IN VITRO EFFECT OF IRON OXIDE NANOPARTICLES ON XANTHINE OXIDASE ACTIVITY

Ritu Hooda and Minakshi Sharma*
Department of Zoology, Maharshi Dayanand University Rohtak (Haryana), India.

Abstract
Recently, the boom of metal oxide nanoparticles synthesis and its biomedical applications have led to emphasize on the possible impact of these nanoparticles on bio-macromolecules. In this study, we studied the impact of green Fe$_2$O$_3$ NPs and chemically synthesized (Commercial), on xanthine oxidase (XO) activity by addition of CuO-NPs in aqueous suspension (1–100 μg/ml at an interval of 10 μg) in the reaction mixture of its spectroscopic assay. The XO assay was also performed in vivo using fresh human serum in place of XO. The XO activity was decreased, as the concentration of Fe$_2$O$_3$ NPs increased. Km value of XO was increased upon addition of both types of Fe$_2$O$_3$ NPs indicating the lower affinity of enzyme towards the substrate in presence of Fe$_2$O$_3$ NPs. Our results provide insight into the interaction of Fe$_2$O$_3$ NPs with enzyme, which can be important for further applicability of nanoparticles in biomedical applications.

Key words: Iron oxide nanoparticles and xanthine oxidase activity.

Introduction
Today nanotechnology is one of the most active research areas of science. It is a branch of science which deals with particles less than 100 nm in size which is known as nanoparticles (NPs). NPs have a wide range of applications in various fields. Most of the nano-products produced on an industrial scale are used in skin care products that use NPs to deliver vitamins deeper into the skin, sunscreens that use NPs to block UV rays without leaving white residue on the skin, toothpastes, textiles, sports equipment, sanitary ware coatings, food products, medicine and diagnostics (Ball, 2001). Despite the wide applications of nanomaterials, there has been a serious lack of information concerning the impact of NPs on human health and the environment. In general, NPs have the ability to interact with whole physiological surrounding once when they enter human body. In order to optimize the beneficial effects of NPs on living organisms, it is essential to understand the fundamental interactions of it with biological systems (Komatsu et al., 2008). In most of the cases, first molecules they interact with are proteins (Ren et al., 2011). All enzymes are proteins therefore, the effects of NPs on enzymes and their activity are important to study. Enzyme kinetics is a field of biochemistry concerned with the quantitative measurement of the rates of enzyme-catalyzed reactions and the systematic study of factors that affect the reaction rates. A complete, balanced set of enzyme activities is of fundamental importance for maintaining homeostasis of body (Randle 1963). Therefore, understanding of enzyme kinetics is important to study (Kennelly and Rodwel, 2009). Effect of NPs on enzyme kinetics can be studied by incubating the enzyme with NPs and determining its enzyme activity to find out that whether the NPs were inhibiting or activating the enzyme activity (Lynch and Dawson, 2008). Till date no data is available to study the effect of NPs on xanthine oxidase. Xanthine oxidase (XO) is a metallo-protein that catalyzes the formation of xanthine which can further catalyze the oxidation of xanthine to uric acid according to the following equations:

\[
\text{Hypoxanthine} + \text{O}_2 \rightarrow \text{Xanthine} + \text{H}_2\text{O}_2
\]

\[
\text{Xanthine} + \text{O}_2 \rightarrow \text{Uric acid} + \text{H}_2\text{O}
\]

XO is widely distributed, occurring in milk, small intestine, kidney and liver. XO is of major medical interest as a target of many drugs against several diseases in
humans, such as gout, hyperuricaemia and chronic heart failure (Stiefel, 1993). Accumulation of uric acid can result in hyperuricaemia, leading to arthritus and gout (Enemark and Young, 1993). The effects of NPs on key mediators of biological functions such as enzymes have been studied very less (Thoenes et al., 1994). Although information about toxicity of metal oxide NPs continues to increase, a significant knowledge gap still exists on a complete toxicological profile of metal oxide NPs proposed for future use in medical applications. Without the data, risk assessment or regulation for safety of the materials shall suffer immeasurably.

The present study describes the comparative effect of green and chemically synthesized (commercial) Fe$_2$O$_3$NPs (which is synthesized and characterized in our paper Hooda et al., 2019) on activity/kinetics of XO in vitro and vivo (in human serum). The study could also help to further evaluate the long-term impact of NPs on XO.

