and 120 min after the injection of insulin (26). ITT was measured once, immediately after inducing diabetes and before starting the treatment, and then it was repeated monthly.

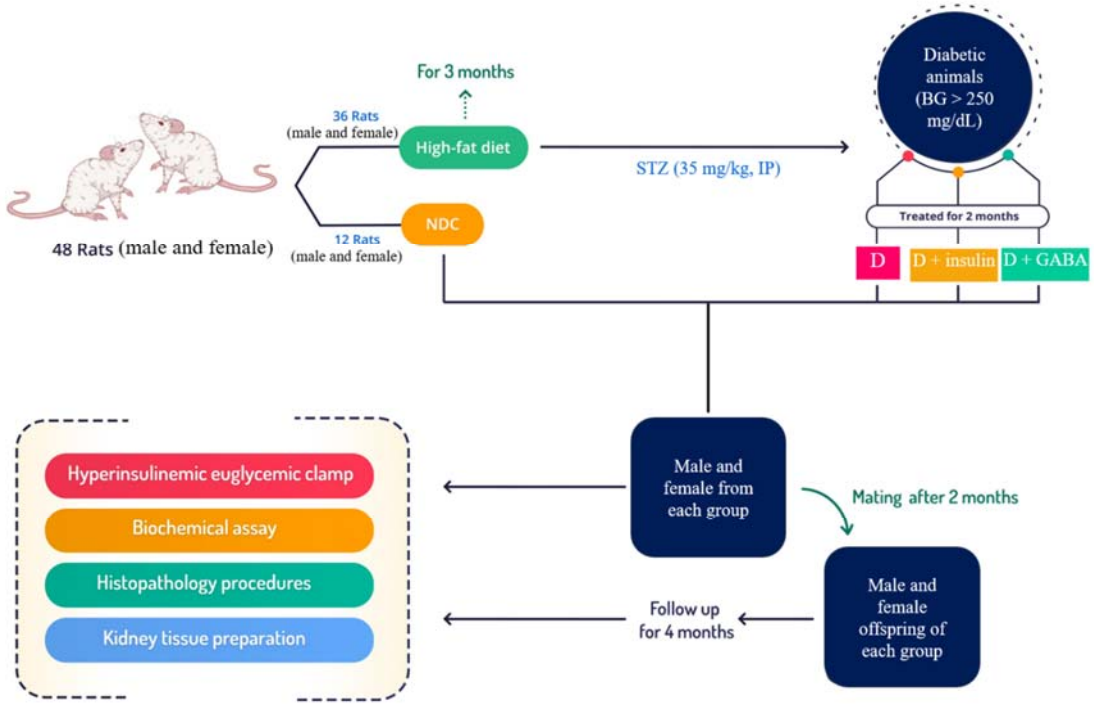


Fig. 1. Experimental design in the study. Four-week-old rats weighing 80-90 g were used in this study. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA. STZ, Streptozotocin; IP, intraperitoneally; BG, blood glucose; D, diabetes; GABA, gamma aminobutyric acid; NDC, non-diabetic control.

***Euglycemic-hyperinsulinemic clamp in the conscious* rat**

All animals were anesthetized with a single dose of ketamine (100 mg/kg, IP) and xylazine (8 mg/kg, IP) (27). Then, the common carotid artery (for drawing blood) and jugular vein (for infusing insulin and glucose) were cannulated, and the tubes were fixed to the back of the animal. Next, rats were housed individually after surgery to prevent wound disruption from cagemates, and the under-surgical areas were cleaned and inspected daily for signs of infection, like swelling, redness, or purulent discharge. Soft bedding was used, and cages were changed frequently to maintain cleanliness. After a 3-5-day recovery period, the animals were used for euglycemic-hyperinsulinemic clamp according to the previous experience (22). In brief, the animals were fasted for 17 h, and the rate of insulin and glucose infusions was calculated based on the body weight of the animals. A Y connection and 2 microinjection pumps (New Era Pump System Inc., Farmingdale, NY, USA) were used to infuse insulin and glucose simultaneously *via* the jugular vein. Also, a blood sample was drawn *via* the carotid artery during the clamp. Based on body weight, insulin (20 mU/kg/min) was administered at a rate of 5 mL/h, and 25% glucose was injected with a variable rate to clamp glucose at euglycemia (22). The clamp lasted for 5 h for each animal, during which blood glucose was measured with a glucometer (ACCU-CHEK Active, Germany) every 5-10 min, and 10 U/mL heparinized saline was replaced. At the end of the study, the blood samples were taken from the tail vein, and then the animals were anesthetized with pentobarbital (150 mg/kg, IP). The right and left kidneys were removed to perform histopathological and quantitative real-time polymerase chain reaction (qPCR) procedures, respectively. Finally, insulin-stimulated glucose uptake was determined by the amount of glucose injected in the last 30 min of the clamp. It was utilized to calculate the glucose infusion rate (GIR; mg/kg/min).

Biochemical assay

Quantitative kits (Pars Azmoon, Iran) were used to measure the serum levels of blood urea nitrogen (BUN), creatinine (Cr), and albumin, as well as the urine levels of glucose, Cr, and albumin. Sodium (Na) serum and urine levels were measured *via* the ion-selective electrodes method. Glomerular filtration rate (GFR) was calculated (28) using the equation (1):

$$CrCl = UF \times \frac{UCr}{PCr} \quad (1)$$

where, CrCl, UF, UCr, and PCr were put instead of Cr clearance, urine flow, urine Cr, and plasma Cr, respectively. Fractional excretion of sodium (FENa) was calculated using equation (2):

$$FENa = \frac{(UNa \times UF)}{(PNa \times GFR)} \times 100 \quad (2)$$

where, UNa and PNa were put instead of urine Na and plasma Na, respectively. Fractional excretion of glucose (FEG) was calculated using equation (3):

$$FEG = \frac{(UG \times UF)}{(PG \times GFR)} \times 100 \quad (3)$$

where, UG and PG were put instead of urine glucose and plasma glucose, respectively. Urine Cr load was calculated using equation (4):

$$UCr\ load = PCr \times UF \quad (4)$$

and urine albumin load was calculated using equation (5):

$$Urine\ albumin\ load = plasma\ albumine \times UF \quad (5)$$

Histopathology procedures

The tissue was immersed in a fixative (commonly 10% neutral buffered formalin) for several hours and then tissue was embedded in a block of paraffin wax and allowed to solidify. Paraffin-embedded tissue of the right kidneys was cut into thin sections (3-5 µm) using a microtome (Genex, Iran) and placed on glass slides. The periodic acid Schiff staining (PAS) and Jones methenamine silver (JMS) were applied to examine the tissue injury. To consider the kidney damage, the presence of tubular atrophy, fibrosis, connective tissue changes, inflammation, degeneration of tubular epithelial cells, congestion, and glomerular damage were evaluated. Based on the damage intensity, the samples were scored as 1-4, while a score of zero was considered to be normal tissue.

Table 1. Primers for quantitative real-time PCR analysis of gene expression.

