

N-Acetyl-L-Cysteine Supplement in Early Life or Adulthood Reduces Progression of Diabetes in Nonobese Diabetic Mice

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ABSTRACT

Background: Oxidative stress contributes to the pathologic process leading to the development, progression, and complications of type 1 diabetes (T1D).

Objective: The aim of this study was to investigate the effect of the antioxidant N-acetyl-L-cysteine (NAC), supplemented during early life or adulthood on the development of T1D.

Methods: NAC was administered to nonobese diabetic (NOD) female mice during pregnancy and lactation, and the development of diabetes was followed in offspring. In an additional set of experiments, offspring of untreated mice were given NAC during adulthood, and the development of T1D was followed. Morbidity rate, insulinitis and serum cytokines were measured in the 2 sets of experiments. In addition, markers of oxidative stress, glutathione, lipid peroxidation, total antioxidant capacity and activity of antioxidant enzymes, were followed.

Results: Morbidity rate was reduced in both treatment protocols. A decrease in interferon γ , tumor necrosis factor α , interleukin 1α , and other type 1 diabetes-associated proinflammatory cytokines was found in mice supplemented with NAC in adulthood or during early life compared with control NOD mice. The severity of insulinitis was higher in control NOD mice than in treated groups. NAC administration significantly reduced oxidative stress, as determined by reduced lipid peroxidation and increased total antioxidant capacity in serum and pancreas of mice treated in early life or in adulthood and increased pancreatic glutathione when administered in adulthood. The activity of antioxidant enzymes was not affected in mice given NAC in adulthood, whereas an increase in the activity of superoxide dismutase and catalase was demonstrated in the pancreas of their offspring.

Conclusion: NAC decreased morbidity of NOD mice by attenuating the immune response, presumably by eliminating oxidative stress, and might be beneficial in reducing morbidity rates of T1D in high-risk individuals. *Curr Dev Nutr* 2019;3:nzy097.

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder in which the immune system specifically attacks the insulin secreting β -cells in the pancreatic islets of Langerhans. Epidemiological studies show that the prevalence of T1D is globally increasing, especially in the young (1, 2), emphasizing the presence of some environmental factors triggering the onset of diabetes in susceptible subjects with a genetic predisposition. Autoantibodies against β -cell-specific antigens, such as glutamic acid decarboxylase, tyrosine phosphatase-like protein (IA-2), and insulin (IAA), can be detected months and even years before the diagnosis of diabetes (3, 4). Moreover, many of the individuals carrying these autoantibodies will never develop diabetes (5), indicating that the self-reactive T cells may stay naïve under the control of the immune system for years or even over the entire



Keywords: type 1 diabetes, offspring, N-acetyl-L-cysteine, oxidative stress, antioxidant

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Abbreviations used: AOX, antioxidant; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; iNOS, inducible NO synthase; NAC, N-acetyl-L-cysteine; NOD, nonobese diabetic; ROS, reactive oxygen species; SOD, superoxide dismutase; T1D, type 1 diabetes; TAC, total antioxidant capacity.

lifespan. Failure of the immune system to further maintain control of these T cells is probably affected by external stimuli, which are not yet fully characterized (6). Several environmental agents have been suggested to promote the outbreak of the disease, including viral infection (7), dietary factors (8–14), and impaired intestinal mucosal immunity (15). In addition, accelerated growth rate in childhood was associated with increased risk of T1D (16).

The role of oxidative stress, which is widely recognized to play an important pathophysiological role in the development of various severe diseases, is not clear in the etiology of T1D (17). A positive correlation does exist between the presence of oxidative stress and diabetes (18). Increased levels of molecules generated by oxidation reactions, such as lipid peroxidation products and protein carbonylation, were found in plasma and urine of T1D patients. In addition, an increase in oxidation products was detected in adipose tissue, liver, and muscle of diabetic animals. However, whereas the contribution of oxidative stress to the development of the severe complications related to diabetes is clearly established (19–22), the role of oxidative stress in the triggering events leading to the progression of the disease has not yet been validated.

Although it is unclear whether oxidative stress is among the primary triggering factors of the disease, it is evident that reactive oxygen species (ROS) are generated and also are crucial to the propagation of the autoimmune attack on β -cells (23). Activated cells of the immune system attack β -cells by several different mechanisms, including activation of FAS-ligand signaling (24), release of perforin/granzymes, and secretion of proinflammatory cytokines. Neutralizing ROS by enhancing antioxidant defense mechanisms was found to protect β -cells against cytokine-induced apoptosis *in vitro* (25, 26). In addition, resistance to alloxan-induced diabetes in mice was correlated with elevated ROS dissipation mechanisms (27). Thus, oxidative stress mediates the damage induced by the autoimmune attack, suggesting that elimination of this stress may limit β -cell destruction. Preventing type 1 diabetes by overexpression of antioxidant enzymes *in vivo* yields conflicting results. Although overexpression of thioredoxin and heme oxygenase-1 was beneficial (28, 29), overexpression of catalase sensitized nonobese diabetic (NOD) mice to diabetes. *N*-Acetyl-L-cysteine (NAC) is the most popular antioxidant used in laboratory experiments, exerting its antioxidative capacities along with a high safety profile (30, 31). Several *in vivo* studies had shown a beneficial effect of chronic treatment with NAC on blood glucose and glucose tolerance in type 2 diabetic mice (32–34), but its benefits on models of type 1 diabetes were barely demonstrated before.

Accordingly, we have attempted in this study to further clarify the role of oxidative stress and the potential benefit of antioxidants in the development of T1D. We investigated the effect of NAC supplemented before the onset of diabetes in early life, during pregnancy and lactation, or in adulthood on the progression of diabetes in NOD mice. This model develops spontaneous disease, as a result of an extensive infiltration of immune cells leading to insulinitis (35), as is also found in affected humans. In addition, there is some similarity in genes of type 1 diabetes between NOD mice and T1D humans (36). Based on these similarities, this model is one of the most commonly used to study the pathophysiology of T1D and potential therapies to this disease (37).

