Novel Approach for Assessment and Mitigation of Heat-Stress Adverse Effects

by

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Abstract

We have characterized the efficacy of a *Bacillus subtilis* probiotic strain for the prevention of heat stress-related complications in rats. It has been shown that pre-treatment of rats with probiotic bacteria prevented microbial translocation from the gut into mesenteric lymph nodes and liver. Heat stressed animals without probiotic treatment had a high level of lipopolysaccharides (LPS) in the blood. In contrast, animals in the probiotic group, exposed to the same level of heat did not show elevation of LPS levels. Additionally, cytokine IL-10 concentration significantly increased in stressed animals without probiotic pretreatment, whereas administration of probiotic treatment before heat stress normalized the level of IL-10. We also found that the elevation of the temperature of the blood during heat stress causes an increase in the shedding of erythrocyte membrane vesicles. The elevation of temperature from 36.7±0.3 to 40.3±0.4 ^{o}C resulted in a significant increase of the concentration of vesicles in blood. At a temperature of 37 ^{o}C, mean vesicle concentrations found in rat blood was $(1.4\pm0.2)\times10^{6}$ vesicles/μL, while after exposure to heat the concentration increased to $(3.8\pm0.3)\times10^{6}$ vesicles/μL in the group of animals without probiotic treatment. Treatment with the probiotic before heat stress prevented vesiculation of erythrocytes in animals. The results have shown a high efficacy of the probiotic *B. subtilis* in the prevention of heat stress- related complications in rats. Additionally, the images of human erythrocytes and associated vesicles have been analyzed by a light microscopy system with spatial resolution of better than 90 nm. The samples were observed in an aqueous environment and required no freezing, dehydration, staining, shadowing,

marking or any other form of manipulation. Temperature elevation has resulted in a significant increase in the concentration of structurally transformed erythrocytes (echinocytes) and vesicles in the blood. The process of vesicle separation from spiculated erythrocytes has been video recorded in real time. Accurate accounting of vesicle numbers and dimensions suggest that 86% of the lost erythrocyte material is lost not by vesiculation but by another, as yet - unknown mechanism. The increase in the number of vesicles associated with elevated temperatures may be indicative of the individual's heat stress level and thereby serve as diagnostic test of erythrocyte stability and heat resistance and level of heat adaptation.

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1. Introduction

All organisms, in order to survive and maintain overall wellbeing must preserve homeostasis, which is constantly affected by internal or external adverse forces termed stressors. Stressors can be in many forms, including environmental (temperature, humidity, ultraviolet, etc.), psychological or nutritional. Temperature represents one of the most challenging of all stressors. Although humans have the capability to withstand large variations in environmental temperatures, relatively small increases in internal temperature can lead to injury, heatstroke and even death (Crandall and Gonzalez-Alonso, 2010). In fact, exposure to hot weather is considered one of the most deadly natural hazards in the United States. It was estimated that between 1979 and 2002, heatstroke claimed more American lives than the combined effects of hurricanes, lightning, earthquakes, floods, and tornadoes (Leon and Helwig, 2010).

Recent studies suggest that elevated internal temperature leads to increased gastrointestinal permeability – termed hyperpermeability – which is thought to be a key factor in producing symptoms associated with heatstroke (Prosser et al., 2004, Moseley et al., 1994, Pals et al., 1997, Travis and Menzies, 1992, Lambert, 2009). Therefore, agents that serve to reduce or prevent gastrointestinal hyperpermeability could provide significant benefit to individuals by mitigating the impact of heat stress.

Beneficial probiotic bacteria have been shown to enhance gastrointestinal barrier function and dampen the pro-inflammatory pathway in several immune mediated diseases and thereby confer a health benefit on the host. Mechanisms of probiotic bacteria action include restoration of microbial homeostasis in the intestines, interference with the ability of pathogenic bacteria to

colonize the mucosa, beneficial modulation of local and systemic immune responses, as well as a stabilizing effect on overall gastrointestinal barrier function (Figure 1). Further, by enhancing gastrointestinal barrier function, probiotic bacteria serve to reduce the probability of enteric bacterial translocation from the gut lumen into the surrounding vascular and lymph systems.

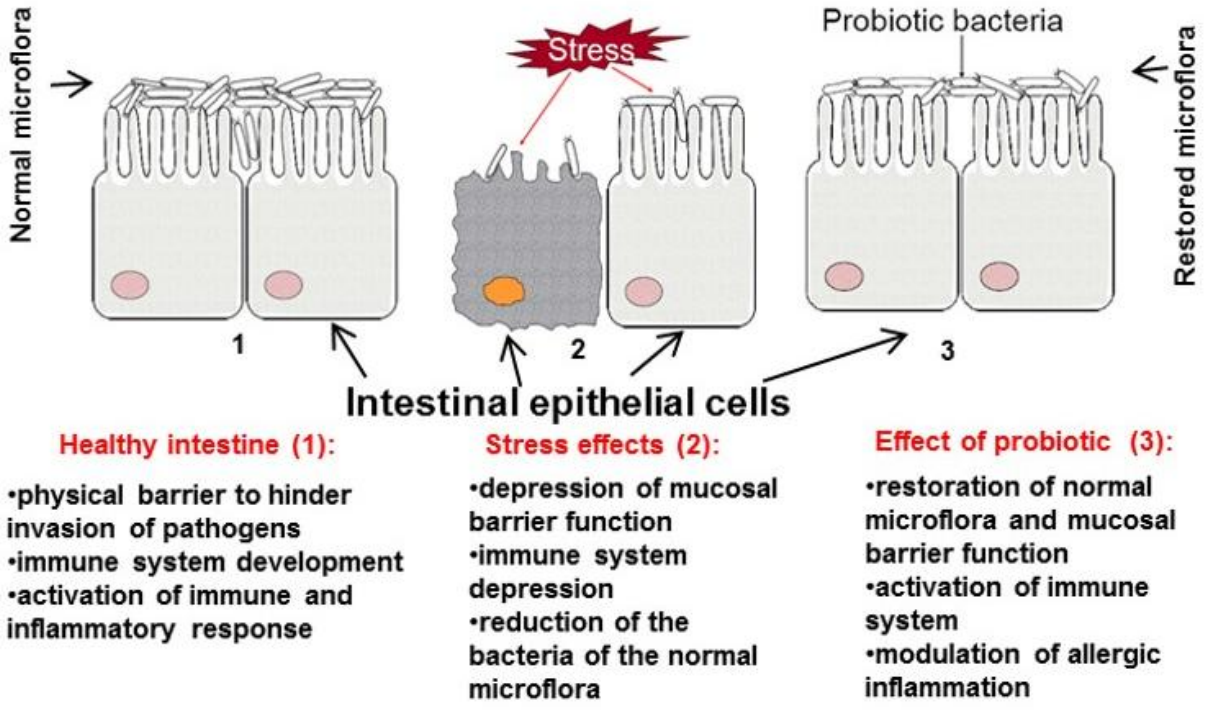

Figure 1. Effect of probiotic bacteria on restoration of stress-inhibited endogenous microflora

Bacterial translocation is one of the suspected causative factors behind fatal heat stroke events. Bacterial translocation is also a strong indicator of extensive gastrointestinal barrier compromise.

In addition to the adverse effects upon gastrointestinal permeability, heat stress also induces or accelerates changes in certain constituents of the blood, most notably in the shape of erythrocyte membranes and the rate of erythrocyte vesiculation. Normally, erythrocytes have a

discoid shape which requires the periodic removal of excess membrane content during an approximately 120 day lifespan. The removal or shedding of excess membrane content has been shown to be affected by elevated temperatures. The elevation of temperature induces the erythrocytes undergo a series of changes that alter the outer membrane shape to create crenated shapes (echinocytes), characterized by convex rounded protrusions or spicules. Gradually, these spicules become smaller and more numerous and eventually bud off irreversibly, forming extracellular vesicles composed of plasma membrane materials (Christel and Little, 1984; Gedde and Huestis, 1997; Glaser, 1979; Glaser and Donath, 1992; Henszen et al., 1997; Muller et al., 1986).

In this work we selected and used a *Bacillus* probiotic strain to assess its ability to prevent and mitigate the undesirable effects on the gastrointestinal barrier associated with heat stress. The *Bacillus* probiotic strain is ideally suited for this role because it has a number of significant advantages when compared to other candidate probiotic bacteria, including: 1) *Bacillus* bacteria are among the most widespread microorganisms found in nature, and as such, humans are constantly exposed to the *Bacillus* species from environmental sources such as air, water, food, etc., thereby lessening the potential for adverse response or allergic reaction; 2) *Bacillus* strains have been documented to produce a variety of antimicrobial compounds that have been shown to be helpful to the host; 3) these bacteria support and promote normal intestinal function and serve to protect the intestinal enterocytes against oxidant-mediated tissue damage and the resulting loss of barrier function; 4) *Bacillus* bacteria are also recognized for their immunomodulation and adjuvant effects. *Bacillus* bacteria are known to also generate antagonistic activity against pathogens which is also beneficial to the host. The above noted attributes lead us to believe that *Bacillus*-based probiotics offer a potentially valuable alternative

in the prevention and treatment of adverse heat stress-induced effects localized in the gut. This proposed approach may result in the development of a product which will protect or restore normal gut function and which will be highly effective in mitigation of heat stress induced complications and do so in an affordable and easy-to-use formulation.

2. Literature Review

2.1 Passive and exercise-induced heat stress

Exposure to high environmental temperatures is considered one of the most deadly conditions, which can quickly lead to human injury and even death. Athletes, occupational workers, and military personnel comprise highly motivated populations which are at risk for heat stroke during intense physical activity in the outdoors (Leon and Helwig, 2010). Heat stroke is a condition that is comprised of a multitude of biological responses during profound heat stress events, which can become lethal if not properly managed (Lim et al., 2007). All scientists agree that heat stress results in a significant morphological damage to the gut. Evidence of this damage has been obtained in human studies (Moseley and Gisolfi, 1993), as well as in animal models (Yu et al., 2010). Through animal studies, it was found that heat stress treatment of pigs caused marked injury to the apical tips of the intestinal villi, inducing epithelial cell shedding, exposing the intestinal mucosa lamina propria, as well as shortening of individual villus height and crypt depth in the small intestine (Yu et al., 2010). Integrity of the gastrointestinal tract serves to protect the internal environment of the body from bacteria and endotoxins (lipopolysaccharides – LPS) of Gram-negative bacteria (Lambert, 2009). Damage to this barrier causes increasing gut epithelial permeability (Travis and Menzies, 1992) and leads to endotoxemia within the gut/portal circulation (Moseley and Gisolfi, 1993). Increased levels of LPS have been registered in patients diagnosed with heat stroke (Leon and Helwig, 2010), in long distance runners (Pals et

al., 1997), and in heat-stressed animals (Hall et al., 2001). The comparison of patients with heat stroke and sepsis shows strong similarity of clinical symptoms, such as septicemia, organ failure, hemorrhage, and systemic inflammation (Lim et al., 2007). The immune system serves to remove LPS from the circulation through the reticuloendothelial system of the liver, high-density lipoproteins and anti-LPS antibodies (Lim and Mackinnon, 2006). Endotoxaemia develops when the rate of LPS clearance cannot keep pace with the rate of LPS translocation. This triggers a systemic inflammatory response which in turn leads to disseminated intravascular coagulation, necrosis of organ tissues, and multi-organ failure, all of which are commonly observed in heat stroke patients (Lim and Mackinnon, 2006). The mechanisms of the systemic inflammatory response syndrome that predisposes an individual to morbidity and mortality were recently summarized by Leon et al. (Leon and Helwig, 2010) (Figure 2 Schematic of heat stress induced GI and organ dysfunction).

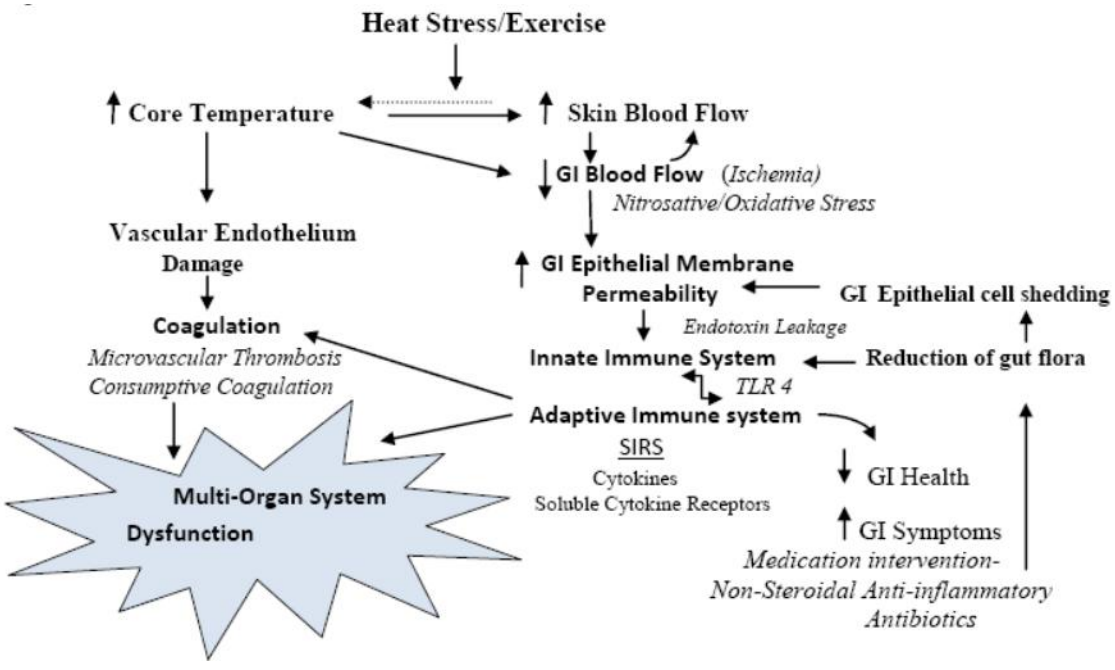

Figure 2. Schematic of heat stress induced GI and organ dysfunction (adapted from (Leon and Helwig, 2010).

The innate and adaptive immune systems sense and respond to the presence of endotoxins (through toll-like receptors, such as TLR4) and consequently stimulate the production of cytokines and other immune modulators (Walsh and Whitham, 2006). Consequently, gastrointestinal hyperpermeability sets the stage to stimulate or over stimulate the immune system of the host due to the release of endotoxins and LPS.

2.2 Heat Stress Complications

2.2.1 Gastrointestinal Changes

Recent studies suggest that increased gastrointestinal permeability is a key factor in the generation of the symptoms associated with heatstroke (Prosser et al., 2004, Lambert, 2009). Therefore, agents that can reduce or prevent gastrointestinal hyperpermeability would offer a significant benefit by mitigating the impact of heat stress on individuals. The gastrointestinal mucosa serves to protect the internal environment of the body from bacteria and bacterial endotoxins (lipopolysaccharides –LPS) typically found in the gut lumen (Moseley and Gisolfi 1993). Dysfunction of this protective barrier results in increased intestinal permeability and the diffusion of toxic bacterial components from the gut lumen into the blood (Lambert 2009). Gut microflora are the key in keeping and maintaining gut mucosal barrier function. There is strong evidence regarding the role and influence of stressors on the depression of overall intestinal microflora and, as a result, on the overall immune resistance of the organism. On the other hand, stability of gut microflora determines the ability of the organism to tolerate a variety of stressors (Berg, Muller et al. 1999). Thus, by modulating the intestinal flora one might offer a novel and non-invasive approach for promoting host well-being by the prevention or reduction of the level of the adverse effects of stress in the gut.

2.2.2 Beneficial bacteria

In animal models it has been shown that there are several therapeutic methods of reducing exotoxemia, including: antibiotics (Moseley et al., 1994), anti-LPS antibodies (Lim et al., 2007), and colostrum (Prosser et al., 2004), all of which can help to protect animals from serious injury or death.

Beneficial probiotic bacteria have been shown to enhance gastrointestinal barrier function and dampen inflammation in several immune mediated diseases thereby conferring a health benefit on the host. The mechanisms of probiotics action include restoration of microbial homeostasis in the intestine, interference with the ability of pathogenic bacteria to colonize the mucosa, beneficial modulation of local and systemic immune responses and a stabilizing effect on overall gastrointestinal barrier function. In animal stress models, probiotic pretreatment of rats completely abrogated stress-induced bacterial adhesion to the intestinal mucosa and translocation of bacteria to mesenteric lymph nodes (Zareie et al., 2006). There is no data about efficacy of probiotic bacteria for prevention of gut-associated adverse effects arising in the gut due to heat stress. This approach seems to be very promising, effective and easy-to-use.

Previously it has been shown that select *Bacillus* strains have significant advantages over other probiotic bacteria, including: 1) *Bacillus* bacteria are among the most widespread microorganisms in nature, and as such, humans are constantly exposed to the Bacillus species in the environment via air, water and food; 2) Bacillus strains produce a variety of antimicrobial compounds as well as other compounds useful for the proper function of the human gut; 3) these cultures are friendly in the gut and they help to protect the intestinal cells against oxidant-mediated tissue damage and the resultant loss of barrier function; 4) they are recognized for their immunomodulating and adjuvant effects; 5*) Bacillus* cultures have also been shown to have

antiviral activity; 6) they are extremely resistant to harsh conditions found in the environment, as well as in the gastrointestinal tract of the organism (Sorokulova, 2008). The unique therapeutic compounds found within the *Bacillus* culture create specific mechanisms for the prevention and treatment of stress-induced adverse effects. It is hypothesized that some of the compounds produced by *Bacillus* cultures are highly effective against pathogenic bacteria without harming normal microflora, while others positively support barrier function of the gut and by extension the immune status of the host. Therefore, the use of a beneficial *Bacillus* strain for the prevention of heat stress-associated adverse effects on the gut is worthy of examination. Modulating the intestinal flora via probiotic therapy presents a novel, non-invasive therapeutic approach for promoting host well-being that can prevent and treat the adverse effects of stress in the gut. Possible mechanisms of probiotic action include restoration of microbial homeostasis in the intestine, interference with pathogenic bacteria's ability to colonize the gut mucosa, beneficial modulation of local and systemic immune responses and a stabilizing effect on gastrointestinal barrier function. In animal stress models, probiotic pretreatment of rats completely abrogated stress-induced bacterial adhesion to the intestinal mucosa and translocation of bacteria into the mesenteric lymph nodes (Zareie et al., 2006). The efficacy of treatment with *Lactobacillus* strain has also been shown to reduce the enhanced neuronal activation in spinal and supraspinal sites which is induced by acute stress (Ait-Belgnaoui et al., 2009).

By contrast, antibiotic-based treatment strategies serve to disrupt the normal bacterial flora of the gastrointestinal tract in an attempt to treat or reduce the symptoms, but not the causes related to gastrointestinal epithelial integrity. Furthermore, these medicinal interventions/treatments rely upon the absorptive ability of these drugs by the dysfunctional gut

that has been compromised by stress. Thus, drug dosing and therapeutic efficacy are dependent upon the drug's ability to successfully move across the gastrointestinal epithelial membrane.

2.2.3 Changes in blood

It has been demonstrated *in vitro* that normal red blood cells (discocytes) undergo shape changes as a result of variation of temperature. Elevated temperature of just a few degrees Celsius induces a series of crenated shapes (echinocytes), characterized by convex rounded protrusions or spicules. Gradually, the spicules become smaller and more numerous and eventually bud off irreversibly, forming extracellular vesicles composed of plasma membrane materials (Christel and Little, 1984; Gedde and Huestis, 1997; Glaser, 1979; Glaser and Donath, 1992; Henszen et al., 1997; Muller et al., 1986). Based upon on a combined volume/density cell fractionation method, it has been shown that throughout the lifespan of the mature erythrocyte, cell volume and hemoglobin content are reduced by 30% and 20% respectively, while the hemoglobin concentration increases by 14% (Werre et al., 2004). Additionally, along with the decrease in the total hemoglobin content, both surface area and lipid content equally decrease by 20%, while the actual surface-to-volume ratio increases (Rumsby et al., 1977). This suggests that the decline of hemoglobin and surface area results from the creation and discharge of hemoglobin-containing vesicles, as well as the amount that vesicles contribute to the erythrocyte surface area reduction. Therefore, a detailed examination of the effects of heat upon vesicle formation may further our understanding of the complexities of the effects of heat on blood.

3.3 Vesicles in human and animal blood

3.3.1 History of vesicles in blood

In the middle of the nineteenth century, scientists observed and described particles that they found in human blood plasma and other liquids. These findings were thoroughly described

and analyzed by the professor of anatomy, H. Charlton Bastian, at the University College in London (Bastian, 1872). Researchers of that era had at their disposal remarkably high resolution microscopes and they described and photographed very small biological objects (Carpenter, 1875). In the beginning of nineteenth century, French microbiologist Antoine Bechamp described small particles he observed in both human and animal blood through the use of a high-resolution optical microscope. He named those small particles "microzymas." He said, "microzymas, anatomical elements, are living beings of a special order without analogue" (Bechamp, 2002). In 1925, Gunter Enderlein also described small particles he termed "protits" that he observed in blood. The size of these particles was calculated to be about 0.01 micron. Further, he observed that under specific conditions, "protits" could evolve into other forms and sizes (Enderlein, 1999). The finding of small particles in blood was later duplicated by Royal Rife (Rife, 1953), Wilhelm Reich (Reich, 1979), and Gaston Naessens (Bird, 1990). These apparently identical particles or forms which were identified by various authors were not characterized to such a level that allowed identifying them as the same or as different entities. Only recently by utilizing methods of biochemistry and molecular biology, was it even feasible to attain a better description and characterization of the nanoforms isolated from blood (Kajander and Ciftcioglu, 1998, Kajander et al., 2001, Kajander et al., 1996, Vali et al., 2001). Kajander claimed to discover small particles, which were named "nanobacteria," in cell culture sera, as well as blood. Nanobacteria of 0. 1 - 0. 5 microns in size were reported to be cultured at 37 °C using a doubling time of approximately one to five days. All researchers mentioned previously and who described nanoforms in blood, suspected that their particular physiological and physicochemical properties would be of special importance in the origin of life. Hence, a better method of cultivation, characterization and classification of the particles in blood, and the

development of a consistent and rapid technique of production of the particles are important for continued research.

3.3.2 Vesicles, exosomes, and ectosomes.

Vesicles constitute a heterogenic population of cell-derived microscopic size particles that participate in a wide range of physiological and pathological processes. They are derived from different cell types including platelets, red blood cells, leucocytes, endothelial, smooth muscle, and cancer cells (Burnier et al., 2009, Castellana et al., 2010, Distler et al., 2005, Greenwalt, 2006, Jayachandran et al., 2008, Miyazaki et al., 1996, Orozco and Lewis, 2010, Piccin et al., 2007, Roos et al., 2010, Rubin et al., 2008, Sadallah et al., 2008, Sadallah et al., 2011, VanWijk et al., 2003). The vesicles released by cells have been described using various names, including exosomes, microvesicles, and ectosomes, and even apoptotic bodies. Ectosomes and axosomes are differentiated by their formation and release. Exosomes are formed in multi-vesicular bodies, whereas ectosomes are vesicles budding directly from the cell surface (Sadallah et al., 2011, Pap et al., 2009). One reason for the release of vesicles from cells is apoptosis, which generates vesicles that differ significantly from the apoptotic bodies (Pap et al., 2009, Théry et al., 2001). While the other vesicles are released in the earlier stages of apoptosis, the apoptotic bodies exit cell at the final stages of programmed cell death (Coleman et al., 2001)

This literature review will be concentrating on the vesicles in blood plasma, focusing on red blood cell vesiculation (Greenwalt, 2006) at conditions of elevated temperatures (Kenney and Musch, 2004, Przybylska et al., 2000b, Samoylov et al., 2005, Wagner et al., 1986, Walsh and Whitham, 2006, Rubin et al., 2008, Burnier et al., 2009, Leonards and Ohki, 1983, Willekens et al., 2003, Willekens et al., 1997, Dumaswala and Greenwalt, 1984).

3.3.3 Origin of vesicles in blood

Vesicles have been identified as arising from a large number of cell types *in vivo* and *in vitro*, including: epithelial, fibroblast, hematopoietic, immune, placental and tumor cells, which include T lymphocytes, B lymphocytes, mast cells, dendritic cells, tumor cells, mesenchymal stem cells, Schwann cells, intestinal epithelial cells, neuronal cells, and endothelial cells. They are found in different bodily fluids such as blood plasma, urine, saliva, breast milk, bronchial lavage fluid, cerebral spinal fluid, amniotic fluid and malignant ascites (Caby et al., 2005, van Niel et al., 2006, Ludwig and Giebel, 2012, Pap et al., 2011, Pap et al., 2009, van Niel et al., 2001).

3.3.4 Role and clinical significance of vesicles

Vesicles are increasingly being considered to play a key role in information transfer between cells (Ludwig and Giebel, 2012, Pap et al., 2009, Atay et al., 2011). Their importance has been demonstrated in several physiological and pathological processes, including immune modulation, regenerative therapies, and neurogenerative diseases (Roos et al., 2010, Piccin et al., 2007). Not only may they serve as prognostic markers in different diseases, but they could be used to be as new therapeutic targets or drug delivery systems (Raimondo et al., 2011).

The clinical importance of the vesicles found in blood plasma needs to be seriously considered for their role in many diseases, including thrombotic pathologies, hemophilia, malaria, diabetes, cardiovascular diseases, endothelial dysfunctions, pulmonary hypertension, ischemic stroke, preeclampsia, rheumatologic diseases—rheumatoid arthritis, polymyositis—dermatomyositis, angiogenesis, and also prion-based diseases and cancer (Roos et al., 2010, Burnier et al., 2009, Simpson et al., 2009).

3.3.5 Composition of vesicles

Vesicles, in most instances, range in size from 0.05 to 1.5 μm, and contain membrane proteins of their parental cells, circulate in the peripheral blood and play active roles in the physiology and pathogenesis of several illnesses (Roos et al., 2010, van Niel et al., 2006). The presence of proteins in vesicles from various cells has been analyzed by a variety of techniques, including: western blotting, fluorescence-activated cell sorting analysis of vesicle-coated beads, and mass spectrometry. Both common and cell-specific proteins might be directed selectively to vesicles. The cell-specific proteins include cytosolic proteins - such as tubulin, actin and actin-binding proteins (cytoskeletal components obtained from T-cells and enterocytes derived vesicles). Vesicles contain cell-specific transmembrane proteins -including α- and β-chains of integrins (such as αMon DCs, β2 on DCs and T cells, and α4β1 on reticulocytes), immunoglobulin-family members (such as intercellular adhesion molecule 1 (ICAM1)/CD54 on B cells, A33 antigen on enterocytes and P-selectin on platelets) or cell surface peptidases (such as dipeptidylpeptidase IV/ CD26 on enterocytes and aminopeptidase N/CD13 on mastocytes) (Thery et al., 2002).

