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Research Article

Erythrocytes Microvesiculations in Health and Diseases

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ABSTRACT

Heterogeneous mixture of vesicular organelle-like structures (microvesicles) is emitted by many cell sources into their surroundings, including blood and body fluids. Microvesicles are small membrane-enclosed sacs, whose size varies from 0.1-1nm; and they are thought to be shed from a variety of cell types, constitutively when stimulated by calcium activation signal . They seem to enhance the potential information transfer between cell types, displaying a large number of proteins and lipids as membrane constituents and as components of the vesicular content. Their isolation and analysis from blood samples have the potential to provide information about state and progression of malignancy and should prove of great clinical importance as biomarkers for a variety of disease states. They are implicated in normal, physiological and pathological conditions and their potential to serve as indicators in the diagnosis, prognosis and surveillance of a variety of health conditions, have indicated the magnitude of interest in this structures.

In this research project report, non-induced microvesicles were isolated from erythrocytes and analysed using Guava Easy flow- cytometer. Considering the fact that microvesicles compartmentalisation consist of proteins, lipid and nucleic acid, the Protein concentration of microvesicles was quantify and estimated using Biuret method of assay; and the protein components were separated using Agarose gel Electrophoresis.

The result of this present report was in consistency with the previous research work done by several authors, which shows that non- induced microvesicles are shed from erythrocytes; and secondary, that microvesicles skeletal structure consist of protein, lipid and nucleic acid.

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I will never forget the academic help rendered to me by my lecturers Dr. Martin Gonzo and late Dr.Paul Matewele. The knowledge they imparted on me on Haematology and Blood group sereology, and Advanced immunology will never be in vain.

INTRODUCTION

The release and phenomenon of microvesicles was first described by Wolf in 1967 who noted procoagulant particulate matter around activated blood platelets (Wolf., 1967). Van Doormaal et al (2009) stated that in 1970's, the concept of microvesicles and its relevance, significance and its' important on several biological process was established. Subsequently, similar organelles, referred to as exosomes, were implicated by Trams as carriers of 5' exonucleotidase associated with glioma cells (Trams et al.,1981). The groups of Johnstone and Stahl established exosomes as a mechanism involved in the removal of spent transferring receptors from differentiating reticulocytes (Johnstone., 2006); (Harding et al., 1983)

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Interestingly, the shedding of small vesicles from cell surface projections such as microvilli appears to be a common phenomenon (Trams et al.,1981). It is very interesting because of it's importance and relevance in this research paper. Microvesiculation is an ubiquitous cellular mechanism that occur as a result of exocytosis releasing exosomes (between 50 and 100nm) in diameter (Simons-Raposo et al,2009), or directly releasing vesicles from the cell membrane, which we refer in this study as plasma membrane derived vesicles, with diameter size ranging from 0.1-1um, (Hugel et al.,2005; Shukla et al.,1978a). Microvesicles being a small membrane-enclosed sacs are shed by a variety of cell types, (Ansa-Addo et al.,2005). Several successful and research groups have demonstrated their presence in body fluids, such as blood, urine and ascites, amniotic fluid, synovial fluid, breast milk and saliva; and their role to serve as indicators and markers in diagnosis, prognosis and surveillance of a variety of health conditions, has improves the level of interest in this structures ((Graves et al.,2004); Piccin et al.,2007); Smalley et al., 2008;Taylor-Gercel, 2008). Growing experimental evidence indicates that microvesicles are underappreciated component of the cell environment and play an important pleiotropic role in many biological processes. Microvesicles being grouped as microparticles, has a clear distinction (through biogenesis and release) from the exosomes , which are also shed by normal and diseased cells. Exosomes originate from multivesicular bodies that are discharged when fused with the plasma membrane, while microvesicles are released by the outward budding, fission and blebbing of the plasma membrane.

Erythrocyte micro-vesiculation involves the release of small haemoglobin containing vesicles from human erythrocytes when treated with calcium (Allen et al., 1980), and the fission and vesiculation of erythrocytes occur by active process involving the uncoupling of the membrane skeleton from the lipid bilayer (Ciechanover et al.,1983). This phenomenon has been studied in erythrocytes by several Authors, indicating the release of microvesicles, following a disarrangement from the normal smooth, biconcave disc to the speculated echinocyte (Bessis & Mandon,1972; Rumsyby et al.,1977). Naturally occurring microvesicles can also be released from erythrocytes without inducing with calcium chloride. Shedding of membrane derived microvesicles is a physiological phenomenon that accompanies cell activation and growth . The naturally occurring microvesicles are released by this cells activation both in normal and pathological conditions, and the dosage of microvesicles discharged during in normal health condition varies when compares with the concentration released in disease situations, as certain factors like elevation of intracellular calcium concentration initiate and double the release by causing fragments of the cell membrane to be shed as microvesicles (Smitch et al., 2001; Ratajczak et al., 2006a).

Further researches carried out by authors' have indicated that treated erythrocytes with calcium induce a wide range of noticeable biochemical and morphological changes including potassium efflux (Lew–Ferreira.,1976 & 1978; Reed., 1976; Romero., 1976); breakdown of polyphosphoinositides with a consequent increase in 1,2-diacylglycerol and phosphatidate concentrations (Allen et al., 1976b; Allan-Michael et al.,1978; Ponnappa et al.,1980) and varieties of alterations in membrane polypeptides pattern. Elevation in cystolic calcium also leads to the aggregation of protein catalysed by transaminases and cytoskeletal arrangement owing to the proteolysis of protein (Allan et al.,1980; Minetti & Low.,1997; Anderson et al.,1997). However, the composition and function of these microvesicles depend on the type,(Piccin et al.,2007) and the health status of the cells from which they originates, (Vandhana et al., 2010). The major function of plasma membrane-derived microvesicles from different cell types will vary due to variations in the nature and individual concentrations of the micro vesicles constituents (Piccin et al.,2007). Micro vesicles released from erythrocytes carry micro-RNA (Hunter et al.,2008), mRNA, numerous membrane proteins, Lipids and Cytoplasmic constituents, retain the features of their parent cells, (Piccin et al.,2007), and being able to transmit such proteins between cells, they are important mediators of cellular communication. Being a gateway that facilitate cell-to-cell communication (Cocucci et al.,2009; Castellana et al.,2010); microvesicles when produce can internalised into other cells and stimulate a potential effects on neighbouring cells or produce changes in several biological functions, which includes intercellular communication and transport, Programmed cell death (apoptosis), modulation of the immune response, inflammation, Angiogenesis, removal of apoptosis inducing proteins and improves cellular survival, tumour invasion and metastasis, coagulations, stem- cell renewal, allow rapid 'defensive' shedding of complement attack complexes, cardiovascular disease, hypertension, neurodegenerative disorders, diabetes, rheumatic disease and involvement in multidrug resistance (Ratajczac, et al.,2006b; Muralidharan-Chari et al.,2010; Cocucci et al.,2009; Pap et al., 2009; Van et al.,2009).

