Chronic oscillating glucose challenges disarrange innate immune homeostasis to potentiate the variation of neutrophil–lymphocyte ratio in rats with or without hidden diabetes mellitus

Background: The neutrophil–lymphocyte ratio (NLR) has been considered as an inflammatory marker in various disorders, but it is not clear whether the NLR is also elevated with hidden diabetes (HD), which is normal in fasting blood glucose (FBG) but abnormal in the oral glucose tolerance test (OGTT).

Materials and methods: An HD animal model for 27 days and an animal model with oscillating glucose (OG) for 7 days were applied on adult female Sprague–Dawley rats. OGTT, leukogram analysis, histology, and immunohistochemistry were carried out.

Results: In HD rats, the percentage of neutrophils increased but the percentage of lymphocytes decreased; hence, the NLR rose relative to sham. This may be a result of the OG levels often experienced by diabetic subjects, as normal rats given OG (6 g/kg/6 h) for 7 days had significantly reduced lymphocyte numbers and increased NLR compared with the values before and 1 h after oral glucose administration during OGTT. Glucose-induced disarrangement of partitions of circulating immune cells and NLR was involved in the increase in oxidative stress, as these changes were totally blocked by the antioxidant glutathione (GSH). GSH (50 mg/kg/6 h) totally blocked the glucose-induced alterations in lymphocyte and NLR values.

Conclusion: HD associated with elevation of NLR values may be partly attributed to a homeostasis disorder of the innate inflammatory state, caused by oscillating hyperglycemia. Acute high glucose administration produced a significant decrease in lymphocyte number. OG administration potentiated this effect and increased the NLR value, which was blocked by GSH, suggesting that reactive oxygen species play a critical role in maintaining lymphocyte numbers.

Keywords: neutrophil–lymphocyte ratio, diabetes, oscillating glucose level, innate immune, rat

Introduction

The neutrophil–lymphocyte ratio (NLR) is considered to be a marker of low-grade inflammation in diabetes mellitus (DM).1,2 Research demonstrates that neutrophils, the first cells to respond to inflammation, can aggravate the inflammatory state by interacting with macrophages and antigen-presenting cells to induce type 1 diabetes (T1D).3 A high NLR represents leukogram shifts to either a higher number of neutrophils or a lower number of lymphocytes, or both, from the basal innate immune homeostasis. However, the pathological development of NLR in DM remains unclear. The literature to date is contradictory in regard to the changes in neutrophil numbers in patients with DM. The levels of circulating neutrophils have been reported to be increased4,5 or decreased6,7 in patients with T1D. The disagreement in alterations of neutrophils
was thought to be due to different studies coming from the discoveries of different stages of the pathological progress of DM, or from multiple unrecognized factors (mild infections and mild chronic disorders associated with inflammation, etc.) that could alter the NLR in clinical studies, or simply that neutrophils may not be the main cause of the increased NLR.

The development and regulation of the homeostasis of the innate immune system is dependent on the daily suicide of lymphocyte clones and, hence, cell death by apoptosis plays a pivotal role in purging the body of dangerous lymphocytes. T1D is an autoimmune disorder which may be related to defects in lymphocyte apoptosis in this disease. In 1948, Jailer et al. found that oral glucose challenge in both diabetics and non-diabetics produced a significant decrease in circulating lymphocytes, which was much greater in diabetic patients. This phenomenon has been confirmed by later studies in which the rate of lymphocyte apoptosis was significantly higher in type 2 diabetic patients than that in the healthy population. These findings suggest that the increase in NLR value may simply result from the decrease in lymphocyte number, which is caused by high-glucose-induced oxidative stress and cell apoptosis.

