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Modulatory Effects of Betaine on D-Galactose-Induced Aging-Related IL-6 and TNF Inflammatory Mediators during Pregnancy in Rats

Ali Habeeb Jaber
Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Al-Qadisiyah, Diwaniyah, Iraq

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Annotation: Age-related illnesses are aggravated by inflammation caused by aging. However, pregnancy introduces a new level of physiological complexity, which might exacerbate inflammatory responses. This study investigated the impact of betaine on the expression of TNF-α and IL-6, two key inflammatory mediators, in pancreatic tissue of pregnant rats with D-galactose-induced aging. We examined utilizing real-time quantitative PCR, looking at To imitate the consequences of aging, we evaluated changes in IL-6 and TNF using a D-galactose model. In pregnant rats' pancreatic tissue, D-galactose treatment significantly increased IL-6 and TNF-e expression levels when compared to untreated controls. Pregnant rats' pancreatic tissue showed significantly higher levels of IL-6 and TNF-α compared to untreated controls. Giving betaine significantly reduced these increases, returning IL-6 and TNF-α levels near to the control group. This shows that, in situations such as aging and pregnancy, betaine may operate as an inflammatory regulator. These findings suggest that betaine could be useful as a drug to help older women who are pregnant manage with the associated increased inflammation. Although these findings are encouraging, additional

research is needed to discover exactly how betaine protects and what long-term implications this has for dealing with inflammation-related disorders during pregnancy and in old age.

Keywords: Betaine, D-Galactose, IL-6, Pancreatic Tissue, TNF, Inflammation, Pregnant Rats.

Graphical abstract:

Introduction

Aging is one of those biological processes that's still full of mysteries. Over time, it causes our bodies to function less effectively, and this happens for a variety of reasons (1). Some changes occur at the molecular level, others at the cellular level, and still others on a larger physiological scale. All these changes pile up, raising the risk of problems that tend to come with age (2). Lately, there's been a lot of buzz about chronic low-grade inflammation (3). It's becoming increasingly clear that this type of ongoing inflammation plays a major role in aging and is tied to a host of diseases things like heart problems, neurological conditions, and metabolic disorders (4).

Pregnancy introduces its own set of challenges, which can make the effects of age-related inflammation even worse. For instance, as we age, certain organs start to struggle (5). Researchers have shown that aging often leads to a drop in beta-cell mass, less insulin production, and higher levels of oxidative stress. These changes are usually tied to a pro-inflammatory environment in the pancreas, which speeds up the decline in how well the organ works (6). Much of this inflammation is driven by specific cytokines, especially interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF). Pregnancy can pile on additional stress, particularly for metabolic processes, and this can further impact pancreatic function, especially in older individuals (7).

When IL-6 and TNF aren't properly regulated, it can lead to serious problems. Insulin resistance goes up, beta cells don't work as well, and metabolic diseases like type 2 diabetes become more likely (8, 9). Figuring out how these cytokines behave during aging and pregnancy could be key to finding ways to manage pancreatic dysfunction.

One compound that's been getting some attention recently is betaine, also known as trimethylglycine (10). Betaine helps by donating methyl groups and keeping osmotic balance in check. On top of that, it seems to have anti-inflammatory properties, which could be really important when it comes to dealing with inflammation linked to aging (11, 12). But here's the thing: there's still not much research on how betaine specifically affects IL-6 and TNF expression in the pancreas, especially in aging pregnant animals.

D-Galactose is commonly used to mimic aging because it induces oxidative stress and inflammation (13, 14). In our study, we chose pregnant rats as experimental subjects due to their physiological similarities to humans and the importance of studying inflammatory responses during pregnancy. qPCR, a highly sensitive method, were widely used in molecular biology for detection the levels of pro-inflammatory mediators. This process helped us figure out whether giving betaine could influence the inflammatory response in the pancreatic tissue of aging pregnant rats. The current study could be really useful for exploring potential treatments aimed at reducing age-related inflammation during pregnancy. In the long run, this research could pave the way for therapies that help lower inflammation and improve metabolic and pancreatic function in vulnerable populations.