Gene	Reveres primer sequence	Forward primer sequence	Reference
Beta-actin	CTGACCCATACCCACCATCAC	CTGACCCATACCCACCATCAC	Designed with NCBI Primer-BLAST
Icam1	CGCTCTGGGAACGAATACACA	AAGCTCTTCAAGCTGAGCGA	Designed with NCBI Primer-BLAST
Nox4	CCTGCTAGGGACCTTCTGTG	TGGGCCTAGGATTGTGTTTGA	Designed with NCBI Primer-BLAST

qPCR of kidney tissue

After hyperinsulinemic-euglycemic clamp, 50-100 mg of the left kidneys was removed, washed in ice-cold isotonic saline. Then, tissues were kept in DNase/RNase-free microtube and immediately put into a nitrogen tank for RNA isolation. According to the manufacturer's instructions, M-MLV RT (AnaCell, lot N. CS0021, Iran) was used to synthesize complementary DNAs (cDNAs) from 5 to 10 ng of the total RNA. To ascertain the expressions of the *Nox4* and *Icam1* mRNA genes, qPCR was carried out in accordance with our prior experience (22). The list of primers designed in this study has been provided in Table 1.

Statistical analysis

All data were expressed as mean ± SEM, and analyzed by SPSS software version 16 using one-way ANOVA followed by Tukey post-hoc test. In addition, ANOVA for repeated measures followed by Tukey post-hoc test was used to evaluate the measured data monthly or weekly. *P*-values $\leq$ 0.05 were considered statistically significant.

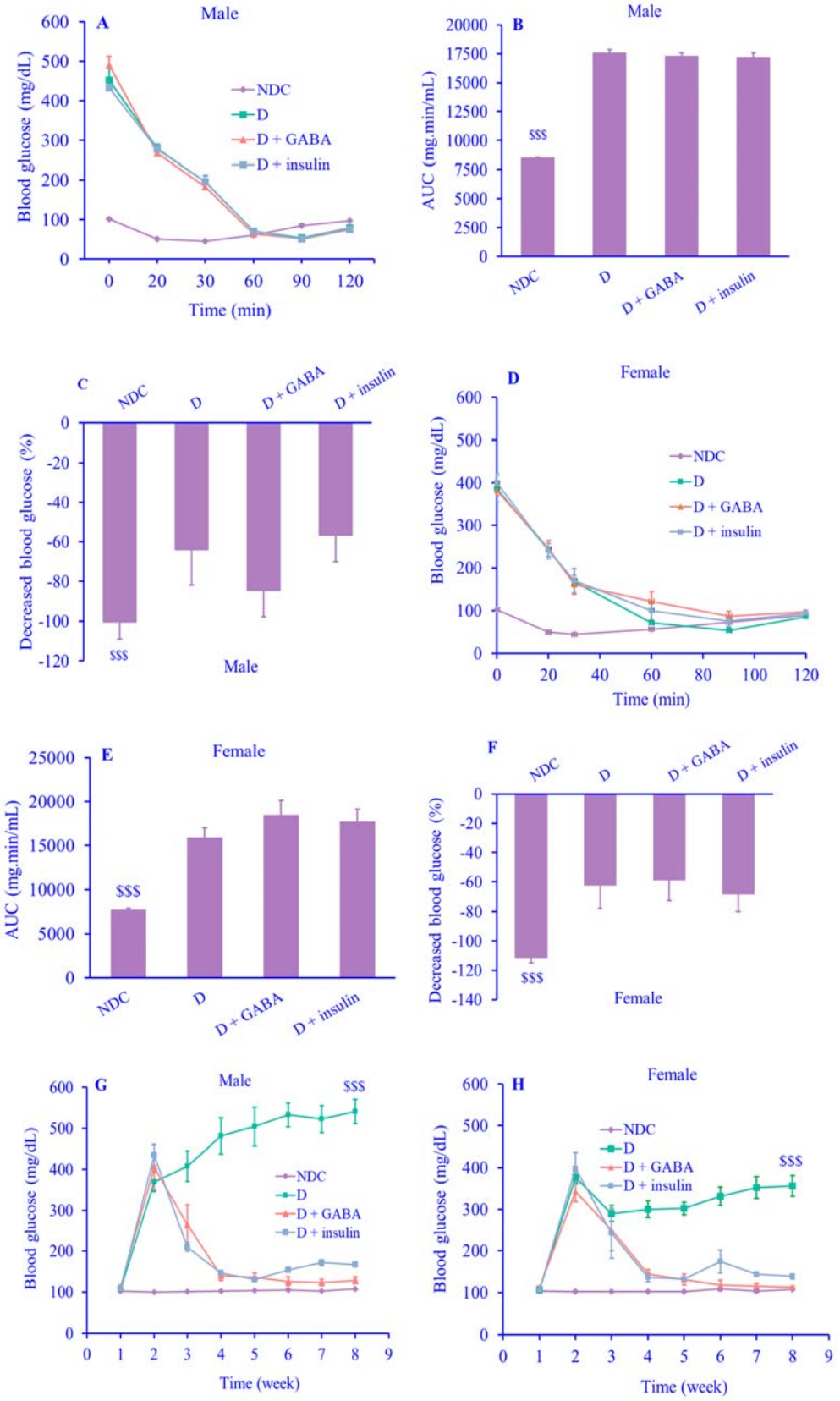
RESULTS

Changes in GIR, ITT, and blood glucose level in parents and their offspring

To confirm the induction of type 2 diabetes ITT, the glucose area under the curve (AUC), and the percentage of hypoglycemia were measured 20 min after insulin administration in all groups. The results showed that AUC in all diabetic groups significantly increased in comparison to NDC groups, and blood glucose could not decrease 20 min after insulin injection (Fig. 2A-F). The results of the present study, illustrated in Fig. 2 G and H, showed that the levels of blood glucose increased in both parents after diabetes induction in D groups compared to NDC groups, and hyperglycemia was maintained until the end of the study in the groups. The administration of GABA or insulin significantly decreased the blood glucose level in both parents in comparison to D group. Significant differences were not observed in blood glucose levels among all offspring in both genders (Fig. 2I and J). The results also showed that GIR decreased in both parents in the D group after diabetes induction compared to the NDC parents. GABA administration in both parents could significantly increase GIR compared to both D groups (Fig. 2K), but insulin could only significantly increase GIR in male parents compared to the male D group (Fig. 2K). The results in offspring groups also showed that GIR in male and female D groups was especially less than one in NDC groups (Fig. 2L). The administration of GABA or insulin in both parents significantly increased GIR in male and female offspring compared to D groups (Fig. 2L).

Changes in water consumption, urine volume, GFR, FENa, and FEG in parents and their offspring

The findings in parental groups (Fig. 3A and B) showed that water consumption in D groups significantly increased in comparison to NDC groups and the administration of insulin or GABA in male gender could significantly reduce polydipsia compared to the D group. As Fig. 3B shows, unlike insulin, the administration of GABA could not significantly decrease polydipsia in female diabetic animals compared to the female D group. Figures 3C and D showed that water consumption in male and female offspring of D parents significantly increased in comparison to the respective NDC ones. GABA administration in diabetic parents could particularly decrease water consumption in both male and female offspring compared to both offspring of D parents (Fig. 3C and D).


