Inhibition of snake venom induced sterile inflammation and PLA2 activity by Titanium dioxide Nanoparticles in experimental animals

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Sterile inflammation (SI) is an essential process in response to snakebite and injury. The venom induced pathophysiological response to sterile inflammation results into many harmful and deleterious effects that ultimately leads to death. The available treatment for snakebite is antiserum which does not provide enough protection against venom-induced pathophysiological changes like haemorrhage, necrosis, nephrotoxicity and often develop hypersensitive reactions. In order to overcome these hindrances, scientists around the globe are searching for an alternative therapy to provide better treatment to the snake envenomation patients. In the present study TiO2 (Titanium dioxide)-NPs (Nanoparticles) has been assessed for antisnake venom activity and its potential to be used as an antidote. In this study, the synthesis of TiO2-NPs arrays has been demonstrated on p-type Silicon Si < 100 > substrate (~30 ohm-cm) and the surface topography has been detected by Field-emission scanning electron microscopy (FESEM). The TiO2-NPs successfully neutralized the Daboia russelii venom (DRV) and Naja kaouthia venom (NKV)-induced lethal activity. Viper venom induced haemorrhagic, coagulant and anticoagulant activities were effectively neutralized both in in-vitro and in vivo studies. The cobra and viper venoms-induced sterile inflammatory molecules (IL-6, HMGB1, HSP70, HSP90, S100B and vWF) were effectively neutralised by the TiO2-NPs in experimental animals.

Biomedical therapy and diagnostics are getting more prominent with the utilization of nanotechnology concept1. The aim of using a nano-medicine is to improve the bio-distribution of therapeutic agent and to allow its accumulation on the target site2. Different types of nano-medicine have been evaluated in recent years, i.e., liposomes, polymers, micelles and antibodies. Several evidences also indicate the ability of these nano-sized carrier materials to improve the balance between the efficacy and the toxicity of therapeutic interventions.

Today, nano-scale research has been emerged as important players in the field of pharmacology and biotechnology. Except few nanoparticles and quantum dots, most of them are toxic in nature and are not applicable for medical or therapeutic applications3. TiO2 has a wide spectrum of properties that varies with different techniques used for its synthesis4 and has applications in the field of biomedical research and treatment such as anti-tumour therapy5,6, manufacturing of bio-products7 etc. Ubiquitous applications and better results of TiO2-NPs, makes them a potential candidate for biomedical research8. In the present study, the synthesis of TiO2-NPs arrays has been demonstrated on p-type Silicon Si < 100 > substrate (~30 ohm-cm) and the surface topography has been detected by Field-emission scanning electron microscopy (FESEM). The size of the deposited TiO2-NPs is maximum in the range of 5–6 nm. The Energy-Dispersive X-ray (EDX) mapping of FESEM image showed the presence...
Involving Animals (CIOMS). Experiments involving animals meet the International Guiding Principles for Biomedical Research Animals (CPCSEA) of the Jamia Hamdard (Deemed University), New Delhi (permission #173/GO/Re/S/2000/...). The use of mice were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for all the animal experiments under the provision of authorized investigators. Experiments involving mice were approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals. All mice were housed five/cages and fed standard laboratory diet ad libitum followed for the care and handling of animals.
by subcutaneous (s.c) injection of various doses of venom. The neutralizing potency of TiO$_2$-NPs was assessed by injection (s.c) of venom (45–225 µg) into groups of six mice followed by immediate injection of fixed dose of TiO$_2$-NPs (5 ng) intravenously²⁴.

**Inhibition of venom haemorrhagic activity.** The minimum haemorrhagic dose (MHD) of venom which when administered into mice causes development of haemorrhagic lesion of 10 mm diameter within 24 hours²³. This lesion was measured and the estimation of neutralization of the haemorrhagic activity was done by mixing a fixed amount of TiO$_2$-NPs (2 ng) with different amounts of venom (5–25 µg). The mixture of TiO$_2$-NPs- venom was incubated at 37 °C for 1 hour, then spin at 2000 rpm for 10 min and finally 0.1 ml of supernatant taken were injected intradermally (i.d)²⁵. After 24 hours haemorrhagic lesion was estimated. To assess the anti-haemorrhagic activity of venom in vivo, various amount of venom (5–15 µg) were injected (i.d) followed by the TiO$_2$-NPs (5 ng, intravenously) and the haemorrhagic lesion measured after 24 hours.

