ORIGINAL ARTICLE

Blood compatibility studies of *Swarna bhasma* (gold *bhasma*), an *Ayurvedic* drug

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ABSTRACT

Swarna bhasma (gold bhasma) preparations are widely utilized as therapeutic agents. However, in vitro biological evaluations of bhasma preparations are needed along with the physicochemical characterization for present day standardization of metallic bhasma preparations to meet the criteria that supports its use. Therefore, an attempt has been made to evaluate the protein adsorption, blood compatibility and complement activation potential of two batches of *Swarna bhasma* preparation, along with its physicochemical characterization. The particle size, morphology, elemental analysis, and in vitro cytotoxicity were evaluated initially. Red blood cell hemolysis, aggregation studies with blood cells, protein adsorption, complement C3 adsorption, platelet activation and tight junction permeability in Caco-2 cell line were investigated. The *Swarna bhasma* preparations with a crystallite size of 28–35 nm did not induce any blood cell aggregation or protein adsorption. Activation potential of these preparations towards complement system or platelets was negligible. These particles were also non-cytotoxic. *Swarna bhasma* particles opened the tight junctions in Caco-2 cell experiments. The results suggest the application of *Swarna bhasma* preparations as a therapeutic agent in clinical medicine from the biological safety point of view.

Key words: Blood compatibility, protein adsorption, *Swarna bhasma*

INTRODUCTION

From as early as 2500 BC, the therapeutic benefits of gold preparations have been reported in Indian, Arabic and Chinese literature.[1] *Swarna* (gold) *bhasma* has been utilized as a therapeutic agent in the traditional Indian *Ayurvedic* medicine for several clinical disorders including bronchial asthma, rheumatoid arthritis, diabetes mellitus, and nervous system diseases.[2-7] *Swarna bhasma* is usually given orally mixed with honey, ghee or milk.

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In recent years, there has been a renewed interest in drug discovery strategies where natural products and traditional medicines are re-emerging as attractive options[8] and hence renewed interests in agents like *Swarna bhasma.* Recent research has revealed that gold nanoparticles exhibit size-dependent absorption through rat skin and intestine, with smaller particles (~15 nm) absorbed more than larger particles (>100 nm).[9] Nanoparticles can also be absorbed through sublingual route directly into the blood stream.[10] Therefore, it can be presumed that some *Swarna bhasma* particles may get absorbed through the sublingual route directly into the blood stream. This has not been experimentally proved for *swarna bhasma*. However, the antioxidant/restorative effects of *Swarna bhasma* against global and focal animal models of ischemia have been reported.[6] Acute oral administration (continuous for 8 weeks on albino mice; 10 mg/20g b.w./day) of *Swarna bhasma* had not reported any toxic effects as assessed by liver function tests and histological investigations.[11]

In modern medicine, gold nanoparticles find significant applications in drug delivery as they are capable of encapsulating active drugs and targeting.[12] Colloidal gold nanoparticles represent a completely novel technology in the field of particle-based tumor-targeted drug delivery. The monolayer of polyethylene glycol (PEG) over gold nanoparticles has been found to improve the cellular internalization

properties.[13] Surface modification of gold nanoparticulate carriers with poly(ethylene glycol) has emerged as a strategy to enhance solubility of hydrophobic drugs, prolong circulation time, minimize non-specific uptake, and allow for specific tumor-targeting.[13] *Swarna bhasma* has been well-characterized physicochemically and since it contains more than 90% of gold particles[14] it may also be therapeutically applied in similar lines like gold nanoparticles. Cellular internalization of *Swarna bhasma* and/or its uptake *via* paracellular pathway have not been established yet.[15] Uptake of nanoparticles can occur not only *via* micro-fold (M)-cells, the highly specialized epithelial cells in the Peyer's patches and isolated follicles of the gut associated lymphoid tissue (GALT), but also across the apical membrane of enterocytes.[15] It has been demonstrated that uptake of gold nanoparticles occurred in the small intestine by absorption through single, degrading enterocytes in the process of being extruded from a villus and gold nanoparticles typically less than 58 nm in size ultimately reaches blood and various organs through blood.[16] Therefore, compatibility with blood is an extremely important factor when these particles are absorbed into the blood stream. Blood compatible materials can be defined as those materials which do not damage blood components when they come in contact with blood.[17] *In vitro* biological evaluations of *bhasma* preparations are also needed along with the physicochemical characterization and clinical evaluation for present day standardization of metallic *bhasma* preparations to meet the criteria that supports its use worldwide.

Therefore, an attempt has been made to study the physicochemical characterization and blood compatibility of two batches of *Swarna bhasma*.