Methods

Materials

NAC was purchased from Mercury. Malondialdehyde was purchased from MP Biochemicals. An Insulin ELISA kit was purchased from Mercodia. Thiobarbituric acid, cumene hydroperoxide, glutathione, glutathione reductase, and NADPH were all purchased from Sigma Aldrich.

Methods

Study design.

The Animal House at the Ariel University operates in compliance with the rules and guidelines set down by Israel's Ministry of Health's Council for Animal Experimentation, based on the US NIH's Guide for the Care and Use of Laboratory Animals, DHEW (NIH, Pub. 78-23). All studies were approved by the institute committee on the use and care of animals, with the institutional license number IL-60-11-14. NOD mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in an animal laboratory with a controlled environment of 20–24°C, 45–65% humidity, and a 12-h (0730–1930) light/dark cycle. All experiments were performed on females, which are known to develop diabetes at higher rates than males (60–80% and 20–30%, respectively).

According to the 3Rs of the use of animals in research, in order to reduce the number of animals used, the study design was built in a way that enabled the use of the same control group for the 2 different interventions: NAC supplemented during gestation and lactation or NAC given during adulthood. For this (see scheme in [Figure 1](#)), breeding females (F_0) were divided into 2 groups, a control group and an NAC-treated group, in which NAC at a concentration of 600 mg/kg/d had been administered to females from breeding, through gestation and the lactation period. Neonates (F_1) had been separated at 3 wk of age and were maintained on standard unpurified diet and water. In order to investigate the effect of NAC given in adulthood on the prevalence of diabetes, female offspring (F_1) of control F_0 female were randomly separated at 6 wk of age into 2 groups—control group (-NAC) and test group supplemented with NAC at 600 mg/kg/d—whereas female offspring of NAC-treated F_0 mice were not given NAC any more. Thus, this study included 3 groups of F_1 female: offspring of control F_0 (control group), offspring of control F_0 given NAC in adulthood (600 mg/kg/d) and offspring of NAC-treated mice. The dose used in this study was chosen according to a preliminary dosing (200–1200 mg/kg/d) experiment, demonstrating a similar effect of all doses used on morbidity rate (data not shown), and on previous studies, demonstrating beneficial effects of NAC on glycemic control in T2D mice, whereas no adverse effects were found (38–40).

In all intervention and experimental groups, the mice consumed *ad libitum* standard rodent unpurified diet (186 g/kg proteins, 442 g/kg carbohydrates, 62 g/kg fat, and 36 g/kg fibers; Envigo, Teklad TD.2018; detailed information on the diet is provided in [Supplemental Tables 1 and 2](#)) and *ad libitum* drinking-water in the control group, or water supplemented with NAC, daily. The oral route of administration was chosen in order to mimic the oral administration of NAC taken as supplement, and as is commonly done by others (41). The mean consumption of water with or without NAC supplementation was measured. The NAC concentration in drinking-water was calculated

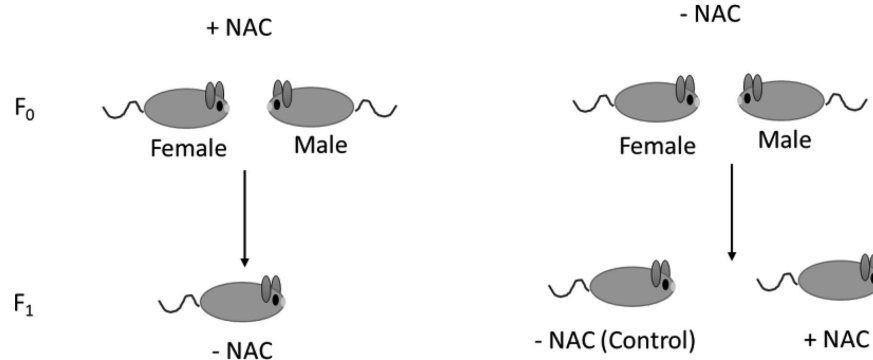


FIGURE 1 Scheme of the study design, as described in Methods.

and prepared according to their measured mean daily intake, in order to reach the dosage of NAC in the different groups.

The rate of development of diabetes and insulinitis was followed as the primary outcome, whereas markers of oxidative stress, activity of antioxidant enzymes, and level of cytokines were measured as secondary outcomes.

For the survival experiments, mice were followed until 30 wk of age. According to expected 70% rate of disease development, in order to obtain a power analysis of 80%, each study group included 40 mice. For all other measurements, 14-wk-old mice ($n = 10$) had been anesthetized by intraperitoneal injection of ketamine + xylazine (ketamine: 100 mg/kg, xylazine: 10 mg/kg), and all efforts were made to minimize suffering. Anesthetized mice were euthanized by terminal bleeding, blood was collected, and serum was prepared and stored at -80°C until assayed for insulin, lipid peroxidation, and total antioxidant capacity (TAC). Liver and pancreas were isolated for histological and biochemical analyses, and protein concentration was measured using the Bradford method.

Diagnosis of diabetes.

Tail blood glucose was monitored every other week, from age 12 to 40 wk, using the Accu-Chek Go glucometer (Roche Diagnostics). Animals with blood glucose concentrations above 250 mg/dL for 2 consecutive measurements were considered diabetics.

Cytokine measurement.

Serum cytokines were measured using the mouse cytokine array panel A kit (R&D Systems). The assay was performed according to the manufacturer's instructions in duplicates.

Lipid peroxidation analysis.

Lipid peroxidation was quantified using the thiobarbituric acid reactive substance assay as previously described (42). OD was measured at 532 nm using a Tecan Infinite F200 microplate reader. Values were calculated (nmol malondialdehyde/mg protein) according to a calibration curve of 1,1,3,3-tetraethoxypropane.

TAC analysis.