A
Male
Blood glucose (mg/dL)
Time (min)
NDC
D
D + GABA
D + insulin
B
Male
AUC (mg.min/mL)
$$$
C
Decreased blood glucose (%)
Male
D
Female
Blood glucose (mg/dL)
Time (min)
E
Female
AUC (mg.min/mL)
$$$
F
Decreased blood glucose (%)
Female
G
Male
Blood glucose (mg/dL)
Time (week)
H
Female
Blood glucose (mg/dL)
Time (week)

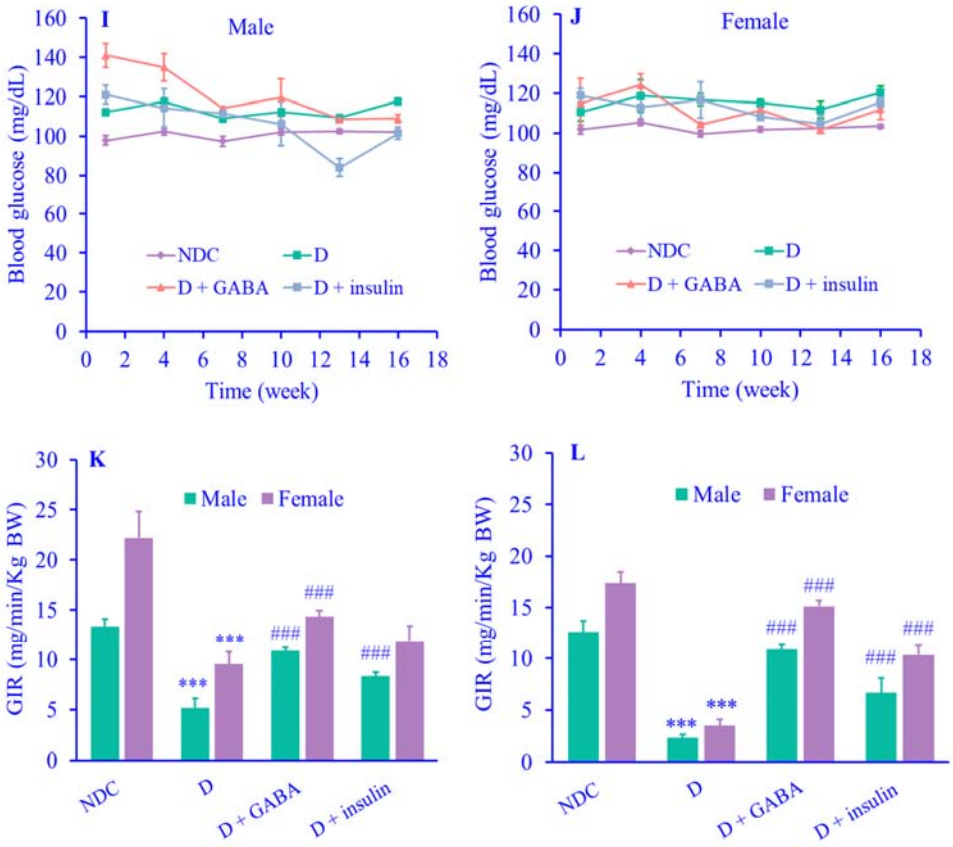
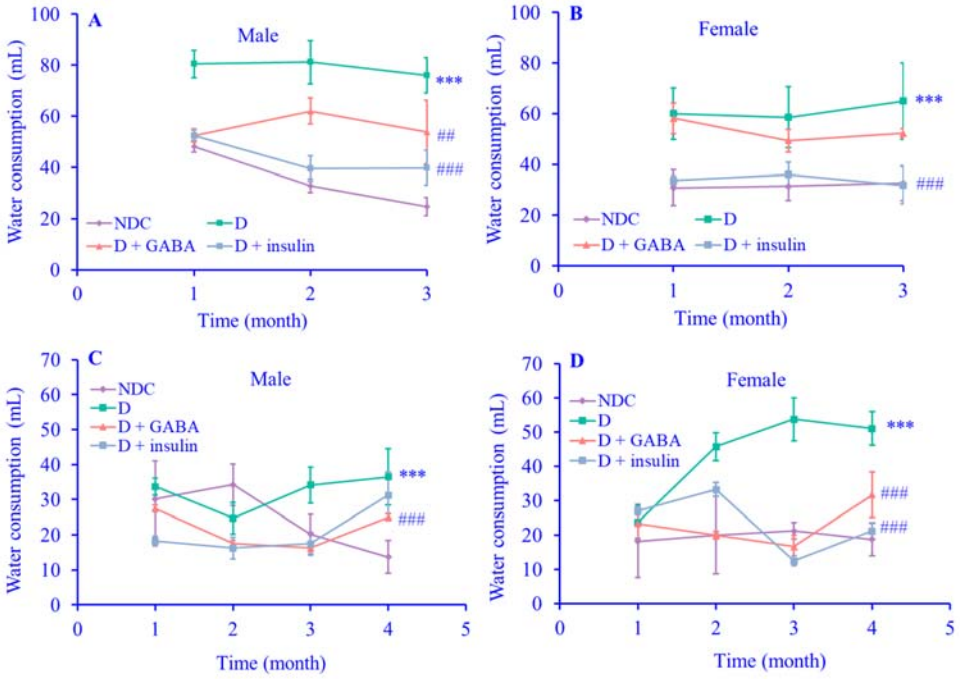
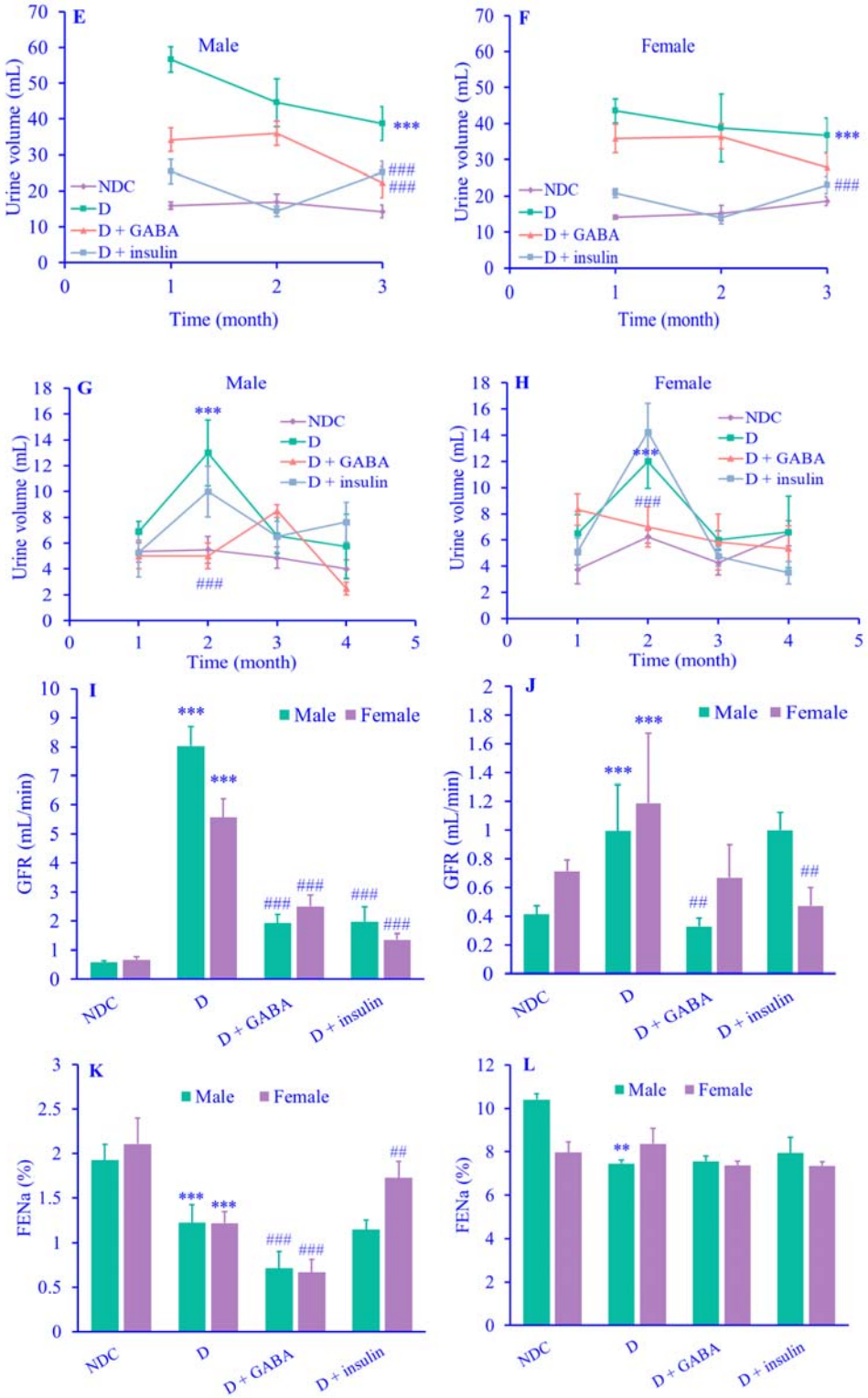