Materials and Methods

Two bottles of *Swarna bhasma* were purchased from The Indian Medical Practitioners Co-Operative Pharmacy and Stores Limited, Chennai, India (*Swarna Bhasma* ED) and designated as SB1 and SB2. Complement protein C3 kit was from Orion Diagnostica, Finland. Platelet factor (PF4) kit, Asserachrom PF4, was from Diagnostica Stago, France. All other chemicals and other reagents used were of analytical reagent grade.

Particle size and zeta potential determination by dynamic light scattering

The particle sizes and the zeta potentials of *Swarna bhasma* samples were analyzed by photon correlation spectroscopy and laser Doppler anemometry, respectively, with a Zetasizer, Nano ZS (Malvern Instruments Limited, UK) at 25°C.[18]

X-Ray diffraction analysis

The X-Ray diffraction (XRD) powder diffraction pattern of *Swarna bhasma* was recorded on X-ray diffractometer (Siemens D5005 Diffractometer) using CuKα radiation, l = 1.5406 Å over the range 30.0–80.0°.

Scanning electron microscopy and energy dispersive spectroscopy

The morphology and elemental composition of the *bhasma* samples were determined by Environmental Scanning electron microscopy (SEM) (FEI Quanta) with energy dispersive spectroscopy (EDAX). A representative portion of each sample was sprinkled onto a double side carbon tape and mounted on aluminum stubs, in order to get a higher quality secondary electron image for SEM and EDAX examination.

In vitro cytotoxicity studies

The L929 fibroblast cells were seeded in 24 well plates at a density of 5×10^5 cells/well, cultured for 24 h in incubator at 37°C under 5% CO_2. The medium was replaced with SB1 and SB2 particle suspension in the medium at a concentration of 5 mg/ml/well and incubated for 20 h. Medium alone was used as control. The particles were removed and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was done.

Blood cell aggregation and hemolysis studies

RBCs were separated by centrifuging fresh blood at 700 rpm. This was washed with saline and diluted in saline in a ratio of 1:4. WBCs were isolated by centrifuging the fresh blood after layering with histopaque for 20 min at 700 rpm. Platelet-rich plasma (PRP) was collected by centrifuging the fresh blood at 1000 rpm for 20 min layered on histopaque solution. To 1 mg each of SB1 and SB2 particles, 100 µl of the diluted RBC, WBC suspension or PRP were added and incubated for 30 min at 37ºC. Polyethylene imine (PEI) and saline were taken as positive and negative controls, respectively, for all studies. Aggregations if any were observed through a phase contrast microscope (Leica DM IRB, Germany) at a magnification of 40×. Hemolysis assay was done on the particles as reported elsewhere.[19] Normal saline was used as negative control (0% lysis) and distilled water as positive control (100% lysis). The absorbance was measured at 541 nm by UV–Vis spectrophotometer (Varian).

Protein adsorption studies

The plasma was separated by centrifugation of fresh blood at 700 rpm. Ten milligram each of SB1 and SB2 particles were dispersed in 200 µl of saline. To this 200 µl of plasma was added and incubated for 1 h. After incubation the plasma was separated by centrifugation at 10,000 rpm and diluted with saline. The proteins in the plasma samples before and after incubation were separated by polyacrylamide gel electrophoresis (PAGE) using discontinuous native-PAGE method of Laemmli.[20] A resolving gel of 12% and a stacking gel of 4% were used. Electrophoresis was carried out at 100 V for 90 min using Mini-PROTEAN II electrophoresis cell (Bio-Rad, CA, USA). The gel was digitalized using an image analyzer (LAS 4000, Fuji) and the densitometry scans were

done with the software Multi-Gauge V3.

Complement activation

Complement activation by *Swarna bhasma* was determined by the turbidimetric method, assessing the depletion of complement protein C3 on incubation with the nanoparticles. The particle suspensions (100 µl) were incubated for 1 h at 37°C with 100 µl of citrated blood. The final concentration of the gold *bhasma* particles in the assay system was maintained at 10 µg/ml of blood. The assay was done as per the protocol provided by the kit manufacturer.

Platelet activation

Human blood (5 ml) was collected from consented voluntary donor in the morning hours after 25-min rest with slight or no stasis. It was immediately placed in the ice/water bath for 20 min. Platelet-rich plasma (PRP) was collected by centrifugation at 700 rpm for 20 min. Ten milligram each of *bhasma* samples were incubated with the fresh PRP for 15 min. This was centrifuged at 2500 g for 20 min. Plasma supernatant was collected by aspiration and PF4 was assayed by enzyme-linked immunosorbent assay (ELISA) kit (Diagnostica Stago, France) according to manufacturer's instructions. Samples were assayed in duplicates. PF4 levels were expressed in IU/ml. Precision of the assay was ±0.7 UI/ml in replicate determinations.

