A study of extracellular vesicle concentration in active diabetic Charcot neuroarthropathy

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Title: A study of extracellular vesicle concentration in active Diabetic Charcot Neuroarthropathy

Short title: Extracellular vesicles in Charcot Neuroarthropathy

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ABSTRACT

It was the aim of this work to determine whether the plasma concentration of extracellular vesicles (EVs) in active Diabetic Charcot Neuroarthropathy (CN) is connected to the inflammatory markers, temperature elevation in the affected foot and concentration of soluble receptor for advanced glycation end products (RAGE). EVs were isolated from peripheral blood of 35 patients with active CN. EVs were counted after repetitive centrifugation and washing of samples, by flow cytometry. Foot temperature was measured by infrared thermometer. Concentration of soluble receptor for advanced glycation end products (RAGE) was determined by enzyme-linked immunosorbent assay (ELISA). We found statistically significant correlations of EV concentration (but not soluble RAGE concentration) with C-Reactive Protein (CRP) and with temperature difference between the affected and the contralateral foot ($r=0.40$, $p=0.032$; $r=0.89$, $p<10^{-8}$, respectively). We provide evidence that the concentration of EVs is related to elevation of markers of inflammation (CRP and foot temperature difference) in acute Diabetic CN. EV-based markers could be considered as a potential aid in early diagnosis of CN.

Keywords: Diabetes mellitus, Charcot neuroarthropathy, Extracellular vesicles, Exosomes, Advanced glycation end products

Abbreviations

AGE, advanced glycation end product; CD, cluster of differentiation ;CN, Charcot Neuroarthropathy; CRP, C-Reactive Protein; ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicles; HbA1c, glycosylated hemoglobin; IL-interleukin; M-CSF, macrophage-colony stimulating factor; RAGE, receptor for advanced glycation end products; RANK, receptor activator of nuclear factor kappa-B**;** RANK-L, receptor activator of nuclear factor kappa-B ligand; TNF-α, tumor necrosis factor alpha; WBC, white blood cells;

1. Introduction

Charcot Neuroarthropathy (CN) is a condition most commonly caused by Diabetes, which is characterized by bone and joint disruption/destruction, subluxation, dislocation and deformity, and, in the acute phase, elevated temperature of the affected foot (Dg and La, 1997; Najafi et al., 2012; Rogers et al., 2011). While several biochemical mechanisms have been suggested to contribute to its development, the etiology of CN remains enigmatic (Petrova et al., 2014). It is currently believed that CN occurs as a consequence of an imbalance of activity of two types of cells: osteoblasts and osteoclasts (Jeffcoate, 2015). This imbalance leads to enhanced bone lysis and fracture and is driven by inflammation (Petrova and Shanahan, 2014, 2014). Indeed, increased amounts of the pro-inflammatory cytokines TNF-α, IL-1 and IL-6, decreased amounts of the anti-inflammatory cytokines IL-4 and IL-10 and increased amounts of the surface molecules CD40, CD80, and CD86, were found in monocytes of patients with CN (Uccioli et al., 2010). Increased concentrations of TNF-α and IL-6 were found also in sera of patients with CN (Petrova et al., 2015).

CN has been associated with an increase in advanced glycation end products (AGEs) in blood that is often caused by poorly controlled diabetes and/or inflammation due to hyperglycemia and oxidative stress (Jeffcoate, 2015). It was found that AGEs stimulate apoptosis of human mesenchymal stem cells (Kume et al., 2005) and osteoblasts (Alikhani et al., 2007) by binding to receptor for AGE (RAGE) on their membranes (Witzke et al., 2011). It was also observed that the concentration of RAGE in blood plasma (soluble RAGE) of patients with CN was lower than in healthy subjects and also lower than in patients with diabetes mellitus (Witzke et al., 2011). Possibly due to a deficiency of soluble RAGE, binding of AGE to soluble RAGE was decreased with a deleterious consequence of AGE binding to membrane fraction of RAGE (Witzke et al.,

2011). As the above processes are regulated by many pathways involving the osteoblast and the osteoclast lineages (Nomura et al., 2000; Petrova and Shanahan, 2014; Schmidt et al., 2001), it is evident that intercellular communication is crucial for bone remodeling.