3.3.6 Morphology of vesicles

Morphology of blood derived vesicles has been studied by electron microscopy and has been shown to be dependent on their type and origin. For example, most exosomes are in the range of 40-100 nm, homogenous in shape (cup-shape), with a density range of 1.1-1.19 g/cm^3 (Théry et al., 2001, Atay et al., 2011, Kesimer et al., 2009, Caby et al., 2005, Denzer et al., 2000, Raimondo et al., 2011). Ectosomes (shedding particles) are normally of 0.1 – 1.5 μM, variable in shape, with a density of ~ 1.16 g/cm^3 (Raimondo et al., 2011, Cocucci et al., 2009).

3.3.7 Induction of vesicle production and shedding

Exosomes and ectosomes have two distinct mechanisms that result in vesicle production and shedding. The exosomes have an endosomal origin. These vesicles are released via exocytosis, in which multivesicular bodies leave via the lysosomal pathway and then fuse with the plasma membrane. Regular or normal exocytosis can be exemplified by synaptic vesicles (or neurotransmitter vesicles) that are released by synapse releasing neurotransmitters. The release is regulated by a voltage-dependent calcium channel (Augustine, 2001).

Ectosome formation can be induced by two major mechanisms: the elevation of intracellular Ca^{2+} concentration and the reorganization of the cytoskeleton (Burnier et al., 2009, Pap et al., 2009). The increase of intracellular Ca^{2+} level changes the phospholipid distribution of the plasma membrane which results in the overrepresentation of phosphatidylserine (PS) and phosphatidylethanolamine (PE) where they are found on the outer side of the cell membrane. This imbalance causes membrane deformation and bulging.

In contrast, the reorganization of the cytoskeleton leads to the detachment of the plasma membrane from the cortical actin. A few processes which initiate this detachment can be considered. One of them is related to the phosphatidylinositol 4,5-biphosphate that regulates the attachment of the membrane to the cortical cytoskeleton. In another process, calcium release leads to activation of two enzymes: *calpain* and *gelsolin*. Calpain hydrolyzes actin-binding proteins that decrease association of actin with membrane glycoproteins (Burnier et al., 2009) In platlets, gelsolin is involved in restructuring of cytoskeleton leading to outer membrane bulging (Pap et al., 2009).

3.3.8 Vesicles related to intestinal cells

The intestinal epithelium is typically considered to be both a binding surface and a physical barrier between the body and its surroundings. The epithelial cells may also be significant in the control of luminal antigen presentation to mucosal immune cells. The intestinal cell-released vesicles in the 30-90-nm-diameter size originate from the apical and basolateral sides of the villus, and this discharge has been shown to be significantly augmented in the presence of interferon γ (van Niel et al., 2001). It has been shown that vesicles produced by the intestinal epithelial dendritic cells and/or enterocytes represent a very wide range of proteins including antigens MHC classes I and II, integrins, immunoglobulins, cell-surface peptidases, cytoskeletal proteins, tetraspanins, heat-shock proteins, membrane transport and fusion proteins, signal transduction proteins, and metabolic enzymes. Given such a broad protein presentation, the vesicles of intestinal origin are likely to be strongly involved in or indicative of the level of intestinal homeostasis.

3.3.9 Vesicles related to heat shock proteins at exercise and hyperthermia

Intracellular heat shock proteins are recognized as universal protein markers of stress whose expression is induced by various cell stressors, including heat, metabolite deprivation, redox imbalances, as well as during physical exercise (Krause and Rodrigues-Krause Jda, 2011). The first confirmation that exercise induced the release of heat shock proteins and their resulting accumulation in the blood was provided by Walsh and colleagues (Walsh et al., 2001). It was demonstrated that 60 minutes of running exercise at 70% of maximal oxygen uptake resulted in a noticeable increase in the circulating level of HSP72. Extracellular heat-shock protein expression during exercise has been shown to increase with the level of hyperthermia attained during physical exercise. Cycling experiments conducted at 40 ^{o}C, 50% humidity, 60% and 75% of

maximal oxygen uptake respectively have revealed that heat shock protein release is related to the core temperature reached during the exercise (Periard et al., 2012).

It has been demonstrated that micropaticles serve as transporters of heat shock proteins from a variety of cells including reticulocytes (Johnstone et al., 1987), dendritic cells (Théry et al., 2001, Thery et al., 1999, Thery et al., 2002, Lancaster and Febbraio, 2005a, Lancaster and Febbraio, 2005b), peripheral blood mononuclear cells (Lancaster and Febbraio, 2005a, Lancaster and Febbraio, 2005b), tumors (André et al., 2002), and enterocytes (van Niel et al., 2001).

3.4 Vesicles generated by red blood cells

3.4.1 History of red blood cell discovery

Antoine van Leeuwenhoek was the first person who published a description of the distinctive physical properties associated with human blood cells (van Leeuwenhoek, 1675). He wrote: "*…the clearer aqueous matter of blood, being that liquor in which the red blood globules swim; … and that I had observed…. I very well remember, that, about two years ago I observed my own blood, and noted, that those blood globules that make blood red, seemed to be firmer and harder than they are my blood now; at which time I fell into a sickness, which held me near three weeks. But now I find those globules of my blood softer and my body in a good state of health... I imagine that those blood globules in a healthy body must be very flexible and pliant, if they shall pass through the small capillary veins and arteries, and that in their passage they change into an oval figure, reassuming their roundness when they come into a larger room.*" In 1682-1684 Leeuwenhoek described the oval shape associated with the red blood cells of fishes, amphibians, birds, and he also published the first known drawings of red blood cells (Bessis and Delpech, 1981).

Leeuwenhoek noted that frog red blood cells have the oval shape and also the presence of a central body. In his letter to Mr. Oldenburg, Leeuwenhoek stated: "*After writing in the year of our Lord 1673 to Mr. Oldenburg of blessed memory, that the matter coloring our blood red consists of globules, I set myself to examining the blood of oxen, sheep and rabbits, and found that there is no difference in size between the globules in the blood of human beings and the said animals. As a result I imagined that the matter of all blood that colors the blood red, consists of globules. But after inspecting the blood of salmon, cod, frogs, etc., and finding that the matter which makes the blood red, consists of flat, oval particles-as mentioned before-I set myself to examine the blood of various birds and then found that the matter in them that makes the blood red also consists of no other parts than flat particles such as I have said the blood of .fishes consists of. Consequently I can now but think that the red matter of the blood in all fishes, birds and animals that live in water, consists of no other figures than flat, oval particles*" (Bessis and Delpech, 1981).

Leeuwenhoek was the first scientist who observed and described unique properties of red blood cells. His remarkable observations made more than 300 years ago appear to still to be accurate. He stated that red blood cells make blood red. He brilliantly foresaw that the physical state of red blood cells can indicate the state of health or disease. He explained that red cell flexibility and deformability were needed in order to pass through thin capillary vessels. He was also the first who noticed that different animals have nucleated and non-nucleated red blood cells. It took 187 years before George Gulliver in his review (Gulliver, 1862) outlined major properties of red blood cells and more than 250 years before Gorter and Grendel in 1925 provided initial information on the structure of the red blood cell membrane (Gorter and Grendel, 1925).

The first observation of blood red cells was erroneously credited to Jan Swammerdam due to his 1668 letters where he claimed to have personally observed and described red blood cells (Mohandas and Gallagher, 2008). After visiting Leeuwenhoek on several occasions during 1674, Swammerdam described in his letter to Mr. Trevenot human red blood globules previously described by Leeuwenhoek without mentioning his name. Swammerdam further observed human blood recovered from the intestines of a louse by way of a glass capillary that was also invented by Leeuwenhoek in 1674. Swammerdam probably observed red blood particles recovered from louse but he stated that they do not exist in blood of live animals. He wrote: "*"It is, however, a matter of doubt, whether the blood in its vessels has any globules, for when drawn from them it may easily acquire that figure; ... Nor shall I positively assert, that there are originally globules in the Louse's blood, for they may be easily formed by the mixture of the blood with the fat, and some wounded particles of the* viscera *or bowels, which consist of a congeries or heap a sit were* of globular *parts"*(Bessis and Delpech, 1981).

In the April 1678 letter to Mr. Trevenot, Swammerdam admitted that he had learned how to use the capillary glass tubes (needed to draw blood) from Leeuwenhoek, though he was very disrespectful and arrogant. *"Sir, I sent you by the last post a complete anatomy of a human louse (pediculus hwnanus). It is a very curious invention I use to anatomize the louse. The first one who used the Tubulus* vitreus *is a Dutchman, living at Delft, named Leeuwenhoek, but all he could do with the microscopes concerns only externa, or things he puts in his glass tubes. It is impossible to go into discussion with him, as he is biased, and reasons in a very barbarical way, having no academic education."*

It is very difficult to verify Swammerdam's contribution and timing because most of his letters were published posthumously, and it is possible that he may have added different

interpretations and revised the dates in his manuscripts just before his death (Bessis and Delpech, 1981).

3.4.2 General properties of red blood cells

Red blood cells or erythrocytes move within the blood vessels and transport O_2 and CO_2 both of which are bound to hemoglobin. A typical concentration of erythrocytes in human blood is 5×10^{12} cells/liter. Gross erythrocyte volume exceeds 2 L or approximately 10% of total cell volume. Erythrocytes, like many other types of blood cells, originate from a pluripotent stem cells found in the bone marrow. During the formation of red blood cells, erythroblasts (a developing red blood cell) extrudes its nucleus to become an immature erythrocyte (reticulocyte), which then exits the bone marrow and moves into the bloodstream. The reticulocyte loses its mitochondria and ribosomes as it becomes a mature erythrocyte within a few days after entering the bloodstream. Aged or old erythrocytes are removed by macrophages in the liver and spleen, both of which phagocytize 2×10^{11} aged erythrocytes per day, representing almost 20 ml of packed cell volume. The erythrocyte cytoplasm contains hemoglobin, the iron-containing oxygen-transport metalloprotein that can bind oxygen and responsible for the red color associated with blood. (Alberts et al., 2002). Each molecule of hemoglobin contains four iron atoms, and each iron atom can bind with one molecule of oxygen (which contains two oxygen atoms, called O_2) for a total of four oxygen molecules ($4\times O_2$) or eight atoms of oxygen bound to each molecule of hemoglobin. A single erythrocyte contains 270 million hemoglobin molecules. Mature erythrocytes lack a cell nucleus and most other organelles in order to provide maximum space for hemoglobin transport. Erythrocytes are simple cells and have a limited number of metabolic pathways. The glycolytic pathway is the sole source of ATP in erythrocytes, and is thus, very important for cell survival (Granick, 1949). Human red blood cells

circulate about 120 days (20 s per circulation) and are normally oval and flexible biconcave discocytes with a diameter of ~8 μm, a surface area of ~140 μm^2 and a volume of ~100 μm^3 (Evans and Fung, 1972). The total erythrocyte area is ~3,400 m^2, which makes it capable of transporting large amounts of oxygen equal to ~270 mL/s or 5.4 L per one blood circulation.

3.4.3 Life, aging and death of red blood cells

3.4.3.1 Erythrocyte functions

After 7 days of maturation, a reticulocyte becomes an adult and fully functional erythrocyte. The primary function of erythrocyte is to transport oxygen. Iron atoms of hemoglobin bind oxygen molecules in the lungs and deliver them throughout the body. Additionally, erythrocytes transport carbon dioxide arising from the tissues and transport it to the lungs in a bicarbonate form (Granick, 1949). Erythrocytes also perform additional responsibilities beyond the transport of oxygen and carbon dioxide. Erythrocytes can influence or change vascular properties and enhance blood flow by way of various mechanisms. When placed under mechanical strain in narrow blood capillaries, erythrocytes release ATP that cause the capillary walls to relax or dilate so as to enhance blood flow (Wan et al., 2008). Another way erythrocytes control vascular properties involves deoxygenated molecules of hemoglobin that cause erythrocytes to release S-nitrosothiols which also serve to dilate vessels, thus resulting in more blood being delivered to areas with an oxygen deficit (Diesen et al., 2008). Erythrocytes are capable of producing nitric oxide, using L-argine as substrate (Kleinbongard et al., 2006), and under shear stress nitric oxide synthase is activated and that results in the release of nitric oxide which can modulate mechanical properties of blood vessels (Ulker et al., 2009). It has been demonstrated that erythrocytes can also produce hydrogen sulfide, that is known to relax vessel walls (Benavides et al., 2007). Erythrocytes also participate in defense against pathogenic

bacteria. When a bacterium lyses an erythrocyte, the released hemoglobin molecule elicits an antimicrobial action in the vicinity of the bacteria by discharge of the reactive oxygen species that in turn kills microbes (Jiang et al., 2007).

3.4.3.2 Eryptosis

Mature erythrocytes undergo aging and eventually they are recognized and cleared by macrophages (Rous, 1923, Rous and Robertson, 1917). The aging erythrocyte (Ghashghaeinia et al., 2012) is characterized by changes in the plasma membrane, that make them selectively recognized by macrophages and then they are phagocytized in spleen, liver, or bone marrow. This process is termed eryptosis or programmed erythrocyte death (Bratosin et al., 2001, Föller et al., 2008, Lang and Qadri, 2012, Vittori et al., 2012).

Eryptosis, is characterized by erythrocyte volume reduction, membrane microvesiculation (release of small vesicles) and cell membrane phosphatidylserine externalization. Phosphatidylserine-exposing erythrocytes are recognized by macrophages, which engulf and ingest the dying cells. The regulated form of eryptosis is induced by Ca^{2+} influx, and prevented by cysteine protease inhibitors (Bratosin et al., 2001).

Known triggers of eryptosis include osmotic shock (Bosman et al., 2011), oxidative stress (Richards et al., 2007), energy depletion (Lutz et al., 1977), ceramide (Lang et al., 2010), hyperthermia (Foller et al., 2010), prostaglandin E2, platelet activating factor, hemolysin, listeriolysin, paclitaxel, chlorpromazine, methylglyoxal, cyclosporine, amyloid peptides, Bay-5884, anandamide, curcumin, valinomycin, aluminum, mercury, lead and copper (Föller et al., 2008).

Diseases and clinical conditions associated with accelerated eryptosis include sepsis, malaria, sickle-cell anemia, b-thalassemia, glucose-6-phosphate dehydrogenase (G6PD)-

deficiency, phosphate depletion, iron deficiency, hemolytic uremic syndrome, hereditary spherocytosis, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome, sepsis, renal insufficiency, diabetes, malaria, mycoplasma infection, and Wilson's disease. (Föller et al., 2008, Lang and Qadri, 2012).

Eryptosis may be inhibited by erythropoietin, adenosine, catecholamines, nitric oxide (NO) and activation of G-kinase (Föller et al., 2008). The oxidative eryptosis is prevented by the antioxidant systems that include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase which withstand glutathione regeneration, and NADH-methemoglobin reductase. Additionally, endogenous non-enzymatic antioxidants also provide protection against oxidative injury: they are lipophilic (vitamin E, carotenoids, melatonin) and water soluble compounds (vitamin C, glutathione, ceruloplasmin, uric acid) (Vittori et al., 2012).

3.4.3.3 Physiological significance of eryptosis

One of the purposes of eryptosis is the avoidance of cellular swelling which would eventually lead to hemolysis. If an erythrocyte is injured (energy depletion, defective Na^+/ K^+ATPase or enhanced leakage from the cell membrane) the eryptotic machinery cannot work. It leads to increase of intracellular of Na^+ and Cl^- ions so that the osmotic pressure moves water into the cell with subsequent cell swelling. The cellular K^+ loss decreases the K^+ equilibrium potential leading to membrane depolarization. The decrease of electrical gradient across the cell membrane slows Cl^- entry, which is followed by water influx driven by osmotic pressure. The resulting cell swelling compromises the integrity of the cell membrane and causes cell hemolysis (Föller et al., 2008). The swelling and rupture of the erythrocyte membrane results in the release of cellular hemoglobin. Released hemoglobin is normally filtered in the renal glomeruli. A high hemoglobin load can cause kidney dysfunction (Aronson and Blumenthal, 1998). Released

hemoglobin is additionally captured by haptoglobin, which is then recognized by hemoglobin scavenger receptors and endocytosed by macrophages (Kristiansen et al., 2001). When haptoglobin is depleted during critically elevated hemolysis (Rother et al., 2005), the released hemoglobin is collected by the endogenous metal nanoparticles (Samoylov et al., 2005). In any event, excessive cell hemolysis is a negative phenomenon (Rother et al., 2005).

Hemolysis is prevented if erythrocyte swelling is preceded by eryptosis. Eryptosis is elicited by an elevation in cytosolic Ca^{2+} activity, which initiates cell membrane vesiculation, cell membrane scrambling and activation of the cysteine endopeptidase calpain, an enzyme that degrades the cytoskeleton and thus causing cell membrane blebbing. Ca^{2+} further stimulates Ca^{2+}-sensitive K^+ channels, the following outflow of K^+ hyperpolarizes the cell membrane, which causes outflow of Cl^-. The cellular loss of KCl combined with osmotically driven water leads to cell shrinkage and phosphatidylserine externalization of eryptotic cells. This leads to macrophage recognition and ingestion of dying erythrocytes (Lang and Qadri, 2012). The erythrocyte is broken down by phagocytic action, and the hemoglobin molecule is split. The globin chains are reutilized, while the iron-containing portion or heme group is further broken down into iron, which is reutilized, and bilirubin, which is conjugated with glucuronic acid within hepatocytes and secreted into the bile (Clark et al., 2000, Llesuy and Tomaro, 1994, Willekens et al., 2005, Rous, 1923).

3.4.4 Erythrocyte membrane

3.4.4.1 Composition of erythrocyte membrane

The membrane of the erythrocyte performs numerous functions which helps it in maintaining its surface deformability, flexibility, adhesion to other cells, as well as immune recognition. These features are dependent on its composition, which in turn specifies its

properties. The erythrocyte membrane consists of 3 distinct layers or tiers: the glycocalyx is the outer most tier and is abundant with carbohydrates; next is the lipid bilayer which includes numerous transmembrane proteins, in addition to its lipidic major constituents; and the inner most tier or layer is the membrane skeleton, which is a structural system of proteins situated on the internal surface of the lipid bilayer. Half of the membrane total mass, in humans and many other mammalian species' erythrocytes, is comprised of proteins. The other 50 percent of the mass is comprised of lipids, specifically phospholipids and cholesterol. (Yazdanbakhsh et al., 2000).

3.4.4.2 Lipids

The red blood cell membrane consists of a regular lipid bilayer, the same as what is present in practically all other animal cells. In other words, the lipid bilayer is comprised of cholesterol and phospholipids in equal amounts by weight. The lipid composition is essential since it defines numerous physical properties, including membrane permeability and fluidity. Furthermore, the function of several membrane proteins is controlled by interactions with lipids within the bilayer.

In contrast to cholesterol, which is equally allocated amongst the internal and external layers, the 5 main phospholipids are asymmetrically or unevenly distributed. The external monolayer is composed of phosphatidylcholine and sphingomyelin, while the internal monolayer is comprised of phosphatidylethanolamine, phosphatidylserine, and small amounts of phosphoinositol. The asymmetric lipid distribution in the membrane is a result of the action of several phospholipid transport proteins. These proteins, known as "flippases," transfer phospholipids from the external to the internal monolayer. While another group of proteins termed "floppases," perform the opposite or antagonistic movement against a concentration

gradient in an energy dependent process. Furthermore, there are also "scramblase" proteins that transfer phospholipids in either direction simultaneously, along their own concentration gradients in an energy independent manner (Mohandas and Gallagher, 2008).

The retention of asymmetric lipid distribution in the membrane (such as the preeminent position of phosphatidylserine and phosphoinositol in the internal monolayer) is vital for the cell's stability as well as functionality because of a number of factors, including:

(1) Macrophages identify and phagocytize erythrocytes that display phosphatidylserine on their external surface. Therefore, the detention or restriction of phosphatidylserine in the internal monolayer is critical if the erythrocyte is to endure its regular interactions with macrophages. (2) Early destruction of erythrocytes in the state of disease (e.g. thalassemia and sickle red cells) has long been associated with breaking of lipid distribution resulting in exposure of phosphatidylserine on the external monolayer. (3) An exposure of phosphatidylserine may activate adhesion of erythrocytes to vascular endothelial cells, blocking normal transport through the blood vessels. Hence it is essential that phosphatidylserine is retained exclusively in the internal layer of the membrane. (4) Both phosphatidylserine and phosphatidylinositol-4, 5-bisphosphate (PIP2) can control membrane mechanical performance, because of their interactions with skeletal proteins such as spectrin and protein 4. 1R (Mohandas and Gallagher, 2008, Yazdanbakhsh et al., 2000).

The existence of specific structures termed "lipid rafts" in the erythrocyte membrane have been described by recent reports. These structures are enriched with cholesterol and sphingolipids related to specific membrane proteins, namely flotillins, stomatins (band 7), G-proteins, and β-adrenergic receptors. Lipid rafts have been implicated in erythrocyte

morphological changes, intercellular signaling, and malaria parasite infection (Kamata et al., 2008, Kriebardis et al., 2007, Motoyama et al., 2009, Murphy et al., 2006).

3.4.4.3 Proteins

There are presently more than 50 recognized membrane proteins which can be found in concentrations ranging from a couple of hundred up to a million copies per red blood cell. About twenty five of these membrane proteins have the blood group antigens, including the A, B and Rh antigens, among numerous others. These membrane proteins are capable of performing a broad range of functions, such as transporting ions and molecules through the erythrocyte membrane, adhesion and interaction with other cells such as endothelial cells, as signaling receptors, along with other presently unknown functions. The blood types of humans are caused by variants in surface glycoproteins of erythrocytes. Disorders of these proteins in the membrane are connected with numerous disorders, including hereditary spherocytosis, hereditary elliptocytosis, hereditary stomatocytosis, and paroxysmal nocturnal hemoglobinuria (Mohandas and Gallagher, 2008, Yazdanbakhsh et al., 2000).

Erythrocyte membrane proteins are arranged in accordance with their particular function:

Erythrocyte transport proteins.

Band 3 - Anion transporter, is an essential structural part of the red blood cell membrane, and it comprises up to 25% of the total cell membrane surface. Every red blood cell includes approximately one million copies of the anion transporter molecules and is inclusive of the Diego Blood Group (Junqueira and Castilho, 2002). When hemoglobin starts to denature, it creates hemichromes which cross-link the main erythrocyte membrane-spanning protein, band 3, into clusters. These clusters supply the recognition site for antibodies directed against senescent red blood cells. Those antibodies interact with the aged erythrocytes and induce their elimination

from blood circulation (Low et al., 1985). It was shown that artificially denatured hemoglobin binds to and clusters the protein, band 3, in the red blood cell membrane. The comparison of the locations of denatured hemoglobin aggregates (Heinz bodies) with band 3 in sickle cells employing phase contrast and immunofluorescence microscopy demonstrated that Heinz bodies and clusters of band 3 are often colocalized inside the membrane. When compared, ordinary red blood cell membranes lacking Heinz bodies exhibit a continuous staining of band 3. Likewise, ankyrin and glycophorin are occasionally seen to aggregate at Heinz body sites, but the level of colocalization is lower as compared to band 3. All these results show that the binding of denatured hemoglobin to the membrane causes a redistribution of a number of important membrane components (Waugh et al., 1986).

Aquaporin 1 - water transporter, identifies the Colton Blood Group (Antonelou et al., 2011, Agre et al., 1995)

Glut1 - glucose and L-dehydroascorbic acid transporter (Montel-Hagen et al., 2009).

Kidd antigen protein - urea transporter (Olivès et al., 1995).

RhAG - gas transporter, most likely of carbon dioxide, becomes Rh Blood Group and the linked uncommon blood group phenotype Rh_{null} (Endeward et al., 2008).

Na+/K+ - ATPase (Raccah et al., 1996);

Ca2+ - ATPase (Guerini et al., 1984);

Na+ K+ 2Cl- - co-transporter (Flatman and Creanor, 1999);

Na-H exchanger (Rutherford et al., 1997);

K-Cl – co-transporter (Lauf et al., 1992);

Gardos Channel (Maher and Kuchel, 2003).

Muscarinic receptor M1 (Tang, 1986, Tang, 1991),

Acetylcholinesterase (Butikofer et al., 1989, Carvalho et al., 2004, Chernitskii et al., 1994, Lutz et al., 1977)

Erythrocyte cell adhesion proteins

ICAM-4 - binds to integrins (Spring et al., 2001);

BCAM - a glycoprotein that identifies the Lutheran blood group and as well referred to as Lu or laminin-binding protein (Parsons et al., 2001).

Proteins playing a structural role in erythrocyte

The following membrane proteins create linkages with skeletal proteins and might play an essential function in controlling cohesion between the lipid bilayer and membrane skeletal system, most likely allowing the red cell to keep its advantageous membrane surface area simply by blocking the membrane from collapsing (vesiculating) (Willekens et al., 2008).

Ankyrin-based macromolecular complex - proteins connecting the bilayer to the membrane skeleton throughout the interaction of their cytoplasmic domains with ankyrin. The link between bilayer and membrane skeletal system, created through the interactions of the cytoplasmic domains of numerous membrane proteins with the spectrin-based skeletal system. Band 3 and RhAG present this kind of links simply by interacting with ankyrin, which in turn binds to β-spectrin. (Mohandas and Gallagher, 2008).

1) Band 3 - also assembles numerous glycolytic enzymes, the presumptive CO_2 transporter, and carbonic anhydrase into a macromolecular complex named a “metabolon, ” which might play a vital role in regulating red cell metabolism and ion and gas transfer function (Mohandas and Gallagher, 2008, Sowah and Casey, 2011).
2) RhAG - also linked to transport, defines associated unusual blood group phenotype Rhmod (Avent and Reid, 2000, Endeward et al., 2008).

Protein 4.1R-based macromolecular complex - proteins interacting with Protein 4.1R. Protein 4.1R is a multifunctional element of the red cell membrane. It generates a ternary complex with actin and spectrin, which identifies the nodal junctions of the membrane-skeletal system, and its connection to the transmembrane protein glycophorin C produces a connection between the protein system and the membrane bilayer. 4. 1R arranges a macromolecular complex of skeletal and transmembrane proteins at the junctional node and that perturbation of this macromolecular complex not simply accounts for the properly recognized membrane lack of stability but could also redesign the erythrocyte surface. (Salomao et al., 2008).

1) Protein 4. 1R - weak expression of Gerbich antigens (Reid et al., 1987);
2) Glycophorin C and D - glycoprotein, specifies Gerbich Blood Group (Reid et al., 1987);
3) XK - identifies the Kell Blood Group and the Mcleod unusual phenotype (lack of XK antigen and significantly decreased expression of Kell antigens) (Kaita et al., 1959);
4) RhD/RhCE - defines Rh Blood Group and the associated unusual blood group phenotype Rh_{null} (Avent and Reid, 2000)
5) Duffy protein - has been suggested to be related to chemokine clearance (Denomme, 2004);
6) Adducin - interaction with band 3 (Gardner and Bennett, 1987);
7) Dematin- interaction with the Glut1 glucose transporter (Azim et al., 1996).