Visualization of tight junctions-actin and zona occludens 1(ZO-1)

Caco-2 cells were seeded (at 20,000 cells/well) onto four well cell culture plates (Nunc). The cells were maintained in incubator at 37°C under 5% CO_2 and used for transport experiments 6 days post-seeding.[21] Medium was replaced with Hank's buffered salt solution (HBSS) transport medium, and cells were equilibrated at least for 2 h before uptake experiments. Cells were treated with 500 µl SB particles at a concentration of 10 mg/ml for 1 h. The particles were removed by washing the cells three times with phosphate-buffered saline (PBS). The cells were fixed with 250 µl of 4% paraformaldehyde for 20 min at room temperature. Then the cells were permeabilized using 0.2% Triton X-100 in blocking solution, made of 1% (w/v) bovine serum albumin (BSA) in PBS, for 20 min, so as to make the cell wall permeable to the stain. The permeabilized cells were then washed twice with PBS and incubated with 250 µl of 1% BSA for 30 min.

For actin filament visualization, the blocking solution was removed and cells were incubated with 200 µl rhodamine phalloidin solution (0.2 µg/ml) for 20 min at room temperature. After removal of rhodamine phalloidin, the cells were treated with 1% BSA as before. The cells were washed with PBS, and dried overnight at 4°C. Images were obtained using Carl Zeiss LSM Meta 510 inverted confocal laser scanning microscope (Carl Zeiss, Germany), equipped with He/Ne laser 543. The visualization of rhodamine phalloidin was done using excitation and emission wavelengths of 543 and 605 nm, respectively. For ZO-1 staining the blocking solution was removed and cells were incubated with 200 µl of ZO-1 antibody (0.1 µg/ml) overnight at 4°C. After removal of ZO-1 antibody the cells were treated with 1% BSA as before. The blocking solution was removed and the cells were incubated with 250 µl FITC anti-rabbit IgG for one hour at room temperature. The cells were washed with PBS, and dried overnight at 4°C. Images were obtained using Carl Zeiss LSM Meta 510 inverted confocal laser scanning microscope (Carl Zeiss, Germany), equipped with Argon2 laser. The visualization of FITC was done using excitation and emission wavelengths of 488 and 505–530 nm, respectively.

Results

The particle size distributions of the two batches of *Swarna bhasma* particles evaluated by dynamic light scattering are

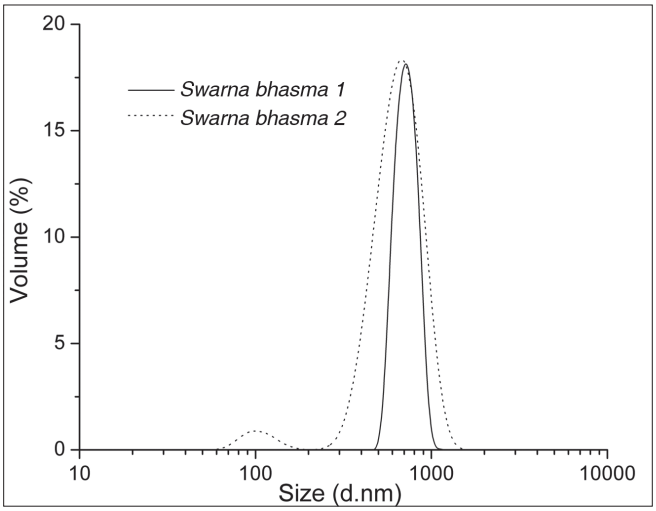

Figure 1: Particle size distributions of *Swarna bhasma* preparations SB1 and SB2