Several scientific studies and researchers have identified the molecular contents of microvesicles as a resemblance with the parental cells (Piccin et al.,2007). Microvesicles cargo includes a variety of distinct combination of lipids, proteins and nucleic acids (messenger ribonucleic acids, mRNA, microRNA, miR, and DNA). The identification of RNA molecules in microvesicles supports the hypothesis that they are biological agents for the transfer of nucleic acids and subsequently modulate the target cells' protein synthesis. MicroRNAs are small non-coding RNA that play important roles in post-transcriptional gene regulations (Bartel,2009) and they regulate their targets by translational inhibition and mRNA destabilization , and conferring new function to the target cell (Valadi et al.,2007), and this may be a new mechanism for genetic exchange between cells (Lewin et al.,2009).

Another significant component of microvesicles is the mRNA, (Valadi, et al.,2007) which carries genetic and coding informations obtained from DNA chains to cellular machine that translate it into polymers of amino (Valadi et al.,2007). Having stated that the constituents and compartmentalization of microvesicles depend on the parent cell type, it is obvious that the functions of microviscles from different cell types will vary due to, perhaps, variation in nature and individual concentrations of the microvesicles' compositions. In response to this, microvesicles originating from several cell types are expected to achieve a different effect, in varying magnitude depending on the parent cells and the cells condition.

Furthermore, cell receptors is also another indicating element to be considered when comparing the effect of microvesicles from various cell types, as cells without the cognate receptors will induce little or no effect on the biological cells while the cells with cognate receptors will induced greater effect.

Erythrocytes

Erythrocytes are important constituent of blood that transport oxygen from the lungs to the cells of the body. The term erythropoisis is used to explain the process of erythrocyte formation, and they originates from their progenitor precursor known as haemopoetic stem cell which are produced as a result of differentiation of common erythroid progenitor in the bone marrow, producing a constant supply of blood cells.

Morphologically, erythrocytes have several morphological features that made them unusual with white blood cells and platelets. Their prominent features include - a bioconcave shape disc , 6-9 micro meter in diameter and 1.5 to 2.5 micro meter thick. They lack nucleus and contain predominately haemoglobin that is distributed to form a dense outer rim with a paler centre that approximately one third of the diameter of the cell. They have the ability to change shape as they squeezes through the capillaries, known as diapedesis. As constituent part of blood cells, they constitute about 45% of the total blood cells and they are active part of human transport system. The biogenesis and maturation of erythrocyte starts when the RBC loses its nucleus and travelled to the bone marrow as a matured reticulocyte, containing some remnants of organelles. Eventually, these organelles leave the cell and a mature erythrocyte is form. Red blood cells last an average of 120 days in the blood stream, and they are removed from the blood stream after their aging by macrophages in the liver and spleen.

However, several pathologic conditions can alter the size, shape and haemoglobin content of red blood cells. Variation in size (Anisocytosis) and in shape (poikilocytosis) can alter the haemoglobin contents. Pathologic red blood cells may be larger or smaller than normal, may be abnormally shaped, or may contain inclusions. In situation of hypoxic conditions (low oxygen level), bone marrow can stimulates for the production of red blood cells; and a hormone called Erythropoitin regulates this synthesis. At low oxygen levels, the proximal region of the kidney respond by releasing a hormone called erythropoietin, which then travels to the red bone marrow to stimulate the marrow to begin red blood cell productions. As these cells are produces and matured, they extrude their nucleus as they slowly fill with haemoglobin until they are bright red reticuloctes ready to escape the bone marrow and squeeze into the blood capillaries to begin circulating around the body. Within a few days, this reticulocytes completely loses all its nuclear material and becomes a full- fledged RBC that is ready to serve the oxygen needs of the body.

More over, in connection with erythrocytes, vesiculations of the matured erythrocyte plasma membrane occurs in certain diseases and during blood storage. In sickle cell anaemia, membranes of circulating erythrocytes actively undergo both exocytosis end endocytosis (Allan & Raval. ,1983; Westerman et al.,1979). Cytoplasmic vesicles present in sickle cells express on their surface plasma membrane calcium-ATPase, suggesting that they arise by endocytosis from the erythrocyte plasma membrane (Williamson et al., 1992). During blood storage, red cells shed vesicles that, like those observed in sickle cell anaemia, containing GPI- anchored proteins (Long et al.,1993) as well as A,B and Rh blood group antigens (Greskovic et al.,1992).

Figure 1: Erythrocytes.

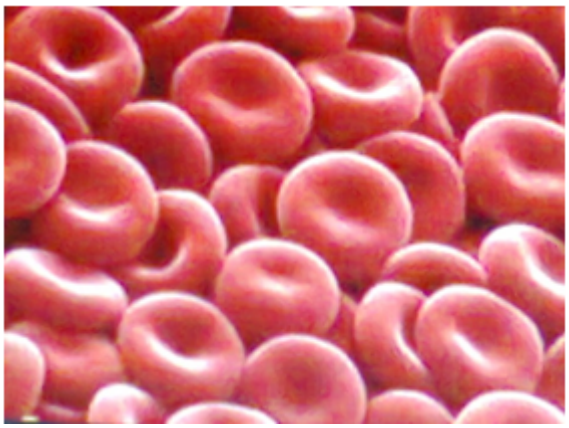
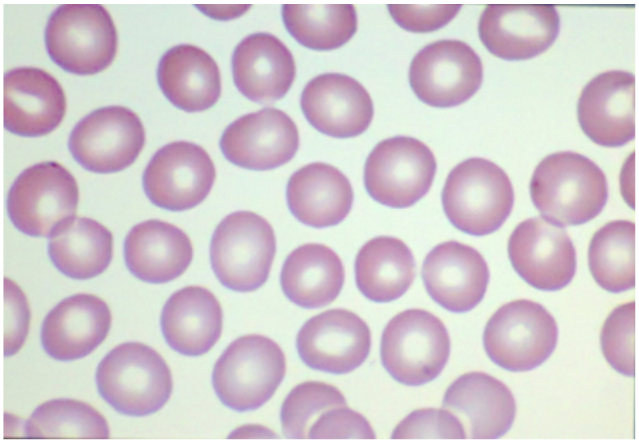


Figure 2: Morphology of Erythrocytes.



