

Investigating the ultrastructure of platelets of HIV patients treated with the immuno-regulator, Canova: a qualitative scanning electron microscopy study

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Summary. The resistance of HIV strains to the available antiretroviral medication has become a major problem in the world today. This has forced researchers to investigate the possible use of alternative drugs such as homeopathic medicine (e.g. immunomodulators) to enhance the immune system of patients infected with HIV. Canova is an immunomodulator of herbal origin which is known to stimulate the host defense against several pathological states through the activation of the immune system. Blood platelets play an important role in homeostasis, thrombosis and the immune response by forming platelet aggregates. The ultrastructure of platelet aggregates of patients with HIV has been studied previously using SEM to determine the effect of HIV on the platelet morphology. Membrane blebbing and ruptured platelet membranes were observed which is indicative of apoptosis, revealing that HIV patients may develop thrombocytopenia as a result of peripheral platelet destruction. The aim of the current study was to investigate the effect of HIV on the morphology of platelets from patients treated with the immuno-modulator, Canova, compared to control individuals and HIV patients not on the Canova treatment. Blood was drawn from the individuals and the coagula were formed by adding human thrombin to the platelet rich plasma. Examination was done using SEM. CD4 counts were also determined. Slight morphological changes were seen when comparing the fibrin networks from the control, untreated HIV patients and the Canova-treated HIV patients, suggesting that HIV does not impact on the fragility of fibrin networks. In HIV patients there are bleb-like bulges on the membrane of platelets as well as

membrane breakages visible on the aggregate, whereas in the Canova-treated patients membrane blebbing is far less pronounced and there are large areas of intact, smooth membranes with visible canalicular areas, suggesting that Canova protects the membranes of platelets and that blebbing does not appear in such great proportions as was found in the untreated HIV group. These results support and provide ultrastructural evidence for the results seen in previous research, where it is seen that Canova protects the immune system of immuno-compromised patients by keeping the ultrastructure intact thereby preventing the devastating cyto-destructive effects of HIV disease.

Key words: Canova, Immunomodulator, HIV, Platelets, Fibrin networks

Introduction

In the world today, it would appear as if more and more strains of HIV are becoming resistant to the available antiretroviral drugs. Although HIV/AIDS has greatly benefited from the availability of these branded and generic medications, the high cost, safe and correct administration and maintenance may prove to be a potential source of more harm than good (Bartlett and Muro, 2007). Constantly, physicians and researchers are forced to think about using and developing alternative medications. This is necessary to avoid the complications such as drug resistance, intolerance or contraindications to the available therapies, and the consequent disillusionment with large-scale antiretroviral rollouts that might ensue (Havlir and Hammer, 2005). Because of all the above mentioned reasons, and possibly due to cultural orientation, countries with high numbers of HIV infected people,

such as South Africa and India, turned to traditional healers for help. In vitro studies on the antiretroviral and immunostimulant/immunomodulatory properties of medicinal plant extracts have shown promising initial results, as have preliminary open-label clinical trials of traditional medicines in both Africa and Asia (Asres et al., 2005; Bodeker et al., 2006).

Canova is such a product of herbal origin that is used to enhance the immune system of patients living with HIV and AIDS. It is an immunomodulator and is known to stimulate the host defence against several pathological states through the activation of the immune system (De Oliveira et al., 2006). Canova was developed in the Canova laboratory in Argentina and today it is produced as a homeopathic medicine in Brazil according to the Hanemannian homeopathic method (Seligmann et al., 2003). The final bottled product is an aqueous, odourless and colourless solution that contains *Thuya occidentalis* (Cupressaceae), *Bryonia alba* (Cucurbitaceae), *Aconitum napellus* (Ranunculaceae), and *Arsenicum album* (arsenic trioxide) in distilled water (Lopes et al., 2006).