Materials and Methods:

Animal and treatment

This study was conducted on 20 pregnant female Wistar rats, eight months old and weighing between 250-300 g (280± 20 g) divided into 4 groups. The first group had received daily subcutaneous injections of 0.9% normal saline for six weeks, together with daily oral normal saline administration for the same duration. The second group designated as D-galactose group was treated with daily subcutaneous injections of D-galactose in a dose of 60 mg/kg for six weeks. The third group was treated with D-galactose and betaine. We added betaine to their drinking water at a concentration of 1% for the entire six-week period. Finally, the fourth group only received betaine, also at a concentration of 1% in their drinking water, for six weeks. At the end of the six-week experiment, we anesthetized them using a mix of ketamine and xylazine. Once they were under anesthesia, we collected samples of their pancreatic tissue, which was essential for further analysis.

Tissue Sample Preparation

Upon collection, tissue samples were promptly immersed in liquid nitrogen to preserve their molecular integrity (15). To ensure aseptic conditions, the samples were finely ground into a powder using a porcelain mortar.

RNA Extraction

The RNA extraction followed a previously established protocol with minor adaptations. Briefly, 50 mg of powdered tissue was transferred to individual microtubes. To these microtubes, 750 µL of RL solution was added, ensuring thorough coverage of the tissue (16). For efficient phase separation, 200 µL of chloroform was introduced to each microtube containing tissue and RL solution. The contents were vigorously mixed by inversion, ensuring comprehensive intermingling of the components. This mixing step was repeated several times to promote optimal separation. After thorough mixing, the samples were delicately placed in a 4°C refrigerator for 10 min, facilitating the partitioning of phases. Following incubation, the microtubes were subjected to centrifugation at 12,000 rpm for 15 min. This centrifugal step enabled the separation of the aqueous and organic phases.

The supernatant, containing the aqueous phase, was cautiously transferred to new microtubes to maintain phase separation. To this solution, 400 µL of cold 75% ethanol was gently added, ensuring even dispersion by inversion. The ethanol-containing solution was incubated at 4°C for 10 min, promoting RNA precipitation. Subsequently, the samples were transferred to chromatography columns, allowing them to settle undisturbed for 5 min to facilitate binding of RNA to the column matrix. Centrifugation of the chromatography columns at 12,000 rpm for 1 min led to the removal of the lower solution containing impurities. This process effectively separated the bound RNA from unwanted contaminants.

A final centrifugation step of 2 min at 12,000 rpm resulted in the release of purified RNA from the columns. The eluted RNA was meticulously collected in 1.5 ml tubes, ready for subsequent

molecular analyses.

cDNA Synthesis

The cDNA synthesis protocol involved the conversion of purified RNA into complementary DNA (cDNA) for subsequent gene expression analysis. Purified RNA samples were annealed with an Oligo(dT) primer, facilitating primer binding. This RNA-primer mixture was then subjected to an enzymatic reaction involving Reaction Buffer, RNase Inhibitor, Revert Aid Reverse Transcriptase, and dNTP Mix. The cDNA synthesis was performed by incubating the reagents in a thermal cycler to terminate the enzymatic reaction by heating, after which the cDNA produced was stored for future analysis of gene expression (IL-6 and TNF).

Real time PCR analysis

The real-time qPCR utilizing SYBR Green dye was employed to identify gene expression levels of the rats IL-6 and TNF. The experiments were conducted a total of five times using the Rotor-Gene equipment from Corbett Research, located in Sydney, Australia. The GAPDH gene served as the internal control. For the GAPDH-rat gene, the forward sequence AGTTCAACGGCACAGTCAAG and the reverse sequence TACTCAGCACCAGCATCACC were utilized. In the case of rat IL-6, the forward and reverse sequences were GTTTCTCTCCGCAAGAGACTTC and TGGTCTGTTGTGGGTGGTAT, respectively. For rat TNF, the forward and reverse sequences used were AACACACGAGACGCTGAAGT and TCCACTCAGGCATCGACATT, respectively. The PCR reaction was performed in a final volume of 12.5 µL using 15-well plates. The reaction mixture consisted of 6.25 µL of Syber-Green Super Mix, 10 µM of each primer (forward and reverse), and 3 µL of cDNA. All samples were run in duplicate. The thermal protocol employed was as follows: an initial denaturation step at 94°C for 5 minutes, followed by 50 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 30 seconds, during which the signal was acquired.

