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Uric Acid Lowering Effect of Xanthine Oxidase Inhibitors, Febuxostat and Allopurinol in an Animal Model

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Original Research Article

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Abstract: We had investigated the xanthine oxidase inhibitory activity and uric acid lowering effect of newly marketed xanthine oxidase inhibitor, febuxostat and compared its effect with allopurinol in vitro. In hyperuricemic animal model serum uric acid, lowering effect of these drugs was evaluated. Serial dilutions of febuxostat and allopurinol were made, ranging from100 µg/ml to 0.75µg/ml. Xanthine oxidase inhibition was carried out in vitro. Hyperuricemia was induced in Wistar rats by potassium oxonate injection on day 1, 3 and 7. Febuxostat and allopurinol (5mg/kg) once daily was given for 7 days. Serum uric acid was measured on day zero, 1, 3 and 7 by uricase method. Michaelis Menten equation was applied to calculate IC50, Vmax and Km. IC_{50} of allopurinol and febuxostat were 9.07 and 8.77 µg/ml respectively. Km and Vmax of febuxostat were 8.89 and 107.13 where as allopurinol showed Km 7.77 and Vmax 194.14.Graded dose response was observed for both allopurinol and febuxostat. Hyperuricemia was successfully induced with potassium oxonate. Treatment with allopurinol reduced serum uric acid levels up to 3.21±0.8mg/dl on day 7, but reduction was less than febuxostat 0.81 ± 0.12 mg/dl. From this study, we have concluded that febuxostat is an effective option for cases of hyperuricemia.
Keywords: Hyperuricemia, potassium oxonate, febuxostat, xanthine oxidase inhibition, in vitro.

INTRODUCTION

Hyperuricemia, serum urate concentration more than 7 mg/dl is one of the common biochemical abnormalities [1]. Oddness in formation and/or excretion of serum uric acid (SUA) may increase its concentration.

Hyperuricemia is constituent of metabolic syndrome, which is characterized by high blood pressure, atherogenic dyslipidemia, dysregulated glucose homeostasis and/or insulin resistance as well as abdominal obesity [2, 3]. The prevalence of metabolic syndrome in USA is approximately 27 % [4].

Uric acid is the final product of purine breakdown in humans. Hypoxanthine and xanthine are the intermediate compounds produced during purine catabolism by xanthine oxidase (XO). Reactive oxygen species are produced simultaneously during these reactions [5]. In most of the animals, uric acid is further metabolized into allantoin. That is highly soluble in water, so excreted easily without crystallization [6]. The human beings and higher primates cannot oxidize uric acid because of nonsense mutation of the enzyme uricase, resulting in higher uric acid levels in humans [7].

Serum uric acid levels are maintained by a balance between hepatic production and excretion through the kidney and gut. Any imbalance in it may precipitate hyperuricemia [8]. Major causes of hyperuricemia include purine rich seafood, overproduction of purines, tumor lysis syndrome, under excretion of uric acid by various drugs (low dose salicylates, thiazide and loop diuretics, cyclosporine, pyrazinamide and ethambutol) [9]. Purine analogue allopurinol and its metabolite oxypurinol were introduced by Elion *et al*., [10] and have been used for hyperuricemia and chronic diseases of gout since 50 years. Febuxostat, a non-purine XO inhibitor is claimed more potent and safer drug in mammalian species [11]. We now report that febuxostat has more XO inhibition in vitro and uric acid lowering effect in rats.

MATERIALS AND METHODS

Drugs and reagents

Xanthine(X7375-25G), xanthine oxidase (X1875-25U), and potassium oxonate (156124-5G) were purchased from Sigma Aldrich Company. Febuxostat and allopurinol were procured from Pharm EVO Pharmaceutical Industries. Reagents, kits compatible to the Chemistry Analyzer (UA Kit, catalogue no., UA 230, Randox Laboratories Limited,

UK) were procured from local venders. Other chemicals and reagents used were of pharmaceutical grade.

Xanthine oxidase inhibitory assay

Method adopted by Alsultanee *et al*., [12] was used with few modifications. Allopurinol and febuxostat were dissolved in 100 µl of dimethyl sulfoxide and further dilutions were made in distilled water. A reaction mixture having final concentrations of allopurinol and febuxostat ranging from 100 µg/ml to 0.75 µg/ml,1.8 ml 50 mM potassium phosphate buffer (pH 7.5), 0.1 ml test sample compounds and 0.1 ml 0.2 units/ml of xanthine oxidase in phosphate buffer (prepared fresh). The mixture was initially incubated at 37°C for 15 minutes. Then, 1 ml of 0.15 mM of xanthine was added into the solution. The test sample was incubated again at 37°C for 30 min. The reaction was stopped by adding of 0.1 ml of 0.5 M HCl. UV/VIS spectrophotometer (model UV-1602, Biotechnology Medical Services, Canada) was used to measure the absorbance at 295nm against a blank prepared in the same way except enzyme solution. Another reaction mixture was prepared (control) having 0.1 ml of distilled water instead of test compounds [12, 13]. All samples were run in triplicate and mean was taken.