Blood platelets play an eminent role in haemostasis, thrombosis and the immune response. There are approximately 300000 platelets/ μl of blood. When aggregated, blood platelets thus display a larger total volume and surface area than the aggregates of all other leukocyte subtypes taken together (von Hundelshausen and Weber, 2007). The effects of HIV/AIDS on leukocytes, erythrocytes and thrombocytes are well documented and usually present as cytopenias (Bamberg, 2002). Although leukocytes are mostly affected, thrombocytopenia is also known to develop with detrimental decreases in the aggregation capacity of blood platelets (Poliakova et al., 1995) (Leissinger, 2001). Previously, the morphology of the platelet aggregates (prepared from platelet-rich plasma (PRP)) was studied using PRP from HIV patients and controls without HIV (Pretorius et al., 2008). The morphology was studied using a scanning electron microscope to determine the effect of the virus on platelet ultrastructure. The results indicated that although the platelets do aggregate, the morphology was altered with membrane blebbing and ruptured cellular membranes indicative of apoptosis. It was concluded that HIV patients may develop thrombocytopenia as a result of peripheral platelet destruction.

The aim of this study was to investigate the morphology of platelets from patients positive for HIV who have been on the homeopathic immunomodulator Canova and to compare the ultrastructure to platelets of normal individuals and other HIV patients not receiving Canova.

Materials and methods

Preparation of fibrin clots

Blood was drawn from controls without the virus, 4 patients with known HIV infection without treatment

and 6 patients with HIV and only using Canova (Ethical clearance was obtained from the Research Ethical Committee of the University of Pretoria, South Africa; ethical clearance number 151/2006 and 115/2006).

Fresh platelet-rich plasma (PRP) was prepared by drawing 40 ml of blood which was centrifuged at 1,000 rpm (maximum RCF = 17,523 \times g; 1,250 g) for 2 minutes. Fibrin clots were prepared in order to obtain platelet aggregates as well as fibrin fibers from both the patients as well as the control groups. Human thrombin (provided by the South African National Blood Service) was used to prepare these fibrin clots from the infected patients as well as the controls. Human thrombin is prepared from a single regular donor by calcium chloride activation of a euglobulin fraction of plasma obtained by apheresis. Each individual unit is tested and has to be non-reactive for hepatitis B surface antigen (HbsAg), HIV-1 antibody, HIV-2 antibody and HIV p-24 antigen, hepatitis C virus (HCV) antibody and antibodies to *Treponema pallidum*. These tests are performed by licensed assay methods. The thrombin solution is at a concentration of 20 U/ml and is made up in a biological buffer containing 0.2% human serum albumin.

When thrombin is added to PRP, fibrinogen is converted to fibrin and intracellular platelet components, e.g. transforming growth factor, platelet-derived growth factor and fibroblastic growth factor are released into the coagulum. A volume of 20 μl of the PRP was mixed with 20 μl of human thrombin on a 0.2 μm millipore membrane to form the coagulum (fibrin clot). This millipore membrane was then placed in a Petri dish on filter paper dampened with phosphate buffered saline (PBS) to create a humid environment and placed at 37°C for 10 minutes. This was followed by a washing process where the millipore membranes with the coagula were placed in PBS and magnetically stirred for 120 minutes. This was done to remove any blood proteins trapped within the fibrin network.

Preparation of washed fibrin clot for SEM

Washed fibrin clots were fixed in 2.5% glutaraldehyde in Dulbecco's Phosphate buffered saline (DPBS) buffer with a pH of 7.4 for 1 hour. Each fibrin clot was rinsed thrice in phosphate buffer for 5 minutes before being fixed for 1 hour with 1% osmium tetroxide (OsO_4). The samples were rinsed thrice with distilled water for 5 minutes and were dehydrated serially in 30%, 50%, 70%, 90% and three times with 100% ethanol. The SEM procedures were completed by critical point drying of the material, mounting and examining the tissue with a JEOL 7500F SEM.

Viral load and CD 4 counts

Viral load and CD4 counts were determined. The HIV viral load was determined by using a NASBA assay (Nuclisens Easy Q HIV version 1.2, from Bio-merieux) with the assistance of the Department of Virology,

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University of Pretoria. The CD4 counts were determined with flow cytometric analysis. The flow cytometric analysis was performed on an EPICS XL-MCL[®] flow cytometer (Coulter Immunotech, Beckman Coulter). Viral load was determined using peripheral blood from each patient was used and analysed with a FLOWCARE[™] PLG Reagent (CD45-FITC/CD4-PE) detection kit from Beckman Coulter. The blood sample were labeled and placed in a 12x75 mm test tube and the red blood cells were allowed to lyse. A volume of 100 μ l of blood was incubated with 10 μ L of phycoerythrin (PE)-CD4. Cells was stained using a direct method. In order to verify instrument alignment, FLOW-CHECK beads will also be ran. This method is according to the instructions for EPICS XL-MCL[®] flow cytometer (Coulter Immunotech, Beckman Coulter).