Statistical analysis

The data were modeled by a parametric distribution and presented as means ± SD. To assess statistical significance, we employed one-way analysis of variance (ANOVA) followed by the Bonferroni's test for multiple comparisons, or the unpaired t-test for comparing two groups. Statistical analyses were conducted using Excel and GraphPad Software, *Prism 8.0* (*GraphPad* Software, San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

Results

Analysis of IL-6 Gene Expression using Real-Time PCR

IL-6 Upregulation in Aging Model

The study revealed a substantial upregulation of IL-6 protein expression in pancreatic tissue from both the DG group (365.36%; $p<0.0001$) and the DG+BET group (182.28%; $p<0.0077$) compared to the control group, aligning with the established role of D-Galactose-induced aging in promoting an inflammatory environment.

Mitigating Effect of Betaine on IL-6 Expression

Notably, the group receiving betaine displayed IL-6 protein levels similar to the control group, suggesting a potential mitigating effect of betaine on age-associated IL-6 upregulation. Betaine supplementation exhibited a marked reduction in IL-6 protein expression within the DG+BET group (39.34%; $p<0.0075$) and the BET group (82.64%; $p<0.0001$) compared to the DG group, indicating its potential regulatory influence on IL-6 gene expression. These trends are visually depicted in Fig. 1, encapsulating the distinct alterations in IL-6 protein expression across the experimental groups.

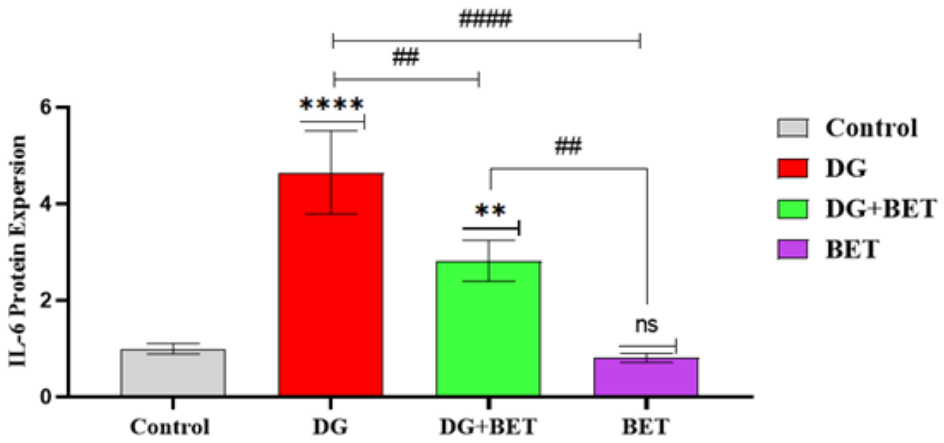

Fig. 1. Graphical illustration of IL-6 expression. expression of IL6 in pancreatic tissues of all analyzed groups was detected by real time PCR using primers specific for IL-6.

Implications for Inflammation and Pancreatic Function

The findings resonate with existing literature on the anti-inflammatory properties of betaine, proposing its role in countering the inflammatory cascade induced by D-Galactose. This suggests a potential for betaine to modulate the age-related inflammatory milieu, contributing to the preservation of pancreatic function.

Analysis of TNF Gene Expression using Real-Time PCR

TNF Upregulation in Aging Model

The study unveiled a significant surge in TNF protein expression in pancreatic tissue subjected to D-Galactose administration (201.30%; $p<0.0038$) compared to the control group, aligning with the understanding of D-Galactose-induced aging promoting inflammatory processes.

Potential Influence of Betaine on TNF Expression

The pancreatic tissue exposed to both D-Galactose and betaine (DG+BET) exhibited a moderate, albeit non-significant, increase in TNF protein expression (65.63%; $p<0.3912$), suggesting a potential influence of betaine in attenuating the TNF response induced by D-Galactose. The betaine-only group displayed TNF protein levels similar to the control group.

Mitigation of TNF Expression by Betaine

A significant reduction in TNF protein expression was observed within the DG+BET group (45.03%; $p<0.0343$) and the BET group (67.94%; $p<0.0034$) compared to the DG group (Fig. 2), implying a potential role for betaine in dampening the heightened TNF levels associated with D-Galactose-induced aging.