A diabetic animal model could be applied to test this hypothesis. Compared to clinical studies, experimental animal models have the advantage of using controlled experimental designs to systematically assess the diagnostic value of NLR during the pathological development of T1D induced by alloxan. Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6-pyrimidinetrione) is a derivative of pyrimidine used to selectively destroy pancreatic β-cells. In the alloxan-induced diabetes animal model, there is a substantial number of rats failed to increase their fasting blood glucose (FBG) level. Alloxan is not commonly used to establish the diabetic model as the percentage incidence of diabetes is quite variable. Alloxan produced only 72% of T1D rats with abnormal FBG. Female animals have been reported to be more resistant than males to the chemically induced β-cell cytotoxicity. Therefore, we chose alloxan-treated female rats with normal FBG but with an abnormal oral glucose tolerance test (OGTT) as a hidden diabetes (HD) model. In the HD model, we determined the dynamic alteration of neutrophil and lymphocyte counts, NLR, and renal damage, to show that NLR together with OGTT can be used as an indicative marker and diagnostic reference for HD. To determine the cause of the increase in NLR in HD animals, whether it results from diabetic inflammation or the oscillating glucose (OG)-induced disorder of innate immune homeostasis which is often experienced in diabetic subjects, we administered oral OG (6 g/kg/6 h) to normal rats for 7 days and examined the leukogram pattern before and 1 h after oral glucose administration during the OGTT period.

**Materials and methods**

Ninety-three Sprague–Dawley female rats with a body weight of 200±10 g (mean±SD) were purchased from the Chinese Academy of Medical Sciences (Shanghai, China). Rats were acclimatized under a controlled environment at 22±1°C and a 12 h light/dark cycle, with free access to food and water, for 2 weeks prior to the experiments. The research protocol was approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University, China. All experiments were conducted according to the guidelines of the committee.

**Induction and management of HD**

Rats were divided into sham (n=14) and alloxan-induced HD (n=12) groups. HD was induced by 2 intraperitoneal injections of alloxan, 100 mg/kg body weight on 2 sequential days. The criterion for inclusion in the HD group was an FBG ≥110 mg/dL but abnormal OGTT during the experimental period. Rats were weighed daily throughout the study and the body weights were recorded. The changes in body weight were calculated as the differences in body weight between postalloxan treatment day and prealloxan treatment day (P0). Before FBG measurements, the rats were fasted for 12 h (22:00–10:00 h). FBG was measured with an Accu-Chek® reflectance meter with Accu-Chek active test strips (Roche Diabetes Care, Mannheim, Germany) on P0, and postalloxan treatment days P5, P7, P16, and P27. During experimental days P24–P25, rats were maintained in metabolic cages for 24 h to collect urine. The water intake, food intake, and urine volume were measured and recorded. The urine aliquots were stored frozen for the subsequent measurement of albumin by enzyme-linked immunosorbent assay (Rat Albumin ELISA Kit, AssayMax™; Assaypro, St. Charles, MO, USA).

**Animal model with OG administration**

Animals were given either 6 g/kg glucose or distilled water (2 mL/100 g body weight, n=7 for both groups) by gavage every 6 h (at 06:20, 12:20, 18:20, and 24:20 h) for 7 days. To test the hypothesis that a disorder of innate immune homeostasis is induced by OG-produced oxidative stress, an antioxidant, glutathione (GSH, 50 mg/kg/6 h, n=7), was intraperitoneally injected into animals with OG challenge as described above. For leukogram analysis, the tail blood was collected preadministration and 1 h post-2 g/kg glucose administration on treatment day 7.

**OGTT**

On experimental days P7 and P27, the OGTT was performed on overnight-fasted (22:00–10:00 h) normal and alloxan-treated rats. A glucose load (2 g/kg body weight) was given...
to each rat orally with a feeding syringe. Blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 min of glucose administration, and the blood glucose level was determined using a glucometer (Accu-Chek Active; Roche, Mannheim, Germany).

Leukogram analysis
To obtain repeated measures in the same animal, blood samples were taken from the tail of each animal at P7 and P27 in both sham and HD groups. To examine the effects of hyperglycemia in the leukogram, blood samples were also collected on P27 in the diabetic group (DM group, FBG: mean±SD 227.10±119.96 mg/dL, n=6). The identification and differential counting of leukocytes were performed with samples stained by Wright’s dye under an optical microscope. As previous studies reported that some lymphocytes and neutrophils were difficult to differentiate and recognize by machine and some neutrophils were counted as monocytes by the LaserCyte,16,17 to determine the leukocyte classifications more accurately, manual differential counts were performed by 2 technicians in a blinded manner. The NLR was calculated by dividing the neutrophil count by the lymphocyte count.