TAC was measured using the QuantiChrom Antioxidant capacity assay kit (BioAssay Systems) according to the manufacturer's instructions. In this assay, Cu^{2+} is reduced by antioxidant to Cu^{+} , which forms a colored complex with a dye reagent. The absorbance was measured using Tecan

Infinite F200 microplate reader at a wavelength of 570 nm. TAC in a sample is then assessed as the Trolox equivalent antioxidant capacity. A value of 1 Trolox equivalent antioxidant capacity in a sample is defined as a concentration that is equivalent to 1 mmol/L Trolox, a water-soluble analog of α -tocopherol.

Reduced glutathione and oxidized glutathione analysis.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using the GSH/GSSG Ratio Detection Assay Kit (Abcam). In this kit, a nonfluorescent dye turned fluorescent upon reacting with glutathione. Serum and tissue extracts were deproteinized by Trichloroacetic acid (TCA), and the pH was neutralized by NaHCO_3 . Total glutathione (GSH + GSSG) and GSH were measured according to the manufacturer's instructions. The level of GSSG was calculated according to the following formula: $(\text{total glutathione} - \text{GSH}) / 2$. The signal was measured using a fluorescence microplate reader at an Ex/Em of 490/520 nm.

Catalase activity assay.

Pancreas and liver were homogenized in HEPES buffer (20 mM). Peroxide removal was measured (240 nm) in phosphate buffer (0.05 M, pH 7.8) to follow catalase activity (43). Catalase activity was calculated according to the following formula:

$$k = \frac{1}{60} \ln \frac{A_0}{A_{60}} \quad (1)$$

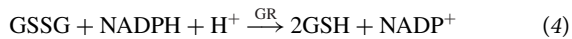
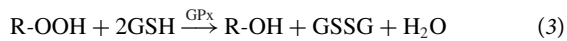
where A_0 is the initial absorbance, and A_{60} is the absorbance at 60 s. Based on the calculation of k , $k_{\text{total/mL}}$ was calculated, and the results were normalized to protein concentration according to the following formula:

$$\frac{k}{\text{mg}} = \frac{k_{\text{total/mL}}}{\text{mg/mL}} \quad (2)$$

Glutathione peroxidase activity assay.

In this reaction, 2GSH are oxidized by glutathione peroxidase (GPx) to the generation of GSSG, in the presence of cumene hydroperoxide (R-OOH), which was used in this assay as a substrate. This reaction was coupled to the reduction of GSSG by glutathione reductase (GR) in the presence of NADPH. NADPH disposal is measured at 340 nm,

indicating the GPx-dependent reaction rate (43):



GPx activity was calculated according to Equation 3:

$$\text{Units/mL} = \frac{\text{mmol}}{\text{min} \cdot \text{mL}} \quad (5)$$

Superoxide dismutase activity assay.

Superoxide dismutase (SOD) activity was measured using an SOD activity kit (Cayman Chemicals). Pancreas and liver were homogenized in HEPES buffer (20 mM). In this biochemical assay, xanthine-xanthine oxidase is used to generate O_2^- . Tetrazolium salt is reduced in the presence of O_2^- to the generation of formazan dye, which has absorbance at 460 nm wavelength, and used as an indicator of O_2^- production. SOD, which neutralizes O_2^- to the generation of H_2O_2 , competes with tetrazolium, leading to reduced formazan production. SOD activity is calculated according to the manufacturer's instructions. One unit activity is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Histology.

Pancreata obtained from mice at 14 wk of age were fixed in 10% formalin and then embedded in paraffin. Paraffin-embedded tissue sections were stained with hematoxylin and eosin. Sections were taken from 3 different levels. For the scoring of insulinitis, each islet was scored as normal (grade 0), peri-islet insulinitis (grade 1), intra-islet insulinitis, covering <50% of the islet (grade 2), or extensive insulinitis, covering >50% of the islet's area (grade 3). Scoring was performed by a reader blinded to the categories of the mice. Eight pancreata per group were stained, enabling the analysis of 228 islets in control, 237 islets in NAC-treated mice, and 219 islets in offspring of NAC-treated mice.

Data analysis

Values are presented as means \pm SEM. Statistical differences between the treatments and controls were tested using an unpaired 2-tailed Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc testing, when appropriate. Analysis was performed using the GraphPad Prism 5.0 software. Fisher's exact test and Kaplan-Meier analysis were used for the statistical analysis of survival rate. A chi-square analysis was used for statistical analysis of insulinitis. A difference of $P < 0.05$ or less in the mean values was considered statistically significant.

Results

NAC reduced morbidity rates of NOD mice

Morbidity rates were followed in female NOD mice supplemented by NAC during adulthood. NAC did not affect morbidity rates at age 12–24 wk (Figure 2A), but significantly reduced morbidity rate from 16/20 mice (81%) in untreated mice to 9/15 mice (45%) in NAC-treated mice at age 30 wk ($P < 0.05$, according to Fisher's exact

test). In addition, the possibility that NAC supplementation given to dams during pregnancy and lactation might affect morbidity rates in offspring was investigated. NAC supplementation significantly reduced the morbidity rate in offspring ($P < 0.05$ according to Kaplan-Meier analysis), from 16/20 (80%) to 10/20 mice (50%) at age 30 wk, as shown in Figure 2A.

In agreement with the lower morbidity rate, the pathological autoimmune attack was also reduced in NAC-treated mice (600 mg/kg/d), according to the 0–3 score (Figure 2B), as shown in Figure 2C and D, which presents the extent of insulinitis in 14-wk-old mice. There was a significant difference ($P < 0.001$, chi-square analysis) between control and NAC-treated mice, either treated in adulthood (NAC/adults) or during early life (NAC/offspring). Although only 23% of islets were free of leukocyte infiltration in untreated mice, 60% of islets in NAC/adults and 44% of islets in NAC/offspring were free of insulinitis. On the other hand, 28% of islets in control mice, but only 6.7% and 13% of islets in NAC-treated/adults and NAC/offspring mice, respectively, were completely invaded by leukocytes. Thus, adult mice treated with NAC and their offspring had a reduced severity of insulinitis at 14 wk, indicating a lower activity of the deleterious autoimmune response in these mice. Attenuation of the immune response was also confirmed by measuring serum cytokines. A major reduction was found in the level of most cytokines measured, mainly $\text{TNF}\alpha$, $\text{IL1}\alpha$, granulocyte-macrophage colony stimulating factor, C-C motif chemokine ligand 1, c-x-c motif chemokine ligand 13 and $\text{IFN-}\gamma$ in both groups supplemented with NAC, either in early life or in adulthood (Figure 2E).