PRODUCTION OF MICROVESICLES

Different biological circumstances under which formation of microvesicles (vesiculations) has been observed reflect the diversity of their biogenesis, structure and function. Thus, cellular transformation, stress, or programmed cell death are associated with a different output and nature of vesicular structure (Rak., 2010).). The biogenesis of microvesicles are derived from at least three distinct mechanisms: (a) breakdown of dying cells into apoptotic bodies, (b) blebbing of the cellular plasma membrane (ectosomes) and (c) the endosomal processing and emission of plasma membrane material in the form of exosomes (Vindelov et al., 1983; Simpson et al., 2009; Johnstone., 2006; Al-Nedawi et al.,2009).

Indeed, it is clear that microvesicles are heterogenous, and this aspect has led to the usage of multiple names for their designation under different experimental settings (Rak., 2010; Al-Nedawi et al., 2009). Some of the most frequently encountered descriptors are microvesicles, ectosomes and exosomes . Although grouped together as microparticles, plasma-

derived micovesicles are quite distinct in several manners, especially in their size, the speed at which they can pellet from the body fluid and their method of production. Plasma membrane derived vesicles are derived from shedding that originate from cell membrane surfaces of endothelial cells, cancer cells, epithelial cells, haematopoietic cells, erythrocytes, platelets, monocytes and all other eukaryotic cell types in both normal and disease conditions,(Piccin et al., 2007; Cocucci et al.,2009). While exosomes are released through invagination of extracellular membrane or due to fusion of intracellular multivesicular bodies with the plasma membrane and subsequent exocytosis, possibly, as a mechanism of intracellular communication (Pilzer et al., 2005; Al-Nedawi et al., 2009); Simpson,et al.,2009).

However, despite the increased focus and attention which has been given to the concept of microvesicle recently, researchers have continued to confuse the two distinct microparticles (membrane-derived microvesicles and exosomes), may be due to lack of detail description of each, (Burner et al.,2009).

BIOLOGICAL SIGNIFICANCE OF CIRCULATING MICROVESICLE

Having been demonstrated that microvesicles originates from the same membrane constituent and cytoplasmic content of parents cells, the molecular contents and compartmentalisations of microvesicles raises the question as to their biological role. The significance of microvescicles has attracted much attention due to their effect on several biological process. In this regard, several non-exclusive hypotheses have been put forward to explain the functional importance of vesiculation in various cellular contexts. As a network of intracellular communication, micro-vesicles have a potential to influence processes as diverse as cell polarity, differentiation, migration, chemotherapy resistance, immune-regulation, inflammation, coagulation, angiogenesis, cancer metastasis (Ratajczac et al.,2006; Thery et al., 2009; Mathivanan et al., 2010; Rak., 2010; Simons- Raposo., 2009; Cocucci et al., 2009) and rapid defensive shedding of complement attack complexes from plasma membrane of cells that have undergone organization, thereby protecting them from destruction (Pilzer et al.,2005).

Recently, many aspects of cancer progression have contributes immensely to the increase of microvesicles. Tumour-associated microvesicles are abundant in the blood, urine, and other body fluids of patients with cancer, and they provides a unique opportunity to noninvasively access the wealth of biological information related to their cells of origin. The quantity and molecular composition of microvesicles released from malignant cells varies considerably when compared with those released from normal cells. Thus, the concentration of membrane derived micro vesicles with molecular markers indicative of the disease state may be used as an informative blood-based bio-signature for diseases such as cancer (Van Doorman et al., 2009). By harnessing specific bioactive molecules such as proteins, RNAs, and miRNAs, and facilitating the cell-to-cell transfer of their cargo, tumours associated microvesicles can affect a variety of cellular events that significantly impact tumour progression. A number of reports have demonstrated that tumour-associated microvesicles release proangiogenic factors that promote endothelial cells proliferations, angiogenesis, and tumour growth; microvesicles shed by tumor cells and taken up by endothelial cells also facilitate angiogenic effects by transferring specific mRNAs and miRNAs (Cocucci et al.,2009). Also, following some data available recently, it has been suggested that microvesicles from the endothelia cells, especially that of tumor cells are capable of inducing endothelia dysfunction (Pfister,2004) due to its ability to upset the Nitric oxide (NO) redox balance of the affected cells (Brodsky, 2004).

In progress with the biological significant of microvesicles, several research investigations have demonstrated the involvement of erythrocyte microvesicles in interference with Antitumour immunity. Microvesicles from various tumour types can express specific cell-surface molecules (e.g Fasl or CD95) that induce T-cell apoptosis and reduce the effectiveness of other immune cells. Microvesicles released from erythrocyte and lymphoblastomer cells express the immune-suppressing protein latent membrane protein-1 (LMP-1), which inhibits T- cell proliferation and prevents the removal of circulating tumour cells. As a consequence, tumour cells can turn off T-cell responses or eliminate the antitumor immune cells, by releasing microvesicles (Muralidharan-Chari., et al., 2010). Further research investigations on the significant role of microvesicles have also demonstrated the potential role of membrane derived microvesicles in clinical trials and clinical practice. Analysis of tumour-associated microvesicles could help investigators track and monitor patients' clinical responses to therapy during the course of a trial. Within this information, clinicians would be able to provide their patients with more timely therapeutic decisions. Clinical research data suggests that tumour-specific markers exposed on membrane derived vesicles are useful as a clinical tool to diagnose and monitor diseases like pancreatic, ovarian and gastrointenstinal malignancies.

AIMS AND OBJECTIVES OF THE STUDY

Considering the above evidence that microvesicles cargo includes a variety of molecular entities, comprising of distinct combinations of lipids, proteins and nucleic (messenger ribonucleic acid, mRNA, micrRNA, miR; and DNA) (Wolf, 1967; Valadi et al.,2007; Skog et al.,2008); Pisetsky et al.,2010); and also having in mind that molecular content of microvesicles is defined by processes of their formation as well as the state and nature of their parent cells. The aim and objection of this report was to demonstrate (using Guava Easy flow-cytometer) that microvesicles are released from erythrocyte membrane naturally in normal and disease conditions, without inducing with calcium chloride; and also to know how many erythrocytes makes a microvesicles .