Histology
To allow the kidney to recover from the acute mildly nephrotoxic effects of toxic chemicals over more than 3 weeks,18 studies examining the effects of treatments on the development of diabetic nephropathy were executed 27 days after alloxan treatment. The rats were killed and tissue samples were taken from the pancreas and kidney for histological studies. The samples were fixed in 2% buffered paraformaldehyde and embedded in paraffin. Then, 5 μm slices were made from the paraffin blocks which were stained with Picro Sirius red with or without hematoxylin as counterstaining. As previous studies reported, the stained tissue sections were scanned using a Leica fluorescence microscope (Leica DM6000B; Leica, Wetzlar, Germany). DAPI was used to stain nuclei. Whole-sample images were scanned and assembled on a Leica fluorescence microscope (Leica DM6000B) using Leica LAS X software. For quantitative immunohistochemistry, the relative expression/mm² determined from 200 fields (40×) ofpancreas from each rat was quantified using ImageJ software. The average β-cell and α-cell number and their ratio per islet of pancreas were quantified by two independent operators in a blinded fashion.

Immunohistochemistry
On experimental days P7 and P27, pancreas samples were fixed in 2% w/v paraformaldehyde for 2 h and processed for immunohistochemistry as described previously.20 After preblocking, pancreas samples were stained with mouse anti-insulin (66198-1-Ig, Proteintech, 1:500) and rabbit anti-glucagon (PB0742, Boster, 1:200) antibodies in 5% v/v goat serum. All secondary antibodies (Alexa Fluor 488 or 594 goat anti-mouse or anti-rabbit) were from Molecular Probes, Waltham, MA, USA. DAPI was used to stain nuclei. Whole-sample images were scanned and assembled on a Leica fluorescence microscope (Leica DM6000B) using Leica LAS X software. For quantitative immunohistochemistry, the relative expression/mm² determined from 200 fields (40×) of pancreas from each rat was quantified using ImageJ software. The average β-cell and α-cell number and their ratio per islet of pancreas were quantified by two independent operators in a blinded fashion.

Statistics
Results are presented as the mean±SD. Statistical significance was determined by Student’s t-test or using analysis of variance (ANOVA) with Tukey tests for posthoc comparisons at a 1% or 5% significance level of difference. The relationships between variables were determined by linear regression analysis. GraphPad Prism 6.0 software (La Jolla, CA, USA) was used to calculate the OGTT area under the curve (AUC) and for statistical analyses.

Results
Animal models
Rats injected with two sequential 100 mg/kg doses of alloxan produced two cohorts of animals. The first group (81%) developed a gradually increasing hyperglycemia from day 7 onward. In contrast, the FBG levels measured on experimental days P5, P7, P16, and P27 were normal (74–110 mg/dL) in the second alloxan-treated cohort (Figure 1A). Alloxan-treated rats with normal FBG but abnormal OGTT were considered as successful HD animals. On treatment day P7,
Figure 1 Alloxan produced the development of HD in female rats (sham n=8, HD n=7).

Notes: (A) FBG levels during experimental period P0–P27. (B) OGTT after 7 days of alloxan treatment. (C) OGTT after 27 days of alloxan treatment. (D) OGTT AUC after 7 and 27 days of alloxan treatment. (E) Body weights were measured daily during experimental period P0–P27. (F) Body weight changes during experimental period P1–P27. The changes were obtained from the difference in body weight between posttreatment day P1–P27 and pretreatment day P0. The data for OGTT AUC were analyzed by unpaired Student’s t-test. The data for FBG, OGTT, body weight, and body weight changes were analyzed by 2-way analysis of variance with Tukey tests for posthoc comparisons. *p<0.05 and ***p<0.001 sham vs HD; +++p<0.001 P7 HD vs P27 HD.

