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Original Research Article

Complex interaction between allopurinol-induced uric acid reduction and glycemic control: a clinical and molecular study

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Abstract

Background: Diabetes mellitus type 2 (DMT2) and hyperuricemia are two prevalent metabolic diseases worldwide, including in Indonesia. Within the Minahasa tribe, the prevalence of these diseases is among the highest in Indonesia. The interaction between hyperuricemia and DMT2 is inconclusive, as previous studies about whether allopurinol and its related uric acid reduction correlate with insulin resistance have shown conflicting results.

Objective: To examine whether allopurinol-induced uric acid reduction can modify insulin resistance in nondiabetic Minahasan male subjects and to study the putative molecular mechanisms of this interaction.

Methods: The clinical part of this research was a pseudo-experiment with a pre-test/post-test design. Twenty nondiabetic Minahasan male subjects were subjected to the daily dose of 300 mg allopurinol for three months. Plasma glucose, uric acid, and insulin levels were measured pre- and post-treatment. Homeostatic model assessment of insulin resistance (HOMA-ir) values was calculated by the Oxford HOMA calculator. For the wet lab experiment, the human embryonic kidney HEK293T cell line was treated tolerable allopurinol concentrations. The expression of glucose transporter 4 (*GLUT4*), glucose transporter 1 (*GLUT1*), insulin receptor isoform A (*IR-A*), and thioredoxin-interacting protein (*TXNIP*) mRNAs were analyzed by quantitative real-time polymerase chain reaction (qPCR).

Results: In nondiabetic Minahasan male subjects, allopurinol administration decreased uric acid serum level, but did not affect plasma glucose and insulin levels. In fact, there is a trend of increasing HOMA-ir among the subjects following allopurinol administration. *In vitro*, allopurinol treatment also did not significantly change the expression of the tested mRNAs, suggesting that allopurinol's effect on diabetes control has other, complex mediative pathways. The limitations of the current study include the small size of the clinical sample and target genes.

Conclusion: Allopurinol administration and its related uric acid plasma reduction does not significantly affect insulin resistance; a trend however exists that allopurinol and uric acid reduction increased HOMA-ir. At the molecular level, mRNA expression of *GLUT1*, *GLUT4*, *IR-A*, and *TXNIP* is not significantly affected by allopurinol.

Keywords: *allopurinol; diabetes mellitus; insulin resistance; hyperuricemia; Glut4*
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INTRODUCTION

Diabetes mellitus type 2 (DMT2) has been a critical and pervasive health issue globally, exerting a significant burden on individuals, healthcare systems, and societies. In Indonesia, DMT2 is also a significant health concern with a growing prevalence and substantial impact on the population's health and economy. The country has experienced a rise in diabetes cases, with an estimated national prevalence of 10.8% in 2018, placing it among the top 10 countries with the highest prevalence of DMT2.[1,2]

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The 2018 nationwide Basic Health Research in Indonesia also reported a varying prevalence of DMT2 based on regions, with North Sulawesi province ranking first on the national highest proportion of obesity and hypertension, and fourth on the diabetes mellitus prevalence, thus reflecting a possible hereditary and environmental effect on DMT2 pathogenesis.[1] Minahasa is a majority tribe in North Sulawesi, with a reported high prevalence of DMT2 and hyperuricemia, partially attributed to their eating habit.[3,4] Their eating habits include high-calorie, high-fat, and high-purine diets, especially during the feasting seasons.[5,6] This population may reflect a unique group of people where researchers can control a single parameter (e.g. uric acid level) while keeping other parameters wild type.

Allopurinol is the first line xanthine oxidase inhibitor used in the management of hyperuricemia and its clinical manifestations, such as gout and kidney stones. This xanthine oxidase inibition decreases the conversion of hypoxanthine to xanthine and to uric acid.[7] Allopurinol is rapidly absorbed after oral administration, with peak plasma concentrations occurring within 1 to 2 hours.[8] The drug has a bioavailability of approximately 78%, and it is only minimally bound to plasma proteins, allowing it to be widely distributed throughout the body.[8] Once absorbed, allopurinol is metabolized in the liver to oxypurinol, its primary active metabolite, which also inhibits xanthine oxidase.[9] Oxypurinol has a much longer half-life (approximately 18 to 30 hours) compared to allopurinol (1 to 2 hours), which contributes significantly to the drug's therapeutic effects. Allopurinol is excreted primarily through the kidneys, with about 80% of the dose appearing in the urine as oxypurinol and other metabolites, while the remainder is excreted unchanged. Allopurinol and oxypurinol bind to the active site of the xantine oxidase, thus preventing the substrate from binding.[10] This mode of action not only decreases uric acid production but also leads to an increase in the concentration of the more soluble xanthine and hypoxanthine, which are more easily excreted.