In comparison with the shedding of microvesicles by erythrocytes, haemolysed red blood cell was used as a negative control method. This report was not trying to repeat the already demonstrated fact that microvesicles contain certain protein which plays a vital role in erythrocyte microvesiculation, but, in addition to the protein components, this report investigates and analysed the concentration of the protein (using Biuret method) and also identify the protein band separations (using agarose gel

electrophoresis). However, due to unavailability of the required monoclonal antibody and resources needed for western blotting analysis, this report did not confirm the protein band separations.

Statistically, this report, however tested the hypothesis and assumption that stated that the mean number of naturally occurring erythrocytes microvesicles released in normal and disease conditions are equal (H0 : U1 = U2), while alternate hypothesis stated that the mean number of non- induced microvesicles released in normal and disease conditions are not equal.

MATERIALS, REAGENTS PREPARATIONS AND METHODS

MATERIALS USED

NHS BTT blood pack was used, which was supplied by the NHS supplied donor units stored in CPDA. Phosphate buffered saline (Fisher- Scientific UK limited) which contains Na2HPO4 = 1.44g/l, anhydrous KH2PO4 = 0.24g/l, anhydrous NaCl = 0.2g/l and distilled water with the final PH of 7.4; Centrifuge (Denley BS 400); Haemocytometer (Abcam technical); Microscope (prior scientific instrument limited). RPMI 1640 and NaHCO3 (Sigma-Aldrich limited); Guava Easy flow-cytometer and Nexin reagent (Guava technology);15ml Working Biuret Reaction;3ml Protein Standard(2mg/ml BSA); DI Water; Bovine Serum Albumin(BSA); Folin and Ciocaiteu's Phenol Reagent (2N); Sodium tartrant: Hydrated Sodium Tartrant; Copper sulphate; Sodium hydroxide and Sodium bicarbonate; Agarose gel; Ethidium bromide; Electrophoresis buffer; Loading buffer;1X TBE buffer solution; Transilluminate. All the laboratory protocols were strictly adheres; and good knowledge of clinical procedures, good laboratory practices and knowledge of laboratory equipments, instrumentations and terminology's were very useful in achieving an accurate, precise and reproducible results of this assay.

REAGENTS PREPARATIONS

Preparations Of Edta

2g of Sodium hydroxide pellets was dissolved in 80mls of deionised water. 18.6g of disodium EDTA was dissolved in the mixture, and more deionised water was added to make up the mixture to 100mls. The EDTA was added for the purpose of chelating calcium. The solutions were allowed to dissolve properly at pH of 8.0, and Autoclaved.

Preparations Of Borate –Edta (Tbe) Buffer (5x Stock Solution)

20ml solution of 0.5M EDTA was added into a conical flask (500ml size). 54g of Tris base and 27.5g of Boric acid was added to the solution and the volume were made up to 500ml of deionised water. The whole mixtures were allowed to dissolved properly using a magnetic stirrer, and autoclaved. The solution were diluted to a working concentration of 1X before use.

Preparations Of 1% Agarose Gel

2g of Agarose powder was dissolved in 100mls of TBE buffer, and microwaved for 8mins until it dissolved completely. 3.5ul of Ethidium bromide was added at temperature of 50 degrees centigrades. The gel was allowed to cool before pouring it on the gel tray.

Preparations Of Phosphate Buffered Saline

2tablets of PBS was dissolved in 400mls of distil water in a conical flask (500ml size). The solutions was autoclaved for 20minutes between 35-37 degree centigrade, and allowed to cool sufficiently. Autoclaving for 20mins was required to achieve a good sterilization.

Isolations And Purification Of Erythrocyte Microvesicles (Emvs)

15mls of blood samples stored in CPDA or SAG-M was provided by NHS blood and transplant, and 10 ml of this blood were mixed thoroughly with 30ml of phosphate buffered saline in a 50ml centrifuge tube, and centrifuge immediately at 600 x g at 4 degrees centigrade for 10 minutes to remove soluble plasma proteins. The supernatant was removed and discarded after centrifugation. This process was repeated three times in order to remove any erythrocyte bound plasma proteins. Erythrocytes were then counted with haemocytometer using x10 objective lense and x40 for magnification). The number of red blood cells countered were recorded and documented. Following washing, the cells are re-suspended in 10ml of RPMI 1640 (to bath the cells) and incubated at 37 degrees centigrade in shaking water bath for 45mins, after which the samples were placed in ice to stop the reaction. The samples were centrifuge at a low speed of 160 x g at 4 degree for 15 minutes to pellet the erythrocytes. The eMV containing the supernatant was transferred to a fresh tube and centrifuge at a higher speed of 4000 x g for 60 minutes at 4 degree centigrade to remove the cell debris and ghost cells, after which the supernatants are transfer to another fresh tube and sonicate in a sonicating water bath for 5 minutes at 4 degree centigrade prior to centrifugation to disperse aggregated exosomes. The supernatant was further centrifuge at 25,000 x g at 4 degree centigrade for 90 minutes to pellet the eMV. The supernatant were removed carefully and discarded; the remaining eMV pellet was re-suspended in 100ul of phosphate buffered saline for immediate analysis. For the flow cytometry analysis, 10ul of the eMV stock sample were diluted in 190ul (1:20) of phosphate buffer saline.

BIURET PROTEIN ASSAY

1.0ml of distilled water was added to the test tube, standard tube and distils water tubes respectively. 4.0mls of Biuret reagent were added to each tube, then votexed. The tubes were incubated at 37 degrees centigrade for 20mins.The tubes were cooled after incubation and read at 540nm.

AGAROSE GEL ELECTROPHORESIS

The samples were run on Agarose gel. 5ul loading dye was added to each. 20ul of the samples were loaded into the wells of the agarose gel; and 10ul of the molecular weight marker were also

loaded and the positions of the protein samples were noted. The gel was run at 100volt for 45 minutes

RESULTS

After washing the whole blood (before high speed ultra centrigugation), erythrocyte cell counts was performed using haematology cell counting chamber and analysed with Guava flow cytometer on three consecutive times; the mean value of the reading were taken and recorded. The number of cells counted was greater than 400cells.The purpose of the counting was to determine how many microvesicles were there, and secondary how many microvesicles are released per RBC. It can be showed below the diagram that 3446666 millions of microvesicles were released by 1ml of the isolated erythrocyte.