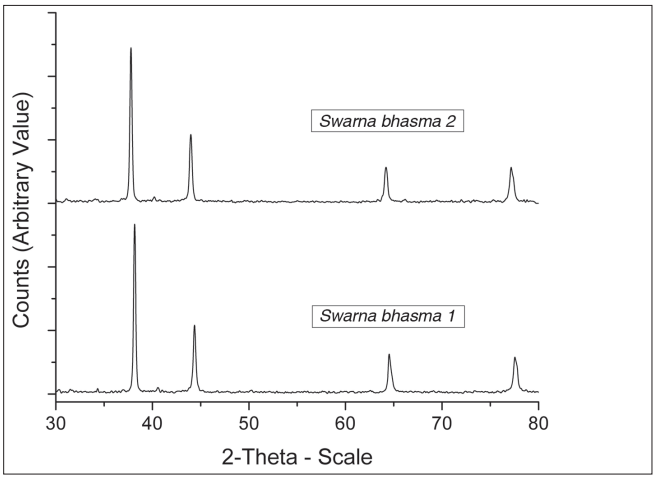

Figure 2: X-ray diffraction patterns of *Swarna bhasma* preparations SB1 and SB2

shown in Figure 1. SB1 had a mean particle diameter of 717 nm and SB2 had a mean particle diameter of 669 nm. The zeta potentials of nanoparticles at neutral pH (pH7.4) were found to be -17.4 ± 0.55 mV and -16.3 ± 0.37 mV, respectively, for SB1 and SB2 preparations.

The XRD patterns of *Swarna bhasma* are shown in Figure 2. The size of gold crystallites in *Swarna bhasma* was calculated from the XRD pattern using the Scherrer formula and determined to be the same (28 nm) for both SB1 and SB2. Morphologies of SB1 and SB2 by scanning electron microscopy are shown in Figures 3a and 3b. The elemental composition of the *Swarna bhasma* samples were analyzed by EDAX as shown in Table 1.

In vitro cytotoxicity of these particles has been done with L929 fibroblast cells as per ISO standard.[22] It has been confirmed by the *in vitro* cytotoxicity studies that the *bhasma* particles are non-toxic. As compared to control (medium) the particles showed 100% cell viability.

The aggregations of the blood cells on interaction with the nanoparticles are shown in Figures 4, 5 and 6, respectively, for RBC, WBC and platelets. It revealed no aggregation of blood cells on incubation of *Swarna bhasma* at a higher interaction ratio of 10 mg/ml. Polyethyeleneimine (PEI) which was used as positive control showed aggregation whereas saline used as negative control did not show any aggregation. The same was

Table 1: Elemental analysis of *swarna bhasma* by EDAX

Element	SB1 (%)	SB2 (%)
As	9.95	9.56
Nb	0.56	0.72
Au	88.10	91.2

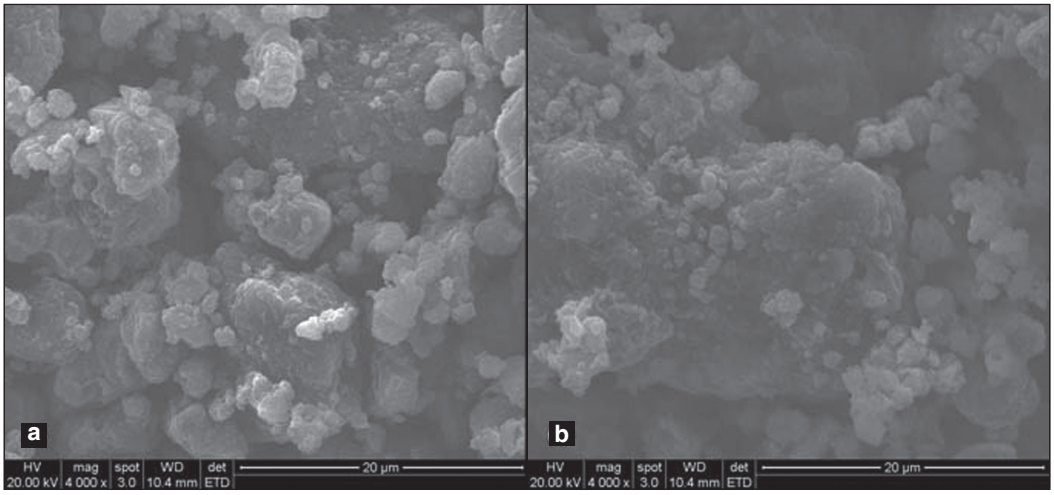

Figure 3: Morphology of *Swarna bhasma* preparations (a) SB1 and (b) SB2 by scanning electron microscopy

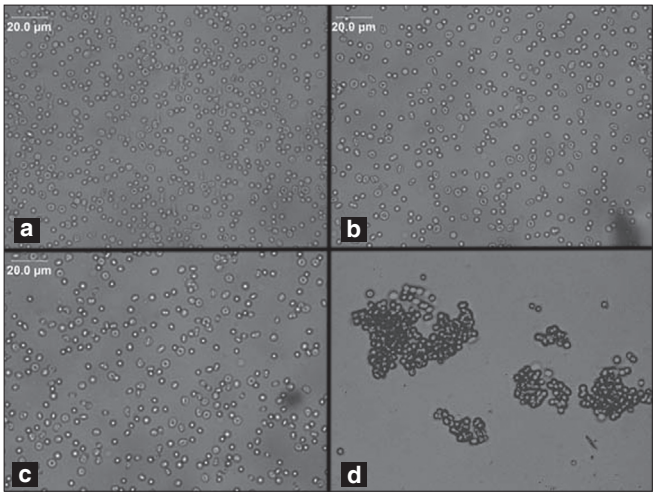

Figure 4: Aggregation of RBC by incubation of (a) SB1, (b) SB2, (c) normal saline (negative control) and (d) polyethylene imine (positive control)