In addition to its wide use in hyperuricemia management, allopurinol has also been studied for its potential effect on glucose levels in patients with diabetes. Several association studies have found a positive association between elevated serum uric acid levels and diabetes,[11,12] while others,[13,14] have reported an inverse relationship between increasing serum uric acid levels and diabetes mellitus. Other retrospective studies reported no correlation between UA and DMT2.[15] Finally, a meta-analysis by Chen et al of randomized controlled trials (RCTs) found that allopurinol use was associated with a significant reduction in fasting blood glucose (FBG) levels in the subgroup of patients without diabetes, but not in those with diabetes.[16] This variability demands population-specific studies to examine the allopurinol-uric acid-DMT2 relationship within each subgroup of people.

Since the previous research yielded conflicting results, the direction of correlation was an open-ended question and need to be addresed separately in different population. Therefore, in this research, we aim to study whether allopurinol administration in healthy, nondiabetic male subjects in Minahasa may lead to the modification of HOMA-ir. Furthermore, using transcriptomic research, we also aimed to elucidate its molecular mechanism, at least partially.

MATERIALS AND METHODS

Clinical study

The clinical part of this study employed a pre- and post-test pseudo-experimental research design, where the parameters of before and after treatments were compared. The patients were conveniently drawn from the community, consisting of 22 subjects with inclusion criteria as follow: male, non-diabetic, age ranged from 20-39 years, and from the Minahasa tribe. DMT2 diagnosis for exclusion was made based on the national criteria (blood glucose >126 mg/dl after a minimum of 8 hours fasting). Five patients were excluded due to uncountable HOMA-ir. Patients received a daily dose of 300 mg allopurinol, to be taken in the morning with food for the next 3 months. Fasting blood glucose (FBG), plasma insulin (Ins) and other demographic characteristics were recorded. HOMA-ir, the parameter for insulin resistance, was calculated based on the Oxford HOMA calculator (https://www.rdm.ox.ac.uk/about/our-clinical-facilities-and-units/DTU/software/homa). The final parameters consisted of plasma UA level, FBG, Ins, HOMA-ir, HOMA-s (insulin sensitivity), and HOMA-b (beta cell function). Two-tailed, paired t-tests were used to calculate significant differences between pre- and post-treatment, with a significance level set to <0.05. An ethical clearance was obtained for the study (003/FIK).

Laboratory study

Human embryonic kidney cell line HEK293T were cultured in standard Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum and pen/strep antibiotics in a humidified 37-degree Celsius incubator with 5% CO2. This cell line was selected because it has been well characterized in the context of glucose metabolism study and can express key protein in response to experimental treatments.[17,18] A low passage (p20-30) cell generations were used for the experiment. At 70% confluency, HEK293T cells were trypsin-dissociated and seeded into 6-well culture plates in DMEM and pen/strep. After 48 hours (~40% confluency), 1 or 4 mm of allopurinol were added into the cultures and incubated for another 24 hours. Another set of wells was left untreated as the control group. Experiments were done in triplicates. Cells from each well were harvested and lysed in 1 ml Trizol for further RNA extraction using the phenol-chloroform method. The resulting RNA pellets were resuspended and diluted in RNAse-free ddH_2O. A reverse transcriptase kit (Takara, Japan) was used to convert mRNA into cDNA. Ten nanograms of cDNA from each sample were used as a template for quantitative real-time polymerase chain reaction (qPCR). The target genes were *GLUT1* (*SLC2A1*) and *GLUT4* (*SLC2A4*) glucose transporters, glucose-dependent protein thioredoxin-interacting protein (*TXNIP*), insulin receptor subunit alpha (*IR-A*), and beta-actin (*ACTB*), a housekeeping gene for internal control. Primer pairs for these genes are presented in Table 1