Fig. 2. Comparison of insulin tolerance test in (A) male and (D) female; AUC in (B) male and (E) female; decreased blood glucose in (C) male and (F) female in parents before intervention; blood glucose in (G) male and (H) female parents; blood glucose in (I) male and (J) female offspring; GIR in (K) parents and (L) offspring. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA. Data were expressed as mean ± SEM, n = 6. $^{***}P < 0.001$ demonstrates a significant difference compared with the respective NDC group in each gender; $^{###}P < 0.001$ versus the respective D group in each gender; $^{\$\$\$}P < 0.001$ versus other groups. AUC, Area under the curve; GIR, glucose infusion rate; NDC, non-diabetic control; D, diabetic; GABA, gamma-aminobutyric acid.

However, insulin therapy in diabetic parents just could significantly decrease water consumption in their female offspring compared to female offspring of D parents (Fig. 3D). The results of this study showed that urine volume in both D parents significantly raised when compared with NDC groups (Fig. 3E and F), and both insulin and GABA in male gender could significantly decreased urine volume compared to D group (Fig. 3E). On the other hand, GABA could not reduce polyuria in the female animals in comparison to female D group (Fig. 3F). The results presented in Fig. 3G and H showed that urine volume significantly increased in both male and female offspring of diabetic parents compared to ones of NDC parents the only 2 months after their follow-up. Insulin therapy in diabetic parents did not have any effect on the urine volume in their male and female offspring compared to the offspring of D parents, but in 2 months after follow-up, GABA administration could significantly reduce urine volume in both sexes of offspring compared to both sexes of male and female offspring of D parents. (Fig. 3G and H). GFR was calculated for all groups, and the results illustrated that diabetes induction seriously increased GFR in both parents compared with respective NDC groups, and GABA or insulin therapy significantly

decreased GFR in both parents compared with respective D groups (Fig. 3I). As the results presented in Fig. 3J, the offspring of D parents in both sexes have a significant increase in GFR compared to the offspring of NDC parents. However, treatment with insulin only in the female offspring and treatment with GABA only in the male offspring were able to significantly reduce GFR compared with the female and the male D groups, respectively (Fig. 3J). Figure 3K showed that FENa in both sexes significantly decreased in D groups in comparison to the respective NDC groups. GABA administration could significantly decrease FENa in both parents compared to both D parents (Fig. 3K). However, insulin therapy could only significantly increase FENa in female parents compared to the female D group (Fig. 3K). Also, the findings showed that diabetes induction could significantly decrease FENa in male offspring compared to the male NDC group. On the other hand, insulin or GABA administration in both parents could not make any significant changes in both sexes of their offspring compared with both sexes of the D offspring (Fig. 3L). The results of the present study (Fig. 3M) showed that FEG significantly decreased in both parents in the D groups compared to the NDC groups. Insulin or GABA therapy significantly increased FEG in both parents compared to both D groups. FEG in the male offspring of the D group significantly decreased compared to the male offspring of the NDC parents (Fig. 3N). In addition, female offspring of parents receiving insulin showed a significant reduction in FEG compared to the female offspring of D parents (Fig. 3N).




E
Male
Urine volume (mL)
Time (month)
NDC
D
D + GABA
D + insulin
***
###
F
Female
G
H
I
GFR (mL/min)
Male
Female
J
K
FENa (%)
L
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##
NDC
D
D + GABA
D + insulin

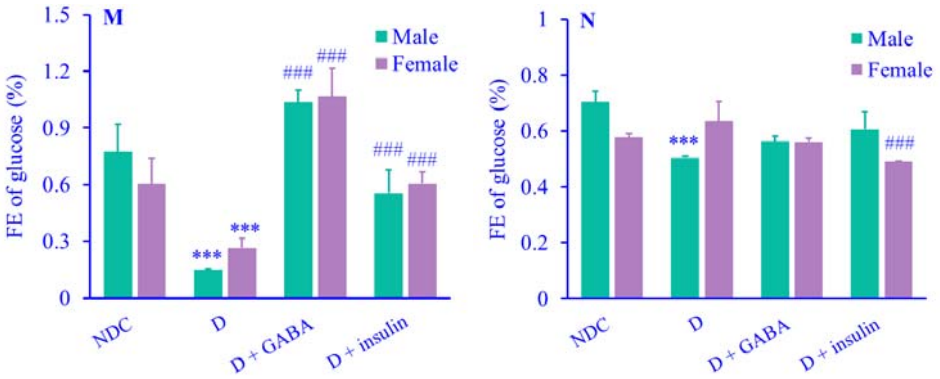
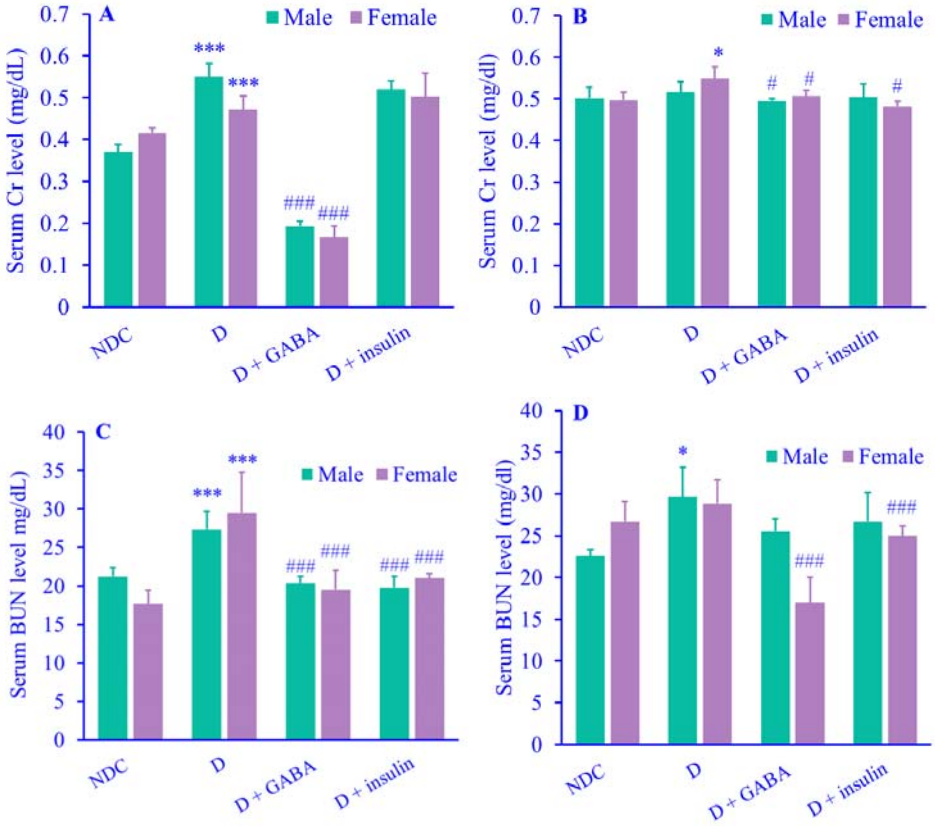