**Inhibition of venom necrotic activity.** To assess the in vitro anti-necrosis effect of TiO$_2$-NPs, various concentration of venom (5–25 µg) were incubated with fixed amount of TiO$_2$-NPs and administered intradermally (i.d) into mice. The necrotic lesion was estimated after 48 hours. In in vivo study, the venom (i.d) (5–15 µg) was injected followed by injection (i.v) of TiO$_2$-NPs and observed after 48 hours.

**Inhibition of venom defibrinogenating activity.** Defibrinogenating activity of venoms or toxins is expressed as the minimum defibrinogenating dose (MDD). MDD of DRV is defined as the minimum amount of venom which when injected (intravenously) into mice causes incoagulable blood 1 hour later. Neutralization of this activity was estimated by mixing different amount of venom (2.5–12.5 µg) with fixed amounts of TiO$_2$-NPs, incubating at 37 °C for 1 hour and centrifugation. The supernatant was injected (i.v) into albino mice (18–20 g) as described above (in vitro). For in vivo studies, the MDD of venom was injected (i.v) followed by the TiO$_2$-NPs (i.v) and the nature of the blood observed after 1 hour²⁶.

**Inhibition of venom PLA$_2$ effect.** For carrying out the PLA$_2$ inhibition activity, 2 ml of egg yolk suspension, 0.2 ml of test material (venom, TiO$_2$-NPs and Venom + TiO$_2$-NPs) were mixed in the different test tube and kept for 1 hour incubation at 37 °C. The test tube containing test materials were kept on a water bath and the time required for coagulation was recorded. A blank was run with normal saline in place of test material. One unit enzyme activity was defined as the amount of venom, which increased the coagulation time of the egg yolk control by one minute²⁷. Estimation of neutralization of the enzyme activity, was done when fixed amount of TiO$_2$-NPs (2 ng) were mixed with different amount of viper venom (2–10 µg) and the mixture was incubated for 1 hour at 37 °C.
Centrifuged at 2000 rpm for 10 minutes, supernatant was tested in a total of 0.2 ml for the enzyme neutralization activity.

**Inhibition of venom-induced mouse paw oedema.** The minimum oedematogenic dose (MOD) of venom/carrageenan is defined as the least amount of venom/carrageenan which when injected into male albino mice produced inflammation (oedema) in the paw. Non-fasted male albino mice (18–20 g) were treated with different doses of venom, carrageenan and TiO₂-NPs. To assess the anti-inflammatory activity of TiO₂-NPs various amount of venom/carrageenan (in 0.01 ml) were injected (intraplantar) followed by the intraperitoneal (i.p) injection of TiO₂-NPs (250 ng/kg) and anti-inflammatory activity was measured. For control, equal amounts of saline were injected (intraplantar). The oedematogenic response was evaluated by the use of a screw gauge at given time intervals. Oedema was reported as the percentage difference between the values obtained from the injected paw with the carrageenan, venom, venom + TiO₂-NPs, carrageenan + TiO₂-NPs and saline, as described by Trebien and Calixto²⁴,²⁸.
Inhibition of Sterile inflammatory Markers. Different pro-inflammatory markers such as heat shock proteins (HSP70 and HSP90), high mobility group box 1 (HMGB1), interleukin 6 (IL-6), von Willbrand factor (vWF), and S100 calcium-binding protein B (S100B) was done by ELISA as described by Engvall et al.29. Briefly, 50 μg of proteins were used with bicarbonate coating buffer to coat the wells in 96-well plates and incubated over night at 4 °C. Nonspecific binding was blocked with 5% BSA and then the samples were washed with PBST (0.05% Tween-20) and incubated for 3 hours with respective primary antibodies (against Hsp70; Hsp90; HMGB1; IL-6; vWF; S100b (1:1000). Next, the wells were washed and incubated with HRP conjugated secondary antibodies for 1 hour, and then washed with PBST and incubated with OPD substrate solution. The reaction was terminated by adding H2SO4 (1 N) and absorbance was measured at 450 nm. Using similar methods, a standard ELISA curve for absorbance versus specific antigen concentration was constructed using respective recombinant proteins.