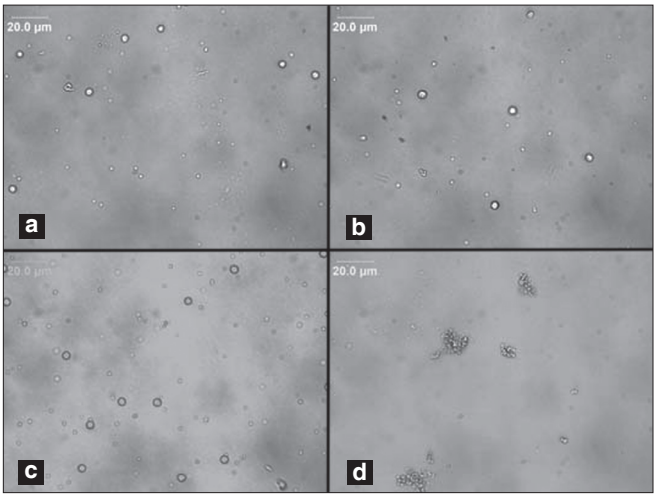

Figure 5: Aggregation of WBC by incubation of (a) SB1, (b) SB2, (c) normal saline (negative control) and (d) polyethylene imine (positive control)

visible with the hemolytic property of the nanoparticles. The hemolysis induced by SB1 was 0.05% and that for SB2 was 0.3% which was well within the acceptable limits of 1%.[23]

The protein adsorption studies evaluated using native-PAGE electrophoresis demonstrated no significant adsorption of proteins occurring onto SB1 or SB2 as shown in the densitometry scan of the treated plasma [Figure 7]. The figure shows the peaks of albumin, globulin region and fibrinogen. Compared to the densitometry scan of control plasma, the peak heights of albumin, fibrinogen or globulins of plasma treated with *bhasma* did not show any change indicating no significant adsorption of blood proteins.

Measuring C3a or C5a in blood or serum after contact with a material has been the most usual way of assessing complement activation. It has been claimed that a surface is biocompatible if these markers are not increased in the fluid phase.[24] Since C3 is cleaved to C3a and C3b by the contact of the surface with blood, irrespective of whether the activation occurs via classical or alternative pathways, and also C3a could be adsorbed on to the material surface just like any other proteins, C3 depletion in the medium can be taken as an indirect measure of complement activation. The amount of C3 in blood (pre-incubation) was 127 mg%. After incubation with SB1 and SB2, it was 126 mg% and 131mg%, respectively, indicating no significant changes in the complement protein level.

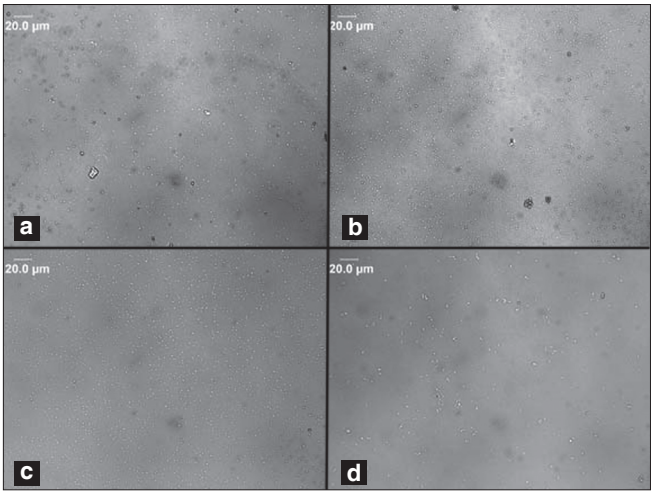

Figure 6: Aggregation of platelets by incubation of (a) SB1, (b) SB2, (c) normal saline (negative control) and (d) polyethylene imine (positive control)