Results

Table 1 shows CD4 counts of the patients (H* = untreated HIV patients; CH* = HIV patients treated with Canova). The coagulum from each patient was systematically viewed with the SEM and not less than 30 platelet clusters were studied. Also, at least 30 areas of fibrin networks were viewed on high magnification from each individual. This was done to ensure that a micrograph from both the platelet aggregates, as well as the fibrin networks were a true representation of the ultrastructure of the controls, naïve HIV and Canova-treated HIV patients. Figure 1a shows a control platelet aggregate with smooth membranes (Label A) and pseudopodia extending from the aggregate. Figure 1b

shows the fibrin network of controls. Major thick fibers are present (Label C) while a few minor, thin fibers are present between thick, major fibers (Label D). Figure 2a shows a platelet aggregate from a HIV patient not on any treatment regimen. Label E shows the membrane of a HIV platelet aggregate. Electron microscopy shows that although the platelets do aggregate, the morphology was changed with membrane blebbing as well as torn cellular membranes. No changes were detected when studying the fibrin network (Fig. 2b). Label F shows thick, major fibrin fibers, similar to that of controls. Sparsely distributed thin, minor fibers are also present (Label G).

Platelets aggregates and fibrin networks from all Canova-treated HIV patients were studied. It seems as if these patients have a platelet aggregate ultrastructure

Table 1. CD4 counts and viral loads of HIV patients HIV.

Patient	Viral load	CD4
H1	89000	201
H2	160000	118
H3	4900	93
H4	17000	133
CH1	14000	251
CH2	47000	140
CH3	42000	407
CH4	300000	200
CH5	3000000	365
CH6	Viral load lower than detectable limit	683

H*: untreated HIV patients; CH*: HIV patients treated with Canova.

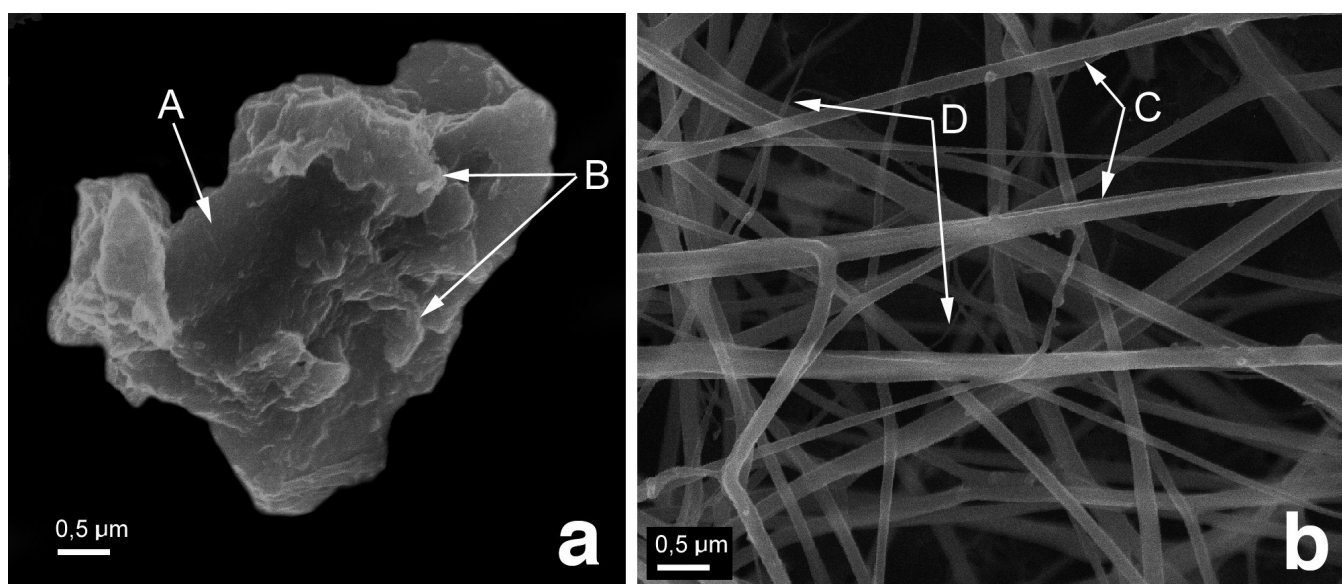


Fig. 1. **a.** Scanning electron micrograph taken with a JEOL 7500F SEM of platelet aggregate from a disease-free control. Label A: smooth membrane; Label B: pseudopodia. **b.** Scanning electron micrograph taken with a JEOL 7500F SEM of fibrin network from a disease-free control. Label C: thick major fibers, Label D: thin minor fibers.