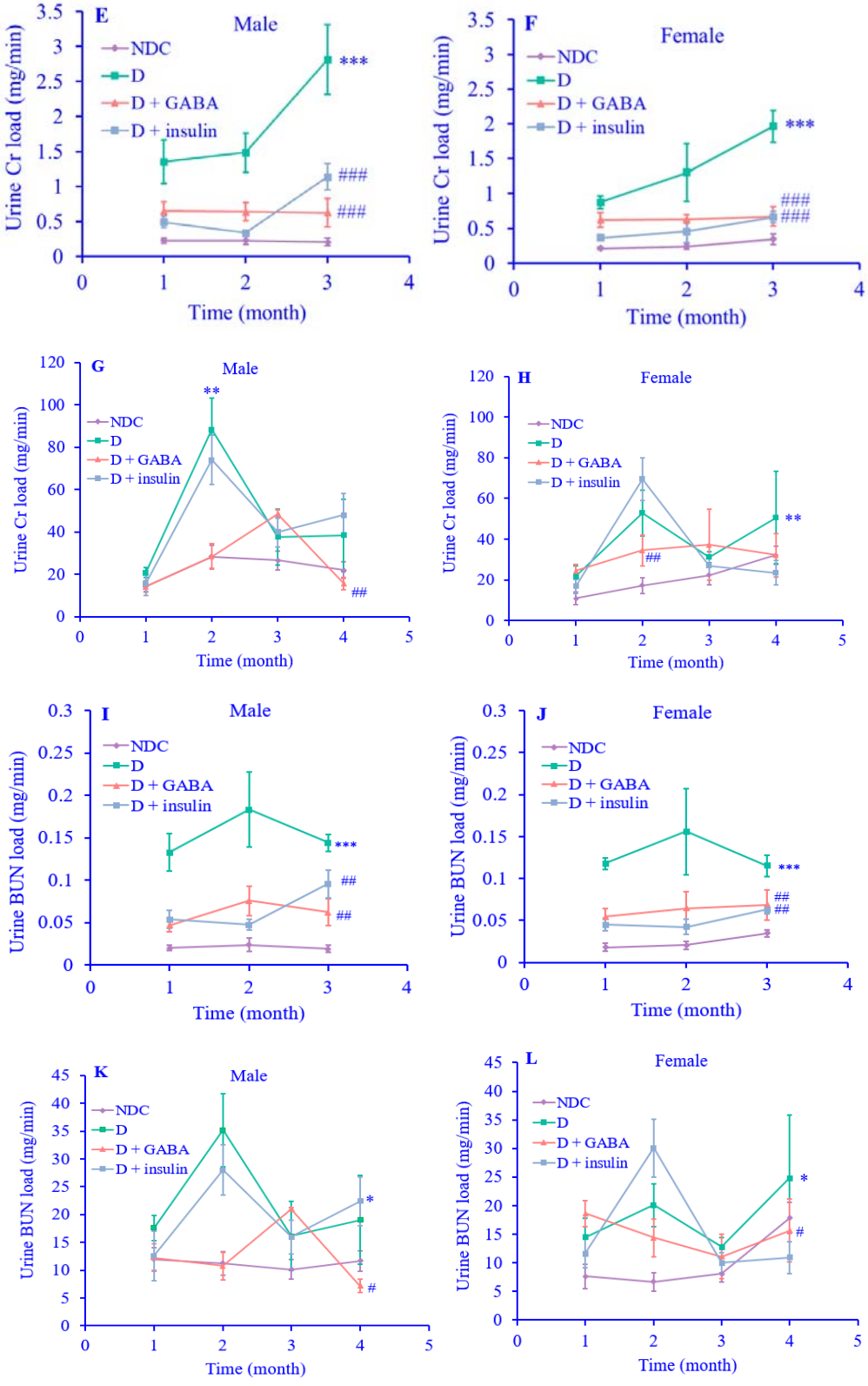
Fig. 3. Comparison of water consumption in (A) male and (B) female parents; water consumption in (C) male and (D) female offspring; urine volume in (E) male and (F) female parents; urine volume in (G) male and (H) female offspring; GFR in (I) male and female parents and (J) male and female offspring; FENa in (K) male and female parents and (L) male and female offspring; FE of glucose in (M) male and female parents and (N) male and female offspring. Water consumption and urine volume were measured every month by using the metabolic cage. To perform this test, each rat was put in a metabolic cage (Tajhizgostar Co, Iran) for 24 h. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA. Data were expressed as mean ± SEM, n = 6. $^{**}P < 0.01$ and $^{***}P < 0.001$ demonstrate significant differences compared with the respective NDC group in each gender; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus respective D group in each gender. GFR, glomerular filtration rate; FENa, fractional excretion of sodium; FE, fractional excretion; NDC, non-diabetic control; D, diabetic; GABA, gamma-aminobutyric acid.

Changes in serum Cr and BUN levels and urine load of albumin, Cr, BUN, and glucose in parents and their offspring

The results of the present study in Fig. 4A showed that diabetes induction increased serum Cr level in both parents in the D groups compared to both the NDC groups. GABA therapy could significantly decrease serum Cr level in both parents compared to both D parents (Fig. 4A). However, insulin administration could not considerably change serum Cr level in both parents compared to both D groups (Fig. 4A). The findings presented in Fig. 4B showed that serum Cr level in female offspring of D parents significantly increased in comparison to the female offspring of NDC parents. GABA therapy in parents significantly decreased the serum level of Cr in both sexes of their offspring compared to both D offspring (Fig. 4B). Female offspring of parents receiving insulin also showed reduced serum Cr level compared to female progeny of D parents (Fig. 4B). As Fig. 4C shown, diabetes induction significantly increased serum BUN level in both D parents compared to both NDC parents. Insulin or GABA therapy significantly decreased serum BUN level in both parents compared to both D parents (Fig. 4C). According to the data presented in Fig. 4D, the serum level of BUN in the male offspring of the D group significantly increased in comparison to the male offspring of NDC parents. Both insulin and GABA administration in parents were able to decrease dramatically serum BUN level in their female offspring compared to female offspring of the D parents (Fig. 4D). The results of the present study showed that the urine Cr load in both D parents gradually increased in comparison to NDC parents from month 1 by the end of month 3, which was significant. Treatment with GABA or insulin in both parents significantly decreased urine Cr load compared to the D groups (Fig. 4E and F). The results shown in Fig. 4G indicated that the urine Cr load in male offspring of the D group significantly increased in the second month compared with male offspring of the NDC group. However, significant differences were not observed between male offspring of groups insulin and D, but a significant difference was observed between GABA groups and D in male sex (Fig. 4G). Diabetes induction in parents caused to significant increase in the urine Cr load in their female offspring compared with female offspring of the NDC group (Fig. 4H).