Recently, extracellular vesicles (EVs) and nanotubules were reported as important participants in cellular processes (Schara et al., 2009). These membranous nanostructures present a cell-cell communication system (Camussi et al., 2010). They were long overlooked due to their small size and fragility, but improved observation techniques and theoretical predictions have led to recognition and appreciation of their physiological and pathophysiological roles (Yáñez-Mó et al., 2015). Nanotubules connect cells and present the most effective transporting mechanism that shields their contents from enzyme decomposition in extracellular space (Rustom et al., 2004). Conversely, extracellular vesicles have a greater range than nanotubules since they may carry the membrane-enclosed cell contents to distant cells, convey molecules to them and influence function of recipient cells or even change their phenotype (Ogorevc et al., 2013). Moreover, EVs can be isolated from body fluids, in particular, from peripheral blood (Diamant et al., 2002). Mechanisms of EV formation and function are not yet completely understood, but it is becoming clear that processes of membrane budding and vesiculation should be considered in attempts to describe and manipulate pathological processes.

Experimental evidence indicates that EV concentration is increased in some diseases including cardiovascular diseases, autoimmune disorders, inflammation and infection (Boulanger et al., 2006) as well as certain cancers (Jansa et al., 2008; Kim et al., 2003). As CN has previously been connected with inflammation, increased temperature of the affected foot (McGill et al., 2000) and with lower values of soluble RAGE (Witzke et al., 2011), we hypothesized that patients with CN might have high concentrations of EVs in isolates from peripheral blood and that the

concentration of EVs might be connected to inflammatory markers in blood, to the difference between the temperature of the affected foot and the temperature of the contralateral foot and/or to the concentration of soluble RAGE in blood plasma.

2. Methods

2.1. Participants

Thirty - five diabetic patients (26 male, mean age 52.8, range 34 to 65 years) with a mean diabetes duration 11.7 years, presenting with active Charcot neuroarthropathy to the Department of Orthopaedic Surgery, University Medical Centre Ljubljana were invited to participate in the study. All presented with a unilateral red, hot (> 2°C difference from the contra-lateral foot), swollen foot in the presence of neuropathy and a normal peripheral circulation (Young et al., 1993). In addition to bilateral loss of sensation, peripheral neuropathy was additionally confirmed by abnormal pressure perception to a 10g monofilament and hallux vibration perception threshold of >25V using a biothesiometer (Biomedical Instruments, Inc, Newbury, OH). All had normal renal function. The studies were performed with ethical permission from the National Medical Ethics Committee (number 82/07/14), and all patients gave informed consent.

2.2. Assessment of extracellular vesicles concentration

For isolation of EVs, venous blood was sampled in 2.7 ml vacutubes (kept at 37 °C) containing 270 µL of sodium citrate at a concentration 0.109 mol/l (Becton Dickinson, NJ, USA). Processing of blood started within 20 minutes from the sampling. During processing, the tubes and blood samples were kept in thermo-blocks at 37 °C.

EVs were isolated by repetitive centrifugation and washing of samples (Diamant et al., 2002) modified as described in detail in (Šuštar et al., 2011a). Visualization of the isolates obtained by this protocole by the scanning electron microscope and by atomic force microscope (Junkar et al., 2009) revealed shapes that are characteristic for particles without internal structure (such as discocytes, dumbbell shapes and "starfish" shapes (Seifert, 1997) that correspond to the minima of the membrane free energy at different ratios between membrane area and enclosed volume (Figure 1). Heterogeneity of the shapes in the isolates can therefore be explained by different membrane areas and different enclosed volumes that are a consequence of different processes taking place before the buds are pinched off from the mother membrane. The population of EVs obtained by this protocole had an average size around 300 nm (Šuštar et al., 2011a) while labelling of isolates with antibodies with anti-CD31-FITC and anti-CD42b-PE yielded about 30% (15 - 45%) of events corresponding to unlabelled particles. Out of these, 15% were ascribed to anti-CD235-FITC labelled particles and 9% to the background, and around 6% of particles where there were no evidences of embedded markers (Šuštar et al., 2011b).