Effects of NAC on oxidative stress and components of the antioxidant system

The major putative activity of NAC is to support antioxidant mechanisms and reduce oxidative stress. Accordingly, we measured the effect of NAC on oxidative stress and the activity of antioxidant enzymes. In order to ensure that the results represent the effect of the treatment, rather than a secondary response to alterations in blood glucose (known to elevate oxidative stress), all measurements were performed on mice at age 14 wk. At this age, clinical signs of diabetes are not yet apparent, as was validated by measuring body weight, fasting glucose, serum insulin, and pancreatic insulin content—all of which were not disturbed at this age in both control and intervention groups (data not shown).

NAC, the most popular antioxidant used in laboratory experiments, undergoes hydrolysis to cysteine, a precursor of GSH, which plays an important role in the antioxidant defense mechanism, and is considered as a redox buffer that maintains a reduced intracellular environment. In this regard, we measured reduced and oxidized GSH in whole blood, pancreas, and liver. Interestingly, NAC given in adulthood differentially affects glutathione level in various tissues; in the whole blood, NAC supplementation was not accompanied by elevation in GSH concentrations. Moreover, the level of reduced GSH was lower in mice given NAC in adulthood than in control mice. Because some reduction in oxidized GSH (GSSG) occurred in these mice, the GSH/GSSG ratio was not affected at all (Figure 3A). Similarly, glutathione levels were almost unchanged in the liver of treated mice (Figure 3B), but total glutathione (both GSH and GSSG) was increased in pancreata of mice given NAC in adulthood (Figure 3C). The glutathione level in NAC-treated offspring was almost unaffected.