**Materials and Methods**

**Chemicals and reagents**

Xanthine, xanthine oxidase (XO) from bovine serum, 4-aminophenazone, ethylenediaminetetra-aceticacid (EDTA), diethylthiocarbamate (DDC), N-ethylmaleimide (NEM), sodium molybdate, sodium thiocyanate, NaCl, MnCl$_2$, CaCl$_2$, ZnSO$_4$, MgSO$_4$, FeSO$_4$, CuSO$_4$, riboflavin, sodium nitrate, FAD, FMN, NAD, sodium azide, ampicillin and peroxidase from horseradish were from SRL Mumbai. Other chemicals were of analytical reagent grade. Distilled water (DW) was used throughout the study.

**Assay of native/free XO**

Assay of XO was accomplished according to Pundir et al., (1998).

**XO assay in vivo**

In the assay mixture, XO was replaced by 1.0 ml fresh human serum. The remaining procedure of the assay was similar to as given in the assay of native/free XO.

**Effect of Fe$_2$O$_3$NPs on XO in-vitro**

To study the effect of Fe$_2$O$_3$NPs (both green and chemical) on XO activity, in the assay mixture 0.1 ml of buffer was replaced by the same amount of aqueous suspension of Fe$_2$O$_3$NPs solution (1–100µg/ml at interval of 10µg). pH of the reaction buffer was maintained at 8.2 and 9.0, when incubated with green and chemical Fe$_2$O$_3$NPs respectively. The remaining procedure of the assay was similar to as given in the assay of native/free XO.

**Effect of Fe$_2$O$_3$NPs on XO in vivo**

To study the effect of Fe$_2$O$_3$NPs (both green and chemical) on human serum XO activity, in the assay mixture 0.1 ml of buffer was replaced by the same amount of aqueous suspension of Fe$_2$O$_3$NPs solution (1–100µg/ml at interval of 10µg). pH of the reaction buffer was maintained at 8.2 and 9.0, when incubated with green and commercial Fe$_2$O$_3$NPs respectively. The remaining procedure of the assay was similar to as given for the assay of XO in vitro.

**Effects of Fe$_2$O$_3$NPs on kinetic parameters of XO**

The kinetic parameters of XO were studied in the presence of both green and chemical Fe$_2$O$_3$NPs.

To determine optimum pH of XO, the pH of reaction mixture was changed from pH 3.0 to 10.0 at 0.5 pH interval, all at a final concentration of 0.05 M. To study the optimum incubation temperature of XO, the reaction mixture was incubated at different temperatures ranging from 25 to 80°C at a 5°C interval. To study the effect of substrate concentration, the concentration of xanthine was varied between 0.1 to 50 mM, Km for xanthine was measured from the effect of substrate on XO as follow: Km = [S] at 1/2Vmax for comparison purpose.

**Effect of metal chelators**

To study the effect of metal chelators, following chelators such as sodium azide, DDC, NEM, EDTA, sodium molybdate, sodium nitrate and sodium thiocyanate, were added in the reaction mixture individually containing green / chemical Fe$_2$O$_3$NPs at a final concentration of 1.0 mM.

**Effect of metals**

To study the effect of metals on XO, the following metals salts CaCl$_2$, FeSO$_4$, MnCl$_2$, ZnSO$_4$, CuSO$_4$, NaCl, and MgSO$_4$ were added in the reaction mixture individually at a final conc. of (1.0 mM), in the presence of both Fe$_2$O$_3$NPs.

**Effect of coenzymes**

To study the effect of coenzymes, FMN, FAD, NAD and riboflavin were added in reaction mixture (0.1 mM) previous to start the reaction, individually in presence of Fe$_2$O$_3$NPs.

**Results and Discussion**

**Effect of Fe$_2$O$_3$NPs on XO activity both in-vitro and in vivo**

When chemically and green Fe$_2$O$_3$-NPs (1-100 µg/ml) were added in the reaction mixture of XO, the enzyme activity was inhibited in both the cases as shown in Fig. 1. The XO activity without addition of Fe$_2$O$_3$-NPs was
**In vitro effect of Iron oxide nanoparticles on xanthine oxidase activity**

**Fig. 1:** Effect of Fe\(_{2}O_{3}\)-NPs on the activity of XO invitro.

**Fig. 2:** Effect of Fe\(_{2}O_{3}\)-NPs on the activity of XO in human serum.

**Fig. 3:** Effect of pH on activity of xanthine oxidase (XO) invitro, in presence of green and chemical Fe\(_{2}O_{3}\)-NPs.