Fresh blood was centrifuged at 1550 g and 37 °C for 20 minutes in a Centric 200/R centrifuge (Domel d.o.o., Železniki, Slovenia). The upper 250 µL of plasma was slowly removed and placed in an 1.8 mL Eppendorf microtube. The residual 500 µL of plasma was gently pipetted into another Eppendorf tube and stored at –80 °C for subsequent evaluation of soluble RAGE. The upper 250 µL of plasma was then centrifuged at 17570 g for 5 minutes in a Centric 200/R centrifuge (Domel d.o.o., Železniki, Slovenia) with a swinging rotor "Lilliput" designed by Domel d.o.o. especially for isolation of EVs, so that EVs were gathered on the bottom of the microtube. The upper 210 µL of plasma was discarded and the remaining 40 µL of pellet was

vortexed at 1200 g and re-suspended in 210 µL of phosphate buffered and citrated saline at pH = 7.4. The samples were centrifuged again at 17570 g for 5 minutes. 210 µL of supernatant was discarded and 40 μL of pellet re-suspended in 60 μL of citrated phosphate-buffered saline at pH = 7.4.

2.3. Flow cytometric analysis

Concentration of EVs in isolates was determined by MACSQuant Analyzer (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) flow cytometer with 405 nm, 488 nm and 640 nm air cooled lasers. The MACSQuantifyTM (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) software version 2.4. was used for data acquisition and analysis of the results. 25 µL of sample was measured. The presence of residual cells and EVs was determined by forward and side scatter parameters. The region of events corresponding to EVs contained more than 90 % of events in all density plots.

2.4. Determination of the concentration of soluble RAGE

Thawed samples of plasma, prepared as described above, were evaluated for soluble RAGE by the Quantikine RAGE enzyme-linked immunoassay (R&D Systems) according to manufacturer instructions. Briefly, 50 µl of samples/standards were added to wells containing 100 µl of dilution buffer and incubated for two hours. Following 4 washes, 200 µl of conjugate was added and incubated 2 hours. After 4 washings, 200 µl of substrate was added to wells, incubated for 30 minutes and mixed with 50 µl of stop solution per well. Absorbance was measured at 450 nm with the correction at 570 nm with Infinite microplate reader (Tecan, Groening, Austria). After subtracting average zero standard optical density, results for samples were calculated from seven

standard samples, ranging from 78-5000 pg/ml using four parameter curve. The average coefficient of variation from replicates was 2.7%.

2.5. Assessment of standard blood parameters

Blood sample analysis was performed for the following hematological parameters:- concentration of white blood cells (WBC), C reactive protein (CRP) and glycosylated hemoglobin (HbA1c). The limit for detection of the CRP assay was 5 mg/l, and therefore levels lower than 5 mg/l were taken as 5 mg/l. Blood cell populations were assessed by the ADVIA® 2120 Hematology System (Siemens, Erlangen, Germany) using ADVIA 2120/2120i BASO reagent and peroxidase cytochemical reactions.

2.6. Measurement of foot temperature

Skin temperatures over the affected joints of the Charcot foot and over the identical area of contralateral foot, were measured using a hand held infrared thermometer (Thermoval duo scan, Hartmann, Heidenheim, Germany).

2.7. Statistical analysis

Methods of descriptive statistics were used to calculate the mean values and standard deviations. Correlations between the respective parameters were assessed by the Pearson correlation coefficient and the corresponding probability p by using p-Value Calculator for Correlation Coefficients Software available at http://www.danielsoper.com/statcalc.