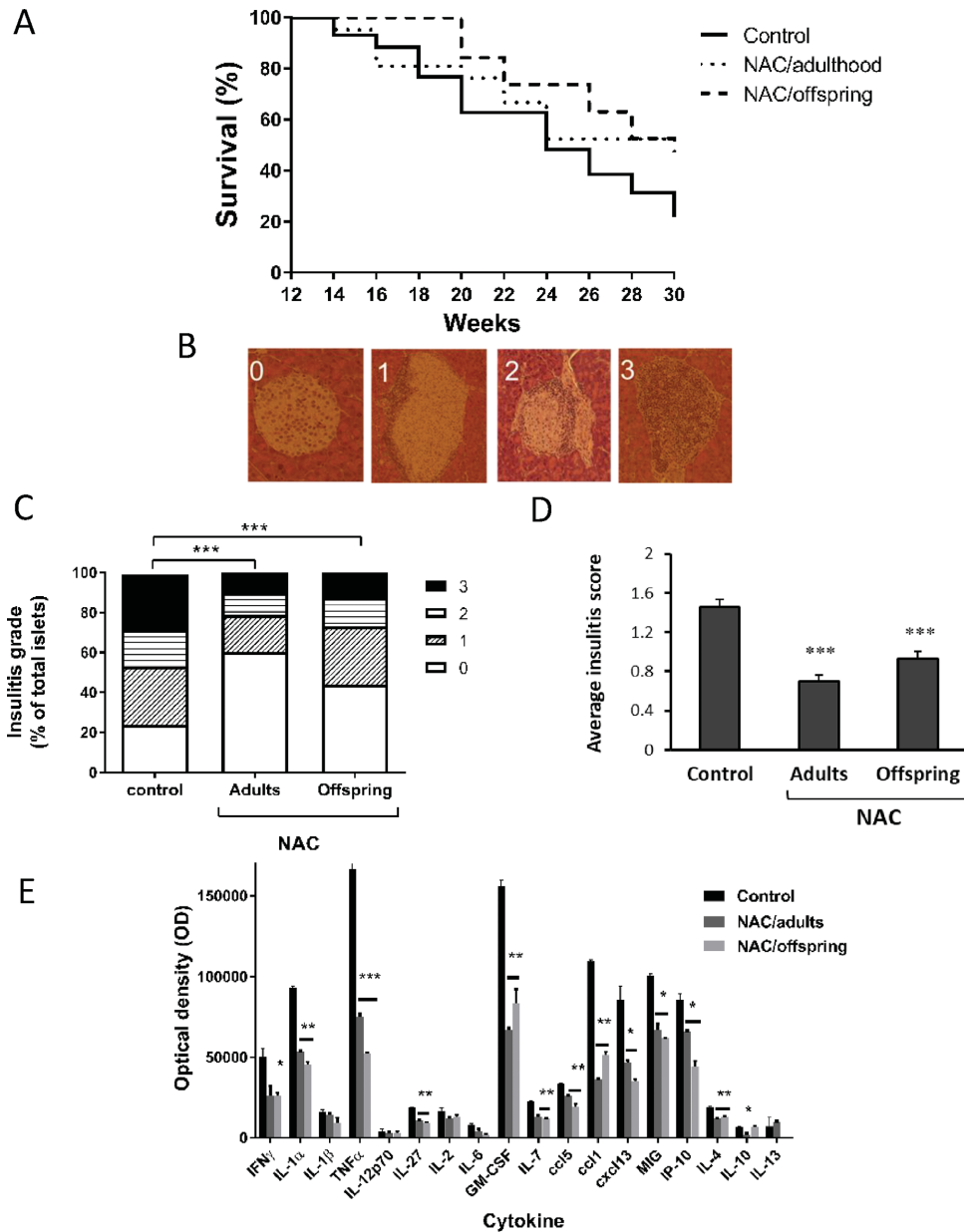


FIGURE 2 Morbidity rate in mice given NAC in adulthood or during early life. (A) Cumulative incidence of diabetes in NAC-treated mice in adulthood ($n = 41$) and offspring of NAC-treated mice ($n = 40$) compared with untreated female NOD mice ($n = 40$). $P < 0.05$ using Kaplan–Meier analysis. Severity of the immune response is reduced in NAC-treated mice and offspring of NAC-treated mice. (B) Islets scored for severity of insulinitis as described in Methods. 0: normal islet; 1: peri-islet insulinitis; 2: intra-islet insulinitis, covering $<50\%$ of the islet's area; 3: extensive insulinitis, covering $>50\%$ of the islet's area. (C) Percentage of islets with various severities of insulinitis (mean \pm SE). Each bar represents the mean \pm SE score of ≥ 50 islets per mouse (8 mice per group). The P values were calculated by chi-square analysis. $***P < 0.001$. (D) Percentage of islets with various severities of insulinitis (mean \pm SE). (E) Serum cytokines level, measured using the mouse cytokine array panel A kit (R&D Systems). Optical density was measured, results are presented as mean \pm SE; $*P < 0.05$; $**P < 0.005$, $***P < 0.001$, by Student's t -test, compared with control.

Lipid peroxidation products, which are generated as a result of elevated free radicals and indicate the presence of oxidative damage, were measured (Figure 4). NAC supplementation significantly reduced lipid peroxidation in serum (Figure 4A) and pancreas (Figure 4B). The level of lipid peroxidation was much higher in liver than in serum and pancreas, and was slightly reduced in offspring of NAC-treated mice (Figure 4C). In order to confirm further the antioxidant effect

of NAC in treated mice and their offspring, TAC was measured. An increase in TAC was detected in serum and pancreas of both groups supplemented with NAC (Figure 4D and E). Liver TAC was not affected by NAC supplementation (Figure 4F).

Antioxidant enzymes are important players in the defense system against oxidative stress. In order to clarify the mechanisms mediating the antioxidative functions of NAC, we measured the activity of

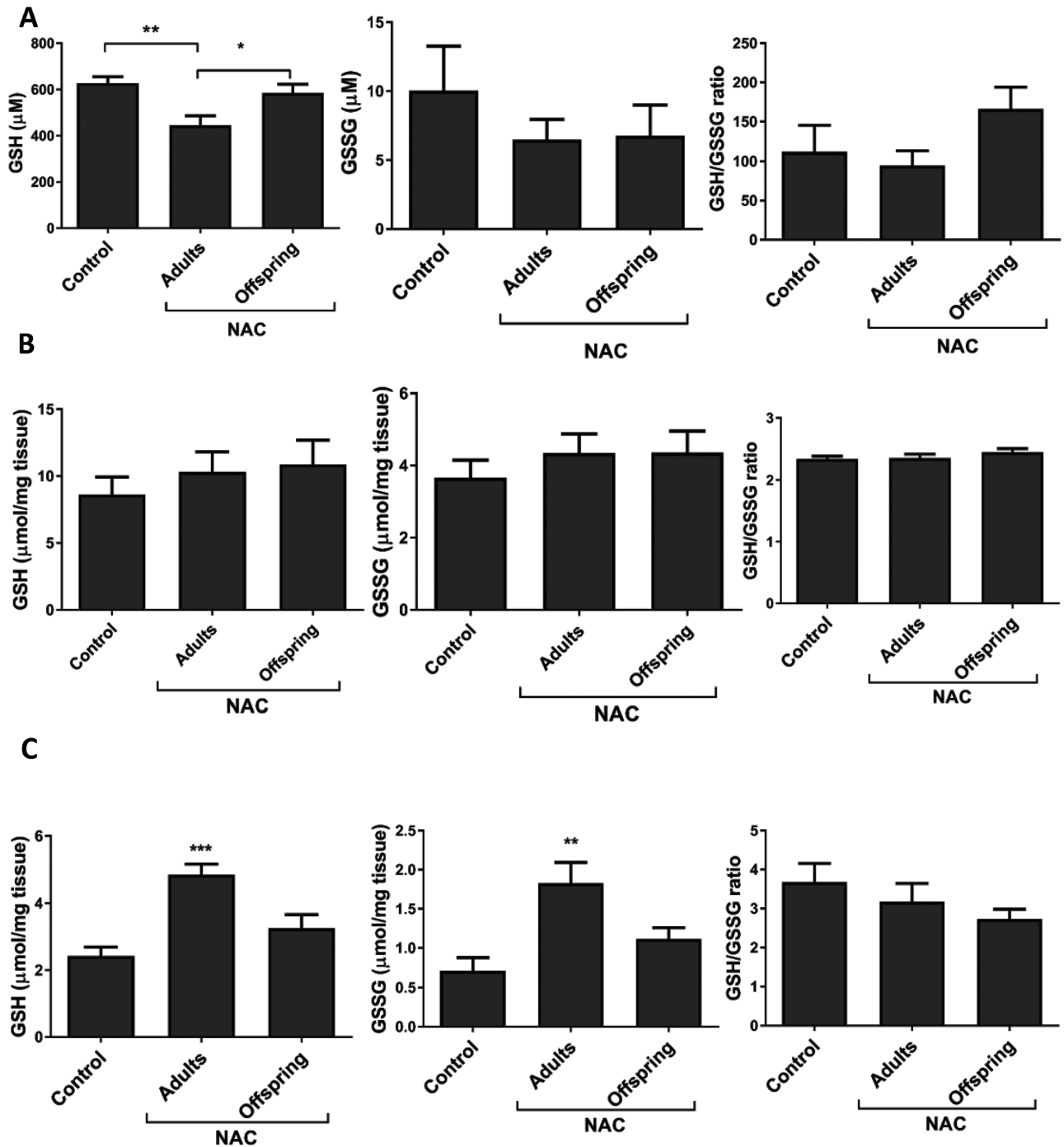


FIGURE 3 Effects of NAC supplementation on GSH level in blood, pancreas, and liver. GSH and GSSG were measured in 14-wk-old control, NAC-treated mice, and their offspring, and GSH/GSSG ratio was calculated in whole blood (A), liver (B), and pancreas (C). Values are means \pm SEs. * $P < 0.05$; ** $P < 0.005$ by 1-factor ANOVA, followed by Bonferroni's test.