Results

Topographic image of TiO2-NPs. In case of GLAD, the TiO2-NPs are formed due to the atomic shadowing effect on the arbitrarily deposited seeds. The diffusivity of the landed atoms on the substrate depends on the rate of evaporation. A lower evaporation rate of the material will enable the landed atoms to diffuse for longer distances before they get impinged by next arriving atoms. This can lead to an increase in the capture radius of the deposited atoms and hence the atoms nucleation area increases. Figure 1A shows a top view of grown TiO2-NPs.
we have noticed that our prepared TiO2-NPs have crystalline nature and defibrinogenation. In in-vivo study, venom (10 μg) against TiO2-NPs (2 ng) of DRV was found to be 2.2 ± 0.1 µg intravenously and 45 ± 4.5 µg when injected subcutaneously in mice (18–20 g); whereas NKV was 2.8 ± 0.2 µg intravenously and 4.61 ± 0.5 µg subcutaneously. The MHD/MND was 5 ± 2 µg per animal (18–20 g) of DRV. The concentration of viper and cobra venoms in terms of protein were found to be 91.5% and 95% respectively. The LD50 of TiO2-NPs [up to 25 ng per animal (18–20 g); whereas 5–6 nm] did not produce any lethal effect up to 48 hours of observations22.

Properties of snake venoms and TiO2-NPs. LD50 of DRV was found to be 2.2 ± 0.1 µg intravenously and 45 ± 4.5 µg when injected subcutaneously in mice (18–20 g); whereas NKV was 2.8 ± 0.2 µg intravenously and 4.61 ± 0.5 µg subcutaneously. The MHD/MND was 5 ± 2 µg per animal (18–20 g) of DRV. The concentration of viper and cobra venoms in terms of protein were found to be 91.5% and 95% respectively. The TiO2-NPs [up to 25 ng per animal (18–20 g); whereas 5–6 nm] did not produce any lethal effect up to 48 hours of observations22.

Lethal activity. In in-vitro study, viper and cobra venoms (1–10 LD50) were incubated with TiO2-NPs (2 ng) and gave protection against venom-induced lethality. In in vivo study, cobra venom (1–5 LD50) was injected (i.p) into male albino mice followed by TiO2-NPs (5 ng/mouse, i.p). Viper and Cobra venom-induced lethality was significantly antagonized by TiO2-NPs as compared with the control animal (venom only) (Table 1). The ED50 of the TiO2-NPs was observed as 2 ng in vitro and 4 ng in vivo respectively against viper venom (Table 1).

Haemorrhagic and necrotic activity. In in-vitro study, Viper venom (20 μg) incubated with TiO2 (2 ng) and injected 0.1 ml (i.d) into mice. It showed protection against venom induced haemorrhagic activity (Table 2). In in-vivo study, venom (10 μg) injected (i.d) into mice followed by the injection (i.v) of TiO2-NPs (5 ng) gave protection against venom-induced haemorrhagic activity. The degree of protection in in-vitro was less than that of in-vitro studies.

Defibrinogenation activity. The TiO2-NPs effectively antagonized the viper venom-induced defibrinogenating activity. In in-vitro study, the TiO2-NPs (2 ng) gave protection up to 2 MDD (5 μg) against venom-induced defibrinogenation. In in-vitro study, venom-induced defibrinogenation was antagonised by TiO2-NPs. The fold of protection was always higher in in-vitro studies (Table 3).

Neutralisation of Venom Phospholipase A2 (PLA2) activity. Egg yolk coagulation method was performed to assess the PLA2 activity of viper venom. 1 unit of venom activity which increased the coagulation time by 1 minute was found to be 2 μg (control 0.9% saline, coagulation time was found to be 45 ± 1.16 seconds). The TiO2-NPs were tested for Phospholipase A2 activity by incubating with different amount of venom (10 μg). The venom PLA2 was effectively neutralized by TiO2-NPs (Table 4).