between that of the controls and the naïve HIV patients, where membranes are less torn and fewer blebbing is present. Platelet aggregates from two different patients and fibrin fibers from HIV patients treated with Canova are seen in Figure 3a-c. Platelet aggregates show slight morphological differences when compared to that of the controls (Fig. 3a,b), although the membranes are not torn (Fig. 3a, Label H) and a few blebs and tearing are present (Fig. 3b, Label I). No differences were seen in fibrin morphology (Fig. 3c, Labels J and K).

When comparing the CD4 counts of the patients to the morphology of the platelets, no pattern can be seen (Fig. 3a), as the patient with the lowest CD4 count (patient CH2 CD4 = 140) possessed platelets that compare well to that of the controls. Also, Figure 3b is the typical platelet morphology of patient CH6 with CD4 count of 683.

Discussion

It is known that an HIV patient that has progressed to full blown AIDS has a CD4 count of 200 or less. In the current study population, all naïve HIV patients can therefore be classified as progressed to AIDS, also their viral loads are significantly high. Canova-treated patients CH2 and CH4 have CD4 counts of 140 and 200. All other Canova-treated patients have CD4 counts higher than 200; these patients can therefore only be classified as HIV positive. Their viral loads, however, show trends that cannot be explained, where patient CH5 has an extremely high viral load of 3,000,000 and a CD4 count of 365; while patient CH1 has a low CD4 count of

251, but relative to patient CH5, a low viral count (14,000). This cannot be explained in the scope of the current article. However, if the known research of Canova is taken into account, the following hypothesis might be true. Canova is a known immunomodulator that boosts the immune cell function in humans. It is known that macrophages proliferate in the presence of Canova and that human monocytes (in the absence of cytokines) change to macrophages or dendritic cells (specific phenotype still under investigation) that are primarily involved in the immune response (Smit et al., 2008). Currently, no research is available to show that Canova might be involved in the decrease of viral load, and from the results presented in the present manuscript, it does not seem that the product has the ability to decrease viral load, but more research should be done to confirm this observation. However, it seems as if the immunomodulatory effect of Canova might be involved in stabilizing CD4 counts – an example is seen in patient CH5, where the viral count is immense (3,000,000) and the CD4 count still above 200. It is suggested that this might be due to the known immunomodulating effects of the product.

In the current research, the ultrastructure of platelets and fibrin networks were investigated, because of the role of platelets in the immune response. On activation, platelets exhibit the ability to release considerable quantities of secretory products and express a multitude of immune receptors on their membrane. Platelets are characterized by an open canalicular system, which may contribute to the engulfment of serum components, pathogens or antigens and platelets are also able to