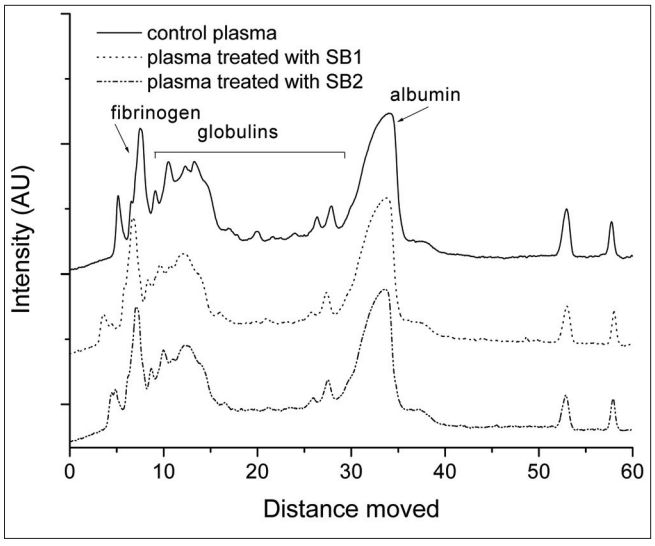

Figure 7: Densitometry scan of native-PAGE of plasma proteins before and after incubation with SB1 and SB2

Platelet factor 4 (PF4) is a platelet-specific protein secreted when a platelet is activated and belongs to the C-X-C chemokine family. Measurements of plasma levels of PF4 have been shown to be the marker of platelet degranulation, and increased level of PF4 is used to detect platelet activation of the circulating pool of platelets.[25] On incubation with *Swarna bhasma* the level of platelet factor 4 in plasma did not change appreciably compared to control plasma. The PF4 level in control plasma was 5.43 ± 0.10 IU/ml and after incubation with SB1 for 15 min it was 4.82 ± 0.4 IU/ml and for SB2 it was 5.07 + 0.3 IU/ml. The platelet adhered onto the bhasma particles were observed through scanning electron microscopy after incubating the SB particles with platelet-rich plasma. There were few cells observed adhering onto the particles. Only one cell was found adhered onto the SB2 sample with no activation or deformation of the platelets as shown in Figure 8.

The control cells stained with rhodamine phalloidin to visualize actin protein showed uniform staining pattern [Figure 9a]. Cells treated with SB1 and SB2 particles showed disrupted staining

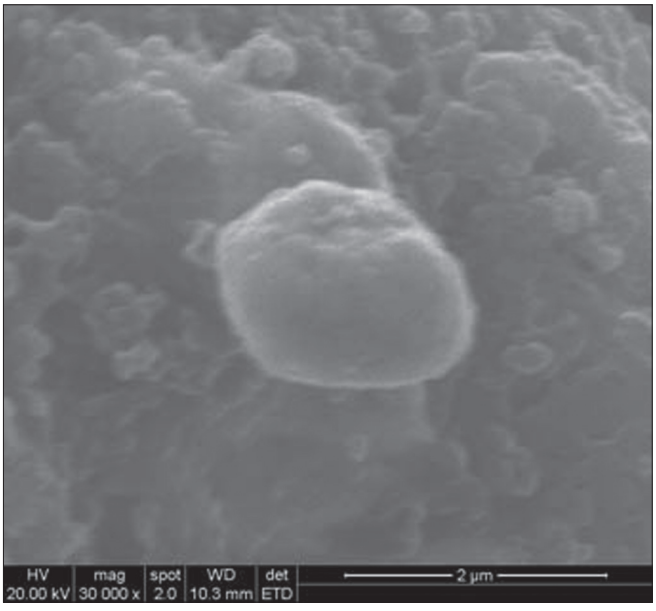

Figure 8: Morphology of platelet adhered onto *Swarna bhasma* preparation by scanning electron microscope

pattern, though the disruption was higher with SB1 as seen from Figures 9b and c. Actin filaments were observed to be discontinuous and disrupted as evidenced from the staining pattern and the clumping. To further investigate the effect on the tight junction proteins immunofluorescence studies using anti ZO-1 was done. ZO-1 is a tight junction associated protein, which plays an important role in tight junction functional regulation. Tight junctions are composed of transmembrane proteins occludin, claudins and junctional adhesion molecules which intercalate with corresponding proteins from adjacent cells to form the intercellular barrier. These proteins associate are with peripheral membrane proteins including the membrane proteins zonula occludens (ZO-1to3) which joins the transmembrane proteins to the actin cytoskeleton. ZO-1 and occluding phosphorylation are associated with stimulus-induced tight junction disassembly and paracellular permeability changes. The effect of SB1 and SB2 particles on ZO-1 tight junctional proteins was evaluated on Caco-2 cell monolayers as shown in Figure 10. In the untreated cells ZO-1 is observed as smooth lines at cell–cell junctions [Figure 10a]. The immunofluorescent staining intensity of *bhasma* particle treated cells were observed to be weaker compared to the control which indicated the loss of ZO-1 from sites of cell–cell contact [Figure 10a and b].