Mouse paw oedema. The assessment of anti-inflammatory activity of the TiO2-NPs was done by mouse paw oedema. Mouse paw oedema was induced by venom, attained its peak at 1 hour of observation. The TiO2-NPs at a dose of 5 μg/mouse (i.p) was found to produce significant inhibition of venom-induced inflammation. Inhibition of inflammation induced by TiO2-NPs was maximum (51.3 ± 2.5%) at 2 hours of observation, as compared with

| Groups          | Venom/Carrageenan μg (MED) | Oedema (%) |
|-----------------|---------------------------|------------|
| Control         | (0.9% saline)             |            |
| Viper venom     | 001(1)                    | 105.3 ± 5.8| 87.5 ± 07.7 | 83.74 ± 5.9 | 78.43 ± 6.5 |
| Carrageenan     | 300(1)                    | 054.4 ± 7.2| 47.9 ± 11.3 | 35.20 ± 7.6 | 34.60 ± 5.3 |
| Inhibition studies (Carrageenan injected 300(1) into intraplantar surface followed immediately by TiO2-NPs) |
| TiO2-NPs (250 ng/kg) | 5(5)                    | 47.5 ± 1.7 | 51.3 ± 2.5 | 52.0 ± 1.2 | 52.8 ± 2.9 |
| Aspirin (10 mg/kg) | 5(5)                | 43.9 ± 0.9 | 42.8 ± 1.2 | 42.2 ± 1.7 | 42.1 ± 0.8 |
| Indomethacin (10 mg/kg) | 5(5)              | 40.8 ± 1.9 | 40.3 ± 1.3 | 40.1 ± 0.5 | 39.9 ± 2.1 |

Table 5. Viper venom induced inflammation and inhibition by TiO2-NPs. Venom and carrageenan were injected intraplantar in the foot pads. Drugs were administered (i.p) immediately after envenomation at 0 hour.
aspirin (42.8 ± 1.2%) and indomethacin (40.3 ± 1.3%). Carrageenan (300 µg in 0.01 ml) injection produced significant inflammation in mouse paws. Pre-treatment with TiO₂-NPs (5 mg/kg, i.p) before carrageenan injection produced a significant reduction in oedema (Table 5).

Sterile inflammatory markers. The sterile inflammatory markers HMGB1, HSP70, HSP90, IL-6, S-100B and vWF were found to increase in Viper and Cobra venoms treated mouse while treatment with TiO₂-NPs significantly reduced confirming the anti-inflammatory effects of TiO₂-NPs (Figs 2 and 3).

Discussion

Injury and death due to snake bite is major health hazards in tropical and subtropical countries. In India more than 250 species of snakes are found, out of which 50 are venomous. Cobra and viper are the common snakes found throughout India32–34. The majority of death occurs due to the bites of these snakes. Snake envenomation is characterized by severe damage caused by the toxic action of venom components. The venom contains multiple components that induce haemorrhage, necrosis, inflammation, nephrotoxicity, cardiotoxicity, hemotoxicity and often leads to death35,36. Viper and cobra venom contains major parts of Phospholipase A₂ (PLA₂) and metalloproteinase. Viper venom is a rich source of PLA₂ enzyme that leads to many pathophysiological disturbances in

![Figure 3](Image). Cobra (*Naja kaouthia*) venom (NKV) induced inflammatory changes in experimental animals. Results expressed as mean ± SEM (n = 6). Results obtained are significantly different from control group (*P < 0.05) and *Naja kaouthia* venom (#P < 0.05).
the victims\textsuperscript{34}. These components directly damage the micro-vessels, with consequential increase in haemorrhage and oedema\textsuperscript{34}. The inflammatory effect induced by viper envenomation is due to the presence of PLA\textsubscript{2} enzyme in venom\textsuperscript{34–37}. The inflammatory effects induced by PLA\textsubscript{2} in response to cobra and viper venoms are primarily related to the action of this enzyme on the membrane phospholipids and the release of eicosanoid precursors\textsuperscript{25,38–42}. In present work, we characterized the oedematogenic, haemorrhagic, lethal and anti-inflammatory effects induced by the viper venom and its neutralization by TiO\textsubscript{2}-NPs (Fig. 4). Nanomedicine is a rapidly evolving field that provides enhanced diagnostic imaging and treatment of diseases known as theranostic. On account of improved health care, theranostic approaches are employed to make drug delivery more efficient and target specific. Theranostic nanomedicine can also be used for different purposes like non-invasive assessment of the pharmacokinetics, bio-distribution and the target site localization of conjugated or entrapped active agents\textsuperscript{43,44}. Therefore TiO\textsubscript{2}-NPs is considered as a suitable target for medicine delivery as well as for detection of target cells due to its high fluorescence property under the excitation of normal white light in \textit{in vitro} condition.