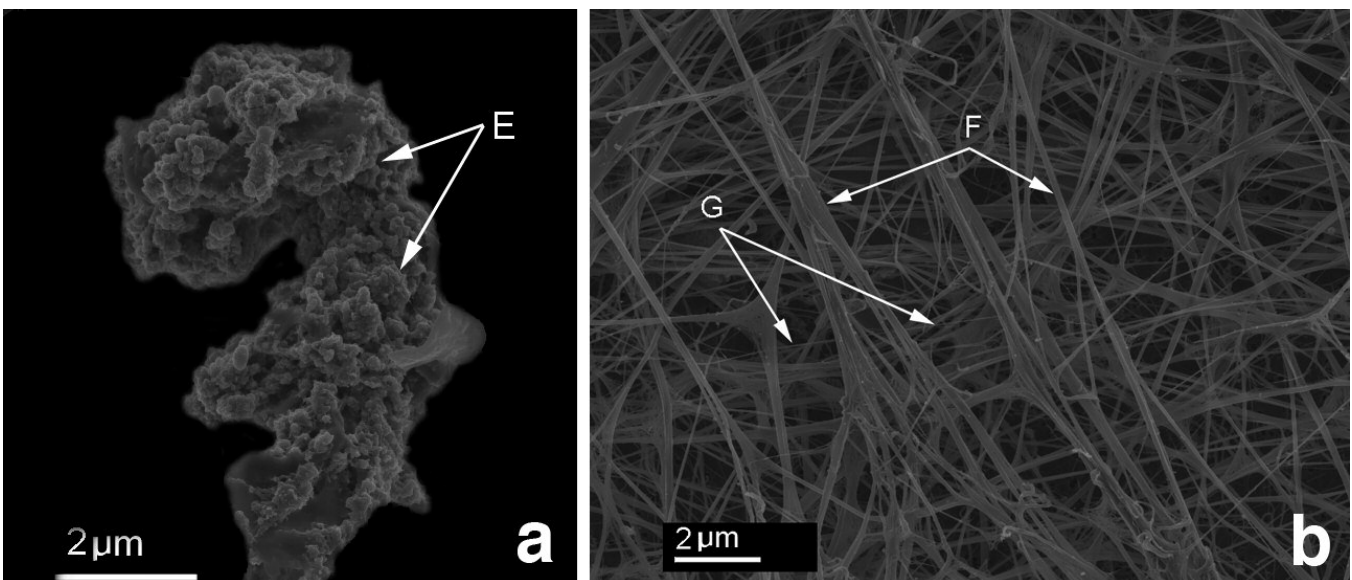


Fig. 2. **a.** Scanning electron micrograph taken with a JEOL 7500F SEM of platelet aggregate from an individual with HIV (untreated). Label E: membrane with blebbing. **b.** Scanning electron micrograph taken with a JEOL 7500F SEM of fibrin network from an individual with HIV (untreated). Label F: thick major fibers, Label G: thin minor fibers.

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internalize HIV. The capture of HIV by these cells amounts to a considerable threat for the immune system, as platelets can remain infectious over a prolonged time, indicating that platelets might facilitate HIV dissemination. Von Hundelshausen and Weber (2007) stated that this characteristic may be targeted in an effort to reduce viral load. It would appear that Canova may just be the plant extract homeopathic medication to assist in stability of platelets.

Control platelet aggregates possess smooth membranes with small pseudopodia projecting from the aggregate (Fig. 1a). Membranes possess small openings formed by the open canalicular system channels opening onto the membrane. Fibrin morphology is typically seen as an arrangement of thick, major fibers, with a few thin

minor fibers arranged in between the major fibers. No changes were seen when comparing the fibrin fiber networks from the untreated HIV patients and the Canova-treated HIV patients. This suggests that, from an ultrastructural point of view, HIV does not impact on the fragility of fibrin networks (Figs. 2b, 3c).

In HIV patients, Pretorius et al. (2008) showed that there are bleb-like bulges on the membrane as well as membrane breakages visible on the aggregate. However, there are areas where the membrane is smooth, similar to that of the controls (Fig. 2a). Research studying virus infected megakaryocytes have shown that there might be an underproduction of platelets and this may possibly represents a major pathogenetic mechanism of HIV-related thrombocytopenia (Davis and Zauli, 1995). It has