Figure 3a: Analysis result : plot 2

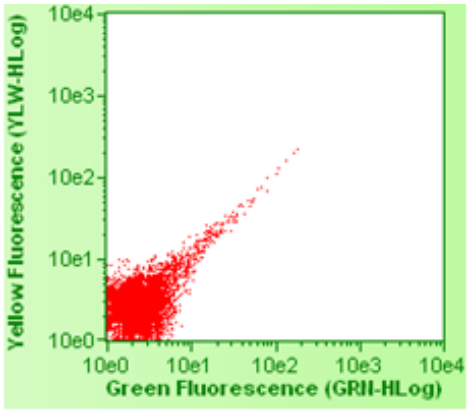


Figure 3b: Analysis result: plot 2

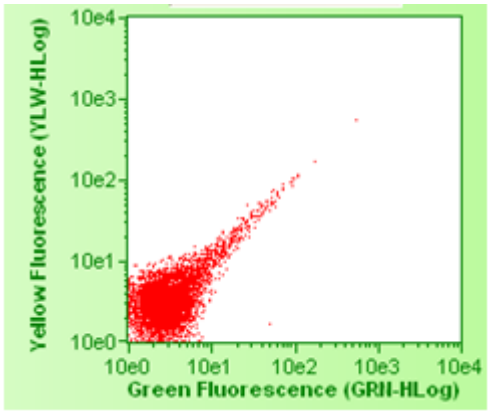


Figure 3c: Analysis result : plot 2

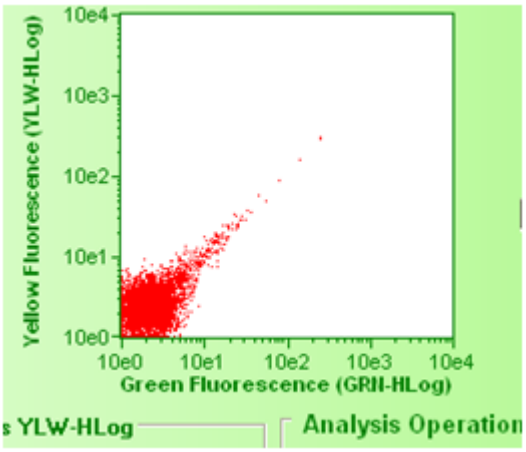


Table 1: The result of the microvesicles produced from peripheral blood Erythrocytes.

ERYTHROCYTE MICROVESICLES (cells/ml)	COUNTS	% OF TOTAL	X-MEAN	Y-MEAN
2270000	5000	100 00	3.55	4.24
3470000	5000	100 00	4.57	4.83
4600000	5000	100 00	4.71	4.93
AVERAGE ERYTHROCYTES MVs				
3446666 cells/ml			4.28	4.67

Figure 4a: Result represents the amount of microvesicles produced by 10ml of peripheral blood erythrocytes (First analysis).

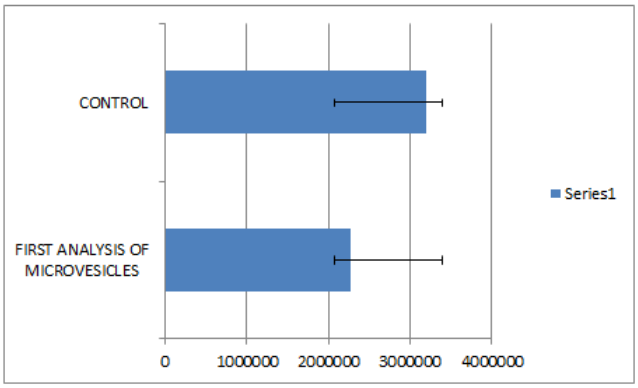


Figure 4b: Result represents the amount of microvesicles produced by 10ml of peripheral blood erythrocytes (Seceond analysis).

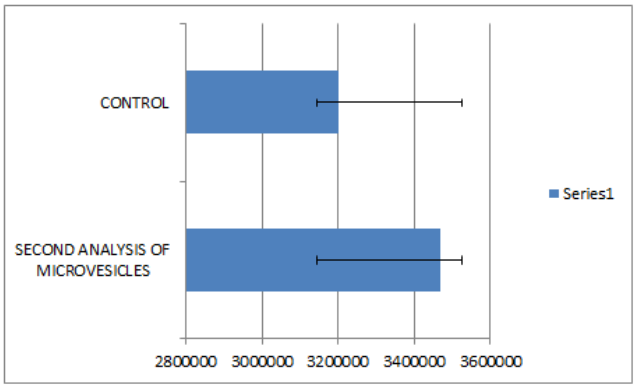
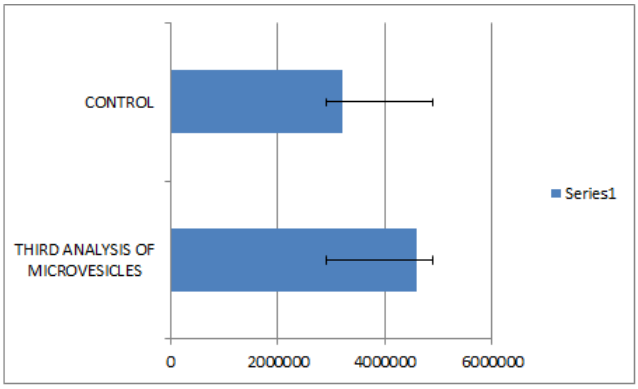


Figure 4c: Result represents the amount of microvesicles produced by 10ml of peripheral blood erythrocytes (Seceond analysis).



However, as previously stated that the microvesicles cargo include a variety of molecular entities comprising of distinct combinations of proteins, lipids and nucleic acid (wolf, 1967; Valadi et.al, 2007; Skog et al; 2008); the analysis of protein was carried out using Buiret assay method and estimated using spectrophotometer set at 540nm. The Absorbance of the sample and the concentrations of Bovine Serum Albumin (BSA) were recorded, tabulated and showed below.

Table 2: The Absorbance of the sample and the concentrations of Bovine Serum Albumin (BSA) was carried out using Buiret assay method.