3.5.4.4 Surface electric potential

The zeta or electrokinetic potential is an electrochemical property of cell surfaces, which depends on the net electrical charge of surface-exposed molecules. The erythrocyte membrane is negatively charged and is enveloped with a fixed layer of cations in the liquid medium. This fixed layer of cations is surrounded by a fog-like diffused layer of cations and anions. Within the diffused layer, movement of erythrocytes in the medium produces a ‘shear’ sliding surface,

which divides unfixed ions from those ions tightly linked to the fixed layer. The potential at the shear surface is termed as the zeta potential (Kruyt, 1952). The normal zeta potential of the erythrocyte membrane is -15.7 millivolts (Tokumasu et al., 2012). This potential appears to be created by the exposed sialic acid residues in the erythrocyte membrane, and their removal results in the reduction of zeta potential. Erythrocytes in the course of microcirculation, and aging, lose sialic acid along with other molecules thus changing cell structures, certain properties and functions. The zeta potential of young erythrocytes is reduced steadily with aging. Fluorescent studies, combined with zeta-potentials provided evidence about the smaller level of charge density and sialic acid on old erythrocytes, and a linear reduction in zeta potentials. A close correlation was observed between the surface charge on an aging red blood cell and its structure and functions. The surface charge has been found to impact cell morphology and membrane deformability (Huang et al., 2011, Jovtchev et al., 2000, Petelska et al., 2012, Dobrzyńska et al., 1999, Sangeetha et al., 2005).

There are few factors that influence a value of zeta potential and a surface density of electric charge. According to the electrochemical theory of double layer (Kruyt, 1952), values of zeta potential and electric charge are reduced when the cell surface is exposed to a higher ion concentration of surrounding media. This reduction is more pronounced for media with multivalent ions. An increase of H^+ ions (pH decrease) also reduces zeta potential and charge. Additionally, a surface binding of positively charged mineral or organic ions reduces zeta potential and a surface density of electric charge. These theoretically predicted properties have been experimentally demonstrated for erythrocytes. The surface charge density was reduced by an increase of ionic strength of electrolytes (Jan and Chien, 1973a), and the membrane becoming more positive at a lower pH and more negative at a higher pH (Petelska et al., 2012).

At certain conditions red blood cells aggregate face-to-face to form long, coin-like cylindrical, chains structures called rouleaux (Samsel and Perelson, 1982). When two erythrocytes come face-to-face at close distance, their interaction is determine by two major forces: longer range capillary and Derjaguin repulsive forces over van der Waals attractive forces (Kralchevsky and Nagayama, 1994, Kruyt, 1952, Oettel and Dietrich, 2008). In order for the aggregation to occur, the attractive forces should overcome the repulsive interactions. It was showed, that in normal erythrocytes at ordinary conditions carry a relatively large negative electric charge (~4.5×10^{-2} C/m^2 (Dobrzynska et al., 2004)) that provides strong repulsive forces that prevent aggregation. However, if the electric charge is reduced by removing sialic acids from erythrocyte membrane, or other external conditions applied that reduce zeta potential (increase ion concentration, pH decrease, binding positively charged molecules), red blood cells aggregate (Jan and Chien, 1973b, Jan and Chien, 1973a). Aggregated red blood cells reduce their capacity for oxygen transport (Marossy et al., 2009). It is remarkable to think that more than 2000 years ago Celsus is reported to have made a statement that corresponds to this condition: "Blood is bad when it is too thin or too thick, livid or black in color, it is best when hot, red, moderately thick, and not sticky"(Celsus, 1814 (ca 25 BC—ca 50)).

3.4.5 Red blood cell discocyte-echinocyte transformations

Earlier observations of blood by Schultz , Ehrlich and Lazarus (Ehrlich and Lazarus, 1900), and Rous and Robertson (Rous and Robertson, 1917) indicated that there is a great diversity of the transitional erythrocyte forms: spheres, capsules, shapes like drumsticks, rods, dumb-bells, pears, balls with strings attached to them, and even short, and thick threads brightly tinted with hemoglobin. The most important erythrocyte forms that are presently recognized are discocytes (normal biconcave discoid), echinocytes (crenated or spiculated discs and spheres,

and stomatocyte (bell-like structure) (Bessis, 1972, Brecher and Bessis, 1972, Kayden and Bessis, 1970, Longster et al., 1972). (Figure. 3).

The excess surface area of red blood cells (which provides them with a flattened shape), along with the elasticity of their membranes, can provide erythrocytes with the flexibility required to go through the capillary vessels. As we have seen in previous sections, the cell membrane consists of a lipid bilayer, with inlayed membrane proteins, and connected to it from the cytoplasmic side is a system of proteins that comprises the membrane cytoskeleton. Both membrane components contribute to mechanical properties of red blood cells (Khairy et al., 2008, Lim et al., 2002, Tachev et al., 2004).

It has been demonstrated that the normal red blood cells (discocytes) go through shape changes toward stomatocytes or echinocytes (Figure 3) at variation of the electrolyte concentration (Backman, 1986, Deuticke, 1968, Elgsaeter and Mikkelsen, 1991, Glaser et al., 1987, Herrmann et al., 1985, Rasia and Bollini, 1998), increase in the medium's pH (Gimsa, 1998, Libera et al., 1997, Gedde and Huestis, 1997, Gedde et al., 1997, Gedde et al., 1995, Rasia and Bollini, 1998), the addition of amphiphiles and other substances (Deuticke, 1968, Bessis, 1972, Brecher and Bessis, 1972, Kayden and Bessis, 1970, Iglic et al., 1998, Glaser et al., 1991, Isomaa et al., 1987, Sheetz and Singer, 1974, Gimsa, 1998), and variations in temperature (Glaser and Donath, 1992, Glaser, 1979). A general relationship was observed between the erythrocyte shape and the transmembrane electric potential (Glaser, 1998, Glaser, 1993, Muller et al., 1986, Bifano et al., 1984, Glaser, 1982). The effect of pH and other substances could be additionally related to their effect on the transmembrane potential (Glaser, 1998).

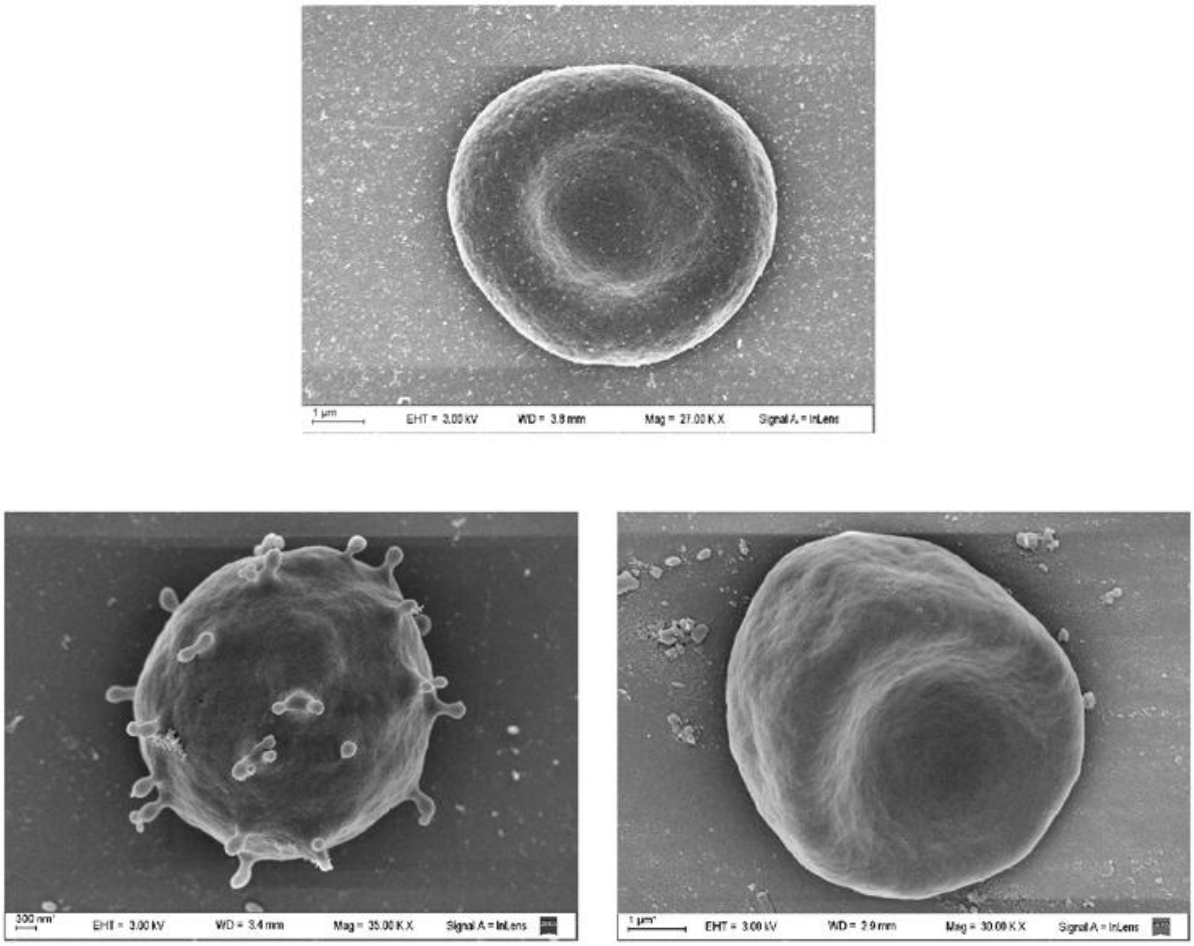

Figure 3. Discoid, ehinocyte and stomatocyte. Altered shapes of erythrocytes subjected to proeryptotic agents. The normal discoid biconcave shape (top) turned to spherocyte with microvesiculation due to increased intracellular calcium concentration (left bottom) or to stomatocyte induced by oxidative stress (right bottom) (Vittori, D., D. Vota, et al. (2012)).

High electrolyte concentration, high pH, and elevated temperature serve to induce a series of crenated shapes, echinocytes, characterized by convex rounded protrusions or spicules. Under further loading, the spicules become smaller and more numerous and eventually bud off irreversibly, forming extracellular vesicles composed of plasma membrane materials (Lim et al., 2002, Allan et al., 1976, Allan and Michell, 1975).

We hypothesize that elevation of temperature during a heat stress may cause transformation of some of the normal erythrocytes into speculated echinocytes that shed membrane vesicles. The increase of the number of membrane vesicles produced during heat

stress is relational to a number of discocyte-echinocyte transitions. Therefore, we hypothesize that the increase of vesicle number following heat stress may be indicative of the stress level.

3.4.6 Vesicles released by red blood cells at normal conditions

The small vesicles released by erythrocytes and obtained from a freshly drawn blood sample at normal conditions and at elevated temperatures were first noted by Schultze (Schultze, 1865) and described in the next section. Ehrlich and Lazarus, in their fundamental work on blood histology (Ehrlich and Lazarus, 1900), described the fragmentation of erythrocytes, and coined the term "schistocytes" for the fragmented parts of human red blood cells. They explained that smallest fragments "microcytes" appear by "budding and division of the red corpuscles"(Ehrlich and Lazarus, 1900). Later, the presence of microcytes in blood were confirmed in the research on the normal fate of erythrocytes, and it was found that a disintegration of erythrocytes takes place constantly in sickness (Robertson and Rous, 1917) and health (Rous, 1923, Rous and Robertson, 1917). The Kupffer cells of the liver were found to phagocytize most of the aged erythrocytes with a daily rate of 2.9% measured by bilirubin yield (Rous, 1923). This rate of erythrocyte destruction/production agrees well with those reported in contemporary findings (Alberts et al., 2002, Granick, 1949). Further the Kupffer cell mechanisms have been confirmed to be a main removal pathway of aged erythrocytes and erythrocyte-derived vesicles (Werre et al., 2004, Willekens et al., 2005).

The alteration in the shape of erythrocytes from the ordinary biconcave disc to a spiculed sphere (echinocyte) (Brecher and Bessis, 1972) can be triggered by an elevation of intracellular concentration of Ca^{2+} (Weed and Chailley, 1972, Allan and Michell, 1975). Vesicles arising from human erythrocytes while in the echinocyte state were collected and analyzed (Allan et al., 1976, Rumsby et al., 1977). The authors speculated that these vesicles budded off from the

echinocyte spicules. Electron microscopy showed about 100 nm vesicles in size and that they contained hemoglobin, 1, 2-diacylglycerol and band 3 membrane protein. Spectrin and actin were noted as being absent in vesicles.

In 1984, large 0.5-1 μm vesicles were obtained by budding from fresh human erythrocytes and visualized by both light and electron microscopy (Leonards and Ohki, 1983). The vesicles were similar to those observed by Schultze and Ehrlich (Ehrlich and Lazarus, 1900, Schultze, 1865), the use of high resolution microscopy allowed the authors to describe the process of vesiculation induced by EDTA and $CaCl_2$ at 45 ^{o}C. Starting with ordinary discoid erythrocytes, the cells first are converted to spherical shape, and then begin to form buds. These buds continue to grow and are finally "released" from the cell surface to become free vesicles. Typically only one bud was produced per erythrocyte, two buds were rare, and three buds were quite seldom. The vesicles were cytoskeleton-free and contained hemoglobin and band 3 membrane protein (Leonards and Ohki, 1983). Very small vesicles of 50 to 200 nm in diameter were found in fresh human blood plasma. These vesicles were separated by a high speed centrifugation and imaged by transmission electron microscopy. The vesicle membranes showed presence of hemoglobin and absence of spectrin (Dumaswala and Greenwalt, 1984) similar to those found in erythrocytes (Rumsby et al., 1977, Allan et al., 1976).

It has been demonstrated that during vesiculation of human erythrocytes in vitro, acetylcholinesterase is redistributed on the cell surface and becomes enriched in the released vesicles. As a result, the remnant cells are depleted of these proteins (Butikofer et al., 1989, Lutz et al., 1977). Although, acetylcholine receptors and acetylcholinesterase are present in erythrocyte membranes, (Tang, 1986, Tang, 1991) their roles are not well understood. It was shown that acetylcholine and choline modulate nitric oxide metabolites on erythrocytes and this

effect is mediated by interactions with erythrocyte membrane muscarinic receptors and membrane enzyme acetylcholinesterase (Carvalho et al., 2004). The authors speculated that acetylcholinesterase participates in the signal transduction mechanism in response to the action of acetylcholine on nitrite and nitrate production in human erythrocyte. We suggest that vesicles enriched in acetylcholinesterase may play signaling role, and thereby serve to indicate a general level of vesiculation in the erythrocyte pool.

Vesicle creation is associated by the degradation of band 3 protein. It has been postulated that elimination of senescent erythrocytes by macrophages is mediated by senescent cell-specific autoantigens originated on band 3, the anion exchanger and the major membrane protein of the erythrocyte (Kay, 2005). In line with this assumption, Willekens et al. (Willekens et al., 2008) verified that vesiculation is not just linked to the removal of membrane bound hemoglobin, but is related to generation of senescent cell antigen, a neoantigen that comes from band 3 immediately after its breakdown in senescent red blood cells.

The overall volume of erythrocytes is reduced as the cell undergoes senescence and it has been noted that a significant amount of hemoglobin is lost during the erythrocyte's total lifetime (Bosman et al., 2008a, Bratosin et al., 2001, Greenwalt, 2006, Vittori et al., 2012, Willekens et al., 2008, Rous, 1923, Willekens et al., 1997, Willekens et al., 2003). This is due to loss of membrane through microvesiculation, resulting in erythrocyte dehydration, membrane protein depletion, elevated density, and decrease in deformability (Bosch et al., 1994).

Depending on a combined volume/density cell fractionation method, it has been shown that throughout the lifespan of the mature erythrocyte, volume and hemoglobin content are reduced by 30 and 20%, respectively, while the hemoglobin concentration increases by 14% (Werre et al., 2004). In addition, the decrease in hemoglobin content, surface area and lipid

content equally decrease by 20%, while the actual surface-to-volume ratio increases. The decrease of hemoglobin and surface area results from the creation and discharge of hemoglobin-containing vesicles. This process takes place throughout the cell lifespan but is hastened in the second half of the 120 day life cycle (Willekens et al., 2003). Supposing that vesicles are spherical and that they contain the same concentration of hemoglobin as the parent red blood cell, the reduction of 20% of total hemoglobin is equal to the loss of 15 μm^3 of membrane. Using an average vesicle diameter of 0.5 μm (Werre et al., 2004), one erythrocyte creates ~230 vesicles in the course of its lifespan. This is equivalent to a membrane area of 180 μm^2, while the erythrocyte surface area is reduced by approximately 30 μm^2 (Bosch et al., 1994). Hence, there is not enough membrane in the entire erythrocyte to package 20% of the hemoglobin into the vesicles. The authors concluded: "Taken together, these data suggest that most vesicles are taken up almost directly by the macrophages of the organ in which they originate before they can reach the venous circulation and be counted. Apparently, the body has developed an efficient mechanism to remove these vesicles..." (Willekens et al., 2008, Werre et al., 2004). Being not aware of the direct removal mechanism of hemoglobin by macrophages, the authors explained the extra loss of surface area in comparison to the hemoglobin loss by incorporation of lipids (Bosman et al., 2008a). This explanation is very unlikely, because during erythrocyte aging, the cell loses 20% of lipids, which is equal to a loss of hemoglobin (Rumsby et al., 1977). We hypothesize, that an alternative mechanism of direct transfer of damaged hemoglobin does exist but remains unknown.

3.4.7 Red blood cell vesicles at elevated temperature

In his work entitled "A heated microscope stage and its use in investigation of the blood" Schultze (Schultze, 1865) used a compound microscope and a specially constructed wet chamber to observe and document changes of blood cells at well controlled elevated temperatures (37-65 ^{o} C) under magnification up to 800 X. Human subjects, dogs, cats, and rabbits were studied. He analyzed a small droplet of live blood collected from humans via fingerpick or venous blood on the glass microscope slide and carefully covered with a coverslip. The work resulted in two plates, 20 figures, and about 140 images of various blood cells and their transformations at elevated temperatures. At the temperature of 37 ^{o} C, most of the red blood cells are oval and flexible biconcave disks of about 8 microns in diameter. He also noted the presence of some amount of small spherical red blood cells that are scattered among ordinary cells. Small granules were also observed among other cells and vesicles. With high precision, Schultze described for the first time, four different types of leucocyte corresponding to what are now recognized as the lymphocyte, the monocyte, the neutrophil- polymorphonuclear leucocyte, and the eosinophil leucocyte. Highly granulated white blood cells with one or a few nuclei were described as "lovely creeping" among red blood cells.

At elevated temperatures approaching 52 ^{o} C, Schultze observed large morphological changes in red blood cells. The red blood cells change from the biconcave discs to the crenated discs, crenated spheres, and then into spheres with very small spicules. Additionally, he illustrated a few other forms that appeared as bells, symmetric and asymmetric bodies with large spicules, and deformed pinched spheres and even one more peculiar form that had a small round head with a flexible tail. He noted that many red blood cells appear to shed multiple vesicles. The population of the small granules that were seen at 37 ^{o} C increased. After 15 minutes at 52 ^{o} C, he noted that even stronger transformations occurred. Most of the structures were represented

by the motile bodies with small heads and long tails and by different size vesicles and granules. The tails of these bodies had been reduced in thickness and then fractured into small pieces like beads. After 20 additional minutes at 52 ^{o}C, only vesicles and granules of various sizes were observed.

More than 100 years later, the major morphological changes in red blood cells at elevated temperatures described by Schultze were confirmed by electron microscopy (Bessis, 1972, Brecher and Bessis, 1972, Kayden and Bessis, 1970, Longster et al., 1972). The authors failed to reference Schultze's groundbreaking paper. It is unfortunate that Schultze's work has not received a proper citation in scientific literature. For example, a comprehensive review by Peyton Rous (Rous, 1923) published in 1923 and entitled "Distraction of the red blood corpuscles in health and disease" covered time period from 1847 to 1922, listed 245 references, and did not mention Schultze's work. A few authors have mentioned Schultze's work only when giving him credit for the thermal fragmentation of red blood cells (Baar, 1974, Brown, 1946, Ham et al., 1948) and his description of white blood cells and thrombocytes (Brewer, 1994, Brewer, 2006).

Studies of osmotic and mechanical fragility of human red blood cells under elevated heat conditions ranging from 47 to 61 ^{o}C demonstrated striking morphologic alterations in the erythrocytes: gradual division with the creation of numerous new forms of different shapes and sizes (Ham et al., 1948). When exposing erythrocytes to heat at 48.6 ^{o}C and the time of exposure is increased from 5 to 30 minutes, the normal discocytes changed into spiculated echinocytes that generated multiple vesicles (Ham et al., 1948).The appearance of spheroid cells coincided with the development of gradual increase in the osmotic and mechanical fragility of the erythrocytes. The transformation in the red blood cells created by high temperature were irreversible and

independent of the nature of the suspension medium whether plasma, serum, or salt solution (Ham et al., 1948).

Wagner et al., studied vesiculation induced by elevated temperatures up to 49 ^{o}C in normal human erythrocytes with the use of a Nomarski differential interference-contrast (DIC) microscope at the original magnification of X1000 (Wagner et al., 1986). This technique allows for the observation of real life cell images. It was observed that the erythrocyte cell membrane goes through spontaneous vesiculation in vivo during and following exposure to heat. The cell turns into a crenated shape or becomes echinocytic, with many small projections (spicules). After some time, the projections extend and pinch off to create small free-floating vesicles. Based upon the presented biochemical data, it is suggested that a localized disruption of the normal membrane skeletal protein and lipid interaction is an essential step in vesiculation. The authors described a detailed sequence of morphologic events of creating red blood cell vesicles. Any time discocytes go through an echinocytic shape transformation, small projections (spicules) developed. These types of projections, which were most likely the sites of disrupted membrane protein-lipid interactions, could fall off and thereby expel myelin structures of various lengths. Over time these types of structures pinched of on their own to create beaded chains and lastly solitary vesicles 300 nm in diameter (Figure 4) (Wagner et al., 1986). A similar observation of erythrocyte vesicle formation through myelin-like structure under energy depletion by incubation in vitro without nutrients or by storage at 4^{o}C under subnormal conditions, were also observed with electron microscopy (Bessis, 1972, Bessis and Mandon, 1972).

A vesiculation process through myelin-like forms represents a different mechanism compared to mechanisms of vesicle shedding by a spiculated echinocyte. The vesiculation by myelin forms

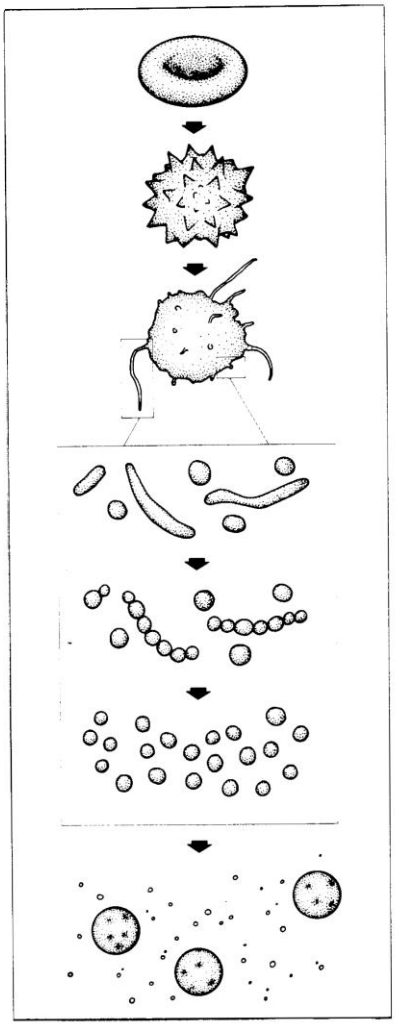

Figure 4. Figure Sequence of morphologic events producing red blood cell vesicles. When discocytes undergo an echinocytic shape change small projections form. These projections, which are probably the sites of disturbed membrane protein-lipid interactions, can pinch off releasing myelin figures of varying lengths. With time these figures themselves pinch off to form beaded chains and finally single vesicles of 300 nm in diameter (Wagner, et al., 1986).

observed with the use of a DIC microscope (Wagner et al., 1986) does not have enough optical resolution to adequately observe the formation of vesicles smaller than 300 nm, while electron microscopy (Bessis, 1972, Bessis and Mandon, 1972) cannot produce an accurate real life sequence of events.

We hypothesize that the observation of the myelin-like vesiculation by high resolution light microscopy may reveal the mechanism of this process with better detail, at higher magnification in smaller scale, and ultimately result in the visualization of vesicles that are much smaller than 300 nm.

3.4.7.1 Burns

It had been demonstrated that burns affecting greater than 15 % of the body surface are often accompanied by the immediate development of moderately severe anemia. Analysis of the morphological changes occurring in the red cells of severely burned patients has revealed that red blood cells undergo fragmentation, as well as other strong morphological changes that occur within a few hours of the injury. The morphological changes that develop in red blood cells in burn patients can be related to the immediate effects of heat on the red blood cells (Brown, 1946).

A blood sample from a patient with burns to approximately 75% of the body surface area showed strong morphological changes in a large fraction of normal red blood cell discocytes were converted into echinocytes. The erythrocyte changes observed in the blood of the burned patient compared well with those detected in samples of whole normal blood heated *in vitro* for 5 minutes at 49 ^{o}C. It was shown also that young red cells are more vulnerable than older cells (Baar and Arrowsmith, 1970, Vtiurin et al., 1987).

3.4.7.2 Proteons

As we discussed in section 3.5.3.3, when erythrocytes escape phagocytosis by macrophages they undergo hemolysis (Föller et al., 2008). When red blood cells are destroyed, hemoglobin is released into the plasma, where it dimerizes, and it immediately binds with the plasma protein haptoglobin. The haptoglobin-hemoglobin complex exposes a neoepitope that is recognized by the hemoglobin scavenger receptor, CD163 on the surface of monocytes/macrophages, which then binds the complex with high affinity and mediates haptoglobin-hemoglobin endocytosis and destruction (Kristiansen et al., 2001). Because haptoglobin is not recycled, formation of massive amounts of haptoglobin-hemoglobin complexes results in fast haptoglobin depletion. Therefore, in extreme hemolytic diseases such as malaria, haptoglobin is normally undetectable (Rother et al., 2005).

In order to examine the fate hemoglobin in blood plasma at conditions that produce high erythrocyte hemolysis, an Auburn University research group subjected samples of whole blood collected from a human, a rabbit, and a dog to osmotic shock with distilled water, and then separated diluted plasma by centrifugation, and incubated it at two different temperature regimes: 37 ^{o}C , ambient pressure, and 120 °C at 140 kPa (Samoylov et al., 2005, Vodyanoy et al., 2007). At low temperatures, within 13 days, small vesicles, termed proteons, were generated in the plasma at a concentration of ~3×10^{12} 1/mL, while it took less than 2 hours to generate the same amount of vesicles at high temperature and pressure. The vesicles could be generated very rapidly at much lower temperature 65 ^{o}C, and ambient pressure, if the plasma samples were subjected to cyclic amplification. The proteon growth turned out to be more efficient, after a small part of an earlier heated sample was added to untreated plasma that was consequently heated. Further rounds of the same process produced a large increase in the proteon

concentration. After having a small but significant increase in proteons throughout the initial two cycles, the third cycle generated a dramatic increase that displayed the saturation level of the system. This cyclic amplification process may be used to detect very low amounts of proteons in samples (Samoylov et al., 2005).