In this study, we have shown that TiO\textsubscript{2}-NPs are effective agents that may be used as a treatment in snake venom induced pathophysiological changes. TiO\textsubscript{2}-NP arrays have been fabricated on p-type Si substrate using a cost effective (GLAD, PVD) technique. The surface topography revealed that the average size of TiO\textsubscript{2}-NPs is in nano-metric range. The element mapping of FESEM image indicates the presence of Titanium (Ti) and Oxygen (O) in the sample. The stability of TiO\textsubscript{2}-NPs synthethised by glancing angle deposition technique was calculated by its zeta potential. The continuous SAED analysis of TiO\textsubscript{2}-NPs reveals that particles are having crystalline behaviour with fine stability. So far, we have seen that TiO\textsubscript{2}-NPs effectively neutralize the viper and cobra venom-induced lethal activities both in \textit{in vitro} and \textit{in vivo} studies. TiO\textsubscript{2}-NPs also effectively neutralized the viper venom induced haemorrhagic activity in experimental animals. The fold of venom induced lethal and haemorrhagic activity was always found to be higher in \textit{in vitro} than \textit{in vivo} studies. The TiO\textsubscript{2}-NPs were also found to be more effective in viper venom induced pathophysiological changes than the cobra venom. The exact mechanism of action is still unclear. Among the several, one of the consequences of snake envenomation is increased DAMP molecules. Here, we have shown for the first time that the sterile inflammatory markers like IL-6, HSP, vWF, S100B and HMGB1 were increased by the administration of both cobra and viper venoms in male albino mice. The venoms may act through the stimulation of arachidonic acid pathway that leads to the generation of ROS and cytokines which leads to haemorrhage and tissue injury\textsuperscript{41,45,46}. Tissue injury caused due to snake envenomation could be because of different mechanism. One of the mechanisms is inflammatory response which entails the generation of Hsp70, Hsp90, HMGB1, IL6, vWF and S100b. They are all generated through the arachidonic acid pathway. The metabolism of arachidonic acid by lipo-oxygenase or cyclo-oxygenase causes the release of pro-inflammatory marker proteins. Presence of PLA\textsubscript{2} in viper venom helps in the conversion of the phospholipids to arachidonic acid which causes release of cytokines and leukotrienes\textsuperscript{25,47,48}. The TiO\textsubscript{2}-NPs inhibits the production of sterile inflammatory proteins in venom-induced experimental rodents. The fold of protection is higher in case of viper venom than the cobra venom may be because of the difference in PLA\textsubscript{2} concentration. PLA\textsubscript{2} is
higher in viper venom than the cobra venom. Further study may provide new biological probes in the treatments of snakebite. TiO$_2$-NPs have shown better results in *in vitro* than in *in vivo* experiments. Our present investigation showed that the TiO$_2$-NPs is effective against venom-induced pathophysiological conditions and could be taken as a potential antidote. The present work may be helpful to treat victims of snakebite especially in rural parts of India where availability of antiserum is limited. We are engaged to explore the mechanism of venom inhibition by TiO$_2$-NPs as a potential therapeutic agent against snake envenomation$^{49,50}$.  

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Author Contributions
The research was conceived and planned by M.I.A. S.C. performed the synthesis and characterization experiments. M.I.A. and G.A.K. helped in designing the animal experiments. M.I.A. and S.B. performed the in vitro animal experiments. M.I.A. and N.D. wrote the manuscript. A.A. helps in constructing the manuscript including figures and tables. M.S.A. provided the intellectual support. All authors read and approved the final manuscript and have no conflict of interests.

Additional Information
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