2. Results

In the diabetic CN patients, the results of standard tests showed WBC $9.2\pm3.3\cdot10^9$ /L, CRP = 19±45 mg/L and HbA_1c = 7.0±1.4 % (54±15 mmol/mol). Apart from CRP (normal < 5 mg/L), the other values were in the normal range. Mean difference between the temperature of the affected foot and the contralateral foot was 2.61±0.29 °C (range 2.1 to 3.6°C). It was found that the concentration of EVs increased with increasing temperature differential between the two feet ΔT (Figure 2a). Best fit of the data was found by exponential dependence. Statistically significant correlation was found brtween the logarithm of concentration of EVs and ΔT ($r = 0.89$, $p < 10^{-8}$ at n = 34) (Figure 2b), and between the concentration of EVs and CRP ($r = 0.40$, $p = 0.032$ at n = 22), but there were no correlations between the concentration of EVs and concentration of soluble RAGE ($r = 0.08$ at n = 33), the concentration of soluble RAGE and the temperature differential ΔT ($r = -0.02$ at n = 32) (Figure 2a) or between CRP and the temperature differential ΔT ($r = 0.03$ at n = 33).

3. Discussion

Early diagnosis of CN is crucial for the success of the treatment (Petrova et al., 2007), however, clinical and radiographic parameters may not be diagnostic. The X-ray may be normal in the acute phase and standard inflammatory markers including the sedimentation rate (Pakarinen et al., 2002) and CRP are helpful, but non-specific. Thus, no predictive markers for CN are available which reflects the fact that the mechanisms of CN are not yet completely understood. Plasma derived RAGE was recently considered as a possible biomarker for CN though the reported data are as yet inconclusive (Oliveira et al., 2013), and the underlying mechanisms were found to be subject to alteration of renal function (Semba et al., 2009). Recently, EVs have

attracted the interest of scientists from different fields of medicine and biology, but to our knowledge, there are no previous reports on EVs in patients with CN.

We demonstrate that in patients with CN, the concentration of EVs in isolates from peripheral blood is strongly connected to the temperature difference between the affected and the contralateral foot ($r=0.89$, $p<10^{-8}$), a recognized marker of disease activity (23). In these patients the concentration of EVs is also weakly connected to the standard inflammatory marker CRP ($r=0.40$, $p=0.032$), in agreement with previous studies indicating elevated concentration of EVs isolated from blood plasma and synovial fluid of patients with inflamed joints (Buzas et al., 2014 and references therein). If increased EV-mediated metabolism is the basic mechanism undrelaying the development of CN and inflammation, then inflammation is not prerequisite to CN, but may be present with higher probability. We found no statistically significant correlation of measured standard blood parameters (including CRP) with soluble RAGE concentration and no statistically significant correlation of soluble RAGE concentration with the difference between the temperatures of the affected and the contralateral foot. Also, the absence of correlation between CRP and the temperature difference ΔT suggests that inflammation is not the cause of the elevated local temperature in CN. There could be basic common mechanisms (e.g. EV-based mechanism) that underlay pathogenesis of CN, inflammation and AGE metabolism.

Due to damage to the sympathetic nerves which causes loss of compliance of arteries in the bone and marrow, turbulent flow of blood with higher shear stresses and higher local temperature is retained in small vessels. Increased shear stresses activate cells in blood, especially platelets, and enhance their microvesiculation *ex vivo* and *in vivo*. The *in vivo* formed EVs are rapidly taken up

by cells (Ogorevc et al., 2014) in the bone and marrow. The effect can be enhanced by trauma that causes changes in vessel geometry or anatomic peculiarities resulting in strong bending of vessels or their (partial) occlusions. Further mechanisms may derive from increased local pressure of oxygen (O_2) (Boulton et al., 1982) which increases probability of formation of oxidized phospholipids in the membrane and nano/micro sized particles containing reactive oxygen species which take part in cell signaling (Manček-Keber et al., 2015).