Concentrations of BSA (mg/ml)	Absorbance of Protein
0	0
0.25	0.009
0.501	0.019
0.749	0.03
1	0.04
1.25	0.052
1.501	0.062
1.999	0.083
2.504	0.104
5	0.206

A graph of the Absorbance of the sample against the concentrations of bovine serum albumin were carefully plotted, and showed below. The determination of unknown protein concentrations was done by tracing the readings from the plotted graph, and was found to be 2.5mg/ml.

Figure 5: Showing a plotted graph of Absorbance of samples against the protein concentrations.

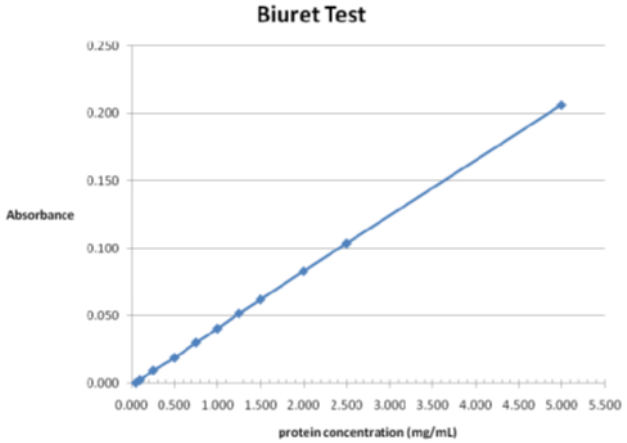
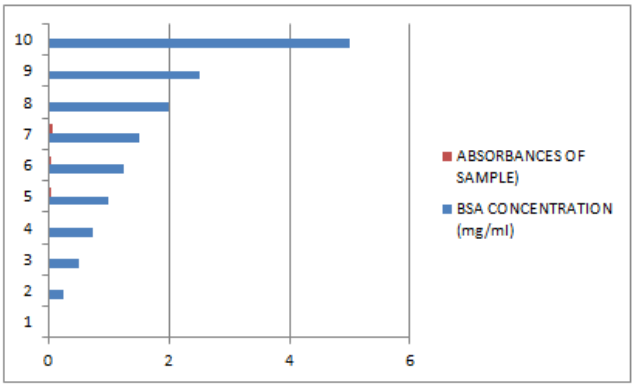
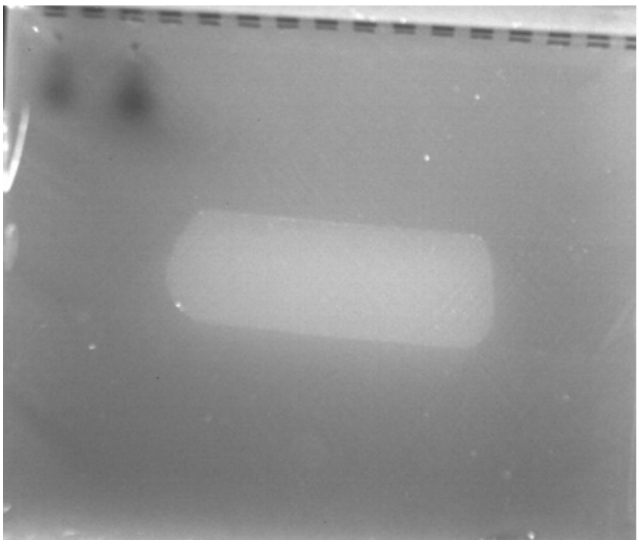


Figure 6: Showing the bar chart of Absorbance of samples against the protein concentration.



In progress with the above analysis, the protein component of microvesicles were further analysed using Agarose gel electrophoresis. The protein bands seperations were carefully identified, noted and showed below. All the laboratory protocols were strictly adheres; and good knowledge of clinical procedures, good laboratory practices and knowledge of laboratory equipments, instrumentations and terminology's were very useful in achieving an accurate, precise and reproducible results of this assay.

Figure 7: The protein component of microvesicles was further analysed using Agarose gel electrophoresis.



Statistically, the null hypothesis stated that the mean values of naturally occurring microvesicles released in normal and disease conditions are equal, while the Alternate hypothesis stated that the mean values of non-induced microvesicles released in normal and disease conditions are not equal. The p value (is 0.032) which is less that 0.05, indicating that there is statistical significant difference; therefore accepting the null hypothesis..

DISCUSSIONS

Microvesicles being a small membrane enclosed sacs are shed by a variety of cell types and has been described as a physiological and biological process which could become significant in pathological and in normal conditions (Ansa-Addo, 2008; Ratajczak et al.,2006). Both the membrane molecular pattern and the internal contents of the vesicle depend on the cellular origin and the molecular processes triggering their formation. The implications of this microvesicles in several biological processes include intracellular communications, promoting Angiogenesis, impact on tumour invasion and metastasis, involvement in multidrug resistance and interference with Antitumur immnunity have been noted (Van et al.,2009; Muralidharam et al.,2010; Cocucci et al.,2009). From these positive research results, this report have compared the ability of red blood cell to released microvesicles in non-induced conditions.

In this present study, microvesicles derived from erythrocytes have been isolated and analysed using flow cytometer. The results of this experimental analysis was in consistency with a documented evidence from other Authors (Berckmans et., 2001; Shet et al., 2003; Hron et al. ,2007; Piccin et al., 2007; Harding et al., 1983); and also the mean diameter (0.5um) of the isolated microvesicles was consistent with the documented value from other researchers (Shukla et al., 1978; Hugel et al., 1978). Further to the above experimental output, the protein component of microvesicles, its concentration and band formations were analyzed using various biochemical techniques. The protein concentration of the isolated microvesicles was found to be 2.5mg/ml , and this confirms the fact that the protein contents of each microvesicle reflect the origin of the cell from which it was released. The results of this reports of the research work is in collaboration and consistency to all the reports of the research work that had done in this field (Ratajczak et al., 2006; Pap, et al.,2009; Piccin, et al,2007). In addition to the proteins specific to the cell type of origin, recent studies have showed that some proteins are common to most microvesicles, and they contain the cytoplasmic proteins tubulin, actin, stomatin, interleukin-6 and band-3 protein; and these were hope to be identified in this research paper. The patchy, fine structure of the microvesicular membrane reflects the inherent lipid organization in raft. Stomatin is an oligomeric integral membrane proteins that is highly concentrated in microvesicles (Salzer, et al., 2001; Snyers, et al., 1998) and it has a very vital function in erythrocyte vesiculation process (Salzer, et al., 2001). A major finding of the current study is that protein sorting during membrane vesiculation is regulated by interactions between integral proteins and the spectrin-based membrane skeleton. In support of this are observations of the molecular behaviour of band 3, an integral protein with known skeletal association, and CD59, a lipid-anchored protein with no linkage to the membrane skeleton. Further support of the importance of skeletal interactions in determining integral membrane protein sorting comes from studies on glycophorine A. (Salzer, et al.,2001). The sickle erythrocytes and the calcium loaded erythrocytes released microvesicles that are selectively enriched in proteins linked to the membrane via a GPI anchor. This observations clearly show that the molecular process of protein sorting of membrane components is regulated, in part, by the interaction with the skeleton.