The urine Cr load in female offspring of parents receiving GABA decreased significantly 2 months after birth in comparison to female offspring of groups insulin and D (Fig. 4H). According to the findings presented in Fig. 4I and J, diabetes induction significantly increased urine BUN load in both sexes compared with NDC groups, and GABA and insulin therapy could significantly decrease urine BUN load in both sexes compared to D groups. The findings of this research showed that urine BUN load in both male and female offspring of D parents significantly increased in comparison with both male and female offspring of NDC parents 4 months after follow-up (Fig. 4K and L). GABA therapy in both diabetic parents significantly decreased urine BUN load in both male and female offspring compared to both offspring of the D group (Fig. 4K and L). Diabetes induction significantly increased urine albumin load in both parents compared to both NDC parents (Fig. 4M and N). As shown in Fig. 4M and N, urine albumin load gradually increased from the beginning of the 1st month to the end of the 3rd month in both diabetic parents compared to both NDC parents. Insulin or GABA therapy significantly decreased urine albumin load in both parents compared to both D parents, and this decrease was not to the extent that urine albumin load reached the level of the NDC groups (Fig. 4M and N). The results of the present study showed that urine albumin load in both male and female offspring of diabetic parents significantly increased in comparison to both male and female offspring of NDC parents (Fig. 4O and P). The administration of GABA or insulin in diabetic parents could not change the urine albumin load in their offspring compared to the offspring of D parents (Fig. 4O and P).




E
Male
NDC
D
D + GABA
D + insulin
Urine Cr load (mg/min)
Time (month)
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D + GABA
D + insulin
Urine Cr load (mg/min)
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D + insulin
Urine Cr load (mg/min)
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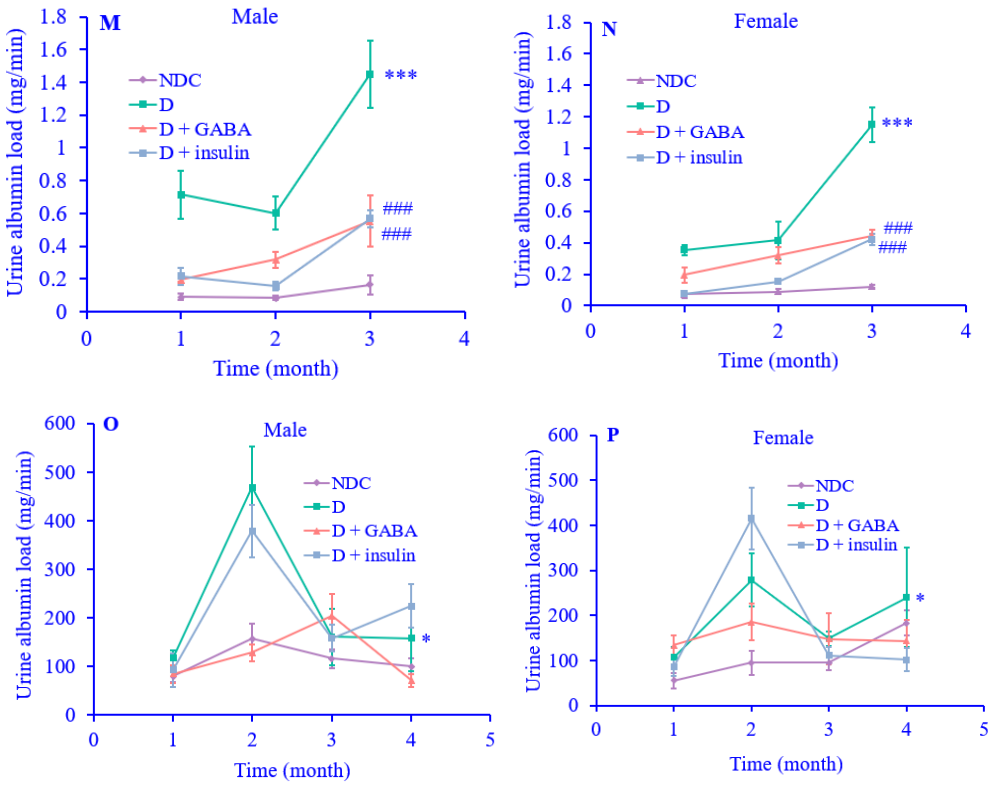

Fig. 4. The comparison of renal function in parents and offspring in all groups. Serum level of Cr in (A) parents and (B) offspring; serum level of BUN in (C) parents and (D) offspring; urine load of Cr in (E) male and (F) female parents; urine load of Cr in (G) male and (H) female offspring; urine load of BUN in (I) male and (J) female parents; urine load of BUN in (K) male and (L) female offspring; urine load of albumin in (M) male and (N) female parents; urine load of albumin in (O) male and (P) female offspring. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA. Data were expressed as mean ± SEM, n = 6. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ demonstrate significant differences compared with the respective NDC group in each gender; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus respective D group in each gender. BUN, Blood urea nitrogen; Cr, creatinine; NDC, non-diabetic control; D, diabetic; GABA, gamma-aminobutyric acid.

Changes in kidney weight, kidney pathology, and the expression of mRNA genes of Nox4 and Icam1 in parents and their offspring

The results of the present study showed that kidney weigh in both D parents significantly increased in comparison to both NDC parents (Fig. 5A). Insulin or GABA administration in both diabetic parents significantly decreased kidney weight compared to both D parents (Fig. 5A). The results illustrated in Fig. 5B showed that kidney weight in male offspring of D parents significantly increased in comparison to male offspring of NDC parents, and GABA therapy just relatively decreased kidney weight in male offspring of GABA group compared with male offspring of D groups. *Nox4* gene expression in both diabetic parents significantly increased in comparison to both NDC parents (Fig. 5C). GABA therapy significantly decreased *Nox4* gene expression in both parents compared to both D groups, but insulin therapy just decreased *Nox4* gene expression in female parent compared to female D parent (Fig. 5C). The results showed that however diabetes induction increased the expression of *Nox4* gene in both offspring of diabetic parents compared to both offspring of NDC parents, this increment was significant only in female diabetic offspring compared with female offspring of NDC parents (Fig. 5D). According to Fig. 5D, the administration of GABA only could significantly decrease *Nox4* gene expression in male offspring of GABA parents compared with male offspring of the D group.

The results of this study indicated that *Icam1* mRNA gene expression significantly increased after diabetes induction in both parents compared to both NDC parents (Fig. 5E). Insulin or GABA administration in both parents significantly decreased the expression of *Icam1* mRNA gene expression compared to both D parents (Fig. 5E). The results also showed that *Icam1* mRNA gene expression in male offspring of D parents significantly increased in comparison to male offspring of NDC parents; however, the expression of this gene significantly decreased only in male offspring of parents receiving GABA compared to male offspring of D parents (Fig. 5F). All of the results were summarized in Table 2.