The authors (Samoylov et al., 2005) further demonstrated that proteons form by reversible seeded aggregation of proteins (mostly Hb) around proteon nucleating centers (PNCs). PNCs are composed of 1- to 2-nm metal nanoclusters that contain 40-300 atoms. Each milliliter of human blood contained around 7×10^{13} PNCs and approximately 3×10^{8} proteons. Subjecting isolated blood plasma to increased temperatures amplified the amount of proteons. The creation of proteons strongly depends on the presence of PNCs. When PNCs were taken from blood plasma by filtration through a 5-kDa filter, the amount of protein in the retentate was equal to that of the non-filtered sample, and no protein was present in the filtrate. However, proteons could not be created from the retentate until an aliquot of the filtrate, containing PNCs, was added back, and proteon production was dependent on the quantity of PNCs added (Samoylov et al., 2005).

Healthy human blood samples are normally found to contain about 3×10^{8} proteons/ml. Proteons appeared as particles of 0.05–5 μm in size when viewed by TEM or scanning EM (Samoylov et al., 2005, Vodyanoy et al., 2007). Similar particles were released by erythrocytes at elevated temperatures (Leonards and Ohki, 1983), elevated intracellular Ca^{2+} (Allan et al., 1976), and ATP depletion (Lutz et al., 1977).

We can summarize now, that there are three known ways for red blood cells to generate vesicles: First, echinocytes shed vesicles by way of their spicules (Christel and Little, 1984, Greenwalt, 2006, Brecher and Bessis, 1972, Vittori et al., 2012), second, vesicles can form from

aggregate of plasma hemoglobin lost after erythrocyte hemolysis by nucleated growth (Samoylov et al., 2005), and third, by way of myelin-like transformation of the erythrocyte membrane. The third method has been briefly described in studies using EM and light microscopy, but it is the most poorly characterized (Bessis, 1972, Bessis and Mandon, 1972, Wagner et al., 1986).

3.4.8 Vesicles originating from red blood cells during storage

The storage of blood originated through Lee and Vincent's efforts in 1913 that showed that citrate could prevent the coagulation of human blood (Stansbury and Hess, 2005).

Erythrocytes can be stored in liquid form in additive solutions for up to 6 weeks and they will experience 0. 4% hemolysis 84% 24 hours *in vivo* recovery, and normal recovery following survival of the cells that last in the circulation for at least 24 hours. Nevertheless, during storage erythrocytes undergo changes, including a decrease in the levels of adenosine triphosphate, diphosphoglycerate, and potassium. Further, erythrocytes will also experience oxidative injury to proteins, lipids, and carbohydrates, loss of overall shape and membrane appearance, elevated levels of adhesiveness, reduced flexibility, reduced capillary flow, and reduced oxygen delivery (Hess, 2010).

Under blood bank storage conditions, erythrocytes become extremely susceptible to a decrease of phospholipid asymmetry caused by hyperosmotic shock and energy depletion (Bosman et al., 2011). As a result, the storage of red blood cells is associated with the creation of vesicles that are characterized by particular proteomic profiles (Bosman et al., 2008b). A 20-fold increase in susceptibility for vesicle formation occurs after 50 days of storage at 4 ° C has been reported (Rubin et al., 2008). This elevated susceptibility could be the cause of the rapid disappearance of a substantial portion of the RBCs over the very first twenty four hours

immediately following transfusion (Bosman et al., 2011). Consequently, the number of vesicles in stored blood can be one of the parameters of a blood preservation quality control (Rubin, 2007).

3.4.9 Diagnostic value of red blood cell vesicles

Vesiculation is a natural process of a normal erythrocyte aging. Because vesiculation can be accelerated in some pathogenic conditions, the number of vesicles in blood can serve, under certain conditions as a diagnostic tool. The increased formation and release of vesicles in cancer cells, as confirmed by their elevated concentrations in blood in the late stages of the disease and their overexpression of certain cancer cell biomarkers, indicates an essential role of vesicles in medical diagnosis. (Simpson et al., 2009). By comparing patients diagnosed with colon cancer against patients diagnosed with other gastrointestinal diseases, a large and statistically significant difference between these two groups was found regarding the number of vesicles in blood (Jansa et al., 2008).

A number of studies have concentrated on the prospective diagnostic, as well as therapeutic applications of vesicles. Despite this interest, only 3 clinical vesicle research projects are registered on government clinical studies. One of these projects is a clinical trial, where tumor antigen-loaded dendritic cell-derived vesicles are employed to induce an anti-tumor reaction of the immune system in lung cancer patients. In another clinical trial, the diagnostic capability of tumor-derived exosomes as markers to observe reactions of neoadjuvant chemotherapy in newly diagnosed breast cancer is now being examined. The third registered clinical study investigates the capability of plant exosomes to deliver the anti-inflammatory agent curcumin to normal and colon cancer tissue (Ludwig and Giebel, 2012).

During physical exercise, the body temperature is elevated (Buono et al., 2005, Ogura et al., 2008). One of the serious consequences associated with a mild increase of temperature (~ 38 ^{o}C) is energy depletion (Kozlowski et al., 1985). This energy depletion in turn results in the accelerated erythrocyte-echinocyte transition (Backman, 1986, Brecher and Bessis, 1972) and increased vesicle shedding from speculated cells (Christel and Little, 1984). At temperatures higher than 38 ^{o}C, cellular hemolysis comes into effect, and vesicle production grows as the temperature increases (Gershfeld and Murayama, 1988). We therefore, hypothesize that the increase of the number of vesicles related to elevated temperatures experienced during exercise may serve as diagnostic of overall erythrocyte stability. Since decreased stability of red blood cells reduces their capability of oxygen transport (Nybo and Nielsen, 2001b, Nybo et al., 2001, Nybo et al., 2002), impairs individual endurance, and causes fatigue (El-Sayed et al., 2005, Periard et al., 2011), the increase in the number of vesicles due to exercise should be examined for its possible diagnostic value of human performance and oxygen capacity.

3.5. Light microscopy of the native blood

The earliest documented use of a light microscope can be traced back more than 4, 000 years ago to the Chow-Foo Dynasty. Ancient Chinese text accurately describes the design of a magnifying tube filled with water. By changing the level of water in this tube, the crude microscope could be adjusted to increase the magnification of an object by about 150 times (Rife, 1954). As previously discussed in section 3.5.1, the first published description of blood and red blood cells rightfully belongs to Antonie van Leeuwenhoek (van Leeuwenhoek, 1675). By the second half of 19th century, researchers had developed highly advanced light microscopes suitable for high resolution examination and photography of wide variety of small objects, including live cells. The microscopes of that era were equipped with a number of important

features, including: high numerical aperture (1.4); apochromatic objectives with iris; high aperture (1.4) achromatic condensers available for bright and dark field imaging with annular illumination (cone of light); with fine vertical adjustment of both the objective and condenser, and accurate and smoothly operated moveable mechanical stages controlled by micrometers. Regular slides, culture growing slides, and cover slips for high magnification imaging with the oil immersion were also available (Carpenter, 1875, Carpenter, 1891, Turner, 1979). The optical characteristics of these early microscopes are very comparable to those associated with modern light microscopes. During the years 1845-73 Friedrich Adolf Nobert used Fraunhofer's optical concept (Born and Wolf, 1999) of creating test plates with groups of lines of increasing fineness to accurately assess resolution and level of magnification, thereby staying one step ahead of the various microscope builders and inventors (Abbott, 1869, Stodder, 1868, Stodder, 1871). Microscope development continued to keep pace with Nobert's test plates until, unintentionally, he created a slide with a series of lines that no one had the ability to resolve (Turner and Bradbury, 1966, Davies et al., 2003). One of the most well-known of Nobert's plates was the nineteen-band test plate with a line spacing of·225 nm. Dr. J. J. Woodward was finally able to successfully to resolve it in 1869 (Woodward, 1869, Woodward, 1871). Following Woodward's success, Nobert produced a more accurate plate containing twenty bands (finest band 112. 8 nm). Dr. Woodward was not able to resolve the finest band of this plate and expressed serious doubts about its existence (Woodward, 1873). The existence of the Nobert's finest band of 113 nm

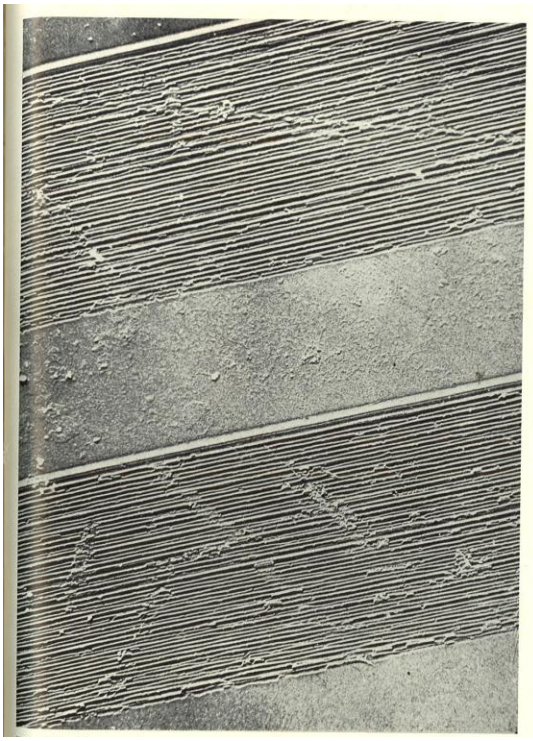

Figure 5. An electron micrograph of a replica of bands nineteen (above) and twenty. These are the finest bands, ruled at an average line spacing of about 0.12 and 0.11 microns respectively (Turner and Bladbury, 1966).

was proven in 1966 only by the use of electron microscopy (Figure 5) (Turner and Bradbury, 1966). Old published images of fine structures of diatoms (Figure 6) and a high resolution Nobert test plate (Figure 7) provide strong evidence that 19th century microscopists had access to microscope systems capable resolving objects separated by ~200-225 nm (Woodward, 1869, Woodward, 1871).

During the last quarter of the 19th century, Ehrlich and Lazarus made an important contribution to support the comprehensive microscopic analysis of blood (Ehrlich and Lazarus, 1900). They developed a complete dry blood specimen test technology that included procedures

for the preparation, fixation, and staining of dry specimens. Using this technology they were able to describe both normal and pathological histology of blood. The technology they developed, is now called “Blood smears” or “Blood films” and it is universally used today to diagnose many diseases (Bain, 2005). Ironically, Dr. Ehrlich, the inventor of this dry specimen technology, made a statement that “he has managed to make a diagnosis in all cases, with the examination of fresh blood”. In spite of Dr. Ehrlich’s personal opinion about the significance of fresh blood examination, the use of blood smears was the preferred method for diagnostics during the past 100 years. Only recently, has live blood cell research become increasingly widespread as new light microscopy methods are introduced and novel nanotechnology-based techniques emerge with the ability to examine the inner workings of live cells (Michalet et al., 2005).

Live blood cell microscopy came back into practice due to the combination of electron and light microscopy. An interference microscope outfitted with Nomarski optics was utilized to examine the red blood cells in the living state, while the scanning electron microscope was used to provide a magnified 3d visual aspect of the fixed erythrocytes (Kayden and Bessis, 1970). Studies that require observation real life cell interactions benefit substantially from live blood

Figure 6. Photo-micrograph of diatom *Pleurosigma angulatum* taken by Dr. R. Zeiss with apochromatic objective NA 1.30 and projection eye-piece 4X. A distance between diagonal rows is ~0.48 μm (Carpenter, 1891).

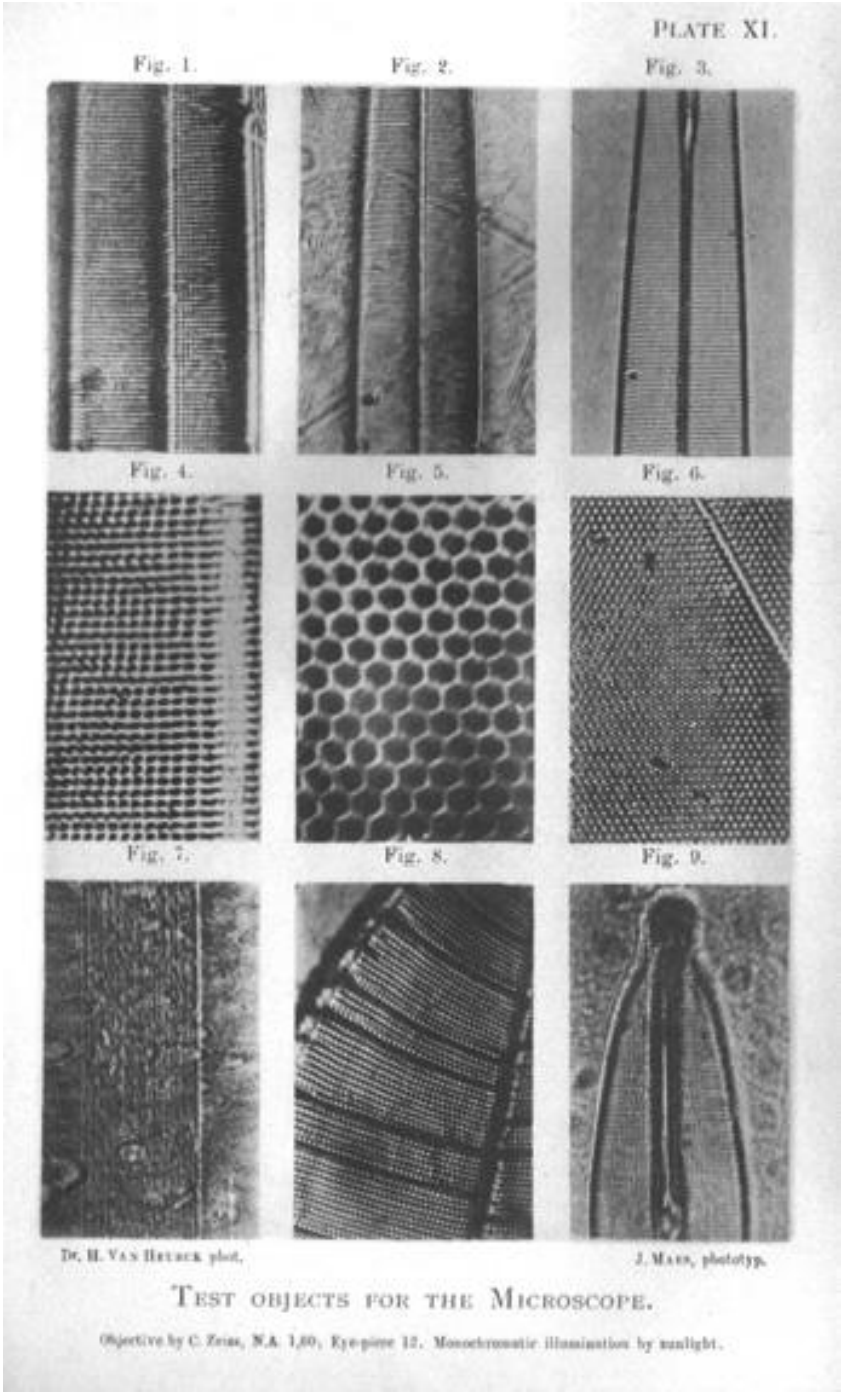

Figure 7. Diatoms photographed by Dr. H Van Heurck by Abbe objective with a NA 1.63. Fig. 1-3. *Amphipleura pellucida*. Fig. 1:3000X, Fig 2:2000X, Fig. 3:2300X. (A distance between transverse lines is ~0.195 μm). Fig. 4. *Ampiphleura* Lindhemeri, Gr., 2500X (A distance between transverse lines is ~0.625 μm. Fig. 5. *Pleurosigma angulatum*, 10,000X (A distance between diagonal rows is ~0.48 μm). Fig. 6. *Pleurosigma angulatum*, 2000X. Fig. 7. The nineteen bands of Nobert's test plate (NA 1.4) (A distance between rows is ~0.225 μm). Fig. 8. *Surirella gemma*, 1000X A distance between rows is ~0.90 μm). Fig. 9. *Van Heurell crassinervis*, 2000X A distance between rows is ~0.28 μm). (Carpenter, 1891).

light microscopy (Franco et al., 1990). Analysis of erythrocyte membrane mechanical properties also involves live cell observations. Erythrocyte shape changes during transformations from normal discocyte into echinocyte were analyzed from 3D confocal microscopy images of live cells (Khairy et al., 2008). Related studies were carried out with a noncontact optical interferometric technique to quantify the thermal fluctuations of erythrocyte membranes (Park et al., 2010). This technology supported researchers ability to quantify the mechanical changes of live erythrocytes as they undergo a transition from the normal discoid shape to the abnormal speculated echinocyte and spherical shapes (Park et al., 2010). Using a quantitative phase microscopy that enables imaging of live erythrocyte membrane fluctuations with nanometer sensitivity and extending quantitative phase imaging to 3-dimensional space (Popescu et al., 2008) were able to characterize mechanical properties of normal and abnormal red blood cells. In spite of high sophistication of the above noted optical methods, the spatial optical resolution was relatively low (250-500 nm). Therefore, many small vesicles budding off of erythrocytes (<250 nm) cannot be resolved and accurately accounted for by light microscopy (Leonards and Ohki, 1983).

The introduction of no-label, high resolution light microscopy gave new opportunity for live cell analysis, including the capability of visualizing and recording cell events occurring at the submicron scale. In the newly developed light system to be used on this work, the object is illuminated by the focused hollow cone of light (instead of beam of light). The effect of the annular illumination serves to narrow the central spot of the diffraction pattern and increase the intensity of the diffraction fringes. There is a similar effect involving the use of an annular aperture is known and used in telescopes (Born and Wolf, 1999) to increase the resolving power.

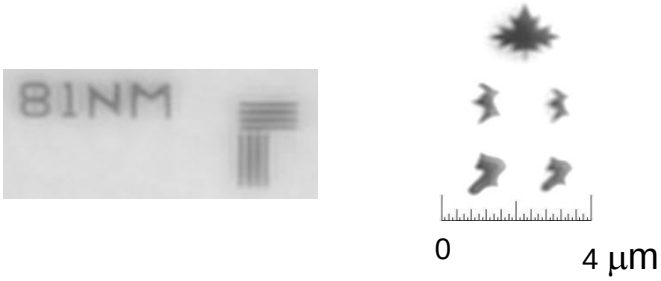

Figure 8. Images of vertical/horizontal bar set (left) at λ = 405 nm and various shapes (right) at λ = 546 nm recorded using the home-built annular illumination system (Vodyanoy, et al., 2007).

With the present scope, a better than 90 nm resolution of the Richardson plate (Richardson, 1988) lines and picture patterns has been achieved (Figure 8). (Vodyanoy et al., 2007, Foster, 2004, Vodyanoy, 2005). The high resolution microscopic illumination system was commercialized and it is known as CytoViva. It is broadly used in live cell and nanoparticle analysis (Weinkauf and Brehm-Stecher, 2009, Asharani et al., 2010, Lim et al., 2002, Rahimi et al., 2008, Shiekh et al., 2010, Carlson et al., 2008, Jun et al., Lim et al., 2012).

4. Hypotheses

1. It is hypothesized that a select *Bacillus* probiotic strain may be suitable for the reduction or prevention of heat stress-related adverse effects upon the gastrointestinal mucosa with the concomitant effect of reducing or preventing endotoxemia and bacterial translocation into the surrounding vascular system and organs.

2. It is hypothesized that the elevation of temperature during a heat stress may cause transformation of some normal erythrocytes into speculated echinocytes that then shed membrane vesicles. The increase of the number of membrane vesicles produced during heat stress is relational to a number of discocyte-echinocyte transitions. Therefore, the increase of vesicle number following heat stress may be indicative of the heat stress level.

5. Objectives

5.1 Main Goals

The main goals of this proposed research effort are to characterize the manifestation of heat stress' adverse effects upon laboratory animals and to develop a new approach for mitigation of heat stress-related complications by using a beneficial probiotic strain.

5.2 Specific aims of this research are:

1) Evaluate the animal model of heat stress and demonstrate feasibility of the proposed probiotic *Bacillus* strain in the prevention of heat stress-induced bacterial translocation.

2) Determine the resulting change in the concentration of LPS and viable bacteria recovered from specific target organs (e.g. spleen, mesenteric lymph structures and liver,

3) Microscopically examine collected blood specimens to assess the effects of heat stress on the on the number of vesicles shed by erythrocytes in blood.

4) Conduct microscopic examination of cross sections of the intestines to examine the condition and size of individual villus between control, probiotic and non- probiotic animals

6. Results

6.1 Article 1. Antagonistic activity of *bacillus* bacteria against food-borne pathogens (Article is accepted for publication in the Journal of Probiotics & Health)

ANTAGONISTIC ACTIVITY OF *BACILLUS* BACTERIA AGAINST FOOD-BORNE PATHOGENS

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Abstract

Bacillus bacteria have attracted the attention of scientists as promising probiotics because of their versatile antimicrobial activity and established health benefits on the host. In this study, seven *Bacillus* strains were identified and analyzed for antagonistic activity against broad spectrum of food borne pathogens. All strains were identified as *B. subtilis,* based on the results of morphological, biochemical characterization and 16S rDNA sequence analysis. *B. subtilis* strains demonstrated antagonistic activity against test-cultures of pathogens, including multiresistant strains. Reference *Bacillus* strains, derived from the commercial probiotics did not show antagonistic activity against tested strains of pathogens. Three of the most active cultures were studied for production of biosurfactants. Crude biosurfactants were isolated and analyzed by oil spread test and inhibition activity against *Salmonella*, *Shigella* and *Staphylococcus* cultures. Biosurfactants from three tested *B. subtilis* strains gave positive oil spread test. Inhibition activity of biosurfactants was found only against *Staphylococcus* strains. Production of biosurfactants depended on the incubation conditions of *Bacillus* culture. Best results were obtained after cultivation of bacilli in starch broth at 30^{o}C. The concentration of produced biosurfactant increased in time with growth of bacteria and reached the maximum at 30 hours of incubation.

Keywords: *Bacillus subtilis,* antagonistic activity, food borne pathogens, biosurfactants

Abbreviations: MRSA- methicillin resistant *Staphylococcus aureus;* SA - starch agar, TBS - trypticase soy agar; NA - nutrient agar; CFU – colony forming unit; OD_{600} – optical density at a wavelength of 600 nm

Introduction

Foodborne pathogens are among the most significant problems in maintaining the health of the population. In 2011 the CDC estimates that each year roughly 1 in 6 Americans (or 48 million people) gets sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. The leading causes of foodborne illnesses in the United States are *Salmonella* and *Shigella* [1, 2]. *Staphylococcus aureus* is among top five pathogens contributing to domestically acquired foodborne illnesses. Staphylococcal food poisoning is estimated to account for 241,148 foodborne illnesses per year in the United States, according to the CDC information (http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html). Foodborne illnesses are routinely treated with several classes of antibiotics. However, the use of these antibiotics has become problematic as over the years there have been numerous reports of cases of multi-antibiotic resistant food borne pathogens, worldwide. [3-5]. In the United States, the proportion of methicillin-resistant *S. aureus* (MRSA) isolates from patients in intensive care units increased from 1992 to 2003 by 3% per year. Moreover, there is a great concern that the continued use of these drugs will result in the emergence of new resistant strains of these bacteria [6-9]. Colonization of the intestinal tract with MRSA may have important clinical implications, such as development of antibiotic-associated diarrhea, environmental dissemination, subsequent risk of infections and toxic shock syndrome [10-14]. Since foodborne infections have a dramatic impact on morbidity and mortality, particularly of infants and children, new approaches for cost effective and easy-to-deliver prophylaxis and treatment of these infections are highly desirable.

One of the growth areas in the control of foodborne infections is the use of probiotics [7]. Probiotic prophylaxes and therapies are gaining wider acceptance as more scientific data emerge regarding the interaction between pathogen and beneficial microbes in the human intestinal tract and molecular mechanisms of probiotics' action. Probiotic bacteria which confer beneficial effect for the host and have pronounced antagonistic activity against these pathogens are expected to present a clear alternative in the prevention and treatment of foodborne infections**.**

Bacteria of the *Bacillus* genus possess a great potential as probiotic cultures. *Bacillus* bacteria are among the most widespread microorganisms in nature. These bacteria are known to be producers of more than 200 antibiotics. *Bacillus* antibiotics differ in their structure, as well as spectrum of activity [15]. Some strains of *Bacillus* synthesize bacteriocines, which are only effective against bacteria of the same species, others produce antibiotics against Gram-negative bacteria and still other strains have a wide spectrum of antibiotic activity (including antifungal and antiprotozoan) [16]. Thus, it is possible to find strains with unique spectrum of activity among *Bacillus* bacteria. The aim of this work was to isolate and characterize *Bacillus* strains with pronounce activity against food borne pathogens.

Materials and methods

Bacterial strains

Seven bacterial strains (16k, M1-1, 11-89, M2-3, 101, BSB, 105), isolated from environment, were used in this study. Morphological characterization of the cultures was done with a high resolution CitoViva microscope [17, 18]. Gram reaction and catalase activity were analyzed in tested strains. Bacterial cultures were identified using API 50 CHB tests (bioMerieux, Marcy-l'Etoile, France). For further characterization, the 16S rRNA gene was PCR amplified using universal 16S primers that correspond to positions 0005F and 0531R. Products

of sequencing reactions were analyzed with an ABI 3100- AVANT Genetic Analyzer in MIDI Labs (Newark, DE). Sequence analysis was performed using BLAST and Sherlock® DNA microbial analysis software and database.

Probiotic strains

Bacillus cultures from commercial probiotics were studied as the reference strains (Table 1).

Test-cultures

Test-cultures of *Salmonella*, *Shigella* and *Staphylococcus* were obtained from the culture collection of Auburn University (Auburn, AL). Stock cultures were maintained at -20^{o}C in NZY medium, supplemented with 25% (v/v) glycerol.

Antagonistic activity of Bacillus strains

Activity of *Bacillus* strains against pathogens were studied by the method of delayed antagonism in solid nutrient medium [19]. Briefly, *Bacillus* strains were inoculated as a line on the surface of a nutrient media. After 72 h of growth at 30 or 37 °C overnight test- cultures were inoculated as a perpendicular line to the *Bacillus* culture. The plates were incubated for 24 h at 37°C. The antagonistic activity was detected as a zone of pathogens' growth inhibition. Different media were tested to assess the antagonistic activity of *Bacillus* strains – NZY, starch agar (SA), trypticase soy agar (TBS) and nutrient agar (NA). Starch agar composed of starch (10g/L), peptone (5 g/L), NaCl (0.5 g/L), agar (15 g/L) was used previously for cultivation of *Bacillus* probiotic strain [20]. Test- cultures of pathogens were grown overnight in NZY medium at 37^{o}C.