Abbreviations: HD, hidden diabetes; FBG, fasting blood glucose; OGTT, oral glucose tolerance test; AUC, area under the curve; P0, prealloxan treatment day; P1, posttreatment day 1; P7, posttreatment day 7; P27, posttreatment day 27.
the OGTT glucose levels after 120 min glucose administration were significantly higher in HD than in sham rats (Figure 1B). The OGTT glucose levels at multiple time points were further increased in HD animals on P27 (Figure 1C). The OGTT AUC value on P27 was also significantly higher than that on P7, as expected (Figure 1D). During the experimental period, there were no differences in body weight between sham and HD animals (Figure 1E). However, relative to sham, animals rendered HD by alloxan injection exhibited a significant reduction in body weight gain (Figure 1F). On treatment day P27, food and water consumption, urine output, and 24 h urinary albumin excretion were significantly increased in HD compared to sham (Table 1).

OG administrations for 7 days in normal rats did not significantly alter body weight, FBG level, or OGTT values compared with sham-treated rats.

**Leukogram**

Alloxan treatment did not alter leukocyte numbers (Figure 2A). In HD rats, the mean percentage of neutrophils was significantly increased (Figure 2B) and the mean percentage of lymphocytes was decreased (Figure 2C); hence, the NLR value was significantly higher in HD compared with sham on P7 and P27 (Figure 2D). The Pearson analysis showed that there were tight correlations between NLR and neutrophils (r=0.98, p<0.001) (Figure 2E) as well as between NLR and lymphocytes (r=−0.89, p<0.001) (Figure 2F). In alloxan-induced DM rats, the same leukogram patterns as in HD rats were found on P27 (Figure 2G–I). With regard to monocytes, eosinophils, and basophils, there were no differences between sham and HD groups (Table 2).

In normal rats, the glucose challenges did not alter leukocyte number, but significantly decreased lymphocyte number and percentage value, relatively increased neutrophil percentage value, and led to the increase in the NLR (Figure 3A–F). Only 1 h post-glucose administration (2 g/kg), lymphocyte percentage values decreased from precontrol 73.88±13.42% and 83.42±5.86% to 59.80±14.34% and 54.32±11.96% in normal sham rats and OG-treated rats, respectively (p<0.05 and p<0.01, respectively). In a comparison of sham with OG treatment, the lymphocyte percentage value dropped further in OG-treated rats at 1 h after glucose administration (p<0.05). This resulted in a 1.97-fold increase in NLR in sham but a 5.64-fold increase in NLR in OG-treated rats. There was a positive correlation ( r=0.58, p<0.05) (Figure 3G) between the change in blood glucose level and the change in lymphocyte numbers, which resulted from the difference between precontrol and 1 h after glucose administration. The disorder of innate immune homeostasis caused by OG may be produced by glucose-induced oxidative stress in lymphocytes, as GSH totally blocked the OG-induced alterations in lymphocyte and NLR values (Figure 3A–F).

**Pancreatic and renal histopathology**

Histopathological changes in rats with HD are shown in Figure 4. The pancreas showed marked β-cell damage. The islets were more irregular and smaller in alloxan-treated rats than in sham controls. The average islet area was smaller after alloxan treatment (Figure 4A). Compared with sham, the average islet area was 3.7 times smaller (p<0.001) and the islet area to pancreatic area ratio was 5.1 times lower (p<0.001) in HD. Immunohistochemistry studies showed that the staining of islet β-cells decreased significantly in alloxan-treated animals. On P27, only one-quarter of residual β-cells remained in alloxan-treated animals (p<0.001) (Figure 4B–D).

Alloxan caused a remarkable change in the kidney weight/body weight ratio (kidney index) on P27 (Table 1). Histological studies showed that both glomerular areas and volumes were higher in HD than in sham controls on P27 (Figure 4E, F).