Table 1. The primer pairs for the qPCR experiments

Gene	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
GLUT1	CTGCTCATCAACCGCAAC	CTTCTTCTCCCGCATCATCT
GLUT4	TGGGCTTCTTCATCTTCACC	GTGCTGGGTTTCACCTCCT
IR-A	TTTTCGTCCCCAGGCCATC	GTCACATTCCCAACATCGCC
TXNIP	GGTCTTTAACGACCCTGAAAAGG	ACACGAGTAACTTCACACACCT
ACTB	AGCCATGTACGTTGCTATCCA	ACCGGAGTCCATCACGATG

Table 2. Respondent characteristics

Parameter	Mean ± SD	Min value	Max value
Age (year)	30±3.52	20	38
BMI (kg/m^2)	27.83±3.05	21.5	34.8
UA (mg/dl), pre-treatment	8.11±1.16	7	11,5
UA (mg/dl), post-treatment	6.81±1.30	4,6	9,5
FBG (mg/dl), pre-treatment	89.00±5.15	78	100
FBG (mg/dl), post-treatment	92.18±9.16	72	110
Ins (mU/mL), pre-treatment	9.15±3.42	2,3	18
Ins (mU/mL), post-treatment	11.42±4.86	2,3	22,1

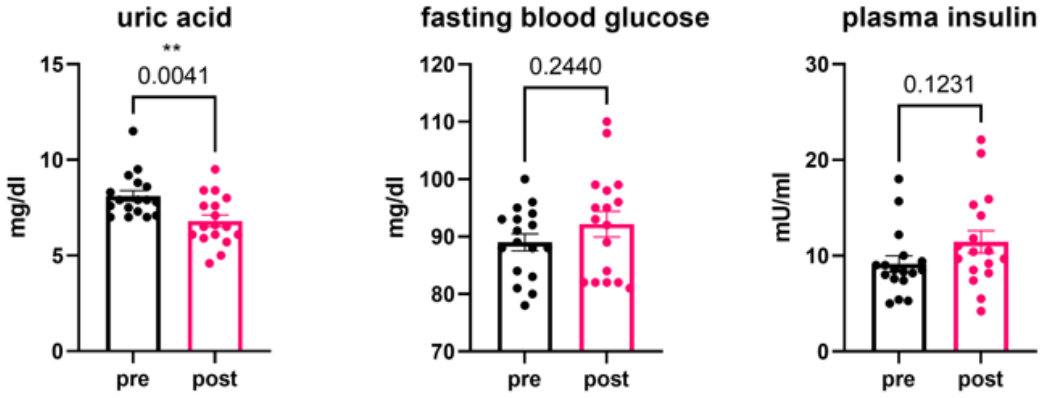

Figure 1. The effect of allopurinol on uric acid, fasting blood glucose, and plasma insulin level; *pre*: before allopurinol treatment; *post*: after allopurinol treatment. Error bars are SEMs.

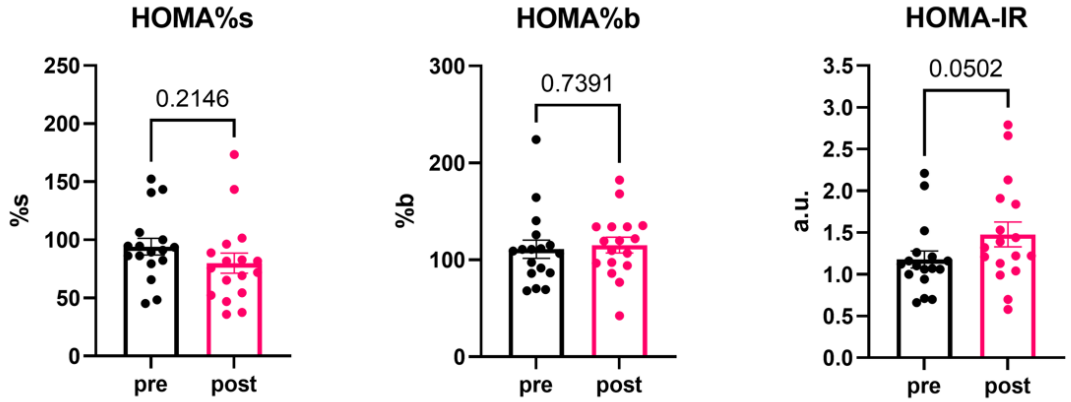

Figure 2. The effect of allopurinol in HOMA-ir parameters showed no difference in insulin resistance before and after allopurinol treatment. A trend of HOMA-ir increase post-treatment is shown in HOMA-ir (right panel, p=0.0502). Error bars are SEMs.