However, in comparison, haemolysed red blood cell was also analysed for microvesiculation, but no microvesicles was displayed on the flow cytometer, indicating the destruction of haemoglobin releasing microvesicles.

FURTHER RESEARCH AND FUTURE PROSPECTIVES

The main role of microvesicles is to promote communication between the cells from which they are derived and their surrounding environments. Whereas the biogenesis and roles of microvesicles have been burgeoning areas of research in the recent past, several pertinent issues require further investigation to better understand the significance and therapeutic potential of these structures.

It would be of interest to know whether microvesicles serves as a general mechanism for intracellular transfer of oncogenic receiptors. Another question that needs to be addressed is whether tumour cells simultaneously release distinct populations of microvesicles that contain discrete sets of molecules. Alternatively, is the composition of shed microvesicles and the nature of the cargo packed within these structures determined by disease stage? In addition, the cellular mechanisms involved in microvesicles formation and release, as well as the targeting of molecules to these sites promise to be a new and exciting area of future investigation. Molecules that regulate microvesicle shedding and proteins on circulating microvesicles that are responsible for tumour growth, progression and survival will be effective targets for anti-cancer therapeutics.

Furthermore, due to unavailability of monochlonal antibody for western blotting analysis, more research needs to be advocated in order to confirm the protein band separation.

Finally, numerous challenges were facing during this experimental analysis. Among them were the inability to collect on time the required materials from the dispensary unit, unavailabity of monochlonal antibody (for western blotting analysis) and some few faulty equipments which delays the experimental approach.

CONCLUSION

Microvesicles shed from both normal and cancerous cells provide a means of intercellular communication allowing for exchange of charged molecules, including RNA and non-secreted proteins between cells.

In this paper, an isolated microvesicles were analysed using Guava Easy flow cytometer. The protein concentration were quantificated using Biuret assay method, and the protein band separation was analysed using Agarose gel electrophoresis. Knowledge of good laboratory practices, clinical procedures and Knowledge of Laboratory safety and infection control procedures including standard precautions and harzadous chemical handling were very useful in the laboratory aspect of this report.

REFERENCES

1. Ansa-Addo Ephraim A. Sigrun Lange, Dan Stratton, Samuel Antwi-Baffour, Igor Cestari, Marcel I. Ramirez, Maria V. McCrossan and Jameel M. Inal.Human Plasma Membrane-Derived Vesicles Halt Proliferation and Induce Differentiation of THP-1 Acute Monocytic Leukemia Cells. J Immunol, (2010): 185:5236-5246.
2. Anderson DR, Davis JL, Carraway KL.Calcium-promoted changes of the human erythrocyte membrane: involvement of spectrin, transglutaminase, and a membrane-bound protease. J Biochem J. (1977). 1977;252: 6617-6623.
3. Allan D, Thomas P, Limbrick AR.The isolation and characterization of 60nm vesicles (nanovesicles) produced during ionophore A23187-induced budding of human erythrocytes. Biochem J. (1980). 188: 881-887. Medline.
4. Allan D, Raval PJ.Biomed Biochim Acta.(1983).1983; 42:11-12.
5. Al-Nedawi K, Meeham B, Rak J. Microvesicles: messengers and mediators of tumour progression. Cell Cycle (2009). 8:2014-2018.
6. Anderson, H.C., Garimella, R. And Tague, S.E. (2005). The role of matrix vesicles in development and biominerazation. Front. Biosc. 10, 822-837.
7. Andreola, G.,Rivoltini, L.,Castelli,C., Huber, V., Perego, P., Deho, P., Squarcina, P., Accornero, P., Lozupone, F., Luugini, L.(2002). Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J. Exp. Med. 195,1303-1316.
8. Aslan D, Gumruk F, Gurgey A, Altay C. (2002). Importance of RDW value in differential diagnosis of hyperchromias.
9. Bain BJ. (2001). Bone marrow aspiration. J Clin Pathol. 2001, 54: 657-663.
10. Bartel, D. P., (2009). "MicroRNAs: target recognition and regulatory functions". Cell 136 (2): 215–233.
11. Brugnara C. (1998). Use of reticulocyte cellular indices in diagnosis and treatment of haematological disorders. Int J Clin Lab Res. 1998, 28:1-11.
12. Castellana D, Toti F, Freyssinet JM. (2010). Membrane microvesicles: Macromessengers in cancer disease and progression. Thromb Res 125: S84-S88.
13. Ciechanover A, Schwartz A L, Dautry- Varsat A, Lodish H F. (1983). Biol Chem. 258: 9681-9689. [Pubmed].
14. Cocucci, Emanuele; Racchetti, Gabriella; Meldolesi, Jacopo (2009). Shedding microvesicles: artefacts no more. Trends in Cell Biology 19 (2): 43-51. Doi.10.1016/j.tcb.2008.11.003. PMID 19144520.
15. Daleke, D. L. (2003). Regulation of transbilayer plasma membrane phospholopid asymmetry. J. Lipid Res. 44,233-242.
16. Graves, L. E., Ariztia, E. V., Navari, J. R., Matzel, H. J., Stack, M. S. and Fishman, D. A. (2004). Proinvasive properties of ovarian cancer ascites-derived membrane vesicles. Cancer Res. 64, 7045-7049.
17. Harding C, Heuser J. Stahl P. (1983). Receptor-mediated endocytosis of transferring and recycling of the transferring receptor in rat reticulocytes. J Cell Biol 97: 329-339.
18. Hugel, B., M. C. Martinez, C. Kumzelmann, and J.M. Freysinnet. (2005). Membrane microparticles: two sides of the coin. Physiology (Bethesda) 20: 22-27.
19. Hunter,M.P., N. Ismail, X. Zhang, B.D. Aguda, E. J. Lee, L. Yu, T. Xiao, J.Schafer, M.L. Lee, T. D. Schmittgen, (2008). Detection of microRNA expression in human peripheral blood microvesicles. Los ONE 3: e3694.
20. Http: //www.nsbri.org/humanphysspace/focus3/erythropoisis.html
21. Htt://science.howstuffworks..com/life/human-biology/blood 1.htm
22. Johnstone RM. (2009). Exosomes biological significance: a concise review. Blood Cells Mol Dis 36:315-321.
23. Lewin, Alfred; Yuan, Alex; Farber, Erica L.; Rapoport, Ana Lia; Tejada, Desire; Deniskin, Roman; Akhmedov, Novrouz B.; Farber, Debora B.(2009). Transfer of microRNAs by Embryonic stem cell microvesicles. PlosONE 4 (3):e4722. doi: 1371/journal.pone. 0004722. PMID 19266099.
24. Long K E, Yomtovian R, Kida M, Knez J J, Medof M E. (1993). Transfusion. 33: 294-300. [Pubmed].
25. Oreskovic R T, Dumaswala U J, Greenwalt T J.(1992). 32: 848-849). [Pubmed].
26. Mathivanan S, Ji H, Simpson RJ (2010). Exosomes: extracellular organelles important in intracellular communication. J Proteomics 73:1907-1920.
27. Minetti G, Low PS. (1997). Erythrocyte signal transduction pathways and their possible functions. Curr Opin Hematol.
28. Muralidharan-Chari, V.; Clancy, J.W.; Sedgwick, A.;D'souza-Schorey, C.(2010). Microvesicles: mediators of extracellular communication during cancer progression. Journal of Cell Science 123 (Pt 10): 1603-11. doi: 10.1242/jcs.064386. PMC 2864708.
29. Pilzer D, Gasser O, Moskovich O, Schifferli JA, Fishelson Z (2005). Emmission of membrane vesicles: roles in complement resistance, immunity and cancer. Springer Semin Immunopathol 27: 375-387.
30. Pan, E.; Pallinger, E.; Paszotoi, M.; Falus, A. (2009). Highlights of a new type of intracellular communication: microvesicle-based information transfer. Inflammation Research 58 (1): 1-8. doi: 10.1007/s00011-008-8210-7. PMID 19132498.
31. Piccin, A., W.G. Murphy, and O.P. Smith. 2007. Circulating microparticles: pathophysiology and clinical implications and clinical implications. Blood Rev.21: 157-171.
32. Rak J (2010). Microparticles in cancer. Semin Thromb Hemost 36:888-906.