Experimental design to study xanthine oxidase inhibition in vivo

Thirty two adult, healthy male Wistar albino rats were taken from the Animal House, University of Health Sciences after approval from the ethical review committee (UHS/REG-17/ERC/4388). The animals were randomly divided into four groups (n=8). Group I (Negative control) group II (Hyperuricemic control), group III allopurinol (5mg/kg) and group IV febuxostat (5mg/kg) [14]. Hyperuricemia was induced by intraperitoneal injection of potassium oxonate (250 mg/kg) on 1st, 3rd and 7thday, one hour before the administration of test compounds [15].

Blood sampling

Blood was taken from tail vein on zero, first and third days of the experiment. On day 7, blood was collected through intra cardiac puncture under light anesthesia three hours after the last dose. Serum was separated and stored at –20°C [16].

Serum uric acid concentration was determined by dry chemistry analyzer on zero, 1st and 3rd days. Diagnostic kits compatible to chemistry analyzer was used for SUA on day seven. [17].

Statistical analysis

Mean ± Standard deviation (SD) was calculated and P value ≤ 0.05 was considered as statistically significant. Data was analyzed with SPSS 20. One way ANOVA and Post hoc Tukey's test was applied to determine the difference among groups. Michaelis Menten equation was used to calculate the inhibitory concentration 50 % (IC_{50}), maximum velocity (Vmax) and dissociation constant (Km).

RESULTS

Xanthine oxidase inhibition in vitro

Febuxostat and allopurinol have graded dose response relationship in XO inhibition. The IC_{50} values of febuxostat and allopurinol were 8.77and 9.07 µg/ml respectively; Km of febuxostat was 9.89 whereas allopurinol presented 7.76. Vmax values of febuxostat and allopurinol were 107.12 and 94.14 respectively (Table-1). Lineweaver-Burk plot was used for graphical representation of data. It reflects the potency of allopurinol is less than febuxostat (Figure-2).

Hypouricemic effect of xanthine inhibitors in potassium oxonate treated rats.

The effect of febuxostat and allopurinol in hyperuricemic rats is shown in Table 2. Potassium oxonate caused 435% rise in SUA as compared to negative control on day seven. Allopurinol significantly reduced SUA levels to 3.21 ± 0.8 mg/dl as compared to negative control ($p<0.001$). Febuxostat treated animals had 0.81±0.12 mg/dl SUA. This group presented highly significant ($p<0.001$) reduction in SUA as compared to control. Allopurinol and febuxostat have comparable reduction of SUA on day one and three but febuxostat has 25% higher SUA lowering effect on day seven ($p<0.001$) as compared to allopurinol (Figure 1).

Table-1: Xanthine oxidase inhibition in vitro by febuxostat and allopurinol

Concentrations µg/ml	**Xanthine oxidase inhibition %**	
	Febuxostat %	**Allopurinol %**
IC $_{50}$	8.77 µg/ml	9.07 µg/ml
Km	8.89 ± 0.79	7.76 ± 1.45
Vmax	107.12 ± 2.58	94.14± 4.67

Results are expressed as percent inhibition (n = 3).
Where: IC_{50} 50 % inhibitory concentration of substrate.
Vmax maximum velocity, Km dissociation constant.

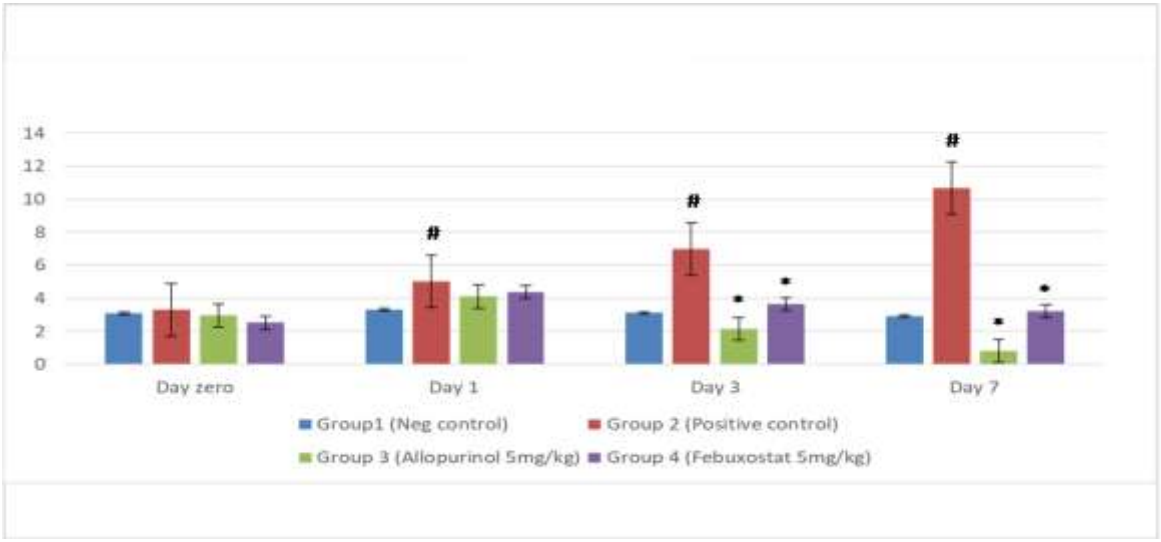

Fig-1. Effect of febuxostat and allopurinol on serum uric acid levels in hyperuricemic animal model.
Results are expressed as Mean ± Standard Deviation (n=8).
Where # p < 0.05 as compared to group 1, * p < 0.05 as compared to group 2.