The qPCR mixture containing the template, primer pair, and SYBRgreen Mastermix reagent (Thermo-Fisher, USA) was amplified in an RT thermocycler (Applied Biosystem, USA). Each sample was loaded onto the 96-well plates in duplicates to account for possible technical errors. The delta-delta Ct value was used to calculate the mRNA expression foldchange. A one-way ANOVA was used to calculate statistical significance, followed by post-hoc Dunnett's test. Graphs were generated using Prism 9.0.

RESULTS

The respondent characteristics are presented in Table 2. As expected, allopurinol treatment decreases uric acid

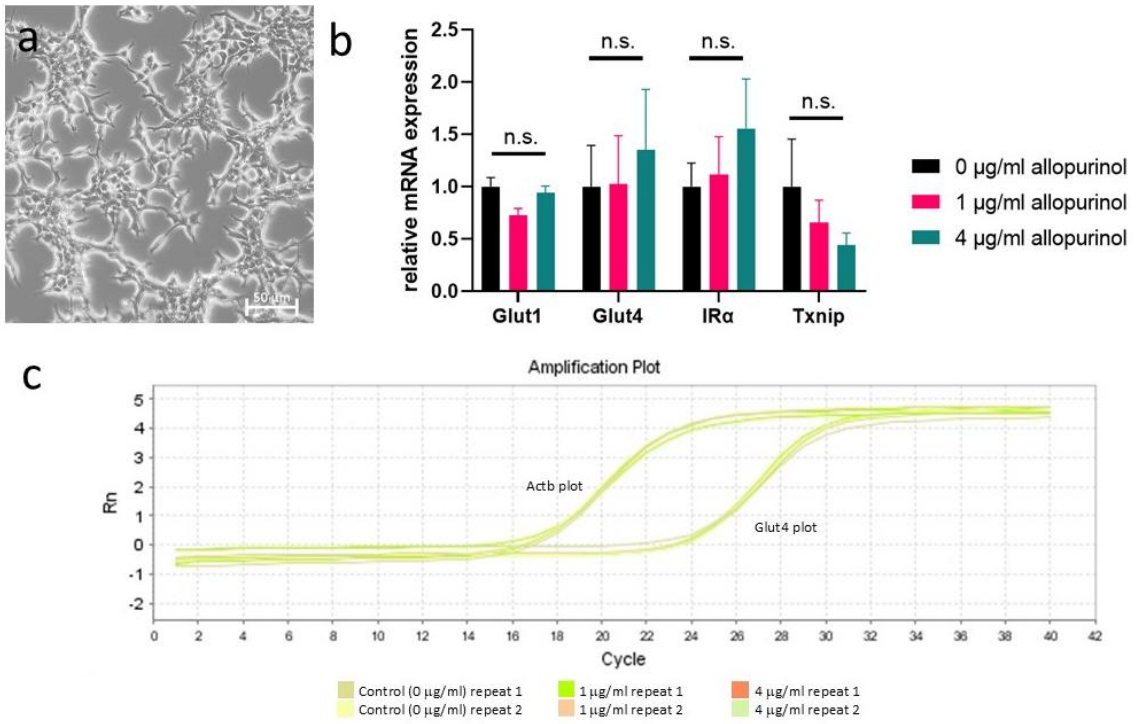

Figure 3. *In-vitro* analysis of allopurinol in HEK293T cell line. (a) The visual viability of the cells following 4µg/ml allopurinol treatment; (b) the relative expression of several glucose metabolism-related mRNA (*GLUT1*, *GLUT4*, IRα, and *TXNIP*) under the influence of allopurinol, showing no significant, but trending differences by one-way ANOVA and post hoc Dunnet's test; (c) a representation of *GLUT4* qPCR amplification plot from one biological repeat (two technical repeats each: 0 µg/ml, 1µg/ml and 4 µg/ml allopurinol treatment) showing overlapping plots (not significant). Error bars are SEMs, n=3 repeats.