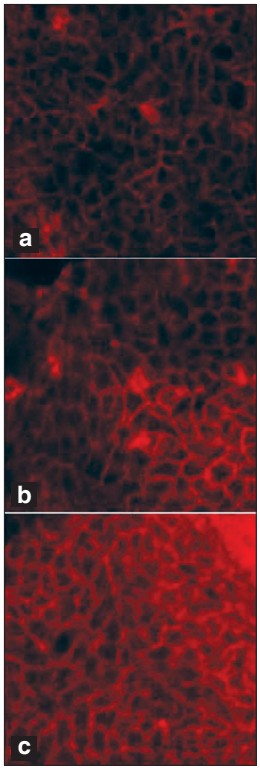

Figure 9: Confocal images (×20) of Caco-2 cell actin. (a) and (b) Caco cells exposed to 5 mg of SB1 and SB2, respectively, for 1 h; (c) Caco cells without any treatment (control)

Discussion

Different methods of preparing *Swarna bhasma* have been reported in various *Ayurvedic* texts.[2,3] This has been done by the incineration of gold with various compounds like mercury, mercury sulfide, sulfur, orpiment (As_2S_3), realgar (AsS), chalcopyrite, etc. out of which the procedure with mercury is considered to be therapeutically effective.[26] Various attempts have been done on the standardization of *Swarna bhasma* preparations for clinical applications.[27] However, the blood compatibility aspects have not been investigated till now.

Although the particle sizes of different batches showed similarity, it seems that these particles are aggregates of much smaller particles. When dispersed in an aqueous medium, gold colloids form a negatively charged hydrophobic particle suspension. This hydrophobicity gives these gold particles a tendency to aggregate together to form larger particles.[28] Both batches of *Swarna bhasma* exhibited larger sizes and agglomeration of the particles. However, the crystallite size calculated from XRD was much smaller. Therefore, the comparatively larger size may be due to the agglomeration of the particles by repeated cycles of calcinations involved in preparation as reported earlier.[29] Zeta potential has been suggested to play an important role in particle uptake because the surface of the intestinal mucosa is negatively charged owing to the presence of glycocalix. Particles with a high positive surface charge like chitosan are usually attracted by

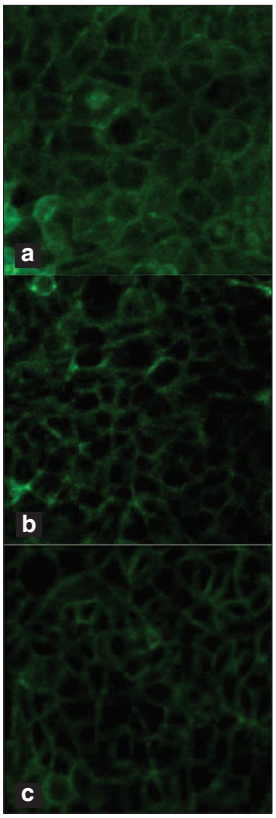

Figure 10: Confocal images (×20) of Caco-2 cell tight-junction protein ZO-1.(a) and (b) Caco cells exposed to 5 mg of SB1 and SB2, respectively, for 1 h; (c) Caco cells without any treatment (control)

the intestinal mucosa, which helps in increasing the intestinal absorption of the encapsulated drug. However, the strong electrostatic interaction between the positively charged particles and the negatively charged glycocalix may slow down the progression and penetration of these particles towards the epithelial cell surface reducing their uptake. Also it has been shown that non-ionized particles have a greater affinity for M cells than for ionized particles[30] and positively charged particles.[31] It has been suggested that gold *bhasma* particles with low negative zeta potential and nanosize may be uptaken by a similar manner.

The X-ray diffraction peaks at 2θ = 38.2°, 44.4°, 64.6° and 77.6° of *Swarna bhasma* were identical with those reported for the standard gold metal (Au°) (JCPDS File No. 04-0784). No other major diffraction peaks were observed confirming that the *Swarna bhasma* is composed of mainly gold nanoparticles. The high intensity of XRD lines in the XRD pattern suggests its crystalline nature. It has been reported that nanoparticles exhibited a size-dependent uptake from the intestine, and its passage via the mesentery lymph supply and lymph nodes to the liver,[32,33] with significant absorption for particles less than 100 nm. Therefore, uptake of *Swarna bhasma* particles of 28 nm through the intestine can be expected.