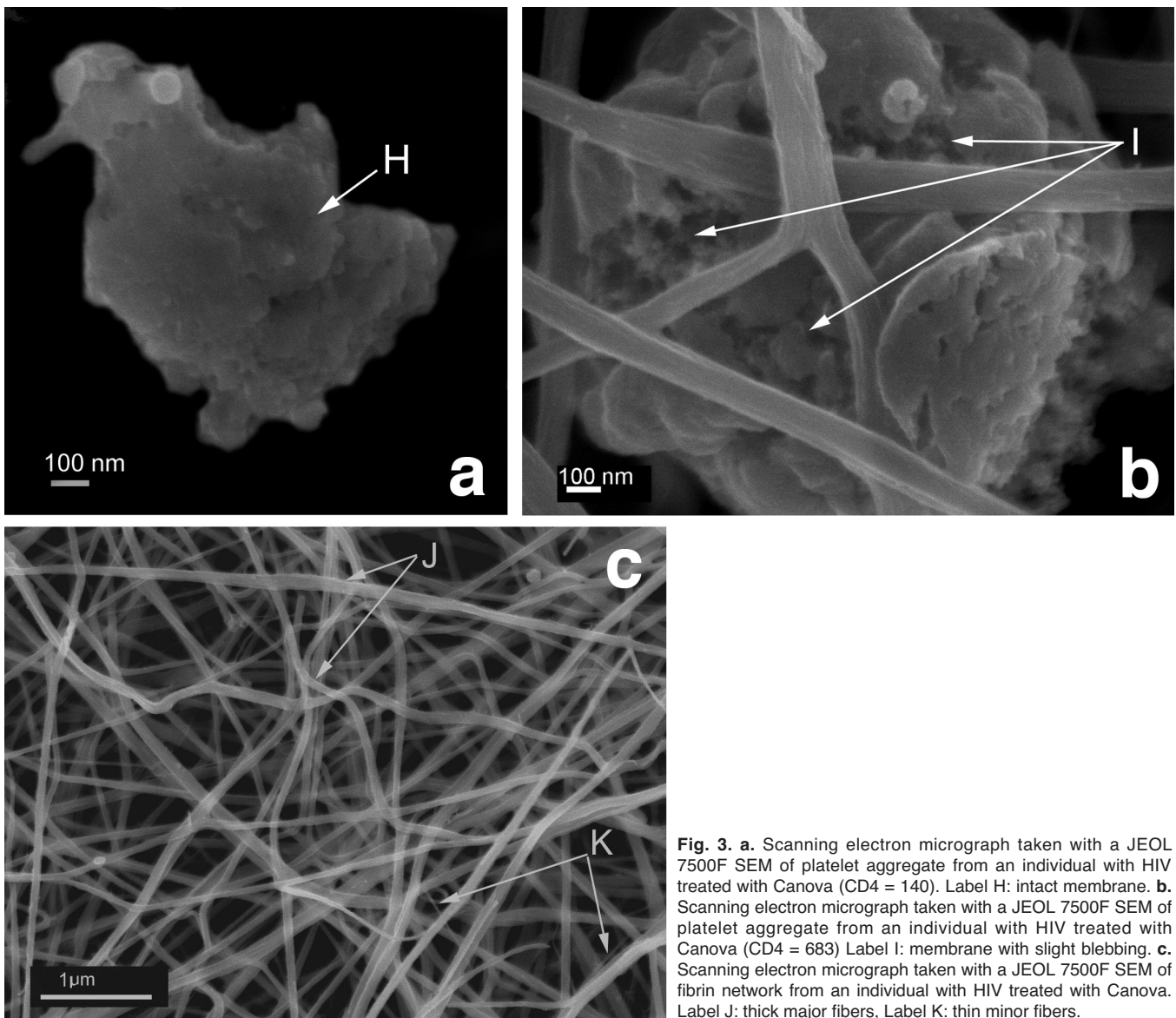


Fig. 3. a. Scanning electron micrograph taken with a JEOL 7500F SEM of platelet aggregate from an individual with HIV treated with Canova (CD4 = 140). Label H: intact membrane. b. Scanning electron micrograph taken with a JEOL 7500F SEM of platelet aggregate from an individual with HIV treated with Canova (CD4 = 683) Label I: membrane with slight blebbing. c. Scanning electron micrograph taken with a JEOL 7500F SEM of fibrin network from an individual with HIV treated with Canova. Label J: thick major fibers, Label K: thin minor fibers.

also been suggested that changes in platelet aggregate ultrastructure is due to altered megakaryocyte morphology or it may be attributable to direct ultrastructural damage by the virus or antibody-induced destruction, perhaps by its normal attaching mechanisms or by entering through the openings of the open canalicular system channels (Zauli et al., 1996). Gottlieb (2006) and Wyllie et al., (1980) also mentioned that the bleb-like protrusions are reminiscent of characteristic apoptotic changes in platelets that occur during pathological settings, resulting in human disease.

In Canova-treated patients membrane blebbing is far less pronounced and there are large areas of intact, smooth membranes with visible canalicular areas. This suggests that Canova protects the membranes of platelets and that blebbing does not appear in such great proportions as was found in the untreated HIV group. Canova is an immunomodulator and therefore protects and supports the immune system of immunocompromised patients. The current results suggest that platelet ultrastructure is kept in tact with the use of Canova and that the product prevents the devastating cyto-destructive effects of the HIV disease.