level (average±SD pre-treatment: 8.11±1.16 mg/dl, post-treatment: 6.81±1.30 mg/dl, p=0.0041). However, there is no difference in fasting blood glucose (pre: 89.00±5.15 mg/dl, post: 92.18±9.16 mg/dl, p=0.2440) and plasma insulin (pre: 9.15±3.42 µU/ml, post: 11.42±4.86 µU/ml, p=0.1231, Figure 1). Following HOMA-ir calculation, we found that there is no significant difference in insulin sensitivity (HOMA%s pre: 94.16±29.53, post: 79.96±35.55, p=0.2146), b-cell predicted function (pre: 111.06±38.52, post: 115.21±33.27, p=0.7391), and insulin resistance (HOMA-ir pre: 1.18±0.42, post: 1.48±0.62, p=0.0502). The statistical calculation of the latter, however, shows that there exists a trend of increasing insulin resistance following allopurinol-induced uric acid decrease (Figure 2).

We then followed up these findings with an *In vitro* experiment to further understand the possible molecular pathway involved in allopurinol-related glucose regulation. HEK293T cell line was cultured with or without allopurinol. Two concentrations (1 µg/ml and 4 µg/ml) were selected based on the in-vivo measurement of allopurinol and its metabolite oxypurinol.[19,20] There is no growth inhibition or massive cell death caused by this concentration of allopurinol *in vitro*, as can be seen from Figure 3a. Among the glucose transporter proteins, glucose transporter 4 (*GLUT4*) and glucose transporter 1 (*GLUT1*) have been well known to be crucial in glucose uptake into the cells.[21] Moreover, thioredoxin-interacting protein (*TXNIP*), a glucose-responsive protein, were also measured to determine the glucose uptake of the cells.[22,23] Insulin receptor isoform a (*IR-A*) were also measured to see whether the glycemic effect is insulin-mediated of insulin-independent. For all these experiments, the mRNA expressions were normalized agaist beta-actin (ACTB) gene expression as the internal control. Compared to the control, both 1 and 4 µg/ml allopurinol did not significantly change the *GLUT1*, *GLUT4*, *IR-A*, and *TXNIP* expression level (Figure 3b, a representative of the qPCR amplification plot is shown in Figure 3c). However, a trend can be observed that allopurinol increases *GLUT4* and *IR-A* mRNA expression, while decreasing *TXNIP* mRNA expression, especially at the higher concentration of 4 µg/ml (Figure 3b). Altogether, these suggest that at the molecular level, allopurinol treatment may increase the *GLUT4* and insulin receptor alpha mRNA expression, which would result in an increase of glucose uptake. On the other hand, allopurinol also seems to decrease glucose uptake, as can be seen by the decreased level of *TXNIP* mRNA, which is sensitive to intracellular glucose concentration.[24]

DISCUSSION

In this current research, we showed that daily administration of 300 mg allopurinol in healthy male subjects has no significant effect in insulin resistance, except for a trend of incresed HOMA-ir approaching statistical significance (p=0.0502. Figure 2 right panel). Likewise, the application of 1 µg/ml allopurinol does not increase the expression of glucose transporters *GLUT1* and *GLUT4*, *IR-A*, and *TXNIP* mRNA expression *In vitro*. There are trends, however, that allopurinol increases *GLUT4* and *IR-A*, but decreases *TXNIP* mRNA expression *In vitro* when applied in supraphysiological

concentration (4 μg/ml).