Bacillus strain	Probiotic	Producer
B. cereus IP 5832	Bactisubtil	Cassenne Marion, Paris, France
B. cereus DM-423	Cereobiogen	Keda Drugs Trade Co Ltd under Dalian university of Medical Sciences, China
B. clausii	Enterogermina	Sanofi -Synthelabo, Milan, Italy

Table 1 Characterization of *Bacillus* probiotic strains

Biosurfactant evaluationFor preparation of the inoculum, *Bacillus* strains were grown in NZY medium overnight at 37^{o}C on shaker-incubator (200 rpm). Seed cultures were inoculated into nutrition media (1 mL of overnight culture into 250-mL flack with 50 mL of tested medium). Two media were used for cultures cultivation: SA and a fermentation media for biosurfactant production by *B. subtilis* natto, composed of 5.0 g/L sucrose, 20.0 g/L peptone, 0.5 g/L yeast extract, 0.02 g/L $MgSO_4 \bullet 7H_2O$, 1.4 g/L $Na_2HPO_4 \bullet 12H_2O$, 0.4g/L KH_2PO_4 [21]. *Bacillus* strains were incubated 24 hours at 30^{o} and 37^{o}C. After incubation the bacterial cells were precipitated by centrifugation at 10,000 x g at 4^{o}C for 10 min. Cell free supernatant was acidified to pH 2.0 with 1N HCl and left overnight at 4^{o}C to precipitate. The resulting precipitate was collected by centrifugation at 10,000 x g for 20 min. The supernatant was discarded and the remaining pellet was resuspended in 5 mL of methanol and left for extraction for 4 hours at room temperature. Methanol extract was centrifuged at 10,000 x g for 30 min and supernatant was transferred into a preweighed 50-mL glass and evaporated overnight under the vacuum in an exicator with silicagel. The glass was weighed again to determine the net weight of the crude biosurfactant . Obtained samples were diluted in deionized sterile water (pH 8.0) for further testing.

Biosurfactant production during Bacillus cultivation

Bacillus strain was cultivated in 250-mL flasks with 50 mL of starch medium at 30^{o}C for 32 hours. At different time intervals, the fermentation medium was sampled for determination of biomass and biosurfactant concentration.

Surface activity of biosurfactants was measured by an oil spreading test [22, 23]. Briefly, 20 µL of crude oil was added to a Petri dish (90 mm diameter) with 50 mL of distilled water to form a thin membrane. Ten microliters of sample was put onto the center of the oil membrane. The diameter of the oil-displaced circle area was measured. Each sample was tested in triplicate.

Antimicrobial activity of biosurfactants was evaluated by an agar well diffusion method [20]. Prepared suspensions of test-cultures in PBS (10^{8} CFU/mL) were inoculated onto the surface of agar medium (100 µL of suspension on each plate). Wells (6 mm diameter) were made with a sterile cork borer. 50 µL of the test solutions were added to each well. Plates were incubated for 24 hours at 37^{o}C. Zones of test-cultures growth inhibition were measured.

Statistics

Statistical analyses (t- Test and ANOVA) were performed using Microcal™ Origin® version 6.0 (Northhampton, MA) to validate the signifigance of the results. The data are presented as means (± SD) of at least three replicates.

Results

Identification of Bacillus strains

The microscopic study of bacterial cultures showed these strains to be Gram-positive rods, less than 1µm in diameter. All strains sporulated aerobically without swelling of the cell and produced catalase. These data indicated that tested strains belong to *Bacillus* genus. Additional testing with API 50CHB kit resulted in identification of all cultures as *B. subtilis*. Partial sequence of 16S rRNA gene confirmed the obtained results of biochemical identification.

Antagonistic activity

Antagonistic activity of *B. subtilis* cultures was tested on different nutrient media at two temperatures: 30° and 37°C. All cultures showed prominent growth on selected media at both temperatures, but no antagonistic activity was indicated at 37°C. *Bacillus* cultures inhibited the growth of pathogenic bacteria only after growth on SA at 30°C (Table2).

Strains BSB, 16K and 105, showed the highest antagonistic activities, were used in further experiments with broad spectrum of *Salmonella* and *Staphylococcus* strains, including clinical isolates. *Bacillus* cultures from commercial probiotics were tested as reference strains. Antagonistic activity of bacilli was studied after growth on SA at 30°C. *B. subtilis* strains were highly effective against all tested strains of *Salmonella* and *Staphylococcus* (Table 3, Figs 1; 2). Commercial *Bacillus* strains showed no antagonistic activity against test-cultures.

Biosurfactant production

Biosurfactant production was tested in two media – starch broth and fermentation medium, used for surfactant production by *B.subtilis natto* [21]. *B. subtilis* strains were incubated in two medium at 30°C and 37°C. Production of biosurfactant was assessed by the oil spreading technique and by inhibition of test-cultures growth. The best conditions for biosurfactant production for all *Bacillus* cultures were cultivation in starch broth at 30°C. Results in Fig.3A indicate that oil spread test for biosurfactants, produced by *B. subtilis* BSB3 and 16k at 30°C gave higher results than for *Bacillus* cultures, grown at 37°C. Study of inhibition activity of these biosurfactants against test-cultures, showed that activity of biosurfactant from *B. subtilis* BSB3, cultivated at 30°C was more pronounced, than at 37°C (Table 4 ; Fig.3B). Biosurfactants from *B. subtilis* 16k, incubated at different temperatures, demonstrated more consistent results. *B. subtilis* 105 produced biosurfactant, as it was confirmed by the oil spreading test, but this

biosurfactant had lack of inhibition activity. Biosurfactants, produced by *Bacillus* strains demonstrated inhibition activity only against *Staphylococcus* cultures. Tested cultures of *Salmonella* and *Shigella* were resistant to these biosurfactants.

Bacillus subtilis strain/ Medium*	Zone of test-cultures growth inhibition, mm					
	Staphylococcus aureus ATCC12600	*Salmonella typhimurium* ATCC 13311	*S. dublin* SA 2424	*S. enteritidis*	*Shigella sonnei*	*Shigella flexneri*
BSB3						
NZY; NA; TSA	0	0	0	0	0	0
SA	28.3±0.3	29.6±0.6	25.6±0.3	20.3±0.8	23.3±0.8	21.3±0.3
M1-1						
NZY; NA; TSA	0	0	0	0	0	0
SA	14.6±1.4	6.7±1.7	5.0±0	7.0±0	11.7±0.3	11.0±0.6
16k						
NZY; NA; TSA	0	0	0	0	0	0
SA	29.3±0.3	25.6±0.3	16.0±0.6	22.0±0.6	21.6±0.3	19.6±0.8
11-89						
NZY; NA; TSA	0	0	0	0	0	0
SA	20.3±0.6	17.7±0.7	15.3±0.3	13.6±0.6	15.7±0.7	14.6±0.3
M2-3						
NZY; NA; TSA	0	0	0	0	0	0
SA	19.7±0.9	23.7±0.8	15.7±1.2	21.7±0.9	20.3±0.3	21.0±0.6
101						
NZY; NA; TSA	0	0	0	0	0	0
SA	17.3±0.7	6.2±0.6	4.3±0.3	18.6±0.9	19.7±0.3	15.3±0.6
105						
NZY; NA; TSA	0	0	0	0	0	0
SA	34.1±1.2	25.7±0.8	16.2±0.3	23.6±0.6	24.3±0.3	21.2±0.3

* NZY – NZY agar; NA- nutrient agar; TSA – trypticase soy agar; SA- starch agar

Table 2 Antagonistic activity of *B.subtilis* strains on different media

#	Test –cultures	Zone of test-cultures inhibition (mm) by *B.subtilis* strains		
		105	BSB3	16k
1	*S. typhimurium* Health 9491	0	23.8±0.7	22.7±0.3
2	*S. typhimurium* DT 104 Dairy	25.6±0.3	25.8±0.8	24.2±0.6
3	*S. diarisonae*	25.3±0.8	27.1±0.9	26.3±0.6
4	*S. panama* SA 3583	22.6±0.3	25.3±0.3	23.1±0.7
5	*S. indica* SA 4401	23.1±0.2	25.6±0.7	25.2±0.3
6	*S. derby* SARB 10	30.2±1.3	31.4±0.8	27.3±0.6
7	*S. typhimurium* LT2	30.1±0.6	32.3±0.7	28.6±0.3
8	*S. mission*	26.6±0.3	25.8±0.6	24.3±0.7
9	*S. Montevideo*	25.0±0.0	25.3±0.7	23.0±0.6
10	*S. typhimurium* 6787	22.3±0.3	24.3±0.7	19.7±0.8
11	*S. typhimurium* Heath 1390	21.7±0.3	22.0±0.6	21.7±0.3
12	*S. bongori* SA 4910	21.7±0.9	25.0±0.6	23.7±0.3
13	*S. typhimurium* Nal 1x fecal	19.7±0.8	20.7±0.3	18.0±1.1
14	*S. Minnesota*	30.7±0.3	31.0±0.7	27.7±0.9
15	*S. salamae* SA 41106	10.0±0.7	23.6±0.3	18.7±0.9
16	*S. typhimurium* 520-96	22.3±0.6	24.0±0.6	21.6±0.7
17	*S.Thompson* 265-4	25.1±0.3	26.3±0.9	23.3±0.6
18	*S. infantis* SARR 27	31.1±0.9	30.3±0.6	27.6±0.3
19	*S. paratyphimurium*	30.2±0.3	30.3±0.6	29.6±0.7
20	*S. typhimurium* DT 104 Swine	21.1±0.2	24.6±0.3	22.6±0,3
21	*S. typhimurium* 9693	22.3±0.3	25.7±0.6	23.3±0.1

Table 3. Anti-*Salmonella* activity of *Bacillus* strains

Figure 1. Anti-*Salmonella* activity of *Bacillus subtilis* BSB3: 1 – *S.paratyphimurium*, 2- *S. infantis* SARR 27, 3 – *S. thompson* 265-4, 4 – *S. minnesota*, 5 – *S. typhimurium* LT2, 6 – *S. derby* SARB 10

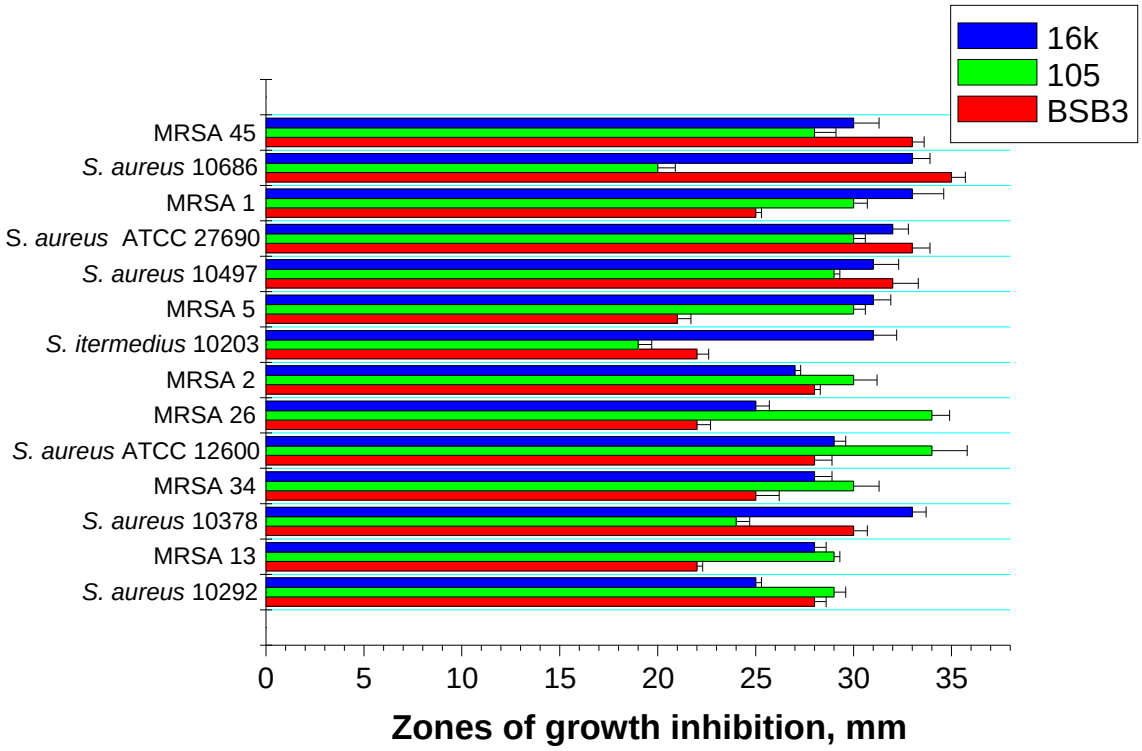
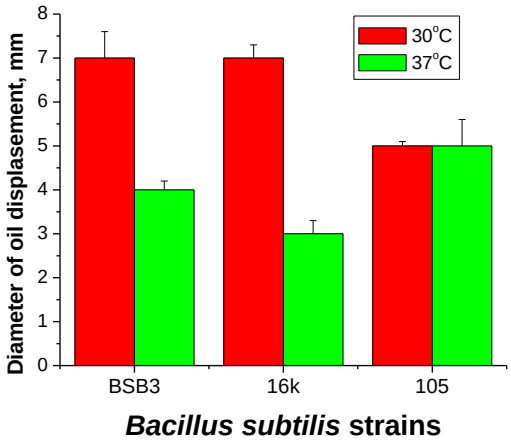
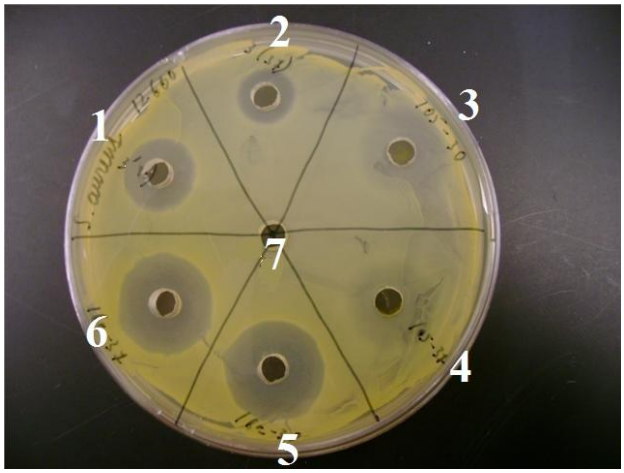


Figure 2. Antagonistic activity of *Bacillus subtilis* strains against *S. aureus.* MRSA – methicillin resistant *S. aureus*
Em: erythromycin; Gm: gentamycin; Km: kanamycin; L: lyncomycin; Met methicillin; N: neomycin; Ofx: ofloxacin; Pn:penicillin G; Sp: spiromycin; Str: streptomycin; Tet: tetracyclin.

Test-cultures	Zone of test-cultures growth inhibition (mm) by biosurfactants from *B.subtilis*					
	BSB3 (30^oC)*	BSB3 (37^oC)**	16k (30^oC)	16k (37^oC)	105 (30^oC)	105 (37^oC)
S. aureus 10292	12	10	17	15	0	0
MRSA 13	10	9	13	11	0	0
S. aureus 10378	15	12	20	19	0	0
MRSA 34	11	8	0	0	0	0
S. aureus ATCC 12600	16	12	20	20	0	0
MRSA 26	15	10	13	13	0	0
MRSA 2	12	10	14	14	0	0
S. itermedius 10203	16	9	25	25	0	0
MRSA 5	12	9	15	14	0	0
S. aureus 10497	13	11	17	17	0	0
S. aureus ATCC 27690	13	11	15	15	0	0
MRSA 1	10	7	15	14	0	0
S. aureus 10686	15	12	18	18	0	0
MRSA 45	13	10	18	16	0	0

Table 4. Activity of biosurfactants against *Staphylococcus* test-cultures





A B

Figure 3. Activity of biosurfactants, produced by *B. subtilis* strains at different temperatures: A- oil spread test; B- inhibition activity against *S. aureus* ATCC 12600; Biosurfactants were isolated from *B. subtilis* strains, incubated at different temperatures: 1- strain BSB3 at 30°C; 2- strain BSB3 at 37°C; 3- strain 105 at 30°C; 4-strain 105 at 37°C; 5- strain 16k at 30°C; 6- strain 16k at 37°C; 7- control (sterile water)

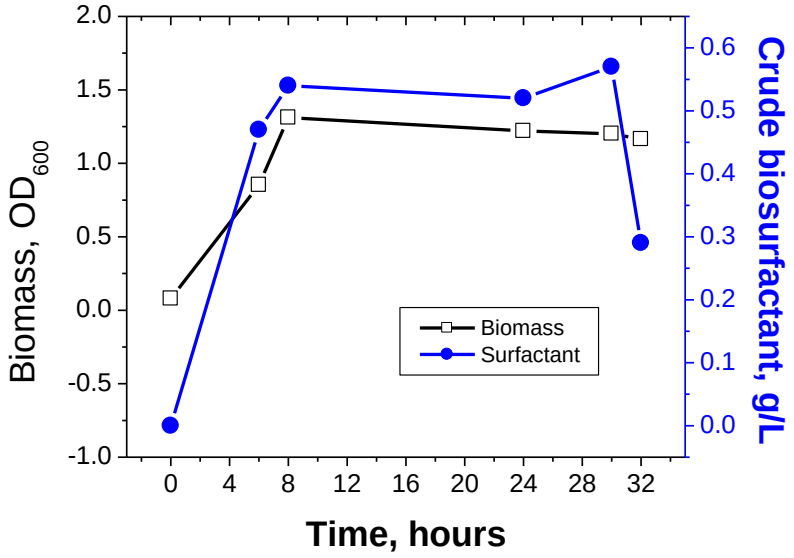


Figure 4. Kinetic of biosurfactant production during growth of *B. subtilis* BSB3

Production of biosurfactant during B.subtilis BSB3 cultivation

Production of biosurfactant by *B. subtilis* BSB3 increased in time and corresponded to the bacterial growth curve. The maximum production of biosurfactant was detected at 30 hours of bacteria cultivation (Fig.4).

Discussion

Bacillus bacteria are known to be effective antagonists of different pathogens [15, 24]. In recent years bacilli were extensively studied as probiotics, due to their health benefits on the host [25, 26]. A search for new *Bacillus* strains with pronounced antagonistic activity against food borne pathogens opens up promising expectations for treatment of these infections.

In the present study, newly isolated *Bacillus* strains were analyzed. All strains, identified as *B. subtilis*, were tested for their activity against disease causing pathogenic strains. Antagonistic activity of *Bacillus* strains was detected only after cultivation on starch agar at 30^{o}C. Incubation of bacilli on starch agar at 37^{o}C, as well as on NZY, NA and TSA at 37^{o}C and 30^{o}C did not result in antagonistic effect. These outcomes are in accordance with our previous findings about

conditions for production of antimicrobial compounds by *Bacillus* cultures [27]. Three *B. subtilis* strains, showed the highest activity against tested pathogens, were studied with broad spectrum of *Salmonella* and *Staphylococcus* cultures, including clinical multi-resistant strains. As reference strains, commercial *Bacillus* probiotic cultures from Bactisubtil, Cereobiogen and Enterogermina were used. None of the reference strains were active against tested pathogens. *B. subtilis* isolates demonstrated high activity of test-cultures' inhibition. Antagonistic activity was detected against all strains of *Salmonella* and *Staphylococcus*, including MRSA. Inhibition of MRSA by *Bacillus* cultures was shown by other authors [28-30], but no anti-*Salmonella* effect was found in the same strains.

Bacteria of the *Bacillus* genus (predominantly, *B. subtilis*) produce various biosurfactants, which have a high potential for biotechnology and pharmacology [31]. These compounds vary in structure and spectrum of activity and usually are responsible for antimicrobial effects of *Bacillus* bacteria [21, 32]. In our study *B. subtilis* strains produced biosurfactants after cultivation in starch broth at 30^{o}C. Incubation of these cultures in fermentation medium, used for *B. subtilis* natto [21], resulted in lack of biosurfactants production. Presence of biosurfactant in cultivation medium was tested by the oil spread test and by inhibition activity against *Salmonella*, *Shigella* and *Staphylococcus* strains. It was shown elsewhere, that the oil spread test correlates with the biosurfactant production [23]. Biosurfactants from three tested *B. subtilis* strains gave positive oil spread test, showing the diameter of oil displacement from 3 to 7 mm. These results are in accordance with data for crude biosurfactants from *B. subtilis* natto [21] and from *B. subtilis* and *B.licheniformis* [33]. Inhibition activity of biosurfactants was found only against *Staphylococcus* strains and depended on the incubation temperature of *Bacillus* culture. Biosurfactant from *B. subtilis* BSB3,

incubated at 30°C, demonstrated higher activity against *Staphylococcus* strains, as opposed to bacteria incubated at 37°C. Activity of biosurfactant, produced by *B. subtilis* 16k did not changed with the change of incubation temperature. Biosurfactant from *B. subtilis* 105 showed no activity against tested pathogens. The inhibitory activity of *B. subtilis* may be the cumulative result of different antimicrobials, known for this bacteria [16]. Identified here biosurfactants play an important role in the anti-*Staphylococcus* activity for at least two *B. subtilis* strains – BSB3 and 16k. Kinetic of biosurfactant production was similar to those reported for *Bacillus* cultures by other authors [33]. The concentration of biosurfactant increased in time with growth of bacteria and reached the maximum at 30 hours of incubation.

In the present study seven *Bacillus* strains were characterized for their activity against *Salmonella, Shigella* and *Staphylococcus* pathogens. Three strains showed pronounced antagonistic activity against broad spectrum of pathogenic cultures, including multi-resistant strains. Inhibitory effect on *S.aures* and MRSA strains was caused by production of biosurfactant, identified for two *B. subtilis* strains.

Further study of antimicrobial compounds, produced by *Bacillus* bacteria, will result in better understanding of the mechanisms of antagonistic activity of bacilli and selection of new strains, promising for biotechnology and pharmacology.

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6.2. Article 2. *Bacillus* Probiotic for Prevention of Heat Stress-Related Complications (manuscript is prepared for publication).

Bacillus Probiotic for Prevention of Heat Stress-Related Complications

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Running title: Microscopy of vesicles shed by erythrocytes
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Abstract

Gut microflora are key in maintaining gastrointestinal mucosal barrier function. There is strong evidence about the influence of stress on the depression of intestinal microflora and, as a result, on the overall immune resistance of the organism. On the other hand, stability of gut microflora determines the ability of the organism to tolerate stress. Thus, by modulating the intestinal flora one might offer a novel and non-invasive therapeutic approach for promoting well-being by prevention and treatment the adverse effects of stress in the gut.

We studied the efficacy of a *Bacillus subtilis* probiotic strain for prevention of heat stress related complications in rats. It has been shown that pre-treatment of rats with probiotic bacteria prevented microbial translocation from the gut into mesenteric lymph nodes and liver. Heat stressed animals without probiotic treatment had a high level of lypopolisaccharides (LPS) in the blood. In contrast, animals in the probiotic group, exposed to heat did not show elevation of LPS level. Cytokine IL-10 concentration significantly increased in stressed animals without probiotic pretreatment. Administration of probiotic before heat stress normalized the level of IL-10. We found, that the elevation of the temperature of the blood during heat stress causes an increase of the shedding of erythrocyte membrane vesicles. The elevation of temperature from 36.7±0.3 to

40.3±0.4 ^{o}C resulted in significant increase of the concentration of vesicles in blood. At a temperature of 37 ^{o}C, mean vesicle concentrations found in rat blood was $(1.4\pm0.2)\times10^{6}$ vesicles/μL, after exposure to heat - $(3.8\pm0.3)\times10^{6}$ vesicles/μL in group of animal without probiotic treatment. Treatment with probiotics before heat stress prevented vesiculation of erythrocytes in animals. The increase in the number of vesicle associated with elevated temperatures may be indicative of the heat stress level and serve as diagnostic test of erythrocyte stability and heat resistance.

Additionally, our results showed that use of the *B. subtilis* probiotic before heat stress exposure prevented damage to the villi structures located in the ileal intestinal area. Animals without probiotic treatment experienced a reduction in the overall intestinal villus height, whereas animals treated with probiotics did not. Animals not treated with probiotics experienced a marked thinning of the mucosal layer as compared to the mucosal layer of animals treated with probiotics. The protection of delicate intestinal structures that directly support water uptake may provide a pathway to reduce the loss of water and minimize dehydration which is often associated with heat stress. Our results showed high efficacy of *B. subtilis* probiotic in prevention of heat stress related complications in rats.

Introduction

Temperature is one of the most challenging stressors, affecting human and animal health. Although humans have the capability to withstand large variations in environmental temperatures, relatively small increases in internal temperature can lead to injury and even death (Crandall and Gonzalez-Alonso 2010). Exposure to hot weather is considered one of the most deadly natural hazards in the United States. It was estimated that between 1979 and 2002, heat stroke claimed more American lives than the combined effects of hurricanes, lightning,

earthquakes, floods, and tornadoes (Leon and Helwig 2010). Extreme heat stress can damage the gastrointestinal mucosa, which protects the internal environment of the body from bacteria and bacterial endotoxins (lipopolysaccharides –LPS) (Moseley and Gisolfi 1993). Dysfunction of this protective barrier results in increased intestinal permeability and diffusion of toxic bacterial components from the gut lumen to the blood (Lambert 2009). Gut microflora is key in maintaining mucosal barrier function. There is strong evidence about the influence of stress on depression of intestinal microflora and, as a result, on the immune resistance of the organism (Gruenwald, Graubaum et al. 2002). On the other hand, stability of gut microflora determines the ability of the organism to tolerate stress (Berg, Muller et al. 1999). Thus, by modulating the intestinal flora one might offer a novel and non-invasive therapeutic approach for promoting well-being by prevention and treatment of the adverse effects of stress in the gut.

Beneficial probiotic bacteria have been shown to enhance gastrointestinal barrier function and dampen inflammation in several immune mediated diseases such as inflammatory bowel diseases or atopic disorders, thereby conferring a health benefit on the host (Dai, Zhao et al. 2012; Gore, Custovic et al. 2012; Khan, Kale et al. 2012; Quigley 2012) . Mechanisms of probiotics action include restoration of microbial homeostasis in the intestine, interference with the ability of pathogenic bacteria to colonize the mucosa, beneficial modulation of local and systemic immune responses and a stabilizing effect on gastrointestinal barrier function. In animal stress models probiotic pretreatment of rats completely abrogated stress-induced bacterial adhesion to the intestinal mucosa and translocation of bacteria to mesenteric lymph nodes (Zareie, Johnson-Henry et al. 2006) and normalized colonic dysfunction (Gareau, Jury et al. 2007). Efficacy of treatment with *Lactobacillus* strain was shown by reducing the enhanced neuronal activation in spinal and supraspinal sites induced by the acute stress (Ait-Belgnaoui, Eutamene et al. 2009).

This study was designed to determine the effect of a *Bacillus subtilis* probiotic strain on prevention of heat stress-inducing complications.