SOD, GPx, and catalase in pancreata of control, NAC-treated mice, and offspring of NAC-treated mice. The activity of these enzymes was also analyzed in the liver, an organ characterized by extensive metabolic activity, leading to a high load of oxidative by-product. To compete with this oxidative load, the liver has a high activity of the antioxidant machinery, including elevated activity of antioxidant enzymes, and the production of some of the circulating antioxidants,

such as glutathione. As expected, the activity of all antioxidant enzymes measured in this study was much lower in the pancreas than in the liver (Figure 5). Although NAC supplementation in adulthood did not affect the activity of SOD, GPx, or catalase, either in the pancreas or in the liver, supplementation during early life enhanced the activity of these enzymes. SOD activity was elevated in both pancreas (Figure 5A) and liver (Figure 5D), catalase activity was enhanced in the pancreas

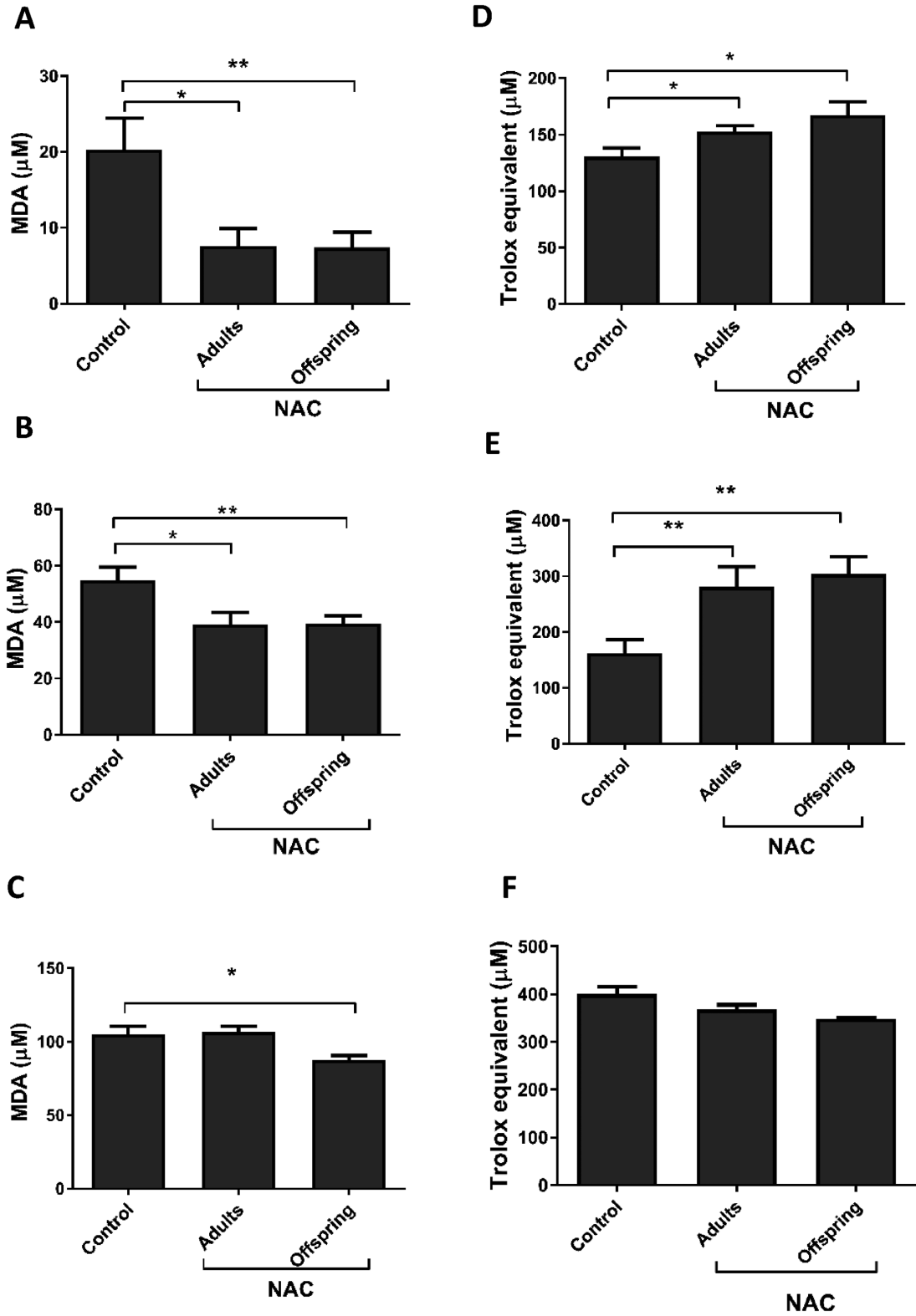


FIGURE 4 Antioxidant capacity, and oxidative stress in NAC-treated mice and their offspring. Lipid peroxidation (A, B, C) and TAC (D, E, F) were measured in serum (A, D), pancreas (B, E), and liver (C, F) of 14-wk-old control, NAC-treated mice, and their offspring. Mean ± SE; **P* < 0.05; ***P* < 0.005 by 1-factor ANOVA, followed by Bonferroni's test.