**Fig. 4:** Effect of substrate on activity of xanthine oxidase (XO) invitro, in presence of green and chemical Fe\(_{2}O_{3}\)-NPs.

**Fig. 5:** Effect of chelators on activity of xanthine oxidase (XO) invitro in presence of green Fe\(_{2}O_{3}\)-NPs.

**Fig. 6:** Effect of chelators on activity of xanthine oxidase (XO) invitro in presence of chemical Fe\(_{2}O_{3}\)-NPs.

When green Fe\(_{2}O_{3}\)-NPs was incubated at different concentrations invitro, the XO activity was inhibited by 13% at 1, 10 and 20µg/ml, 30% at 30µg/ml, 55% (significant) at 40µg/ml, and 60-80% at rest of the concentrations. Likewise chemical Fe\(_{2}O_{3}\)-NPs inhibited the XO activity by 50% (significant) at even low dose 1 to 10 µg/ml, than inhibited in gradual manner from 55 % to 88 % at 30, 40, 50, 60, 70, 80, 90 and 100µg/ml.
than 75% at rest of the concentrations (Fig. 2).

The XO activity was decreased as the concentration of Fe$_2$O$_3$NPs was increased which might be due to the interaction of Fe$_2$O$_3$NPs with the surface of enzyme through H-bonding (Esfandfar et al., 2016; Chauhan et al., 2013). Such a binding of Fe$_2$O$_3$NPs with the enzyme could bring the change in the conformation of enzyme and hence the activity of enzyme is decreased as the concentration is increased.

**Effects of Fe$_2$O$_3$NPs on kinetic properties of XO**

**Effect of pH**

XO showed arise in optimum pH from 7.4 to 8.0 and 8.9, when incubated with green and chemical Fe$_2$O$_3$NPs respectively (Fig. 3). The increase in optimum pH of the enzyme in presence of Fe$_2$O$_3$NPs could be due to release of Fe$^{2+}$ from Fe$_2$O$_3$NPs, which might deprotonate buffer (Chauhan et al., 2013).

**Effect of incubation temperature**

XO showed no significant change in its optimum temperature (40 $^\circ$C), when incubated with both commercial and green Fe$_2$O$_3$NPs.
Effect of metals

Among the various metal salts tested such as NaCl, MnCl₂, CaCl₂, ZnSO₄, MgSO₄, FeSO₄, and CuSO₄ at 1.0 mM concentration, only FeSO₄ caused slight stimulation of enzyme. It might occur due to the possibility that Fe SO₄ salt may react to the Fe-S cluster of XO enzyme and thus stimulate the electron transport (Fig. 7 & 8) in presence of green/chemical Fe₂O₃NPs.

Effect of coenzymes

Of the coenzyme tested, only FAD, at 1.0 mM concentration in the presence of Fe₂O₃NPs stimulated the activity, similar to native enzyme. Comparatively green Fe₂O₃NPs stimulated the enzyme activity significantly more than the commercial Fe₂O₃NPs. There may be the possibility that the plant biomolecules such as proteins, carbohydrates and secondary metabolites like flavonoids, terpenoids and phenolic compounds that are attached to NPs interact better than the chemical NPs (Fig. 9 & 10).

Conclusion

Fe₂O₃NPs which was green synthesized inhibit the XO activity comparatively lower than that by chemical Fe₂O₃NPs, when added in reaction mixture and serum in a spectrophotometric assay at rate of 1, 10 and 20ug/ml. But the high dose of both green and chemical Fe₂O₃NPs (90 and 100 µg/ml) caused nearly complete inhibition of XO activity. Km value of XO was increased upon addition of both Fe₂O₃NPs indicating the lower affinity of enzyme towards the substrate.

Acknowledgement

Author (RH) is grateful to Maharshi Dayanand University, Rohtak for the award of university research scholarship during the tenure of this study.

References