Methods

***Bacillus* probiotic strain**. *B. subtilis* BSB3 was cultured on the plates with Difco sporulation medium (DSM) at 37^{o}C for 5 days. Bacteria were harvested by flooding the surface of the culture with sterile phosphate buffered saline (PBS) followed by scraping with a sterile cell spreader. Bacterial suspension was diluted to 1 x 10^{8} CFU/mL in PBS.

Animals. Adult male Sprague–Dawley rats weighing 250–300 g were used. The animals were housed two in a cage under standardized conditions of temperature (20 ± 1^{o} C), humidity (50 ± 5%) and lighting (12-h day/12-h night) with free access to food and water. All experimental procedures were approved by IACUC of Auburn University, Auburn, Alabama.

Experimental design. 32 rats were used in this study. One group (16 rats) was treated by oral gavage with probiotic bacteria (10^{8} CFU in 1 mL of PBS per rat) (probiotic), the other group (16 rats) received 1 mL PBS by oral gavage at the same time (PBS). Animals were treated twice a day (6 hours between doses) for two days prior to the start of heat stress (Fig.1).
Rats in each group were subdivide (8 rats in each group): 1) control (PBS/25^{o}C), 2) probiotic (probiotic /25^{o}C), 3) stress (PBS/45^{o}C, 25 min), and 4) probiotic + stress (probiotic/45^{o}C, 25 min). Animals from group 3 and 4 were exposed to 45^{o}C heat stress, relative humidity 55% for 25 min in a climatic chamber (Environmental Chamber 6020-1, Caron, OH, USA). Control animals (groups 1 and 2) were exposed to identical conditions as the heat stressed animals, but at 25^{o}C. Rectal temperature was measured in each rat before and after exposure to heat with electronic digital thermometer. At the same time that the rectal temperature was measured in the control rats. Four hours after the stress experiments, rats were anesthetized with isoflurane and

euthanized by rapid decapitation. Trunk blood was collected from each rat to obtain serum. Samples of blood were immediately taken for microscopic examination.

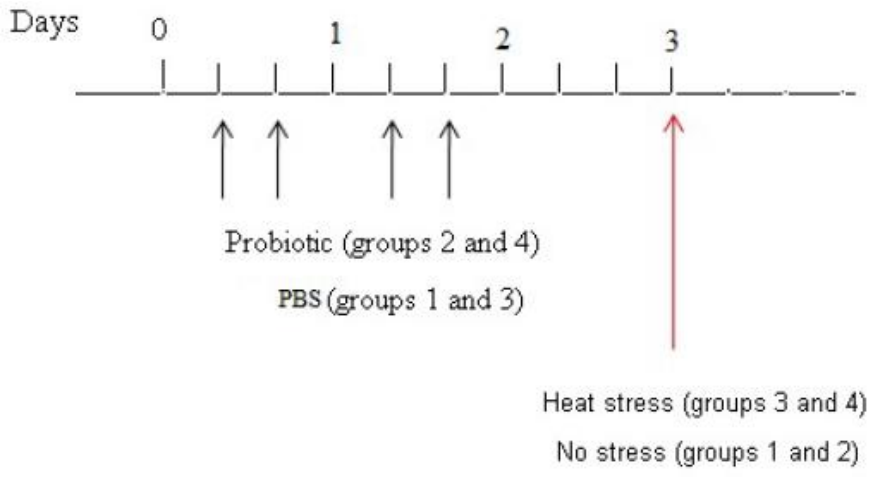

Figure 1. Experimental design.

Bacterial Translocation. After death, mesenteric lymph nodes, liver, and spleen were removed under sterile conditions and placed in the pre-weighing sterile tubes and weighed. After homogenization, aliquots (0.1 mL) of serial 10-fold dilutions of the suspension were plated onto 5% blood and MacConkey's agar plates for recovery of aerobic bacteria and Brucella blood agar plates supplemented with vitamin K1 and hemin for anaerobic bacteria. After 24 and 48 hours of incubation at 37°C, for aerobic and anaerobic cultures, respectively, colonies were counted. Quantitative culture results were expressed as the number of colony-forming units per gram. Bacterial strains were identified by Gram stain, biochemical tests, and sequence of 16S rDNA.

Histological analysis. Ileum samples were removed from each rat, fixed in Bouin's Fixative (Electron Microscopy Sciences, Hatfield, PA, USA) subsequently processed and stained with hematoxylin and eosin. Intestinal villi height and total mucosal thickness were measured for each sample using a Cyto Viva microscope. All samples were coded and examined in a blinded

fashion. Twenty measurements of each parameter in each sample were taken and expressed in nanometers.

Cytokine assay. Serum was obtained and stored in 50 μL aliquots at -20°C until assay. Cytokine determinations were performed on duplicate samples. Serum levels of cytokines were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits for IL-1β; IL-6; TNF-α; INF-γ (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions.

Lypopolisaccharide assay. Serum concentration of LPS was analyzed by commercially available ELISA kit (NeoBioLab, Cambridge, MA, USA) according to the instructions of the manufacturer.

High resolution light microscopy of blood. The microscope system produces the highly oblique hollow cone of light (N.A. 1.2-1.4). Coupled with a high aperture microscope objective with iris, this system provides two different regimes of illumination. When the iris is closed so that no direct light enters the objective after passing through the object, only refracted, scattered, or diffracted light goes in the objective. If the iris is open in such a way to allow the direct entrance of light into objective the front lens of the objective is illuminated by the annular light produced by the empty cone of light entering the objective. In this case the mixed illumination is produced that combines the darkfield and oblique hollow cone brightfield illuminations. The cardioid condenser is an integral part of the illumination system so that the system comprises a collimation lenses and a first surface mirror that focus light onto the annular entrance slit of the condenser. As a part of the illumination system, the condenser is pre-aligned and therefore additional alignment is unnecessary. The illumination system is positioned in Olympus BX51 microscope by replacing a regular brightfield condenser. The illumination system is connected

with a light source (EXFO120, Photonic Solution) by a liquid light guide. The objective used for this work is infinity corrected objective HCX PL APO 100×/1.40-0.70, oil, iris from Leica. The image is magnified with a zoom intermediate lens (2×-U-CA, Olympus), a homebuilt 40× relay lens, and captured by a Peltier-cooled camera (AxioCam HRc, Zeiss) and Dimension 8200 Dell computer. The microscope is placed onto a vibration isolation platform (TMS, Peabody, MA). Live images were recorded with Sony DXC-33 Video camera and Mac OS X Computer (Vainrub, Pustovyy et al. 2006). Test images were calibrated using a Richardson slide (Richardson 1988). A small droplet (7 μL) of freshly drawn blood was placed on a glass slide and coverslipped and positioned on the microscope stage with oil contacts between condenser and objective lenses. Ten image frames of 72 × 53.3 μm^2 in each sample were photographed, videotaped, and concentrations of discocytes, echinocytes, and vesicle count and diameter were measured by Image-Pro Plus software (Media Cybernetics), providing high-resolution direct-view optical images in real time. The samples were observed in an aqueous environment and required no freezing, dehydration, staining, shadowing, marking or any other manipulation. At least 20 image frames were analyzed for each experimental condition. Each frame contained at average between 50 and 200 vesicles (depending on conditions).

Statistics. All results were presented as mean and standard deviation. T-test, linear regression, frequency and cumulative frequency, curve fitting and graph plotting were carried out using Microcal™ Origin version 6.0 (Northhampton, MA) and 2010 Microsoft Excel.

Results

Body temperature. Exposure of rats to high temperature resulted in significant increase of body temperature. The mean body temperature of rats before heat stress was 36.7±0.07^{o}C., which increased immediately to – 40.3 ±0.17^{o}C ($P<0.05$).

Histology. Morphometric study of the intestines showed that heat stress significantly inhibited villi height and total mucosal thickness in rats (Fig.2, 3). Villi height and total mucosal thickness in rats before stress were 611.4±0.8 nm and 740.4±0.7 nm accordingly. After heat exposure these parameters for rats in group 3 (PBS/Stress) were 358.3±2.5 nm and 525.9±3.1 nm. The probiotic treatment prevented the damaging effects of heat on the intestinal morphology. The parameters measured for villi height and total mucosal thickness in the intestines of rats pre-treated with probiotics and exposed to the high temperature (group 4-Probiotic/Stress) did not differ from the same parameters observed in the control rats.

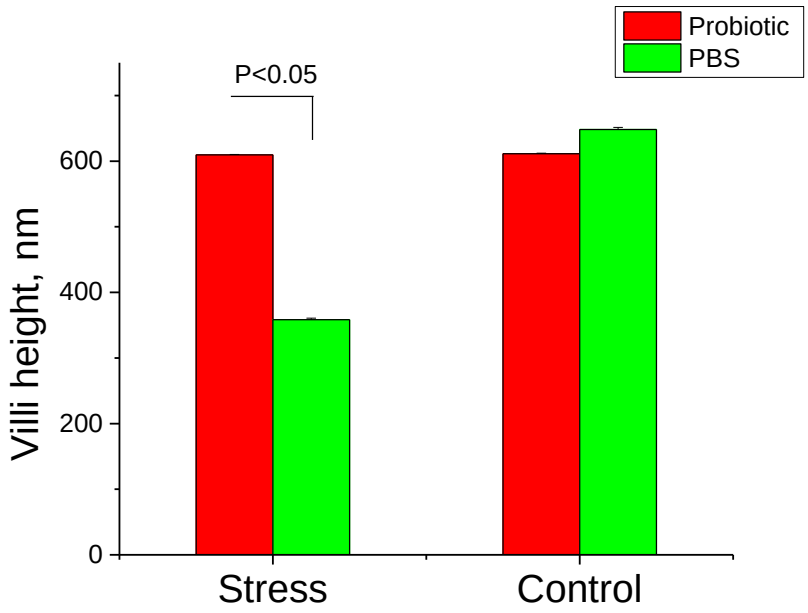

Figure 2. Intestinal villi height in rats from different experimental groups. Rats were pre-treated with PBS or probiotic by oral gavage for 2 days before exposure to 45^{o}C (Stress) or to 25^{o}C (Control).

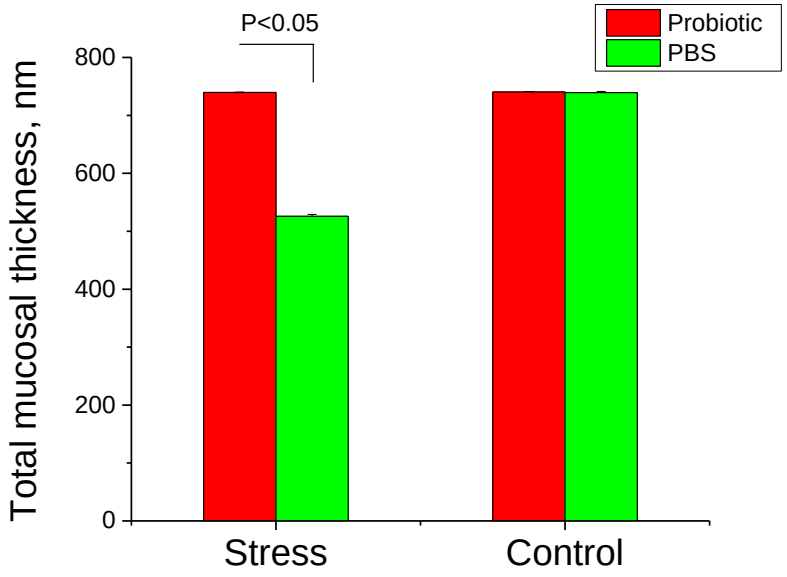

Figure 3. Total mucosal thickness in rats from different experimental groups. Rats were pre-treated with PBS or probiotic by oral gavage for 2 days before exposure to 45^{o}C (Stress) or to 25^{o}C (Control).

Translocation of bacteria. Bacteriological analysis of mesenteric lymph nodes (MLN), liver and spleen were performed for each rat to determine the translocation of bacteria from the gut. All samples analyzed from the control animals (groups 1 and 2) and in heat-stressed animals, pre-treated with probiotic (group 4) were sterile. Bacterial cultures were isolated from MLN and liver of heat-stressed animals (group 3). The concentration of bacteria was $1.7x10^{3}\pm4.6x10^{2}$ CFU/g tissue (Fig. 4)**.** The spleen from all animals was sterile. The isolated bacteria belonged to different species: *Neisseria meningitidis; Rothia dentocariosa; Rothia mucilaginosa; Streptococcus mitis; Lactococcus garviesae; Lactobacillus johnsonii; Lactobacillus acidophilus; Lactobacillus crispatus; Enterococcus hirae.*

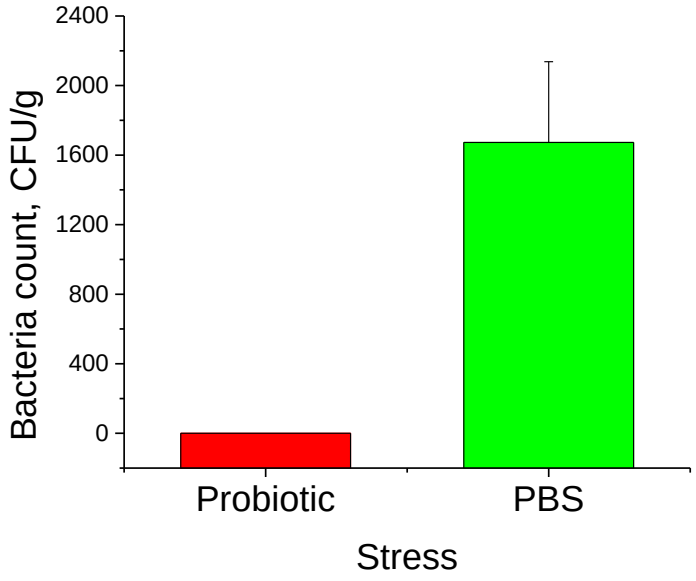

Figure 4. Protective effect of probiotic against bacterial translocation. Rats were pre-treated with PBS or probiotic by oral gavage for 2 days before exposure to 45^{o}C.

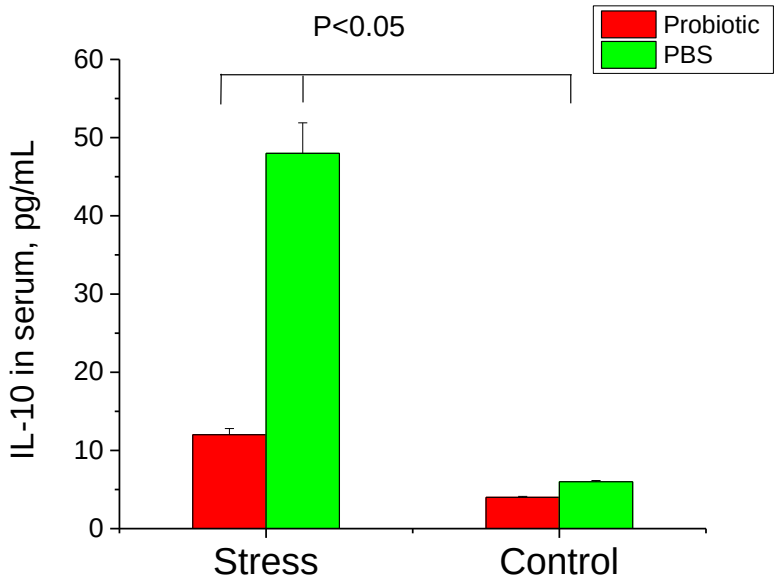

Figure 5. Serum IL-10 concentration in animals of different groups. Rats were pre-treated with PBS or probiotic by oral gavage for 2 days before exposure to 45^{o}C (Stress) or to 25^{o}C (Control). Exposure to heat significantly increased IL-10 level in serum of animals (PBS/Stress)($P<0.05$). Probiotic pre-treatment normalized IL-10 concentration ($P<0.05$) in heat-stressed animals (Probiotic/Stress).

Cytokines. Heat stress did not result in change of IL-1β; Il-6, TNF-α, INF-γ cytokine levels. Concentration of these cytokines was stable in rats from all groups (data not shown). A significant increase of IL-10 level was found in rats, exposed to heat and pre-treated with PBS (group 3- PBS/Stress) – Fig.5. No change of IL-10 concentration was determined in animals treated with probiotics before heat stress (group 4 – Probiotic/Stress).

Serum LPS concentration. Level of LPS was significantly increased in the serum samples of heat-stressed animals, which received PBS before stress exposure (Fig.6). Thus, LPS concentration in serum of rats before stress was 5±0.01 ng/mL, which increased after exposure to heat – (14±0.1 ng/mL) ($P<0.05$). Concentration of LPS in the serum of animals pre-treated with probiotics before heat stress, was not significantly changed in comparison with non-stressed animals (4±0.03 ng/mL and 2±0.02 ng/mL correspondingly) ($P>0.05$).

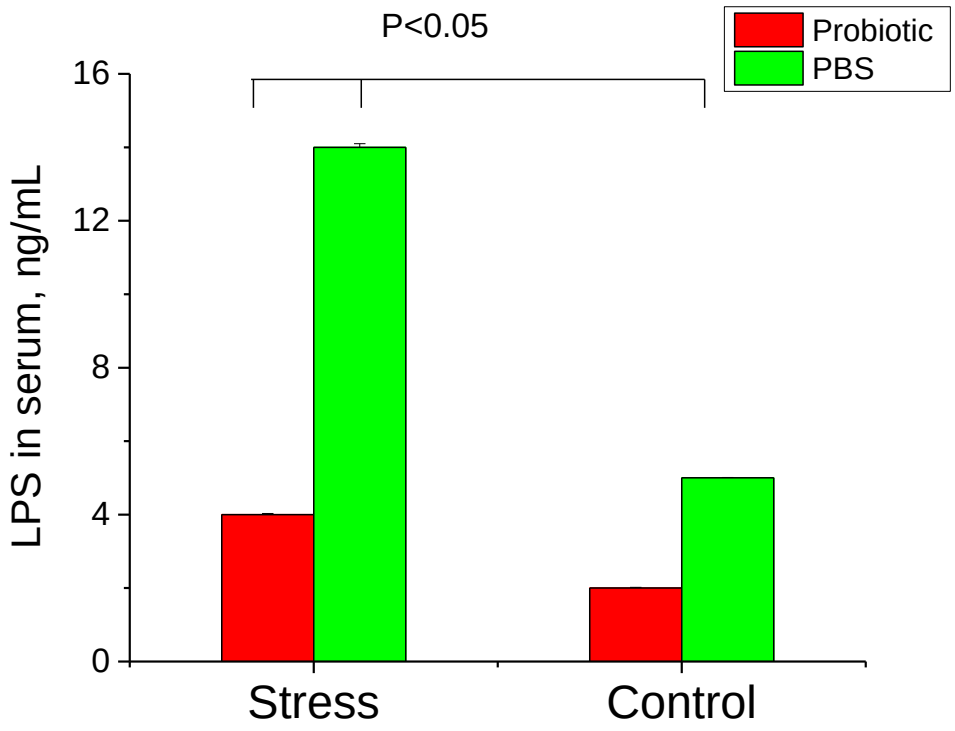

Figure 6. Serum LPS concentration in animals of different groups. Rats were pre-treated with PBS or probiotic by oral gavage for 2 days before exposure to 45^{o}C (Stress) or to 25^{o}C (Control). Exposure to heat significantly increased LPS level in serum of animals (PBS/Stress) ($P<0.05$). Probiotic pre-treatment prevented increase of LPS concentration ($P<0.05$) in heat-stressed animals (Probiotic/Stress).

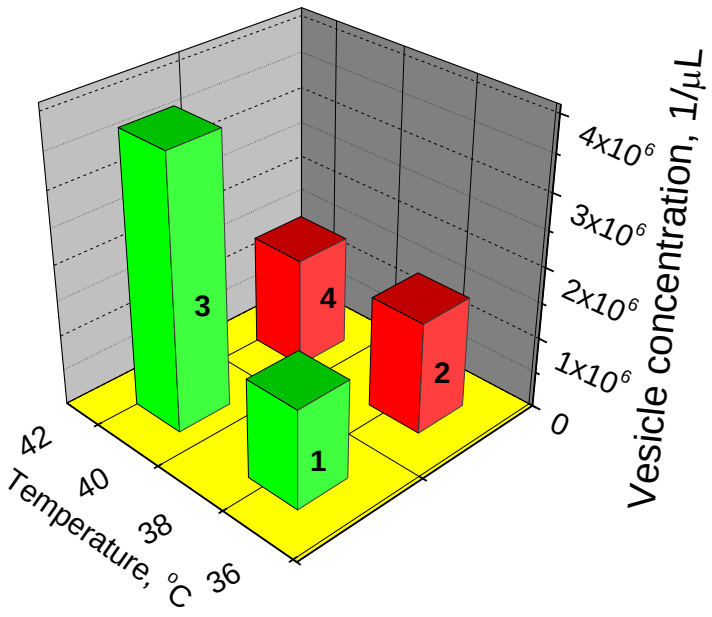

Figure 7. Vesicles concentration in blood of rats. Rats were pre-treated with PBS or probiotic by oral gavage for 2 days before exposure to 45°C (Stress) or to 25°C (Control). A small droplet (7 μL) of freshly drawn blood were placed on a glass slide and coverslipped and positioned on the microscope stage with oil contacts between condenser and objective lenses. Ten image frames of 72 × 53.3 μm^2 in each sample were photographed, videotaped, and concentrations of discocytes, echinocytes, and vesicle count and diameter were measured by Image-Pro Plus software (Media Cybernetics), providing high-resolution direct-view optical images in real time
Groups of rat: 1- PBS /Control; 2- Probiotic/Control; 3 – PBS/Stress; 4 – Probiotic/Stress.

Vesiculation of rat erythrocytes after exposure to heat (passive hyperthermia)

The concentration of free vesicles increased after exposure to heat stress from (1.4±0.2)×10^6 (group 1) to (3.8±0.3)×10^6 (group 3) vesicles/μL in rats, pre-treated with PBS (Fig. 7). The values are significantly different at the 0.001 level. In probiotic treated animals, exposed to high temperature no significant change in concentration of free vesicles was found (Fig. 7, group 2 and 4).

Discussion

Exposure to high environmental temperatures is considered to be one of the most deadly conditions, leading to human injury and even death. Athletes, occupational workers, and military personnel are populations at risk for heat stroke during intense physical activity in the outdoors (Leon and Helwig 2010). Early detection and prevention of the adverse effects of heat stress is of great importance.

In this study we analyzed the effect of probiotic pretreatment on the mitigation of complications following heat stress in the rat model. Exposure of rats to 45^{o}C for 25 min resulted in body temperature increase from 36.7±0.07^{o}C to 40.3 ±0.17^{o}C ($P<0.05$). Significant change of temperature influenced the morphology of intestinal cells and structures in stressed rats. Villi height decreased in comparison with indexes for non-stressed (control) rats (358.3±2.5 nm and 648.2±3.2 nm correspondingly; $P<0.05$). Total mucosal thickness was also notably affected by heat stress (525.9±3.1 nm for stressed animals vs 739.4±2.0 nm for control animals; $P<0.05$). Previously it has been shown that heat stress causes major morphological changes in the gut. For example, it has been found that heat exposure in pigs caused marked injury to the tips of the apical intestinal villi, inducing epithelial cell shedding, exposing the intestinal mucosa lamina propria, as well as shortening individual villus height and crypt depth in the small intestine (Yu, Yin et al. 2010). Our results showed that treatment of rats with the *Bacillus* probiotic before heat stress prevented traumatic effects of heat on the ileal region of the intestine. Rats in the probiotic group, exposed to the heat had the same indexes of villi height and total mucosal thickness, as the control, non-stressed rats.

Morphological changes in the intestines alter the integrity of the gastrointestinal tract - the main factor in protection of the host from the bacteria, endotoxins and lipopolysaccharides

found in the Gram-negative bacteria located in the gut (Lambert 2009). In this study we showed that morphological abnormalities of the intestines of rats exposed to heat stress were accompanied by translocation of gut bacteria into the MLN and liver. Concentration of isolated bacteria was $1.7x10^3 \pm 4.6x10^2$ CFU/g tissue. Treatment of rats with *Bacillus* probiotic before heat stress prevented bacterial translocation. Liver and MLN of rats from probiotic group were sterile. Obtained results are in accordance with the data reported by Zareie et al. (Zareie, Johnson-Henry et al. 2006). Authors identified translocation of gut bacteria into MLN of stressed rats. It has been found, that treatment of rats with *Lactobacillus* probiotic for seven days before stress prevented bacterial translocation.

In our study, heat stress resulted in the elevation of LPS serum levels in untreated rats rats. Probiotic pre-treatment was effective in prevention of LPS release into the circulatory system of rats exposed to heat. Heat-induced elevation of LPS has been shown to be associated with severe complications and multi-organ dysfunction in humans (Moseley and Gisolfi 1993; Smith, Erlwanger et al. 2012). Bacterial LPS were identified as a triggering factor causative of the onset of insulin resistance, obesity, and diabetes (Cani, Amar et al. 2007). Nonalcoholic fatty liver disease also was shown to be associated with the elevated level of serum LPS (Ruiz, Casafont et al. 2007). Lowering of the LPS concentration in blood is discussed as a potent strategy for the control of metabolic diseases (Cani, Amar et al. 2007). A recent study with the human proximal colonic microbiota model demonstrated that the administration of probiotic bacteria (*B. bifidum, L. rhamnosus, B. longum*, and *B. longum* subsp. *infantis*) significantly reduced LPS concentrations in a time-dependent manner (Rodes, Khan et al. 2013). The authors concluded that only specific probiotic strains can decrease LPS concentrations and as a result might reduce the proinflammatory tone. Probiotic administration in the elderly normalized the

LPS level, modulated activation markers in blood phagocytes and reduced low-grade, chronic inflammation (Schiffrin, Parlesak et al. 2009).

Hyperthermia induces the production of pro-inflammatory cytokines, such as IL-1; IL-6; TNF-α, INF-γ (Robins, Kutz et al. 1995; Leon and Helwig 2010). Levels of these cytokines increase with the severity of heat stress up to lethal outcome (Bouchama, Alsedairy et al. 1993). The present study showed no change in the levels of IL-6; IL-1ß, IL-6; TNF-α, INF-γ in rats, exposed to heat stress. But the concentration of IL-10 cytokine was significantly higher in the blood of heat-stressed rats, pre-treated with PBS. Administration of the probiotic before exposure to heat normalized the level of serum IL-10 in rats. Significant elevation of the IL-10 level at high temperature was found in previous mice experiments (Leon 2006) and in heat stroke patients (Bouchama, Hammami et al. 2000). IL-10 cytokine was found to be a potent inhibitor of pro-inflammatory cytokines (Bogdan, Vodovotz et al. 1991; Bouchama, Hammami et al. 2000). In our study the level of IL-10 in rats pretreated with PBS and exposed to heat stress was 8 times higher in comparison with rats of control group (48±3.9 vs 6±0.15 pg/ mL) and 4 times more than in probiotic group (48±3.9 vs 12±0.8 pg/mL).