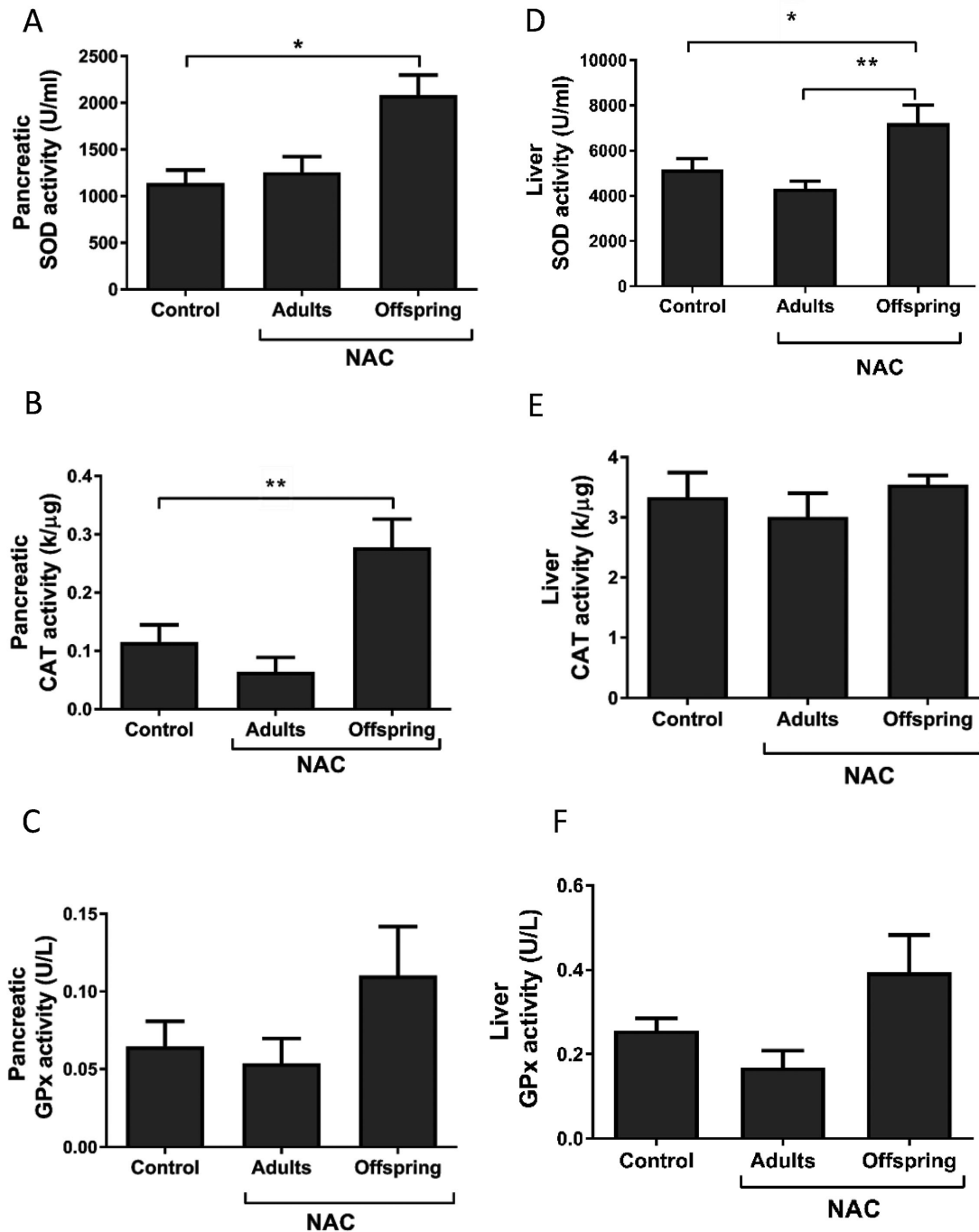


FIGURE 5 Activity of antioxidant enzymes in offspring of NAC-treated mice. Activity of SOD (A, D), catalase (B, E) and GPx (C, F) was measured in pancreas (A, B, C) and liver (D, E, F). Mean ± SE; **P* < 0.05; ***P* < 0.005 by 1-factor ANOVA, followed by Bonferroni's test.

(Figure 5B), and GPx activity was slightly elevated, although not significantly, in the pancreas and liver of offspring (Figure 5C and F).

Discussion

In this study, we investigated the effect of NAC on the development of type 1 diabetes in NOD diabetic prone mice. We showed reduced

diabetes rates in mice supplemented with the antioxidant and in offspring of NAC-treated mice exposed to NAC during their fetal period and lactation. All measures of oxidative state were made in euglycemic mice, suggesting that the elimination of oxidative stress observed following NAC administration might be a cause rather than a consequence of the reduced prevalence of hyperglycemia in these mice. Although the elevation in total antioxidant capacity and reduction in lipid peroxidation is expected in mice given NAC in adulthood, these

results are not obvious in mice given this antioxidant (AOX) in early life (i.e., until 3 wk of age), whereas the measurement of TAC and lipid peroxidation was performed 11 wk later. These results show that early exposure to NAC has a long-lasting beneficial effect on the activity of several components of the antioxidant system.

The pancreatic β cells are highly susceptible to oxidative-stress-related damage as a result of low antioxidant defense mechanisms (23). Accordingly, our results show that the levels of GSH and TAC and activity of SOD, catalase, and GPx are much lower in the pancreas than in the liver. However, although showing only minor effects on the liver, NAC administration, either in adulthood or during early life, succeeded to eliminate oxidative stress in the pancreas. TAC was enhanced, whereas lipid peroxidation levels were reduced in both intervention groups. Total glutathione was increased in mice given NAC in adulthood, and the activity of antioxidant enzymes was elevated in offspring of NAC-treated mice. These results support the use of NAC as an agent that can improve oxidative stress in the pancreas. The results also emphasize that as various organs are at different risks of developing oxidative stress, AOX supplementation might differentially affect target tissues. Thus, measuring serum levels of oxidative stress-related biomarkers as well as antioxidant status does not reflect the redox state in a specific organ, as was also suggested by others (44, 45).