Platelet components are affected during the infection stage of HIV/AIDS. The key factors in the pathogenesis of hemocoagulatory disorders are the reduced count, abnormal function and morphology of platelets (Poliakova et al., 1995). Platelet morphology from peripheral blood smears in HIV/AIDS patients indicated general altered morphology with agranular platelets in 80% of the patients. Some platelets (76%) indicated a fragmented morphology whereas others (31%) were vacuolated (Bamberg, 2002). When examining at the electron microscopy level, Youssefian et al. (2002) found that platelets can function as phagocytes and internalize HIV. Engulfing vacuoles and the open canalicular system (OCS) were observed at the site of prominent α -granule release. Endocytic vacuoles could be identified close to the plasma membrane, surrounding HIV particles located within the OCS. However, the phagocytic function of platelets attracted some opposite opinions. White (2004) revealed that the engulfment vacuoles were made up entirely of invaginated surface membrane. The author suggested that the platelets could only move particles across the surface up to the size of α -granules, and instead, the platelets spread over the organism causing openings of the OCS channels. Thus the author argued that the platelet was a coverocyte and not a phagocyte, as suggested previously by Youssefian et al. (2002).

Taking all this into consideration, as well as the fact that integral glycoproteins are detected on the lining of the plasma membrane as well as the luminal face of the OCS of platelets (Cramer et al., 1994) it could be possible that an internalized HIV virus could still be detected. With this information, however, and the fact that α -granules that are released with the uptake of the HIV virus also release cytokines (King and Reed, 2002); a possible explanation for the mechanism by which

Canova protects platelets and the viral load could be offered. Canova facilitates the morphological appearance of the cell to remain rather normal, and this in turn causes the α -granules to release cytokines that recruit leukocytes to help fight infection. The platelet however, did take up the HIV virus particle and therefore no change to the viral load can be made, but due to the fact that normal morphology are maintained, cytokines can help resist further infections and keep the CD4 counts on T-lymphocytes at bay.

Previous research has shown that Canova has an effect on cells of the immune system *in vitro*, specifically macrophages with activation of these cells. Activation could be observed as spreading of the cell and development of numerous cellular projections. A total of 86% of macrophages indicated activation with this immunomodulatory ethnopharmacological product, whereas activation of only 15% could be detected in the control group (Da Rocha Piemonte and De Freitas Buchi, 2002). Another study done by Sato et al., (2005) indicated that Canova was neither toxic nor mutagenic and plays an important role in the immune system of cancer patients by increasing the amount of lymphocytes in cancer patients. This is of significance, since like HIV patients, cancer patients tend to become immunosuppressed due to chemotherapy. In this case Canova engaged cells like lymphocytes and macrophages to secrete cytokines and provided regression of the tumour (Sato et al., 2005). Clinical studies also indicated that Canova is successful as an immunomodulator as a decrease in the viral load was found after 6 months of treatment and this was maintained during use (das Graçac da Mota Silveira Sasaki, 2001). In a similar study by Bebert et al., (2002), patients suffering from HIV and exposed to Canova for 6 months showed the following results: a total of 58% of the patients indicated a decrease in the viral load, 15% indicated an increase in viral load and 12% indicated a very low reduction. Of the group, 8% indicated no change at all. Castanheira et al., (2002) then also evaluated cancer patients' weight and found that Canova resulted in muscular mass gain. Histological evaluation of blood smears from these patients indicated that they also maintained normal platelet and leukocytes morphology.

The current qualitative results therefore support and provide ultrastructural evidence for results seen in previous research, where it is seen that Canova protects the immune system of immunocompromised patients. However, perhaps it would be of interest to perform *in vitro* aggregation to evaluate the effect of Canova on platelet activation.

Acknowledgements. We would like to thank Chris Botha who arranged the use of the JEOL 7500F at Element 6 (Johannesburg, South Africa) and also Chris van der Merwe from the Laboratory for Microscopy and Microanalysis, University of Pretoria (South Africa) for technical support. We are also indebted to the patients who kindly filled in informed consent to this study.

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Accepted October 8, 2008