We found, that the elevation of the temperature of the blood during heat stress causes an increase of the shedding of erythrocyte membrane vesicles. The elevation of body temperature from 36.7±0.3 to 40.3±0.4^{o}C resulted in a significant increase of the concentration of vesicles in blood. At a temperature of 37^{o}C, mean vesicle concentrations and diameters found in rat blood were (1.4±0.2)×10^{6} vesicles/μL and 0.436±0.03 μm, respectively. The concentration of free vesicles increased after exposure to heat to (3.8±0.3)×10^{6} vesicles/μL in the group of animals without probiotic treatment. This fact is in a very good agreement with previous *in vivo* experiments involving blood and isolated erythrocytes subjected to heat (Schultze 1865;

Rumsby, Trotter et al. 1977; Wagner, Chiu et al. 1986; Glaser and Donath 1992; Chernitskii, Slobozhanina et al. 1994; Repin, Bobrova et al. 2008); and also with erythrocytes collected from burn patients (Brown 1946; Baar and Arrowsmith 1970; Loebl, Baxter et al. 1973; Vtiurin, Kaem et al. 1987). Treatment with probiotics before heat stress prevented vesiculation of erythrocytes in animals. The increase in the number of vesicles associated with elevated temperatures may be indicative of the heat stress level and serve as diagnostic test of erythrocyte stability and heat resistance and adaptation.

Our results showed high efficacy of the *B. subtilis* probiotic in the prevention of heat stress- related complications in rats, including: bacterial translocation from the gut; elevated cytokines level; increasing LPS concentration in the blood; vesiculation of erythrocytes; reduction of villi height and mucosal layer thickness. Probiotic treatment can be effective approach to prevent adverse effects of heat stress.

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6.3 Article 3. Microscopic evaluation of vesicles shed by erythrocytes at elevated temperatures (Article is accepted for publication in the Microscopy Research and Techniques)

Microscopic evaluation of vesicles shed by erythrocytes at elevated temperatures.

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Keywords

Light microscopy, super-resolution, real time, echinocytes, vesicles

Abstract

The images of human erythrocytes and vesicles were analyzed by a light microscopy system with spatial resolution of better than 90 nm. The samples were observed in an aqueous environment and required no freezing, dehydration, staining, shadowing, marking or any other manipulation. Temperature elevation resulted in significant concentration increase of structurally

transformed erythrocytes (echinocytes) and vesicles in the blood. The process of vesicle separation from spiculated erythrocytes was video recorded in real time. At a temperature of 37 ^{o}C, mean vesicle concentrations and diameters found were (1.50±0.35) $\times 10^{6}$ vesicles/μL, and 0.365±0.065 μm, respectively. The vesicle concentration increased approximately 3-fold as the temperature increased from 37 to 40 ^{o}C. It was estimated that 80% of all vesicles found in the blood are smaller than 0.4 μm. Accurate account of vesicle numbers and dimensions suggest that 86% of the lost erythrocyte material is lost not by vesiculation but by another, as yet - unknown mechanism.

Introduction

The introduction of no-label high resolution light microscopy has provided a new opportunity for live cell analysis, including the capability of visualizing and recording of cellular events at the submicron scale. In the newly developed microscope illumination system, the object is illuminated by a focused hollow cone of light (instead of beam of light). The effect of the annular illumination promotes the narrowing of the central spot of diffraction pattern and increases the intensity of the diffraction fringes. The similar effect of annular aperture is known and has been used in telescopes (Born and Wolf, 1999) to increase resolution. A better than 90 nm resolution of the Richardson plate (Richardson, 1988) line and picture patterns has been achieved (Vodyanoy et al., 2007, Foster, 2004, Vodyanoy, 2005, Vainrub et al., 2006). This high resolution microscopic illumination system is broadly used in live cell and nanoparticle analysis (Asharani et al., 2010, Rahimi et al., 2008, Shiekh et al., 2010, Carlson et al., 2008, Jun et al., Lim et al., 2012, Weinkauf and Brehm-Stecher, 2009).

All organisms in order to maintain wellbeing must preserve homeostasis, which is constantly affected by internal or external adverse forces which are termed stressors.

Temperature represents one of the most challenging of all stressors. Although humans have the capability to withstand large variations in environmental temperatures, even relatively small increases in internal temperature can lead to injury, heatstroke and even death (Crandall and Gonzalez-Alonso, 2010). Exposure to hot weather is considered one of the most deadly natural hazards in the United States. It has been estimated that between 1979 and 2002, heatstroke claimed more American lives than the combined effects of hurricanes, lightning, earthquakes, floods, and tornadoes (Leon and Helwig, 2010).

Vesicles constitute a heterogenic population of cell-derived microscopic size particles that participate in a wide range of physiological and pathological processes (Gyorgy et al., 2011). They derive from different cell types including platelets, red blood cells, leucocytes, endothelial, smooth muscle, and cancer cells (Burnier et al., 2009, Castellana et al., 2010, Orozco and Lewis, 2010, Roos et al., 2010, VanWijk et al., 2003, Willekens et al., 1997, 2003, 2008, Willekens, 2010). In this work we concentrated on the vesicles found in blood plasma, focusing specifically on the red blood cell vesiculation (Greenwalt, 2006) at conditions of elevated temperatures (Samoylov et al., 2005, Wagner et al., 1986, Walsh and Whitham, 2006, Leonards and Ohki, 1983, Gershfeld and Murayama, 1988, Glaser, 1979, Ham et al., 1948, Bessis, 1972, Longster et al., 1972, Loebl et al., 1973, Baar, 1974, Araki et al., 1982, Christel and Little, 1984, Przybylska et al., 2000a, Ivanov et al., 2007, Chernitskii and Iamaikina, 1996, Schultze, 1865).

An elevation in cytosolic Ca^{2+} activity, is known to initiate cell membrane vesiculation, cell membrane scrambling and activation of the cysteine endopeptidase calpain, an enzyme degrading the cytoskeleton and thus causing cell membrane blebbing or bulging. Ca^{2+} further stimulates Ca^{2+}-sensitive K^+ channels, the following outflow of K^+ hyperpolarizes the cell membrane, which causes outflow of Cl. The cellular loss of KCl with osmotically driven water

leads to cell shrinkage and phosphatidylserine externalization of eryptotic cells. This leads to macrophage recognition and ingestion of dying erythrocytes (Lang and Qadri, 2012).

In the course of storage under blood bank conditions, erythrocytes become extremely susceptible to decrease of phospholipid asymmetry caused by hyperosmotic shock and energy depletion (Bosman et al., 2011). As result, storage of red blood cells is associated with the creation of vesicles characterized by particular proteomic profiles (Bosman et al., 2008b). A 20-fold susceptibility increase after 50 days of storage at 4 ° C was detected (Rubin et al., 2008). This elevated susceptibility could be the cause of the fast disappearance of a substantial portion of the RBCs over the very first twenty four hours immediately following transfusion (Bosman et al., 2011). Consequently, the number of vesicles in stored blood can be one of the parameters of a blood preservation quality control (Rubin, 2007).

Mature erythrocytes undergo aging and eventually they are recognized and cleared by macrophages (Rous, 1923, Rous and Robertson, 1917). More recently, it was shown that the aging erythrocyte (Ghashghaeinia et al., 2012) is characterized by changes in the plasma membrane, that make them selectively recognizable by macrophages, followed by phagocytosis in the spleen, liver, or bone marrow. This process is termed eryptosis or programmed erythrocyte death (Bratosin et al., 2001, Föller et al., 2008, Lang and Qadri, 2012, Vittori et al., 2012).

It has been demonstrated *in vitro* that the normal red blood cells (discocytes) go through shape changes due to a variation of temperature. Elevated temperature, induces a series of crenated shapes (echinocytes), characterized by convex rounded protrusions or spicules. Gradually, the spicules become smaller and more numerous and eventually bud off irreversibly, forming extracellular vesicles composed of plasma membrane materials (Glaser and Donath, 1992, Glaser, 1979, Christel and Little, 1984, Gedde and Huestis, 1997, Henszen et al., 1997,

Muller et al., 1986). We hypothesize that the elevation of temperature during a heat stress may cause transformation of some of the normal erythrocytes (discocytes) into speculated echinocytes that shed membrane vesicles.

The spiculated erythrocytes and mechanism of vesicle separation has previously only been visualized by electron microscopy (Christel and Little, 1984, Dumaswala and Greenwalt, 1984, Bessis, 1972, Orozco and Lewis, 2010, Repin et al., 2008, Brecher and Bessis, 1972, Longster et al., 1972) which is prone to artefacts arising from sample preparation that involve purification, freezing and dehydration. Structure and chemical composition of erythrocyte vesicles were characterized in our previous EM study (Samoylov et al., 2005). The process of erythrocyte spiculation and vesicle budding in live cells were imaged and characterized as function of temperature in the present work.

Methods

High resolution light microscopy of blood

The microscope system we utilized produces a highly oblique hollow cone of light (N.A. 1.2-1.4). Coupled with a high aperture microscope objective with iris, this system provides two different regimes of illumination. When the iris is closed so that no direct light enters the objective after passing through the object, only refracted, scattered, or diffracted light goes in the objective. When the iris is open in such a way to allow the direct entrance of light into the front lens of the objective is illuminated by the annular light produced by the empty cone of light entering the objective. In this case the mixed illumination is produced that combines the darkfield and oblique hollow cone brightfield illuminations. The cardioid condenser is an integral part of the illumination system so that the system is comprised collimation lenses and a first surface mirror that focus light onto the annular entrance slit of the condenser. As a part of

the illumination system, the condenser is pre-aligned, thereby making additional alignment unnecessary. The illumination system is positioned in an Olympus BX51 microscope by replacing a regular brightfield condenser. The illumination system is connected with a light source (EXFO120, Photonic Solution) by a liquid light guide. The objective used for this work is an infinity corrected objective HCX PL APO 100×/1.40-0.70, oil, iris from Leica. The image is magnified with a zoom intermediate lens (2×-U-CA, Olympus), a homebuilt 40× relay lens, and captured by a Peltier-cooled camera (AxioCam HRc, Zeiss) and Dimension 8200 Dell computer. The microscope is placed onto a vibration isolation platform (TMS, Peabody, MA). Live images were recorded with Sony DXC-33 Video camera and Mac OS X Computer (Vainrub et al., 2006). Test images were calibrated using a Richardson slide (Richardson, 1988). A small droplet (7 μL) of freshly drawn blood was placed on a glass slide and coverslipped and positioned on the microscope stage with oil contacts between condenser and objective lenses. Ten image frames of $72 \times 53.3\ \mu m^2$ in each sample were photographed, videotaped, and concentrations of discocytes, echinocytes, and vesicle count and diameter were measured by Image-Pro Plus software (Media Cybernetics), providing high-resolution direct-view optical images in real time. The samples were observed in an aqueous environment and required no freezing, dehydration, staining, shadowing, marking or any other manipulation. At least 20 image frames were analyzed for each experimental condition. Each frame contained at average between 50 and 200 vesicles (depending on conditions).

Subject and protocol

One healthy male volunteer (age 59) was recruited. VO_2 max 45±5 ml/kg/min, height 190.5 cm, weight 106 kg, and 27% fat. VO_{2max} was obtained utilizing a modified Bruce protocol and computed from expired air samples using an oxygen analyzer (Model S-3; Applied

Electrochemistry, Pittsburg, PS, USA) and a CO_2 analyzer (Model CD-3-A; Applied Electrochemistry, Pittsburg, PS, USA). Gas results were corrected to standard temperature, pressure-dry (McAnulty et al., 2005, Bruce et al., 1949a, 1949b). One week prior to beginning of the heat protocol the volunteer was invited for assessment of maximal oxygen consumption (VO_{2max}) and anthropometric data (body weight, body composition, height, etc.). Once a week, after overnight fast at euhydrated state, the volunteer recorded a nude body weight, and obtained a finger prick blood sample (~7 μL) for microscopic examination. The subject then inserted a rectal thermometer 10 cm past the anal sphincter for continual monitoring of core body temperature, and the subject then exercised on the treadmill at a work rate corresponding to 70% of the VO_{2max}. Exercise continued each day until core temperatures was elevated by 1, 2, and 3°C, respectively, above resting temperature. The subject has been running at 2.8 m/s for approximately 20, 25, and 35 minutes to achieve desired elevations of core temperature. Body weight, and a finger prick blood sample were taken again after exercise. The protocol was approved by the Auburn University Internal Review Board.

Statistical analysis

T-test, linear regression, frequency and cumulative frequency, curve fitting and graph plotting were carried out using Microcal™ Origin version 6.0 (Northhampton, MA) and 2010 Microsoft Excel. Geometric mean (μ_g) and geometric standard deviation (σ_g) for set of measured vesicle diameters ($d_1, d_2, \ldots, d_n$) were calculated using the following equations (Daly and Bourke, 2000):

$$\mu_g = (d_1 d_2 \ldots d_n)^{1/n} \quad (1)$$

$$\sigma_g = exp\left(\sqrt{\left(\Sigma_{i=1}^{n} (ln d_1/\mu_g)^2/n\right)}\right) \quad (2)$$

Surface mean diameters (d_s) and volume mean diameters (d_v) of the vesicles were calculated from the count mean diameters (d) measured by light microscopy by using the following equations (Kethley et al., 1963):

$$\log d_s = \log d + 2.303 \log^2 \sigma_g \quad (3)$$

$$\log d_v = \log d + 6.908 \log^2 \sigma_g \quad (4)$$

Results

Temperature

In a human subject the initial core temperature before exercise was 37.10±0.13. The actual body temperatures achieved during exercise were: 38.10±0.14; 39.2±0.2; 40.0±0.3 ^{o}C.

Erythrocyte-echinocyte transformation in blood

At the basal human core temperature of ~ 37 ^{o}C, erythrocytes in freshly drawn blood appear as oval and flexible biconcave discocytes with a diameter of ~7 μm (Fig. 1 a). When temperature rises due to physical exercise, some of the erythrocytes go through size and shape changes, being transformed into echinocytes. Starting with normal discocytes, they become crenated, and then assume spherical shape of smaller diameter with convex rounded protrusions (buds) (Fig. 1 b and c). The buds grow and finally fall off from the cell surfaces to become free vesicles. Some of the erythrocytes become spherical and grow spicules (Fig. 1 d). Vesicles bud off from the spicules. As the temperature increases, the number of echinocytes and free vesicle increases. When the core temperature increased from 37 ^{o}C to 40 ^{o}C the number of free vesicles is significantly increased (Fig. 1 e and f).

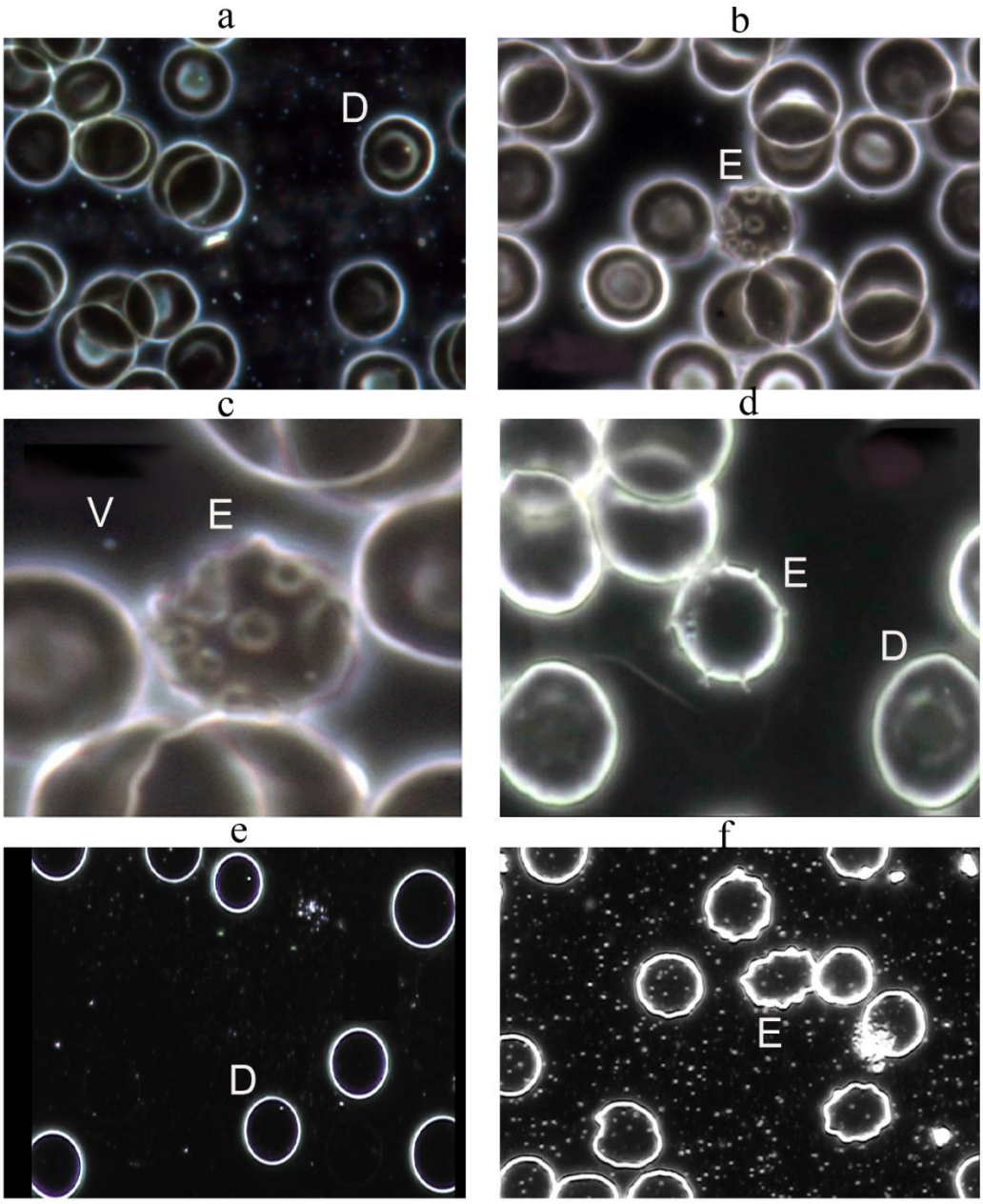

Fig. 1. Human erythrocytes transformation in blood samples at elevated temperatures induced by physical exercise. **a**. Erythrocytes before exercise at core temperature 37 ^{o}C. Most of the erythrocytes are double concave discs of ~7 microns in diameter. D- discocyte (normal biconcave discoid). **b**. Erythrocytes taken at core temperature of 38 ^{o}C after physical exercise. Many erythrocytes are transformed into echinocytes (E) (crenated discs and spheres). c. Enlarged part of the frame **b** with echinocyte (E) in the middle. A free vesicle (V) is shown in a dashed circle. **d**. Another picture frame of erythrocytes taken at 38 ^{o}C after physical exercise. E – echinocyte (spiculated sphere). **e**. A dark field frame of blood video image before exercise at core temperature 37 ^{o}C. **f**. A dark field frame of blood video image after exercise at core temperature 40 ^{o}C. Human erythrocytes are 7 micrometers in diameter and therefore they serve as natural scale bars.

Statistics of vesicles shed by red blood cells

The mean free vesicle concentration in blood after exercise as function of the human core temperature is shown in Fig. 2 a. The concentration is increased approximately 3 times as temperature rises from 37.1 to 40.0 ^{o}C. The percent of echinocytes in blood is also increased with elevated temperature (Fig. 2 b), so that there is a log-log linear correlation between vesicle concentration and percent of erythrocyte-echinocyte transitions (Fig. 2 c).

Fig. 3 a and b show the frequency and cumulative frequency distributions of vesicle concentration and diameters at 37.1 ^{o}C, respectively. A mean vesicle concentration is (1.51±0.35) ×10^{6} vesicles/μL with a mean diameter (d) is 0.365±0.065 μm. A geometric standard deviation calculated by Eq 19 is equal to 1.20. The surface mean diameter (d_s) and volume mean diameters (d_v) of vesicles calculated by Eqs 19 and 20 are 0.377 and 0.403 μm, respectively.

Fig. 3 c compares the cumulative frequency distribution of vesicle concentration at 37.1 ^{o}C (before exercise) and those at temperatures of 38.1, 39.2 and 40.0 ^{o}C (after exercise). When the temperature increases the distribution moves towards higher vesicle concentrations. Vesicle concentration and diameter are shown in comparison with literature data in Table 1.

Condition	*Method*	*t, ^{o}C*	*C, μL^{-1}*	*d, μm*	*Reference*
Live	Light microscopy	37.1	1.5×10^{6}	0.365	Present work
Storage, 5 days	Flow cytometry	4	3,370	<1	(Rubin et al., 2008)
Storage, 50 days	Flow cytometry	4	64,850	<1	(Rubin et al., 2008)
Fixed	Flow cytometry	37	170	0.5	(Willekens et al., 2008)
Centrifuged	Flow cytometry	37	28	ND	(Berckmans et al., 2001)
Centrifuged	Flow cytometry	37	192	ND	(Shet et al., 2003)

Table 1 Human blood vesicle concentration and diameter

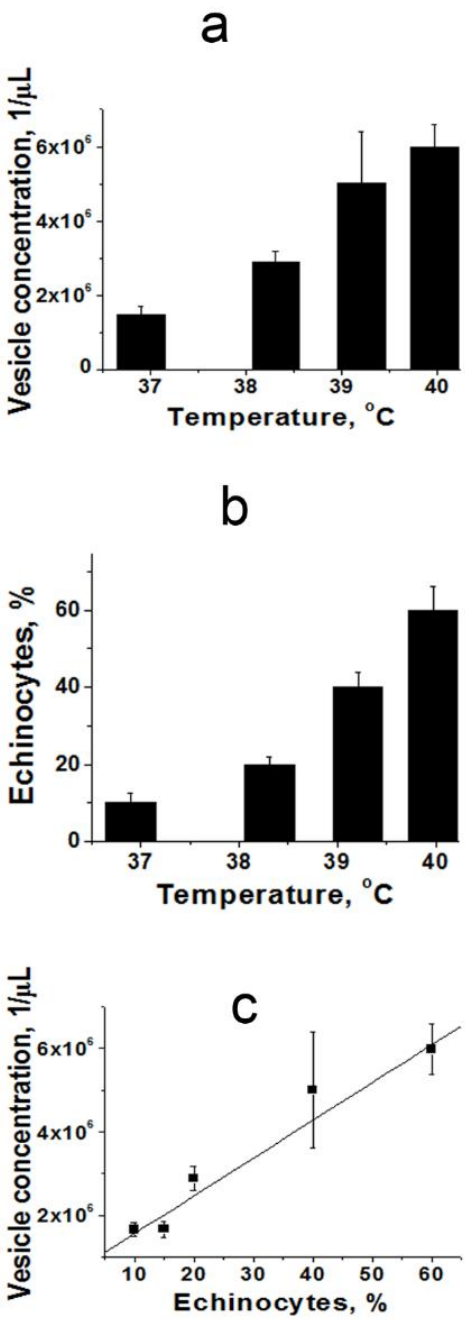

Fig. 2. Number of free vesicles is a function of echinocytes population in human blood. **a**. Mean number of vesicles in 1 μl of fresh blood taken before (at ~37 ^{o}C, core) and after physical exercise at maximal temperatures of ~ 38, 39, and 40 ^{o}C, core, respectively. Count obtained by optical recording of non-fixed 7 μL blood sample with high resolution light microscope system. **b**. Percent of echinocytes estimated in fresh blood taken before (at ~37 ^{o}C, core) and after physical exercise at maximal temperatures of ~ 38, 39, and 40 ^{o}C, core, respectively. **c**. Linear correlation between number echinocytes and vesicle concentration in blood. Points represent experimental data, while line is a linear fit ($R=0.95$, $P<0.012$).

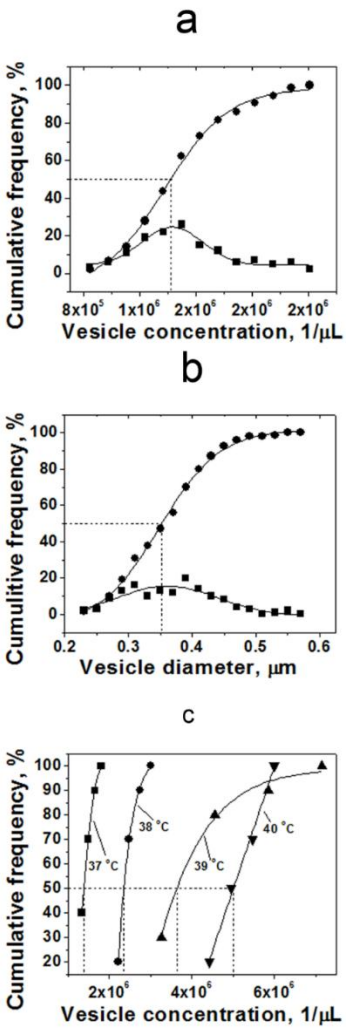

Fig. 3. Vesicle count and diameter distribution for human blood. **a**. Vesicle count distributions. Cumulative frequency distribution (circles) and frequency count distribution (squares) calculated from light microscopy images of non-fixed human blood at 37 ^{o}C. The vesicle concentration at the maximum frequency count corresponds the 50% of the cumulative frequency. The points are experimental data while the cumulative frequency and the frequency count curves are the sigmoidal (Boltzmann) and the Gauss fits, respectively. Sigmoidal fit: Chi^2 =4.5, R^2 =0.997; Gauss fit: Chi^2 =4.0, R^2 =0.948. **b**. Vesicle diameter distributions. Cumulative frequency distribution (circles) and frequency count distribution (squares) calculated from light microscopy images of non-fixed human blood at 37 ^{o}C. The vesicle concentration at the maximum frequency count corresponds the 50% of the cumulative frequency. The points are experimental data while the cumulative frequency and the frequency count curves are the sigmoidal (Boltzmann) and the Gauss fits, respectively. Sigmoidal fit: Chi^2 =4.0, R^2 =0.998; Gauss fit: Chi^2 =7.7, R^2 =0.828. **c**. Cumulative frequency distribution of vesicle concentration as function of temperature. . The data were calculated from light microscopy images of non-fixed human blood at 37, 38, 39, and 40 ^{o}C, respectively. The points are experimental data while the cumulative frequency curves are the sigmoidal (Boltzmann) fits. R^2 =0.996, 0.987, 0.993, and 0.994 for 37, 38, 39, and 40 ^{o}C, respectively.