From the EDAX results it was confirmed that 90% of *Swarna bhasma* contains pure gold and is in correlation with XRD data. EDAX provide good estimate of the concentration of main elements in the sample in a significantly faster way and provides useful information on the distribution of the element forming the sample and their possible chemical form.[34]

The *Ayurvedic* multi-ingredient compounds are formulated in a way that the ingredients are capable of counter-balancing toxic effects, if any, present in the herbs or metals (*bhasma*).[35] These particles pass through extensive processing before they are declared fit for internal use. The processes consist of *Shodhan* and *Marana.* Culture of various cell types with colloidal gold showed no evidence of cytotoxicity.[36-39] No *in vivo* cytotoxicity has been reported with the use of colloidal gold administrated intravenously to ponies and pigs at doses of 400 mg of gold.[40] The initial event when a material comes in contact with blood is the adsorption of proteins. The nature of protein and amount of protein adsorbed will directly influence the compatibility of the particles with the blood. There was a correlation of the adsorption of the globular proteins with the blood cell aggregation showing no activation or aggregation of cells on incubation with *Swarna bhasma*. Activation of platelets initiates the deformation of the cells with pseudopod formation and ends with blood coagulation or thrombus formation.[41] In the present study platelets seem to be not activating and adhering onto the *bhasma* particles and even the very few platelets adhered are not activated as seen from their round shape. This is an indication of the very high platelet compatibility of *Swarna bhasma* preparations.

One of the negative effects of the clinical application of various blood-contacting materials is the activation of the complement system induced by the foreign surface. The response of blood in contact with the material depends on physicochemical features such as surface area, surface charge, hydrophobicity/hydrophilicity etc. The response depends directly on the surface area. Adsorption of C3 triggers complement activation.[41] It has been demonstrated in this study that the adsorptions of C3 on *Swarna bhasma* preparations are insignificant indicating that these preparations do not induce any complement activation when it reaches the systemic circulation.

Pharmacological effects exerted by the therapeutic agents depend upon its ability to cross the biological membranes into the systemic circulation and reach the site of action. This is usually occurred by one of the two pathways; paracellular or transcellular. Most drugs are transported transcellularly depending on their physicochemical properties; however, the paracellular route is usually the main route of absorption for nanoparticles. This is governed by the tight junctions (TJs). TJs are a multiple unit structure composed of multiprotein complex that affiliates with the underlying apical actomyosin ring. TJ proteins identified include transmembrane proteins; occludin and claudin, and cytoplasmic plaque proteins; ZO-1, ZO-2, ZO-3, cingulin, and 7H6. Although the adaptive mechanisms and specific regulation of these tight junctions are areas of active investigation and remain incompletely understood, it is known that some polymers can promote their widening, facilitating absorption of the particles into the systemic circulation. It has been established in this study by the tight junction visualization studies that the *Swarna bhasma* particles are capable of opening tight junctions, thus facilitating the *bhasma* particles to be absorbed into the systemic circulation and comes in direct contact with blood. Thus the *Swarma bhasma* particles should be highly compatible with blood.

Conclusion

Bhasmas are *Ayurvedic* metal-based preparations made by many systematic processes with herbs, converting raw metal into its therapeutic form. *Swarna bhasma*, a therapeutic form of gold metal of nano-sized particles found to be with a crystallite size of 28–35 nm and was 90% pure gold as visible from X-ray diffraction and elemental analysis. They had a low negative zeta potential in a physiological pH. The *Swarna bhasma* preparations did not induce any blood cell aggregation or any protein adsorption. Activation potential of these preparations towards complement system or platelets was negligible. These particles were also non-cytotoxic. Caco-2 cell experiments on tight junction integrity in the presence of *Swarna bhasma*

particles demonstrated their ability to open the tight junctions. It has been demonstrated that uptake of gold nanoparticles occurred in the small intestine by absorption through single, degrading enterocytes in the process of being extruded from a villus and gold nanoparticles typically less than 58 nm in size reaching various organs through blood,[16] which suggests the importance of the blood compatibility studies for the standardization of *bhasma* preparations. Since gold in the *Swarna bhasma* is approximately 28–35 nm in size, it can reach the affected site on oral administration via intestinal absorption and possibly can release Au(I) ions in a sustained manner required for therapeutic action.[42] These results reinforce the application of *Swarna bhasma* as a therapeutic agent in clinical medicine from the safety point of view. These testing protocols may be adopted as a screening test for all *bhasma* preparations to meet the criteria that supports its use worldwide.

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