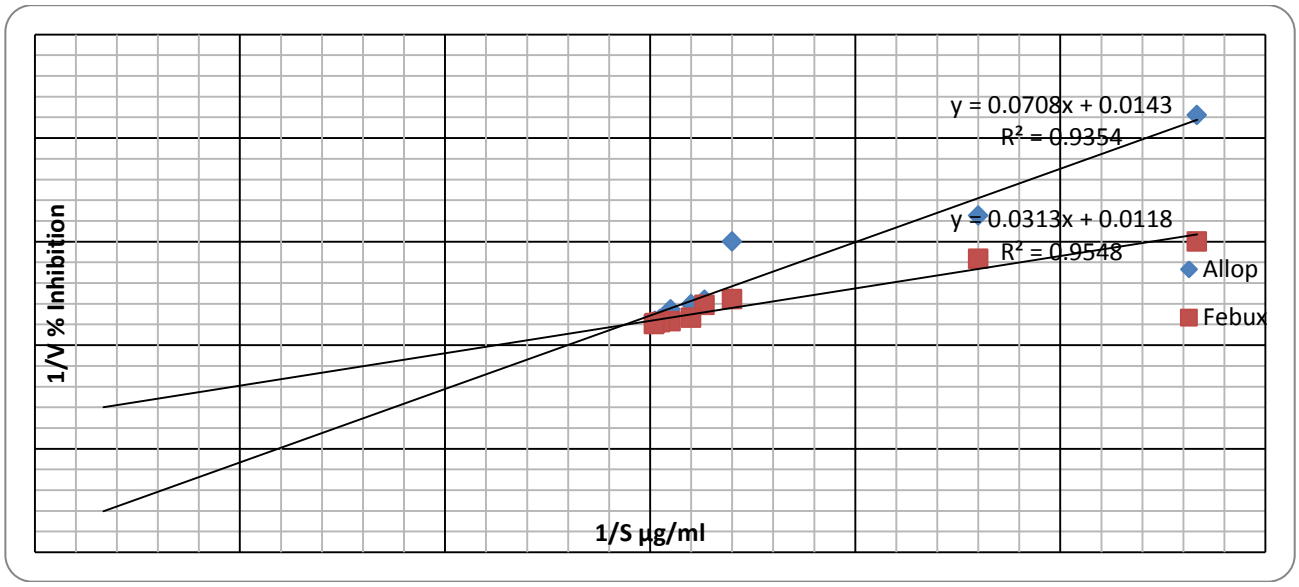

Fig-2: Lineweaver Burk plot of xanthine oxidase inhibition by febuxostat and allopurinol in- vitro
Results are expressed as Mean ± S.D (n=3)

DISCUSSION

Allopurinol, a hypoxanthine isomer was introduced in 1963 and approved by FDA in 1966 and it was the sole XO inhibitor prescribed for hyperuricemia and gout [10]. Allopurinol itself is oxidized into oxypurinol by xanthine oxidase or xanthine dehydrogenase. Oxypurinol tightly binds to xanthine oxidase and truly speaking causes death of xanthine oxidase [18].

Significant progress has been taking place for the discovery of new XO inhibitors. Febuxostat is the second xanthine oxidase inhibitor available in the market after 40 years rule of allopurinol [19].

Xanthine oxidase inhibition by febuxostat was more than allopurinol during in-vitro study (Table 1, Figure 2). Allopurinol has competitive xanthine oxidase inhibition whereas mixed type of xanthine oxidase inhibition by febuxostat has been reported [18, 20].

Xanthine oxidase inhibition of febuxostat and allopurinol stated by Osada *et al*., [21]were in nanomoles whereas higher IC_{50}values observed during in vitro study may be due to the drugs under observation were of pharmaceutical grade instead of analytical grade. Moreover reactions were run at 37ºC instead of 25 ºC reported by earlier worker [21].

Uric acid lowering capacity of allopurinol and febuxostat was observed in rats. Metabolism of uric

acid was blocked by intraperitoneal injection of potassium oxonate. Febuxostat presented more potency than allopurinol in this model (Figure-2). These results are in conformity to the data presented by Osada *et a*l., [20]. Febuxostat has shown 25% more reduction of uric acid as compared to allopurinol.

A disparity is observed during data analysis; the values of Km and Vmax derived by Michaelis-Menten equation are much higher (Table1) than derived from Lineweaver- Burk plot (Figure-2).

CONCLUSION

Correlation of hyperuricemia and metabolic syndrome is a subject under extreme discussion Febuxostat is recommended as a standard and experimental tool during investigation of new uric acid lowering drugs.

Conflict of Interest

The authors have no conflict of interest. The work was funded by Pharmacology Department UHS Lahore. Pakistan.

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