The results from the current literature are already heterogeneous with conflicting results reported from different researchers. Association studies from different populations have found a positive association between elevated serum uric acid levels and diabetes,[11,12] while others[13,14] have reported that serum uric acid levels is negatively correlated with glycaemic control. Other retrospective studies reported no correlation between uric acid level and DMT2.[15] When analysed together, meta-analysis of randomized controlled trials (RCTs) found that allopurinol use was associated with a significant reduction in fasting blood glucose (FBG) levels in the subgroup of patients without diabetes, but not in those with diabetes.[16] In our samples, there exists a trend that allopurinol administration and its related uric acid lowering actually increased insulin resistance, although it did not reach statistical significance. Our population (adult, non-diabetic males from the Minahasa tribe) is unique in the way that they have a high-calorie, high-fat, and high-purine diet.[3–6]. When allopurinol treatment was initiated, we did not give specific instructions on how to control the subjects' diet, (i.e. the subjects were told "Please eat like you usually do"). Although this may increase the confounding variables, it is superior in reflecting the conditions in the community settings. Indeed, we found that allopurinol without any other diet modification had already shown a decrease in uric acid level, which is expected to be shown by an established drug. Interestingly, as a side effect, insulin resistance seems to be increased in our samples. Due to the nature of this pseudo-experiment, it is not possible to elucidate whether this increase reflects a physiological dynamic, the natural history of an ongoing disease progression, or a direct consequence of allopurinol and uric acid lowering. Moreover, DMT2 is a multifactorial disease, with both genetic and lifestyle contributing to its pathogenesis. Therefore, in different populations, the effect of allopurinol and urate-lowering therapy may be variable, thus similar research in different population settings is mandatory to tailor a comprehensive approach to DMT2 management.

In vivo, allopurinol is rapidly metabolized to oxypurinol. After oral administration, allopurinol may rise to 1 ug/mL within the first several hours, before metabolized to oxypurinol.[19] The plasma concentration may rise to 4 μg/mL when administered intravenously in experimental setting.[16] We chose these as the working concentrations. At both 1 μg/ml and 4 μg/ml, the growth of HEK293T cells was not affected by allopurinol, suggesting the nontoxicity of the treatment. A trend of change can be observed in *GLUT4*, *IR-A*, and *TXNIP* expression over increasing allopurinol concentration, but they did not reach statistical significance. The results from our experiment may be explained by several things. First, allopurinol shows a weaker xanthine oxidoreductase inhibition compared to its metabolite, oxypurinol,[10] while *In vivo*, allopurinol is rapidly metabolized to oxypurinol. This pharmacokinetic response could not be replicated *In vitro*, thus, an *In vivo* experimental study in animal models is needed. Results from animal studies mostly highlighted the effect of allopurinol on the complications of diabetes. For example, allopurinol decreases kidney injury in diabetic mice,[25] ameliorates hepatic steatosis,[26] and alleviates cardiac anomaly in insulin resistance,[27] but does not affect insulin resistance *per se*. Whether these effects are mediated by allopurinol, oxypurinol, or due to the uric acid lowering is not fully understood.

Although both our clinical and molecular studies did not reach statistical significance, interesting trends emerged from these current results. Allopurinol seems to increase insulin resistance in healthy, nondiabetic male subjects. At the molecular level, the expressions of *IR-A* and *GLUT4* also tend to increase, suggesting that the glucose uptake should actually increase, resulting in less insulin resistance. However, the expression of *TXNIP* also tends to increase, thus suggesting a failure in uptaking the glucose from the culture medium.[24] Together, these results suggest that the effect of allopurinol on the expression of key protein related in glucose metabolism is versatile, depending not only on the cellular context *in vitro*,[28] but also on the physiological condition at the organismal level,[29] e.g. one gene may be expressed differentially in different individuals as a response to allopurinol. This complex molecular response can also explain the heterogeneity in the results of previous clinical research results[11-16] in determining whether allopurinol increase or decrease insulin resistance.

Further research is necessary to fully capture the complex molecular mechanism of DMT2 and insulin resistance at the molecular levels. For example, controlling the dietary intake of the clinical experiment subjects for the duration of allopurinol administration is a way to control for confounding factors. More samples from more heterogenous populations and longer follow-up duration will also increase the power of future studies. In terms of molecular studies, including more genes involved in DMT2 pathogenesis will also provide a clearer explanation about the effect of allopurinol in DMT2. These include glucagon receptors, other subunit of insulin receptors, IL1, glucocorticoid receptor, among others.

CONCLUSION

The administration of allopurinol 300 mg daily for three months without any diet modification does not have a significant effect on insulin resistance. However, a trend existed indicating that allopurinol administration and uric acid lowering increase insulin resistance, which may be explained by the complex interaction between allopurinol and glucose metabolism-related genes. Further research involving larger clinical subjects and more molecular pathways is necessary to fully understand the mechanism of uric acid-related glycemic control.

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