Although administration of NAC in adulthood increased pancreatic glutathione, hepatic glutathione levels were not affected, and blood glutathione was reduced. Although NAC is known as a precursor of glutathione production, conflicting evidence exists about the effect of NAC supplementation on glutathione levels. A tissue-specific effect of NAC on glutathione concentration was already reported (45), demonstrating either an increase or a decrease in glutathione level in various tissues. It was suggested that NAC enhances GSH production in states of depletion, but has no effect on plasma GSH in the absence of such demand. In our study, NAC was supplemented before the onset of diabetes, and, therefore, no depletion of glutathione pools was expected to occur other than in the pancreas, which is well known to have a low antioxidant system and is highly vulnerable to oxidative stress (23). Our results, showing improved oxidative state in NAC-treated mice in adulthood, a tissue-specific increase in GSH, and the absence of alterations in the activity of AOX enzymes, suggest that NAC exerts its antioxidative effects by enhancing glutathione production in specific tissues. Other mechanisms of NAC action, independent of GSH synthesis, might be involved as well (46–48). On the other hand, whereas no change in glutathione levels was demonstrated in offspring of NAC-supplemented mice, the improved oxidative state was accompanied by elevated activity of AOX enzymes. These results suggest that NAC induces some kind of programming of the antioxidant system and/or the immune system that delays the onset of the disease. The molecular mechanisms mediating the effect of NAC either when given in early life or in adulthood should be investigated further.

A significant reduction in morbidity rate was observed following NAC administration, accompanied by lower severity of insulinitis and reduced level of serum cytokines. These results indicate that NAC attenuates the autoimmune response and are in accord with other evidence supporting the notion that NAC directly improves immune function (49). In addition, elimination of hyperglycemia in alloxan-induced diabetes was accompanied by reduced activity of the proinflammatory transcription factor, NF- κ B, and lower production

of NO in the pancreas of NAC-treated mice (50). Similarly, NAC inhibited NF- κ B, inducible NO synthase (iNOS), and NO production in activated macrophages (51). NAC treatment abrogated the immune response in other autoimmune diseases such as multiple sclerosis, myocarditis, lupus erythematosus, and Sjögren's syndrome, showing a lower production of NO, proinflammatory cytokines, and infiltration of immune cells (48, 52–54).

The immuno-modulatory effects of NAC may be exerted via elimination of oxidative stress, which is known to be involved in several events related to the emergence and progression of T1D. It was suggested that oxidation by ROS may generate new epitopes, which are not recognized as autoantigens by the acquired immune system, and thus may evoke an autoimmune reaction (55). Cysteine-containing peptides are highly sensitive to alteration in the redox environment. When such oxidative modifications occur on MHC-bound peptides, alterations in T-cell recognition may occur (56), suggesting an explanation for the role of NAC, which is known to be involved in thiol modifications, in reducing the risk to initiate the autoimmune process. ROS are also involved in the propagation of the autoimmune disease, being crucial mediators of cellular cytotoxicity and cytokine secretion (25, 57), and worsening the pathological tissue damage (58). Because ROS are both mediators and by-products of the immune process, uncontrolled oxidative stress leads to an escalation of the autoimmune damage in the attacked pancreatic islets. Accordingly, NAC was found to limit the immune response in isolated leukocytes (59–61). An earlier study showed that NAC administration (200 mg/kg/d) delayed, but did not prevent, the onset of T1D in diabetic rats (62). Our data showing that NAC (600 mg/kg/d) has a preventive effect in mice treated with NAC in adulthood are in accord with these results. In addition, our results demonstrating lower severity of insulinitis and major reductions in serum cytokines support the ameliorating effect of NAC on the immune process in type 1 diabetic mice and reinforce the need for further studies in order to clarify the potential beneficial effects of this agent. Although NOD mice are the common model for the study of T1D, there are several limitations for this model, and several therapeutic agents, found to prevent the progression of the disease in these mice, failed to demonstrate such results in humans; thus, the potential benefits of NAC should be further validated on additional models of the disease, such as the IDDM diabetic rats (63).

Our study shows for the first time that NAC administration in early life can delay the onset of diabetes in adulthood. It is well accepted nowadays that the intrauterine environment affects health in subsequent adulthood (64). The concept of trans-generational transmission of diseases claims that the risk of developing disease may be transmitted from mother to offspring in the absence of any genetic susceptibility, infective agents, or environmentally induced congenital defects via an epigenetic mode of inheritance (65). This idea may explain the current increase in prevalence of diabetes in the younger generation. Oxidative stress may be a key link underlying the fetal "programming" leading to elevated risks for the development of various disorders in offspring. The rationale for this hypothesis is based on the involvement of oxidative stress in many risk factors of inhibited fetal growth and/or preterm birth such as preeclampsia, diabetes, smoking, malnutrition, or excessive nutrition (66). High correlations between maternal and fetal plasma levels of antioxidants and oxidative stress markers have been observed, suggesting that maternal oxidative stress levels can transfer to the fetus.

However, little is known of the role of oxidative stress or antioxidants in fetal health. To our knowledge, this is the first study to demonstrate this phenomenon, although further investigations are needed to clarify the molecular mechanisms involved (67).

Conclusion

This study shows a preventive effect of NAC against the development of T1D in NOD mice. NAC, known to be a well-tolerated and safe molecule at doses of ≤ 3 g/d (68), may be considered as a beneficial supplement to reduce or delay T1D morbidity rates in high-risk individuals, based on family history, genetic analysis, or the presence of relevant autoantibodies. However, because preventive treatments are difficult to demonstrate in clinical trials, these promising results should be taken with caution. In addition, our findings provide support for the involvement of oxidative stress in the pathogenesis of T1D and emphasize the need to develop strategies to accurately monitor oxidative states, define appropriate biomarkers, and identify or develop other efficient agents to strengthen antioxidant mechanisms in order to reduce the risk of developing T1D.

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