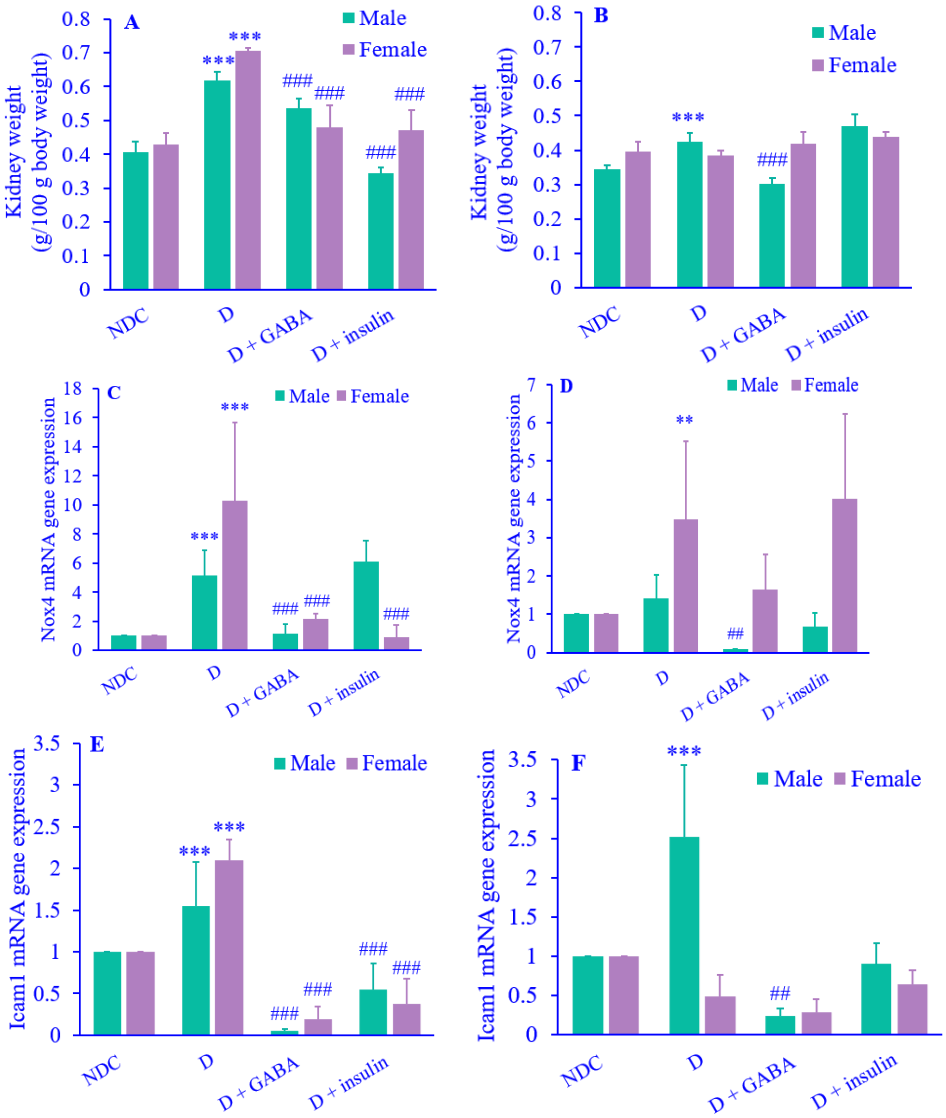

Fig. 5. Comparison of kidney weight in (A) male and female parents and (B) male and female offspring; Nox4 mRNA gene expression in (C) male and female parents and (D) male and female offspring; Icam1 mRNA gene expression in (E) male and female parents and (F) male and female offspring. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA. Data were expressed as mean ± SEM, n = 6. $^{**}P < 0.01$ and $^{***}P < 0.001$ demonstrate significant differences compared with the respective NDC group in each gender; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus respective D group in each gender. Nox, NADPH oxidase; Icam1, intercellular adhesion molecule-1; NDC, non-diabetic control; D, diabetic; GABA, gamma-aminobutyric acid.

Table 2. Summary of study results. Yellow colour was assigned for normal changes; red colour for increasing changes; green colour for decreasing changes; and white colour for without changes. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA.

Group	**NDC**				**D**				**D + insulin**				**D + GABA**			
Generation	**P**		**O**		**P**		**O**		**P**		**O**		**P**		**O**	
Gender	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Glucose infusion rate (mg/min/Kg body weight)																
Blood glucose level (mg/dL)																
Water consumption (mL)																
Urine volume (mL)																
Glomerular filtration rate (mL/min)																
Fractional excretion of Na (%)																
Fractional excretion of glucose (%)																
Serum level of creatinine (mg/dL)																
Serum level of blood urea nitrogen (mg/dL)																
Urine load of albumin (mg/min)																
Urine load of creatinine (mg/min)																
Urine load of blood urea nitrogen (mg/min)																
Kidney weight (g)																
NOX4																
ICAM1																

NDC, Non-diabetic control; D, diabetic; GABA, gamma aminobutyric acid; P, parents; O, offspring; M, male; F, female; Nox, NADPH oxidase; Icam1, intercellular adhesion molecule-1.

Changes in kidney tissues in parents and their offspring

The pathology results were presented in Fig. 6. The findings in the renal tissues of parents showed that renal tubules in both male and female parents of D groups were damaged, and casts were observed in some parts of tubules in comparison to both male and female parents of NDC groups. Tubular size and cellular diameter in both male and female parents of D groups were smaller than both male and female parents of NDC groups. Bare basement membrane of renal tubules was seen in both male and female parents of D groups compared to both male and female parents of NDC groups (Fig. 6A). Renal tubule cells in both male and female offspring of D groups atrophied and damaged in comparison to both male and female offspring of NDC groups, and tubular size and cellular diameter in both male and female offspring of D groups were smaller than both male and female offspring of NDC groups. Bare basement membrane of renal tubules was seen in both male and female offspring of D groups compared to both male and female offspring of NDC groups (Fig. 6B).

No atrophy, damage, and tubular casts were seen in renal tubule cells in both male and female parents of insulin or GABA groups and their offspring compared to both male and female parents of D groups and their offspring, and tubular size and cellular diameter in both male and female parents of insulin or GABA groups and their offspring were bigger compared to both male and female parents of D groups. Ordinary basement membrane of renal tubules was seen in both male and female parents of insulin or GABA groups and their offspring, compared to both male and female parents of D groups and their offspring (Fig. 6).