**Correlations among neutrophils, lymphocytes, NLR, OGTT AUC values, and kidney index**

Correlation analysis showed that both neutrophil and NLR values were positively correlated with OGTT AUC (r=0.62 and r=0.65, respectively, both p<0.01) (Figure 5A, B) and

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**Table 1** Kidney weight, kidney index, heart weight, heart index, blood glucose, water intake, food intake, urine output and 24 h urinary albumin excretion in sham and HD groups on experimental day P27

| Variable                      | Sham (n=8) | HD (n=7) |
|-------------------------------|------------|----------|
| Kidney weight (g)             | 1.46±0.03  | 1.85±0.13*|
| Kidney index                  | 6.91±0.20  | 9.07±0.61*|
| Heart weight (g)              | 0.66±0.02  | 0.62±0.01 |
| Heart index                   | 3.10±0.10  | 3.06±0.16 |
| Blood glucose (mg/dL)         | 75±2       | 83±5     |
| Water intake (mL/day)         | 28±3       | 158±42*  |
| Food intake (g/day)           | 15±1       | 33±7*    |
| Urine volume (mL/day)         | 9±1        | 138±43*  |
| Urinary albumin (μg/day)      | 18.96±14.52| 83.09±56.23*|

**Notes:** Values are expressed as mean±SEM. Kidney index: ratio of kidney weight/body weight (mg/g); heart index: ratio of heart weight/body weight (mg/g). *p<0.05 sham vs HD.

**Abbreviations:** HD, hidden diabetes; P27, posttreatment day 27.
Figure 2 Leukogram of female rats with or without alloxan treatment on P7 (n=5 and n=6, respectively) and P27 (n=7 and n=8, respectively).

Notes: (A) Leukocyte counts after 7 or 27 days of alloxan treatment. (B) Percentage neutrophils after 7 or 27 days of alloxan treatment. (C) Percentage lymphocytes after 7 or 27 days of alloxan treatment. (D) NLR resulting from (B) and (C). (E) Correlation between percentage neutrophils and NLR on P27. (F) Correlation between percentage lymphocytes and NLR on P27. (G) Percentage neutrophils in sham, HD, and DM groups on P27. (H) Percentage lymphocytes in sham, HD, and DM groups on P27. (I) NLR resulting from (G) and (H). The data from the leukogram were analyzed by analysis of variance with further tests for posthoc comparisons. Relationships between parameters were compared using Pearson correlation analysis. *p<0.05 and **p<0.01 sham vs HD.

Abbreviations: P7, posttreatment day 7; P27, posttreatment day 27; NLR, neutrophil–lymphocyte ratio; HD, hidden diabetes; DM, diabetes mellitus.
kidney index ($r=0.67$ and $r=0.69$, respectively, both $p<0.01$) (Figure 5C, D). In contrast, lymphocyte values were negatively correlated with OGTT AUC ($r=-0.57$, $p<0.05$) (Figure 5E) and kidney index ($r=-0.56$, $p<0.05$) (Figure 5F). OGTT AUC had a significant tight correlation with kidney index ($r=0.76$, $p<0.001$) (Figure 5G).

Discussion

Rats with HD have usually been considered as unsuccessful candidates for experiments and discarded from studies. Here, in an HD animal model with a restricted and controlled experimental design, our data have shown that alloxan treatment produced 19% of animals with normal FBG but with abnormal OGTT, diabetic nephropathy, higher neutrophil and lower lymphocyte levels, and a higher NLR. To investigate the mechanism of NLR alteration, we gave rats an acute 2 g/kg glucose, although the leukocyte number was not be the cause of NLR changes. Only 1 h after administration to maintain glucose homeostasis. The OG challenge may be the cause of NLR changes. Only 1 h after administration of 2 g/kg glucose, although the leukocyte number was not changed the lymphocyte number had decreased significantly, which resulted in the decrease/increase in the percentages of lymphocytes/neutrophils and an increase in the NLR.

Many studies on the relationship between NLR and DM have been conducted in patients. Mertoglu and Gunay reported that NLR values were significantly higher in prediabetic, newly diagnosed diabetics by OGTT (HD), and clear diabetic groups compared to a normal group. They suggest that NLR values may be reliable predictive markers in prediabetes and DM. Here, in an animal HD model, we confirm that NLR was indeed elevated in HD animals. In addition, NLR positively correlated with both the OGTT AUC and the kidney index, suggesting that NLR can be taken as additional information for early diagnosis of DM and is also an independent marker for predicting DM, as reported in clinical studies. It will be possible in the future to make an early diagnosis of HD when screening DM patients, using a standardized threshold NLR level.