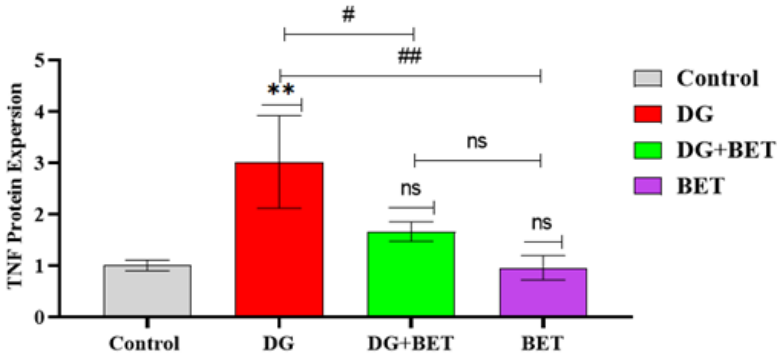

Fig. 2. Graphical illustration of IL-6 expression. expression of TNF in pancreatic tissues of all analyzed groups was detected by real time PCR using primers specific for TNF.

Significance for Inflammation and Age-Related Pathologies

The results underscore betaine's potential role in attenuating age-related inflammation, particularly in modulating inflammatory cytokines like TNF. The study aligns with existing literature on TNF's role in inflammation and emphasizes the promise of betaine supplementation in counteracting TNF-associated inflammation within pancreatic tissue. Further mechanistic investigations are warranted to unravel betaine's underlying mechanisms and its broader implications for age-related inflammatory conditions.

Discussion

Betaine, a naturally occurring compound, has potential anti-inflammatory effects, supports cellular methylation, and protects cells from osmotic stress. Despite the growing interest in betaine, little was known about its specific impact on IL-6 and TNF expression in pancreatic tissue during aging until now (17-19). This study conducted to fill that gap by exploring the effect of betaine supplementation on the regulation of IL-6 and TNF in rats experiencing D-Galactose-induced aging (20, 21).

The D-Galactose group (DG) and D-Galactose plus beta group (DG+BET) show a significantly higher level of IL-6 protein as measured by Real-Time PCR in comparison to the control group. The results are in agreement with the previous studies, which note that the aging induced by D-Galactose creates a more inflammatory environment. Increased levels of IL-6 can be considered a hallmark of inflammation, low-grade and chronic, associated with numerous aging-related health issues. The betaine-treated group had IL-6 levels closely resembling those of the control group. This indicates that betaine may help counteract, or prevent, the rise in IL-6 that is associated with aging (22).

Our findings present betaine as a possible regulator of IL-6 gene expression in pancreatic tissue, thereby alleviating the inflammatory effects induced by D-Galactose. Importantly, the fact that IL-6 levels were lower in both the DG+BET and BET groups compared to the DG group indicates that betaine may have an anti-inflammatory effect. If this were to translate into the clinical domain, it would be a major breakthrough. The current results are consistent with previous studies, adding to the increasing knowledge regarding betaine's ability to affect IL-6 in aging models (23, 24).

In the present study, TNF levels were significantly lower in both the DG+BET and BET groups compared to the DG group. This suggests that betaine could help in reducing TNF levels elevated due to D-Galactose-induced aging. Here lies the evidence with respect to betaine imparting control over inflammatory markers like TNF, thus confirming its role in checking age-oriented inflammation. When viewed against previous studies, betaine seems to have real promises in checking TNF-associated inflammation within pancreatic tissue (25,26).

Conclusion

The current investigation focused on a comprehensive examination on the possible effect of betaine on pancreatic tissue inflammation occurring with age using the D-Galactose-induced rat aging model. The data from this study appeared to suggest that the D-Galactose treatment stood for an upregulation of IL-6 and TNF protein expression in pancreatic tissues so immune in nature that inflammation is considered age-related, which were counteracted by betaine to bring down the levels of IL-6 and TNF proteins to that of the control. In addition, betaine showed promise in reducing the expression of IL-6 and TNF raised by D-Galactose. Our study indicates that the potential of betaine in minimization within the pregnancy-linked exacerbation of inflammatory mediators like IL-6 and TNF may also be considered and specifically in the setting of D-Galactose-induced aging. This indicates that not only can betaine act on age-related inflammation, but it may also have some indication for treatment in inflammation around the time of pregnancy, an important consideration for maternal and fetal health. These findings characterize the insights into age-related inflammatory phenomena and the possible therapeutic

effect of betaine.

Ethical Approval

All the ethical standards with regard to animal care and use at the relevant international, national, and institutional levels were followed in preparation for the study. All ethical standards required by the institution of research were followed. The complete discussion of all ethical issues with regard to animals includes fine details in Materials and Methods.

Conflict of Interest: "The authors declare that they hold no competing interests."

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