A potential mechanism in CN by which EVs and nanotubules are involved in signaling between osteoblast and osteoclast lineages is illustrated in Figure 3. Membrane budding can result in long thin protrusions or shorter globular buds (Schara et al., 2009). The former develop into nanotubules while the latter may pinch off from the membrane to become EVs which are free to move in the surrounding liquid. Osteoblasts shed EVs while osteocytes can reach neighboring osteocytes and cells on the bone surface through canaliculi (Shah et al., 2015) by nanotubules. It was suggested that integration of donor EVs into the host membrane takes place at the tips of protrusions since these parts have similar curvature to EVs that is favorable for the molecules in the EVs (Kralj-Iglič, 2015). The nanostructures enable the cells of the osteoblast and osteoclast lineage to form a network. The existence of such network also suggest the controlling role of osteocytes in regulation of osteoblast/ osteoclast cycle (Nakashima et al., 2011).

When AGE bind to RAGE, AGE-poly-RAGE complexes tend to form (Hegab et al., 2012) which are, due to their bulkiness, expected to be depleted in the buds (Kralj-Iglič, 2015). Consequently, pinched off vesicles are likely to contain less RAGE when AGE is present in higher concentrations while correspondingly larger portion of RAGE connected to AGE would

be retained in the osteoblast membrane. On the other hand, many different types of molecules (in particular, the inflammation products) may promote the budding and vesiculation process, on the basis of the shape of the molecules that constitute the membrane and interactions between them (Kralj-Iglič, 2015).

Osteoclast progenitor cells contain in their membrane molecules called receptor activator of the NF-κB (RANK). Numerous cells including T-lymphocytes, dendritic cells, endothelial cells, fibroblasts and osteoblasts secrete the RANK ligand (RANK-L). However, only osteoblasts secrete macrophage-colony stimulating factor (M-CSF), an enzyme that stimulates osteoclast precursor cells to express RANK on their surface membranes (Mabilleau et al., 2008). Actually, osteoblast progenitor cells contain in their membrane the ligands that can bind to these receptors (RANK-L) (Mabilleau et al., 2008). Osteoclast precursors that express RANK recognize RANK-L through cell–cell interaction (Katagiri and Takahashi, 2002). Binding of RANK-L to RANK signals the development of osteoclast progenitor cells into multinuclear osteoclasts (Katagiri and Takahashi, 2002) which regulates the balance between bone deposition and resorption. In this way, the function and secretory cascade of osteoblasts are crucial to the development and maturation of osteoclasts. Also the concentration of RANK-L in osteoblast lineage is controlled by the osteocytes (Nakashima et al., 2011).

Most recently it was observed that EVs shed from cultured osteoblasts contain RANK-L and can transfer it to osteoclast precursors that contain RANK; RANK-L–RANK signaling then promoted osteoclast formation (Deng et al., 2015). Since osteoblasts express RANK-L as a membrane-associated factor (Boyce and Xing, 2007), RANK-L molecules are expected to be

present in the buds and EVs. RANK-L/RANK signaling through EVs regulates the formation of multinucleated osteoclasts from their precursors (Deng et al., 2015) as well as their activation and survival in normal bone remodeling and in a variety of pathologic conditions (Boyce and Xing, 2007). EVs may explain the previously observed correlations observed by McGill et al. (McGill et al., 2000).

Since the discovery of the physiological and patophysiological significance of EVs and with development of methods for their isolation from body fluids, especially blood, there is a great interest for the use of EVs for diagnostic purposes. Elevated concentrations of EVs have been observed in many diseases (Boulanger et al., 2006; Diamant et al., 2002; Jansa et al., 2008; Kim et al., 2003), therefore the concentration of EVs is a non specific marker and it is necessary to find and define additional EV-borne markers in order to provide the solutions for the needs of particular diseases. In order to better understand the mechanisms underlying CN, it is necessary to consider the role of possible candidates that play a key role in membrane vesiculation, as well as in signaling. Such molecules might then be used as EV-connected markers for diagnostic purposes and for following the status of CN patients. Further studies in this direction are necessary to distinguish CN from infection.

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FIGURE LEGENDS

Figure 1. Scanning electron micrograph of an isolate from peripheral blood with numerous nanoparticles found. Characteristic shapes of submicron vesicles with no internal structure, dumbbell (**A**), discocyte (**B**), and the corresponding modeled shapes calculated by minimization of the membrane free energy (**C** and **D**)- also represented in picture **E** and depicted in C (white arrow) and D (black arrow) erythrocytes E - images were taken at Åbo Akademi University, Åbo/Turku, Finland and are adapted from Šustar et al. (2011a).