The NLR is widely believed to reflect the inflammatory status in T1D. The present studies show that the causes of NLR alterations may be produced by the oscillations in glucose levels that are often experienced by diabetic subjects. In HD rats, the residual $\beta$-cells were able to keep the FBG within the normal range. However, the insulin secretion from residual $\beta$-cells was limited and unable to face the OGTT challenge. The animals with abnormal OGTT were unable to maintain glucose homeostasis. The OG challenge may be the cause of NLR changes. Only 1 h after administration of 2 g/kg glucose, although the leukocyte number was not changed the lymphocyte number had decreased significantly, which resulted in the decrease/increase in the percentages of lymphocytes/neutrophils and an increase in the NLR. The antioxidant GSH totally blocked the effects of the OG challenge, suggesting that reactive oxygen species (ROS) production play a key role in the pathogenesis of diabetic nephropathy.
Figure 3 Leukogram of female rats with or without OG and OG+GSH treatments on P7 (n=7 for all groups).

Notes: (A) Leukocyte counts in PC or P1h treatment. (B) Lymphocyte number after PC or P1h treatment. (C) Percentage lymphocytes after PC or P1h treatment. (D) Neutrophil number in PC or P1h treatment. (E) Percentage neutrophils in PC or P1h treatment. (F) NLR of PC or P1h treatment. (G) Correlation between changes in blood glucose and changes in lymphocyte number. The data from the leukogram were analyzed by 2-way analysis of variance with Tukey tests for posthoc comparisons. Relationships between parameters were compared using Pearson correlation analysis. *p<0.05, **p<0.01, and ***p<0.001 PC vs P1h.

Abbreviations: OG, oscillating glucose; GSH, glutathione; P7, posttreatment day 7; PC, precontrol; P1h, 1 h post-glucose; NLR, neutrophil–lymphocyte ratio.
In the alloxan-induced DM model, as the cytotoxic effects of alloxan last for a very short time, the early nephropathy in alloxan-treated animals may be mainly induced by the alloxan but the late (>21 days) renal disorders would be mainly produced by hyperglycemia. A substantial number of epidemiological studies and animal experiments demonstrate that diabetes is associated with chronic inflammation, which may contribute to the development of DM. The results of the present study suggest that the OG level itself can alter the NLR. Therefore, the increase in neutrophil number is a critical indicator of whether there is an existing inflammatory state. A higher NLR value is usually associated with higher neutrophil numbers and kidney damage. Clinical studies show that patients with an NLR of 1.6–2.4 were almost

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Figure 5 Relationships among leukogram data, glucose metabolic changes, and kidney index values after 27 days of alloxan treatment.

Notes: OGTT AUC values were positively correlated with the percentage of neutrophils (A) and NLR (B). Kidney index values (kidney weight/body weight ratio) were also positively correlated with the percentage neutrophils (C) and NLR (D). The percentage of lymphocytes was negatively correlated with OGTT AUC (E) and kidney index (F). The kidney index values were positively correlated with OGTT AUC (G). Relationships between parameters were compared using Pearson correlation analysis.

Abbreviations: OGTT, oral glucose tolerance test; AUC, area under the curve; NLR, neutrophil–lymphocyte ratio; KW/BW, kidney weight/body weight ratio.
four times more likely to have damaged renal function. Although we have to be wary in directly applying data from HD animal models to human disease, it is concluded that the use of the alloxan-induced HD rat is suitable for the study of the mechanisms and diagnostic markers contributing to the development and progression of HD and DM.

Conclusion
Alloxan treatment caused a progressive loss of β-cell mass in 19% of female rats with HD. HD associated with elevated NLR values may be partly attributed to a homeostasis disorder of the innate inflammatory state, which was caused by oscillating hyperglycemia. Acute high glucose administration produced a significant decrease in lymphocyte number. OG administration potentiated this effect and increased the NLR value, which was blocked by GSH, suggesting that ROS play a critical role in maintaining lymphocyte numbers. Currently, DM cohorts are mainly screened and judged by the FBG levels, which risks HD being missed. Thus, we recommend that the standard and threshold of NLR value for detecting HD could be clinically established and thus used as a convenient and indicative screening marker.

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Disclosure
The authors report no conflicts of interest in this work.

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