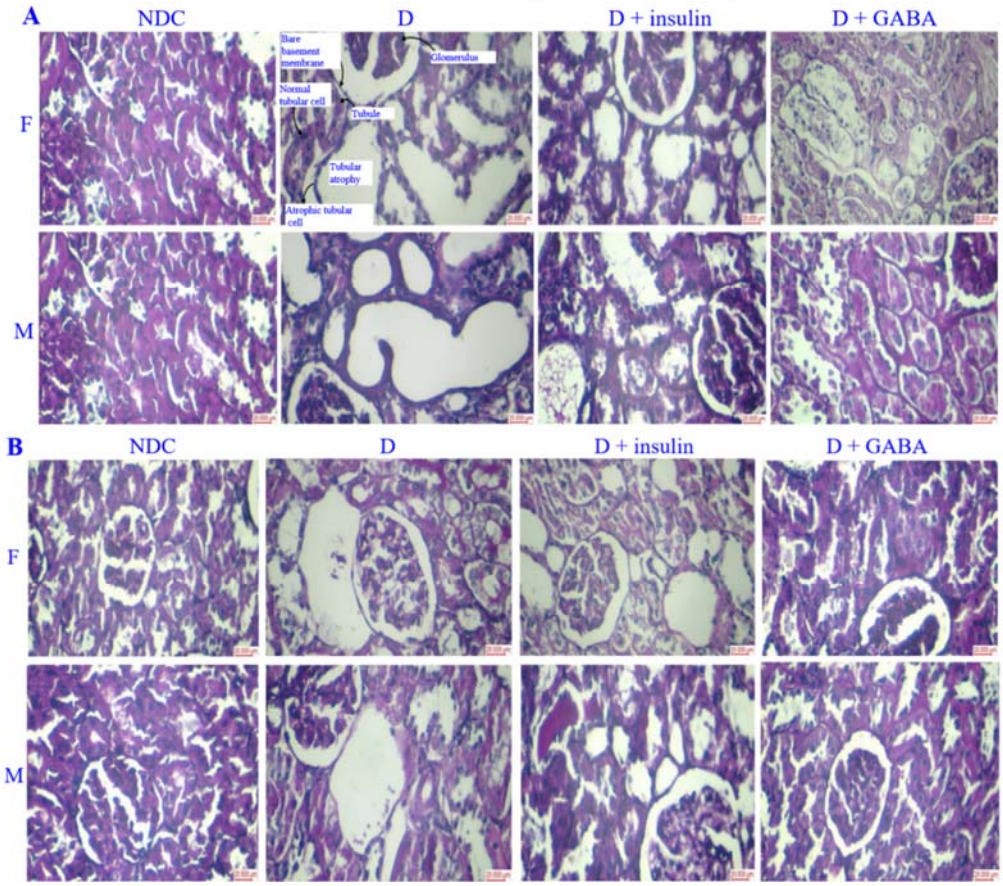

Fig. 6. Comparison of pathology images (magnification ×100) in (A) parents and (B) offspring in the experimental groups. In parental groups, the NDC groups were fed with a normal diet, and diabetes was induced by receiving a high-fat diet and streptozotocin (35 mg/Kg). In addition, D + insulin and D + GABA parental groups received insulin at the dose of 2.5 U/Kg and GABA at the dose of 1.5 g/kg, respectively. Offspring groups were followed for 4 months with a regular diet (standard chow) and without receiving insulin or GABA. NDC, non-diabetic control; D, diabetic; GABA, gamma aminobutyric acid.

DISSCUTION

This study aimed to examine the administration of GABA in the male and female diabetic rat model and prevent DN in the first and second generations of the diabetic rat model and improve kidney function *via* decreasing insulin resistance and the expression of *Nox4* and *Icam-1* genes. In comparison to D parents, the results showed that both parents could benefit from GABA administration in terms of hyperglycemia, insulin resistance, and kidney function. The findings also demonstrated that male and female offspring in the GABA group had better glycaemic parameters and kidney function than both sexes of D offspring. The majority of end-stage renal disease cases are caused by DN, and there are 3 main risk factors for DN, including diabetes, elevated plasma glucose levels, and insulin resistance (29-32). Insulin resistance is closely associated with microalbuminuria, which is one of the main symptoms of DN, and it seems that renal tubule epithelial cells are novel insulin-sensitive cells (30). The current results indicated that type 2 diabetes induction, as previously shown (33), in both parents decreased GIR (a standard gold method to measure insulin resistance), and increased blood glucose levels, and the AUC of ITT. On the other hand, diabetes had a reduced sensitivity to insulin 20 min after insulin administration compared to both NDC parents. Also, kidney function, including GFR, FENa, and FEG, was likely decreased in both diabetic parents by high blood glucose levels and insulin resistance. Increased GFR above normal values is associated with early phases of kidney disease (34). The results showed that urinary albumin load in both diabetic parents increased compared to both NDC parents. A part of the albumin excretion in the urine is related to increased GFR, and another part is related to damage to the glomerular basement membrane, as shown in the pathology results. The literature has suggested that urinary excretion of albumin is a problem in diabetic patients (35). Finding drugs for enhancing kidney function in diabetic people is crucial since DN is a major contributor to end-stage renal disease and a significant risk factor for cardiovascular disease. Angiotensin receptor blockers (ARBs) have been suggested as first-line treatments for DN (36), and reported that high doses of ARBs are needed to treat DN; however, in some cases, aldosterone escape may reverse the beneficial effects of ARBs on DN (36). GABA was selected to prevent DN in HFD-induced diabetes in parents and their offspring. Literature has documented that GABA is present in peripheral organs, including the kidneys, and may alter kidney function and prevent chronic kidney disease (37,38). Takano *et al*. discovered GABA receptors, GABA-producing enzymes, transporters, and GABA-degrading enzymes in the kidney (37). In addition, it has been shown that GABA treatment has preventive effects on ischemia/reperfusion in the kidney (39). GABA ameliorates kidney injury by reducing macrophage infiltration in renal tissue (40). Kim *et al*. showed that some physiological changes caused by acute renal failure such as body weight, kidney weight gain, BUN and Cr elevation, GFR, FENa, and urine osmolality decrease in rats were significantly improved by oral administration of GABA (41). In addition, one study revealed that GABA has potential as a therapeutic agent against the renal damage involved in acute renal failure (41) as well as tubular fibrosis and atrophy. The treatment of GABA in the experiment also enhanced all renal function, including GFR, FENa, and FEG. In the present investigation, GABA treatment reduced kidney weight, an inflammation indicator, in both diabetic parents and their offspring. Researchers proposed that kidney hypertrophy and atrophy can occur in DM patients (42). Renal hypertrophy occurs in DN, and large kidneys predict poor outcomes in patients with diabetes, and renal hypertrophy predicts microalbuminuria in patients with type 1 diabetes (43). The present findings showed that GABA administration decreased albuminuria in both diabetic parents and their offspring. Also, GABA, in addition to reducing insulin resistance, increased urinary glucose excretion. As the results of renal pathology showed, urinary glucose excretion did not cause kidney damage, but reduced blood glucose levels and improved the symptoms of hyperglycemia in the diabetic parents and their offspring.

The molecular mechanisms involved in DN include high blood glucose levels, insulin resistance, and the activation of the renin-angiotensin system (44,45). On the other hand, oxidative stress is crucial for DN, and rat kidneys express *Nox4*. The expression of *Nox4* is quickly induced by DN, and *Nox4* is a significant ROS source linked to kidney damage in diabetes (46-48). The results of this study showed that *Nox4* mRNA gene expression increased in both parents after diabetes induction compared to NDC parents, and GABA therapy decreased the expression of *Nox4* in both parents compared to D parents. Moreover, insulin could reduce *Nox4* expression in females only compared to diabetic females. Researchers showed that GABA decreases *Nox4* gene expression in cultured microglia *via* the $GABA_A$ receptor (49). Gao *et al*. reported that GABA agonists *via* $GABA_B$ receptor could improve the gastric ischemia-reperfusion injury in rats by the inhibition of *Nox4* gene expression in the gastric mucosa (50).

Icam-1 plays a vital role in endothelial dysfunction (51). *Icam-1* is an acute-phase protein marker of inflammation. Recently, some researchers have reported that increased serum *Icam-1* levels are correlated with albuminuria in T1DM and T2DM patients (52), and it was associated with the risk of DN. *Icam-1* might help physicians in the early diagnosis of DN (13). Both mRNA and protein levels of *Icam1* were significantly increased in animal models of DN with T1DM and T2DM (16). The findings of the study demonstrated that both diabetic parents and their male pups had higher levels of *Icam1* mRNA after diabetes induction, as compared to NDC groups. GABA may reduce *Icam1* levels in the arteries and has anti-inflammatory effects, according to the literature (53). The current findings also indicated that GABA administration might lower *Icam1* mRNA gene expression in both parents and their offspring, as compared to the D groups.

CONCLUSION

The results of the study showed that GABA could decrease the risk of DN in both diabetic parents and their offspring, and it could improve hyperglycemia and insulin resistance in both parents and their offspring *via* improving kidney function. According to the findings, maybe the kidney considers a new target of insulin action.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Author's contributions

N. Soltani designed the study, wrote and approved the manuscript, and analyzed the data; H. Rezazadeh performed the experiments and wrote the manuscript; S. Maghareh-Dehkordi, M.V. Touliat, and A. Talebi performed the experiments. All authors read and approved the final version of the manuscript.

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