Figure 2. (a) EV concentration (c_{EV}, circles) and soluble RAGE concentration (c_{RAGE}, stars), according to the difference between the temperatures of the affected and contralateral foot ΔT. (b) Logarhitm of c_{EV} in dependence on ΔT, the best fit linear dependence $\ln(c/\mathrm{nl}) = -3.79 + 1.80\ \Delta T/\mathrm{K}$ (solid line) and 0.99 % confidence curves (broken lines) (Montgomery and Runger, 1999).

Figure 3. Cellular communication through nanotube-nanotube and nanotube-extracellular vesicle contacts between the osteoblast lineage (mesenchymal stem cells – osteoblast progenitors – osteoblasts - osteocytes (blue)) and the osteoclast lineage (haematopoetic stem cells – white blood cell progenitors – osteoclasts (orange)). Other cells and particles in the marrow are not shown.

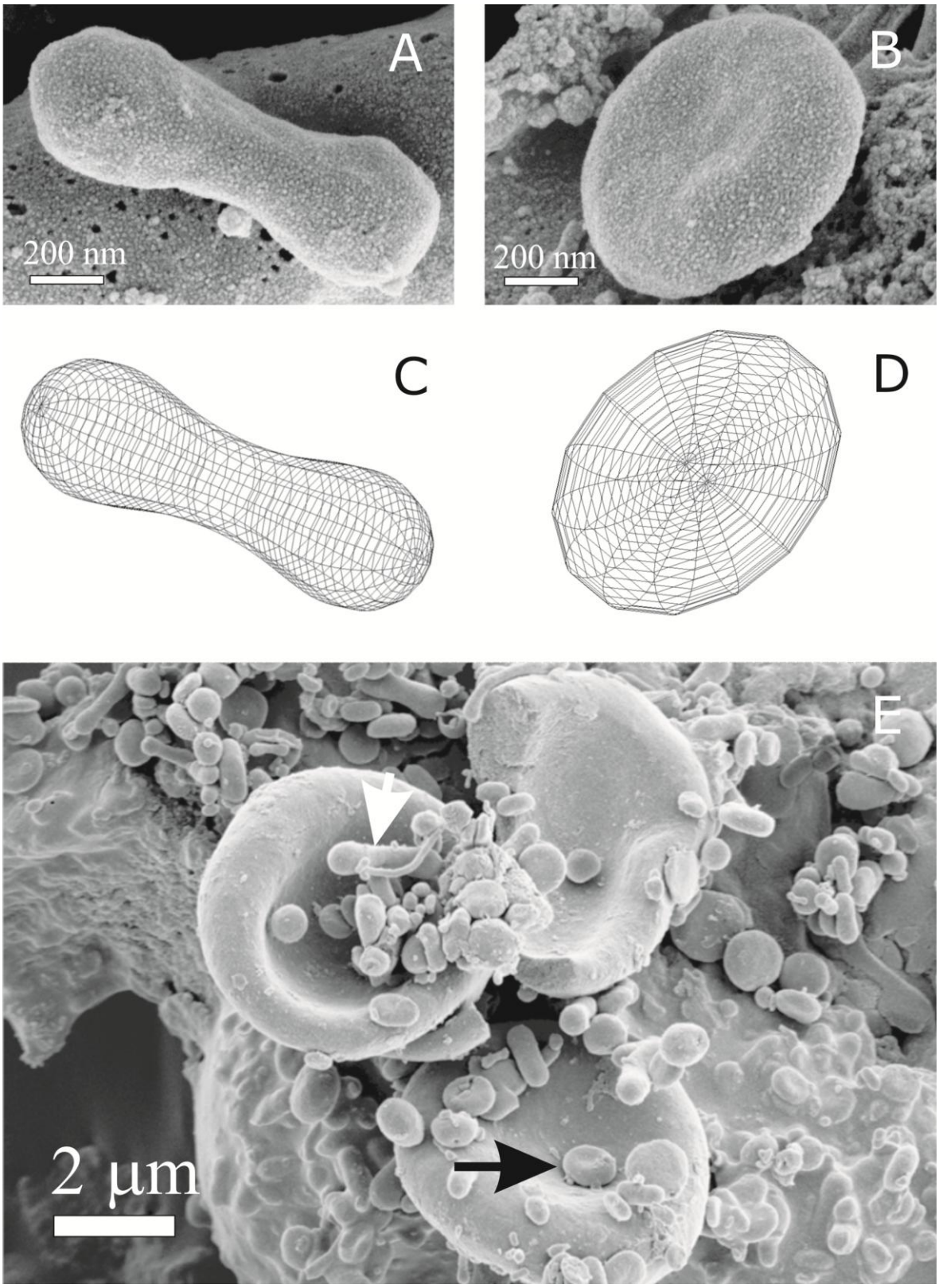


Figure 1

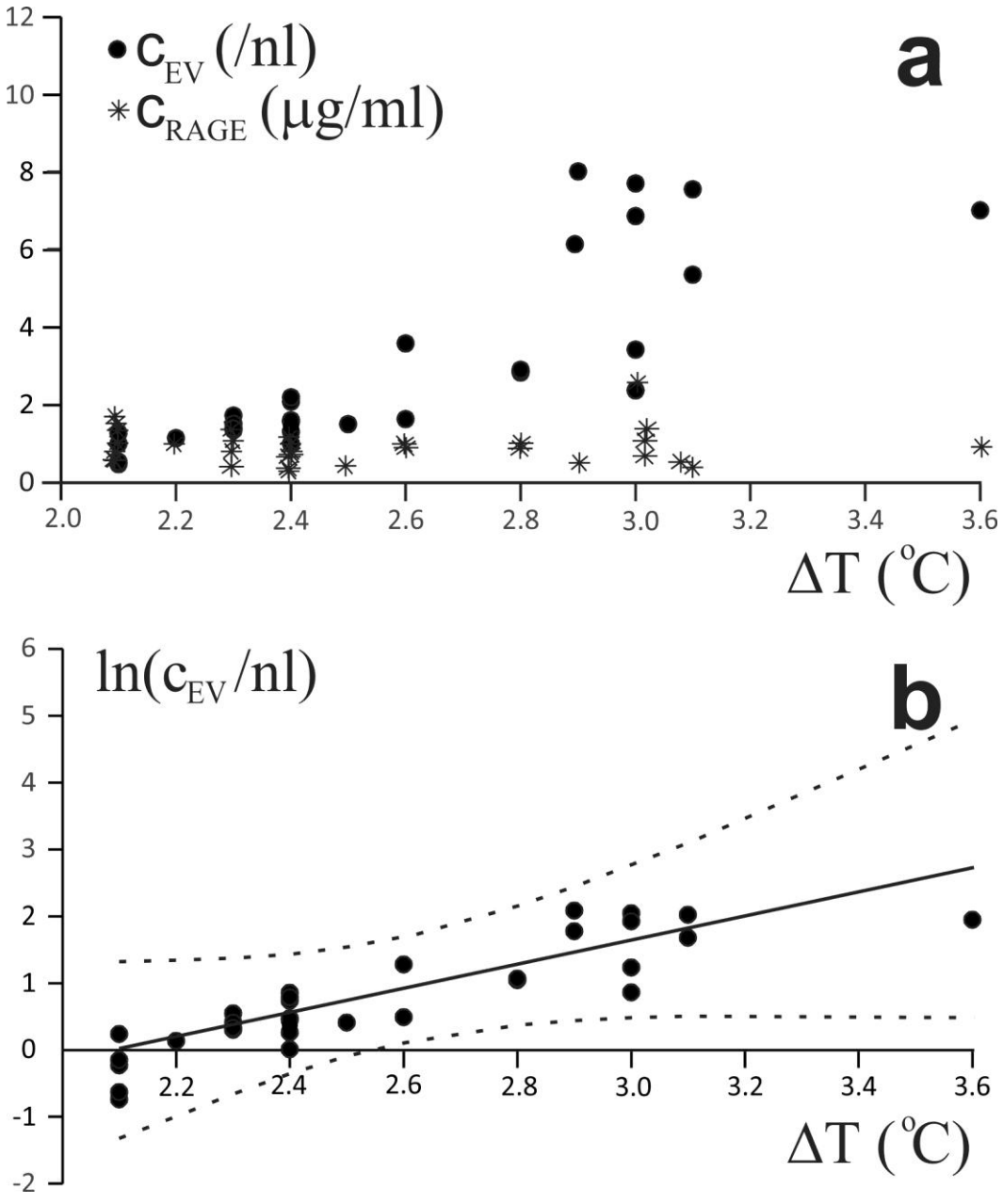


Figure 2

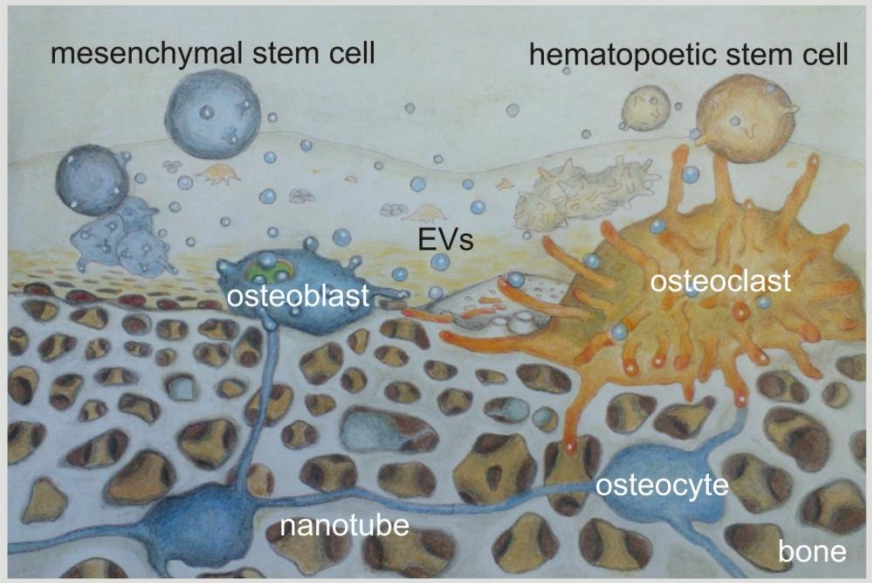
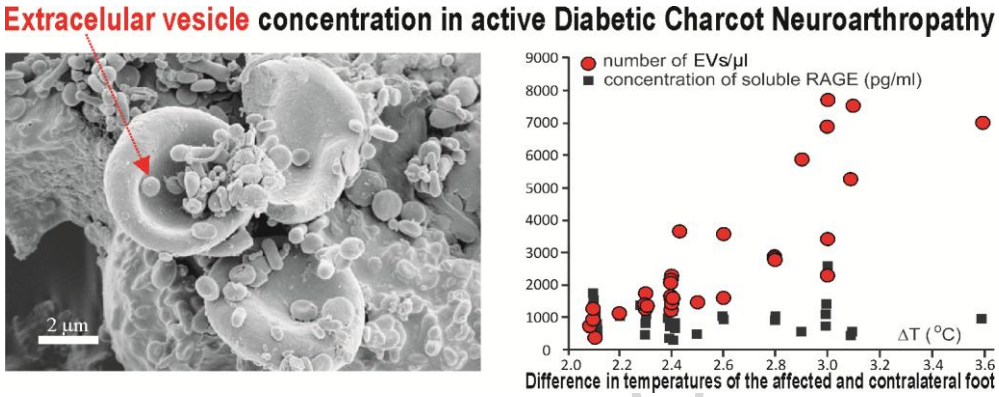


Figure 3



Graphical abstract