33. Ratajczak J, Wysoczynski M, Hayek F, Janowska-Wieczorek A, Ratajczak MZ (2006). Membrane-derived microvesicles of cell-cell communication. Leukemia 20:1487-1495.

34. Ratajczak, J; Miekus, K; Kucia, M; Zhang, J; Reca, R; Dvorak, P; Ratajczak, MZ (2006). Embryonic stem cell-derived microvesicles reprogram hemotopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia 20 (5): 847-56. PMID 16453000.

35. Skog J, Wurdinger T, Van Rijn S et al (2008). Glioblastoma microvesicles transport RNA and proteins that romote tumours growth and provide diagnostic biomarkers. Nat Cell Bio 10:1470-1476.

36. Smith Sk, Farnbach AR, Harris FM, Hawes AC, Jackson LR, Judd AM, Vest RS, Sanchez S, Bell JD. (2001). Bio Chem.276: 22732-22741.

37. Piccin A, Murphy WG, Smith OP. (2007). Circulating microparticles: pathophysiology and clinical implications. Blood Rev.; 21(3):157-71.

38. Pap, E., E. Pa´llinger, M. Pa´szto´I,, and A. Falus, (2009). Highlights of a new type ofintercellular communication: microvesicle-based information transfer. Inflamm.Res. 58: 1–8.

39. Pisetsky DS, Gauley J, Ullal AJ.(2010). Microparticles as a source of extracellular DNA. Immunol Res. doi : 10.1007/s12026-010-8184-8.

40. Simpson RJ, Lim JW, Moritz RL, Mathivanan S.(2009). Exosomes: proteomic insights and diagnostic potential. Expert Rev Proteomics 6:267-283.

41. Simons,M., and Raposo G.(2009). Exosomes: vesicular carriers for intracellular communication. Curr. Opin. Cell Biol. 21: 575-581.

42. Simons M, Raposo G. (2009). Exosomes-vesicular carriers for intracellular communications. Curr Opin Cell Biol 21:575-581.

43. Taylor, D. D. and Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol. Oncol. 110, 13-21.

44. Trams EG, Lauter CJ, Salem N Jr, Heine U (1981). Exforliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim Biophys Acta 645: 63-70.

45. Thery C, Ostrowski M, Segura E .(2009). Membrane vesicles as conveynors of immune responses. Nat Rev Immune 9:581-593.

46. Vandhana Muralidharan-Chari, James W. Clancy, Alanna Sedgwick and Crislyn D'Souza-Schorey (2010) Microvesicles: mediators of extracellular communication during cancer progression. Journal of Cell Science 123, 1603-1611.

47. Valadi H, Ekstrom K, Bossios A. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9:654-659.

48. Vindelov LL, Christensen IJ, Keiding N, Spang-Thomsen M, Nissen NI. (1983). Long-term storage of samples for flow cytometric DNA analysis. Cytometry 3:317-322.

49. Valadi, Hadi; Ekstrom, Karin; Bossios, Apostolos; Sjostrand, Margareta; Lee, James J; Lotvall, Jan O (2007). Exosome-mediated transfer of mRNAs is a novel mechanism of genetic exchange between cells. Nature cell Biology. PMID 17486113.

50. Van Doormaal, FF., Kleinjan, A., Di Nisio, M; Buller, HR; Nieuwland, R (2009). Cell-derived microvesicles and cancer. The Netherlands journal of medicine 67(7): PMID 19687520.

51. Wolf P. (1967). The nature and significance of platelets products in human plasma. Br J. Haematol 13:269-288.

52. Williamson P, Puchula E, Penniston J T, Westerman M P, Schlegel R A.(1992). J Cell Physiol. 152: 1-9. [pubmed].

53. Westerman M P, Diloy P M, Streczyn M. (1979). Biochim Biophys Acta. 557: 149-155. [Pubmed]