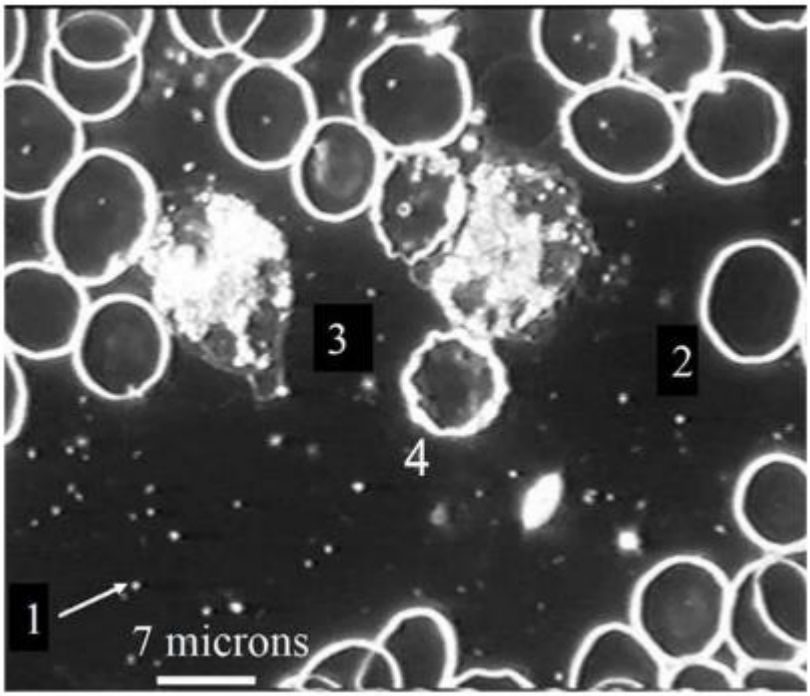

Fig. 4. Direct contact of white and red blood cells in human blood at ~38 ^{o}C, core. 1-vesicle; 2-erithrocite; 3-neutrophil, 4-echinocytes; 100X objective, oil.

Discussion

Light microscopy of live cells and vesicles

Light microscopic images of human blood cells shown in this work were taken within a few minutes after small volume blood samples (7 μL) were drawn. The samples are not subjected any chemical treatments. The cells are videotaped in the natural environment and selective frames are shown in Figs. 1 and 4. The high resolution optical images of human erythrocytes, echinocytes, and free vesicles are consistent with those obtained by electron microscopy (Christel and Little, 1984, Dumaswala and Greenwalt, 1984, Bessis, 1972, Orozco and Lewis, 2010, Repin et al., 2008, Brecher and Bessis, 1972, Longster et al., 1972). The chief advantages of high resolution light microscopy utilized in this work compared to scanning electron microscopy lies in the ability to image live cells in real time at a lower cost. This

technique is especially important for free moving vesicles because it allows for their easier discrimination from background. The present work on vesicles shed by erythrocytes is consistent with our previous publications describing structural and biochemical properties of erythrocyte vesicles produced at elevated temperatures in human, rabbit, and dog blood (Vodyanoy et al., 2007, Samoylov et al., 2005)

In this work, vesicle concentrations found in freshly drawn blood samples at basal conditions were found to be at the level of 10^6 vesicles/μL. This concentration is a few orders of magnitude higher than those reported in the literature (Table 1). It is well recognized that there is a substantial amount of variation between researchers regarding the preparation procedures used including blood drawing techniques, isolating erythrocyte vesicles, with essential differences in anticoagulants, filtration, centrifugation and flow cytometry, all of each which frequently results in data variability (Dey-Hazra et al., 2010, Hind et al., 2010, Huica et al., 2011, Grant, 2003, Rubin et al., 2010, Minetti et al., 2013). Most of the erythrocyte vesicles ranged in size between 0.1 and 1 μm (Hind et al., 2010), whereas the most traditional detection technique, flow cytometry, has a detection threshold in the range of 0.4-0.5 μm. The cumulative frequency analysis of vesicle diameters showed that 80% of all vesicles found in blood are smaller than 0.4 μm (Fig. 3 b). It is therefore concluded that majority of vesicles are lost during preparation and subsequent detection.

Erythrocyte vesiculation at elevated temperatures

Flow cytometry studies demonstrated that vesicles derive mostly from erythrocytes (approximately 65%) (Willekens, 2010). It is well established that physical exercise results in an increase in the core temperature in human and animals (Briese, 1998, Greenleaf, 1979, Nybo and Nielsen, 2001a, Parrott et al., 1999, Periard et al., 2012, Sawka and Wenger, 1988, Walsh and

Whitham, 2006, White and Cabanac, 1996). In this work, the elevated temperature resulting from physical exercise in humans is accompanied by both an increase in the number of transformed erythrocytes (echinocytes) and an increase in free vesicle concentration. A human erythrocyte loses 15 μm^3 of volume during its lifespan (120 days) (Bosman et al., 2008a).

The volume of a single vesicle (*v)* is estimated as $v=\pi d_v^3/6= \pi\ (0.403)^3=3.43\times10^{-2}$ μm3. If all lost erythrocyte volume (*L*) was converted into vesicles, the number of produced vesicles *(n*) during the erythrocyte lifespan would be $n=L/v=15\ \mu m^3/3.43\times10^{-2}\ \mu m^3=437$.

If the surface area of a single vesicle is, $s=\pi d_s^2 =\pi\ (0.377)^2=0.446\ \mu m^2$, and the total area (*S*) of 437 vesicles would be equal to $437\times0.446\ \mu m^2=194\ \mu m^2$ which is much higher than the whole area of erythrocyte (136 μm^2). During a lifespan, erythrocyte reduces its surface area by 20% (Werre et al., 2004), which amounts to the area loss $Ls=0.2\times136\ \mu m^2=27.2\ \mu m^2$.

Comparing the estimated total area $S=194\ \mu m^2$ to the experimental loss $L_s= 27.2\ \mu m^2$, one can conclude that only 27.2/194≈14% of lost area are converted to vesicles. On average, human red blood cells shed one vesicle every two days during their lifespan. This means that 86% of the lost human erythrocyte materials are lost not by vesiculation but by another unknown mechanism.

A similar analysis of the lifespan loss of human erythrocyte volume and surface area was carried out based upon experimental results obtained by centrifugation and flow cytometry (Werre et al., 2004, Willekens et al., 2008). The authors found a strong discrepancy between estimated and observed loss of erythrocyte surface area. The authors concluded: "Taken together, these data suggest that most vesicles are taken up almost directly by the macrophages of the organ in which they originate before they can reach the venous circulation and be counted. Apparently, the body has developed an efficient mechanism to remove these vesicles..."

(Willekens et al., 2008, Willekens, 2010). Being not aware of the direct removal mechanism of hemoglobin by macrophages, the authors explained the extra loss of surface area in comparison to the hemoglobin loss by incorporation of lipids (Bosman et al., 2008a). This explanation seems unlikely, because during erythrocyte aging, the cell loses 20% of lipids which is equal to the loss of hemoglobin (Rumsby et al., 1977). We hypothesize, that an alternative mechanism of direct transfer of damaged hemoglobin does exist but remains unknown.

During observation and video recording of fresh samples of live blood, direct and prolong contacts between echinocytes and white cells are frequently observed. In contrast, white cells were not observed in contact with intact erythrocytes. When white cells approach intact erythrocytes, the cells repulse and scatter. We speculate that during such contacts with echinocytes, peripherally located membrane buds are directly scavenged by white cells. Fig. 4 shows a single frame of a video recording of echinocytes that may be engaged in process of removal of surface vesicles by white cells. The figure also shows many free vesicles and erythrocytes. The facts of the selective interaction of white cells with echinocytes and the avoidance of intact erythrocytes are substantiated by the difference in surface electric charges. It has been demonstrated that the ζ-potential of erythrocytes decreased gradually with aging indicating a lesser amount of negative charge density (Huang et al., 2011). White blood cells have a negative surface charge (Gallin, 1980), therefore the reduction of the aging erythrocyte charge reduces repulsive forces and promotes adhesiveness. It has been also shown that the phosphatidylserine (PS) externalization in erythrocytes after stimulation by a variety of substances or aging, led to increase of the intercellular adhesion (Steffen et al., 2011). Though PS may cause a partial compensation of lost negative charges, the cell-cell adhesiveness was increased probably due to PS-Ca^{2+}-PS interactions between contacting cells.

Conclusions

1. High resolution light microscopy of live blood cells is an appropriate technique for imaging heat induced structural erythrocyte transformations and subsequent vesicle release.
2. Vesicle release accounts for only a small part of the erythrocyte's surface reduction during its lifespan. It is suggested that a large part of the aged erythrocyte membrane is removed by direct contacts with white cells.

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6.4 Article 4. Microscopic evaluation of vesicles shed by rat erythrocytes at elevated temperatures

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Microscopic evaluation of vesicles shed by rat erythrocytes at elevated temperatures.

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Abstract

It is hypothesized that the elevation of the temperature of the blood during heat stress may cause an increase of the shedding of erythrocyte membrane vesicles. Therefore, the increase of vesicle numbers following heat stress may be indicative of and proportional to the level heat stress. In order to test this hypothesis, erythrocytes and the vesicles shed by erythrocytes were collected from rat blood and analyzed after the elevation of body temperature by exposure to external heat. The images of erythrocytes and vesicles were analyzed by a custom light microscopy system with spatial resolution of better than 90 nm. The samples were observed in an aqueous environment and required no freezing, dehydration, staining, shadowing, marking or any other manipulation. The elevation of temperature from 36.7±0.3 to 40.3±0.4 ^{o}C resulted in significant increase of the concentration of vesicles in blood. At a temperature of 37 ^{o}C, mean vesicle concentrations and diameters found in rat blood were (1.4±0.2)×10^{6} vesicles/μL and 0.436±0.03 μm, respectively. The concentration of free vesicles increased after exposure to heat

to $(3.8\pm0.3)\times10^6$ vesicles/μL. It was estimated that 80% of all vesicles found in rat blood are smaller than 0.45 μm. The increase in the number of vesicle associated with elevated temperatures may be indicative of the heat stress level and serve as diagnostic test of erythrocyte stability and heat resistance.

Keywords

Erythrocytes, vesicles, microscopy, heat stress

Introduction

Vesicles constitute a heterogenic population of cell-derived microscopic size particles that participate in a wide range of physiological and pathological processes. They derive from different cell types including platelets, red blood cells, leucocytes, endothelial, and cancer cells (Burnier et al., 2009; Castellana et al., 2010). In this work we concentrated on the vesicles in blood plasma, focusing on the red blood cell vesiculation (Greenwalt, 2006) at conditions of elevated temperatures (Araki et al., 1982; Przybylska et al., 2000; Schultze, 1865; Wagner et al., 1986).

It has been demonstrated *in vitro* that the normal red blood cells (discocytes) go through shape changes as a result of variation of temperature. Elevated temperature, induces a series of crenated shapes (echinocytes), characterized by convex rounded protrusions or spicules. Gradually, the spicules become smaller and more numerous and eventually bud off irreversibly, forming extracellular vesicles composed of plasma membrane materials (Christel and Little, 1984; Gedde and Huestis, 1997; Glaser and Donath, 1992). Hyperthermia stimulates Ca^{2+} entry into erythrocytes leading to cell shrinkage and phosphatidylserine (PS) exposure. PS-exposing erythrocytes are rapidly cleared from circulating blood (Foller et al., 2010). Elevation in cytosolic Ca^{2+} activity is known to initiate cell membrane vesiculation, cell membrane

scrambling and activation of the cysteine endopeptidase calpain, an enzyme degrading the cytoskeleton, and thus causing cell membrane blebbing or bulging. Ca^{2+} further stimulates Ca^{2+}-sensitive K^+ channels, resulting in an outflow of K^+ which hyperpolarizes the cell membrane, and causes outflow of Cl. The cellular loss of KCl along with osmotically driven water leads to cell shrinkage and phosphatidylserine externalization of heat-stressed cells. This leads to macrophage recognition and ingestion of dying erythrocytes (Lang and Qadri, 2012).

Depending on a combined volume/density cell fractionation method, it has been shown that throughout the lifespan of the mature erythrocyte, volume and hemoglobin content reduce by 30 and 20%, respectively, while the hemoglobin concentration increases by 14% (Werre et al., 2004). Additionally, the surface area and lipid content decrease by 20%, while the actual surface-to-volume increases (Rumsby et al., 1977). If the decline of hemoglobin and surface area results from the creation and discharge of hemoglobin-containing vesicles, one can estimate the vesicle contribution in the erythrocyte surface area reduction. Supposing that vesicles are spherical and that they contain the same concentration of hemoglobin as the parent red blood cell, the reduction of 20% of hemoglobin is equal to the loss of 15 μm^3 membrane. Using an average vesicle diameter of 0.5 μm (Werre et al., 2004), one erythrocyte creates ~230 vesicles in the course of its lifespan. This is equivalent to a membrane area of 180 μm^2, while the erythrocyte surface area is reduced by approximately 30 μm^2 (Bosch et al., 1994). Hence, there is not enough available membrane in the entire erythrocyte to package 20% of the hemoglobin in vesicles. The authors concluded: "Taken together, these data suggest that most vesicles are taken up almost directly by the macrophages of the organ in which they originate before they can reach the venous circulation and be counted. Apparently, the body has developed an efficient mechanism to remove these vesicles." (Werre et al., 2004; Willekens et al., 2008; Willekens, 2010). Being

not aware of the any other mechanism, the authors explained the extra loss of surface area in comparison to the hemoglobin loss by incorporation of lipids (Bosman et al., 2008). This explanation is very unlikely, because during erythrocyte aging, the cell loses 20% of lipids which is equal to the loss of hemoglobin. We hypothesize, that an alternative mechanism of direct transfer of damaged hemoglobin exists but remains unknown.

It is well recognized that there is substantial variation between researchers regarding the preparation procedures used in isolating erythrocyte vesicles, with essential differences in anticoagulants, filtration, centrifugation and flow cytometry, which frequently result in data variability (Dey-Hazra et al., 2010; Hind et al., 2010; Huica et al., 2011). Most of the erythrocyte vesicles range in size between 0.1 and 1 μm (Hind et al., 2010), however the most traditional detection technique, flow cytometry, has a detection threshold in the range of 0.4-0.5 μm .
It is hypothesized that the elevation of the temperature of the blood during heat stress may cause an increase of the shedding of erythrocyte membrane vesicles. Therefore, the increase of vesicle numbers following heat stress may be indicative of and proportional to the level heat stress. The evaluation, using high resolution light microscopy, of vesicles shed by erythrocytes in rats exposed to external heat has been a primary goal of our work. It is anticipated that high resolution microscopy allows for improved vesicle counting, which in turn may shed some light on the fate of the hemoglobin lost by erythrocytes. This a new method, not used before and therefore, a validation and comparison with results obtained by other techniques has been a secondary goal of our research.

Methods

Animals

All procedures involving animals were reviewed by the Auburn University Institutional Animal Care and Use Committee and complied with current humane standards for animal use in research. Adult Sprague-Dawley male rats weighing 250-300 g were used. The animals were housed two in a cage under standardized conditions of temperature (20 ± 1^0 C), humidity (50 ± 5%) and lighting (12-h day/12-h night) with free access to food and water. Three groups of rats (10 rats in control, 10 rats in stress group, and 5 rats in temperature control) were studied. These groups are: (1) control (25°C), 2) stress (45°C, 25 min), and (3) temperature control. Animals from group (2) were exposed to 45°C heat stress, relative humidity 55% for 25 min in a 1610 Caron Environmental Chamber. Control animals (group 1) were exposed to identical conditions as the heat stressed animals, but at 25°C. Four hours after the stress experiments small samples (7 μL) of blood from tail vein were taken from each rat for live cells and vesicles and examined with light microscopy. Temperature of each rat was measured with an infrared digital thermographic camera (Flir B360 High-Sensitivity Infrared Thermal Imaging Camera) placed 0.5 m from the animal. This camera has a thermal sensitivity of approximately 0.1 ^{o}C and a spatial resolution of 320×240 pixels. The field of view of the camera is 25° x 19° and minimum focus distance of 0.4 m. The camera is equipped with a laser pointer that pinpoints a reference spot and aligns a marker to it on the image. In the "*Measure Area*" camera mode, the rat IR image is created and the measurement box was positioned at the rat ear space. The camera automatically located the maximum temperature of the area, showed a marker crosshair and a digital temperature value. In all of our experiments with rats, the maximum temperature was shown for the laser pointed inside the rat ear canal (Chan et al., 2004; Ng and Acharya, 2009;

Poole and Stephenson, 1977; Vianna and Carrive, 2005). The temperatures in the Groups 1 and 2 were measured by IR camera only. The temperature in group (3) was measured with calibrated IR and rectal thermometers to determine the correlation between rectal and ear temperatures in rats. A rectal thermometer was inserted 4 cm past the anal sphincter (Lomax, 1966). Five rats in the group (3) were subjected to 45 ^{o}C heat stress, relative humidity 55% for 25 minutes. The ear and rectal temperatures were measured simultaneously before and after heat stress.

High resolution light microscopy of blood

The new microscope system used for this study produces a highly oblique hollow cone of light (N.A. 1.2-1.4). It is coupled with a high aperture microscope objective with iris, thus allowing this system to provide two different regimes of illumination: 1. - When the iris is closed so that no direct light enters the objective after passing through the object, only refracted, scattered, or diffracted light goes in the objective, and 2. - If the iris is open in such a way to allow the direct entrance of light into objective the front lens of the objective is illuminated by the annular light produced by the empty cone of light entering the objective. In this case the mixed illumination is produced that combines the darkfield and oblique hollow cone brightfield illuminations. The cardioid condenser is an integral part of the illumination system so the system is comprised of a collimation lenses and a first surface mirror that focus light onto the annular entrance slit of the condenser. As a part of the illumination system, the condenser is pre-aligned and therefore additional alignment is unnecessary. The illumination system is positioned in Olympus BX51 microscope by replacing a regular brightfield condenser. The illumination system is connected with a light source (EXFO120, Photonic Solution) by a liquid light guide. The objective used for this work is infinity corrected objective HCX PL APO 100×/1.40-0.70, oil, iris from Leica. The image is magnified with a zoom intermediate lens (2×-U-CA,

Olympus), a homebuilt 40× relay lens, and captured by a Peltier-cooled camera (AxioCam HRc, Zeiss) and Dimension 8200 Dell computer. The microscope is placed onto a vibration isolation platform (TMS, Peabody, MA). Live images were recorded with Sony DXC-33 Video camera and Mac OS X Computer. It was demonstrated that this system has a detection limit smaller than 50 nm and the spatial resolution of ~90 nm (Vainrub et al., 2006). Test images were calibrated using a Richardson slide (Richardson, 1988). A small droplet (7 μL) of freshly drawn blood was placed on a glass slide and coverslipped and positioned on the microscope stage with oil contacts between condenser and objective lenses. Ten image frames of 72 × 53.3 μm^2 in each sample were photographed, videotaped, and vesicle count and diameter were measured by Image-Pro Plus software (Media Cybernetics), providing high-resolution direct-view optical images in real time. The samples were observed in an aqueous environment and required no freezing, dehydration, staining, shadowing, marking or any other manipulation. At least 20 image frames were analyzed for each experimental condition. Each frame contained at average between 50 and 200 vesicles (depending on conditions).

Statistical analysis

T-test, linear regression, frequency and cumulative frequency, curve fitting and graph plotting were carried out using Microcal™ Origin version 6.0 (Northhampton, MA) and 2010 Microsoft Excel. Geometric mean (μ_g) and geometric standard deviation (σ_g) for set of measured vesicle diameters ($d_1, d_2,\ldots,d_n$) were calculated using the following equations (Daly and Bourke, 2000):

$$\mu_g=(d_1 d_2 \ldots d_n)^{1/n} \quad (1)$$

$$\sigma_g=exp((\sqrt{\Sigma^n_{i=1}(lnd_1/\mu_g)^2/n})) \quad (2)$$

Surface mean diameters (d_s) and volume mean diameters (d_v) of vesicles calculated from the count mean diameters (d) measured by light microscopy by using the following equations (Kethley et al., 1963):

$$\log d_s = \log d + 2.303 \log^2 \sigma_g \quad (3)$$

$$\log d_v = \log d + 6.908 \log^2 \sigma_g \quad (4)$$

Results

Ear and rectal temperatures

The 5 rat's average ear and rectal temperatures before heat stress were 37.4 ±0.5 and 37.3±0.3 ^{o}C, respectively. After heat stress, the average ear and rectal temperatures measured simultaneously were 39.8±0.6 and 40.5±0.3 ^{o}C, respectively. The average temperatures of 10 control and 10 heat stress test rats that were measured by IR camera only were 36.7±0.3 and 40.3±0.4 ^{o}C. There was no significant difference between the simultaneously measured ear/rectal and the ear only temperatures at the level of 0.05 (Table 1). These results are consistent with human studies that showed that infrared ear thermometers provided a closer estimate of core body temperature than equilibrated rectal temperature (Rotello et al., 1996). We conclude that infrared ear temperature used in this work provides an adequate presentation of core temperature and also eliminates some shortcomings of rectal temperature measurements (Poole and Stephenson, 1977).

Condition	Before heat stress		After heat stress	
Rat #	IR camera	Rectal thermometer	IR camera	Rectal thermometer
1	37.7	37.1	39.1	40.7
2	38.0	37.7	40.4	40.5
3	37.1	37.4	40.5	40.3
4	37	37.1	39.4	40.1
5	37.1	37.2	39.6	40.7
Average	37.4	37.3	39.8	40.5
SD	0.5	0.3	0.6	0.3

Table 1. Comparison of rat ear and rectal temperatures (^{o}C) measured by IR camera and rectal thermometer, respectively

Vesiculation of rat erythrocytes after exposure to heat (passive hyperthermia)

The lifespan of rat erythrocytes is only 50% that of human (Table 2) in spite of the fact that natural human's lifespan is about 20 times longer (Alemáan et al., 1998). Rat and human erythrocyte diameters are very comparable, volume and surface area about 25% larger in humans, but erythrocyte concentration is 60% greater in rats. A general appearance of rat blood cells under high resolution microscope is very similar to one observed in human samples (data not shown).The concentration of free vesicles increased after exposure to heat stress from $(1.4\pm0.2)\times10^{6}$ to $(3.8\pm0.3)\times10^{6}$ vesicles/μL (Fig. 1a). The values are significantly different at the 0.001 level.

The IR ear rat temperature increased during a heat exposure from 36.7±0.3 to 40.3±0.4 ^{o}C. Fig. 1b and c show frequency and cumulative frequency distributions of vesicle concentration and diameter. The figures indicate a significant shift to higher vesicle concentrations and diameter after a heat stress. The mean vesicle diameter at 36.7 ^{o}C is 0.436±0.03 μm, and at 40.3 ^{o}C is 0.559±0.03 μm. A geometric standard deviation calculated by Eq 3 is equal to 1.06. At 36.7 ^{o}C, the surface mean diameter (d_s) and volume mean diameters (d_v) of vesicles calculated by Eqs 3 and 4 are 0.438 and 0.441 μm, respectively.

Subject	Condition	Method	t, ^{o}C	C, μL^{-1}	d, μm	Reference
Rat	Live	Light microscopy	36.7	1.4×10^{6}	0.436	Present work
Rat	Storage, 16 days	Light microscopy	4	163	0.3-0.6	(Giuliani et al., 2000)
Human	Storage, 5 days	Flow cytometry	4	3,370	<1	(Rubin et al., 2008)
Human	Storage, 50 days	Flow cytometry	4	64,850	<1	(Rubin et al., 2008)
Human	Fixed	Flow cytometry	37	170	0.5	(Willekens et al., 2008)
Human	Centrifuged	Flow cytometry	37	28	ND	(Berckmans et al., 2001)
Human	Centrifuged	Flow cytometry	37	192	ND	(Shet et al., 2003)

Table 2. Comparison of properties of fresh intact erythrocytes from rat and human blood.

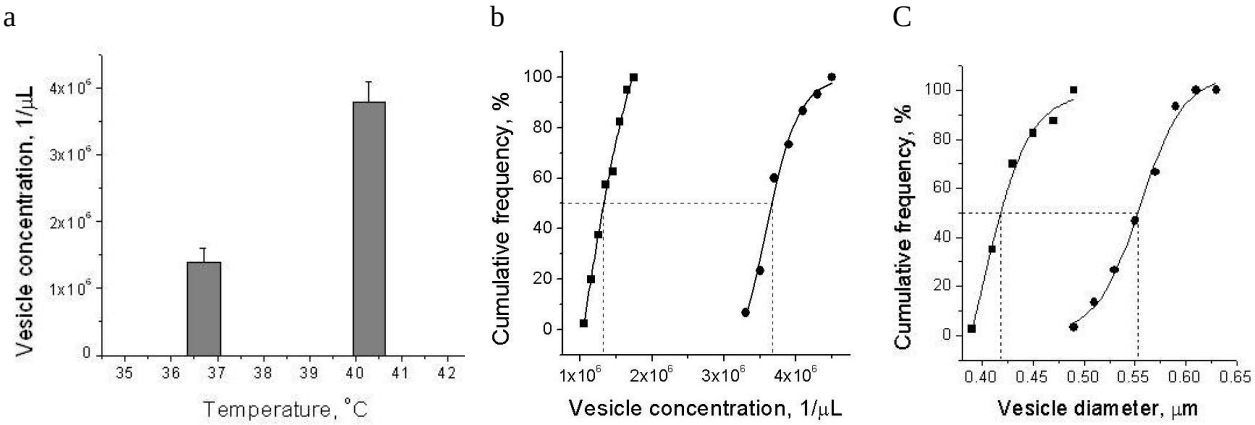

Fig. 1. Vesicle count and diameter distribution of rat blood **a**. Mean number of vesicles in 1 μl of fresh blood taken rat blood before and after 25 min at 45 ^{o}C. The values are significantly different at the level of 0.001. **b.** Cumulative frequency distribution of vesicle concentration in rat blood at 36.7 (squares) and 43.2 (circles) ^{o}C. The points are experimental data while the cumulative frequency curves are the sigmoidal (Boltzmann) fits: R^2 =0.993 and 0.990, respectively **c**. Cumulative frequency distribution of vesicle diameter in rat blood at 36.7 (squares) and 43.2 (circles) ^{o}C . The data were calculated from light microscopy images of non-fixed rat blood before and after 25 min at 45 ^{o}C. The points are experimental data while the cumulative frequency curves are the sigmoidal (Boltzmann) fits: R^2 =0.991 and 0.994, respectively.

Discussion

Erythrocyte vesiculation at elevated temperatures

The main findings of this work can be summarized as following: (1) The number of vesicles shed by erythrocytes increases at elevated temperatures; (2) Eighty percent of erythrocyte released vesicles are smaller than 0.45 μm; (3) Freshly drawn rat blood contains more than 1 million vesicles per one microliter.

Flow cytometry studies demonstrated that vesicles in blood derive mostly from erythrocytes (approximately 65%) (Willekens, 2010). Rat and human erythrocytes have similar physical properties (Table 2). Basal properties including core temperature, vesicle concentration and diameter are very comparable (Table 3). When the rat is exposed to environmental hyperthermia, the core temperature increases and the echinocytes number and concentration of free vesicles in the blood increases. This fact is in a very good agreement with *in vivo* experiments involving blood and isolated erythrocytes subjected to heat (Chernitskii et al., 1994; Glaser and Donath, 1992; Repin et al., 2008; Rumsby et al., 1977; Schultze, 1865; Wagner et al., 1986); and also with erythrocytes involved in burns (Baar and Arrowsmith, 1970; Brown, 1946; Loebl et al., 1973; Vtiurin et al., 1987).

Rat erythrocytes lose 30% of volume during their lifespan (Willekens, 2010). Taking rat erythrocyte volume from Table 2, the lost volume is calculated to be $L_v = 0.3 \times 64.9\ \mu m^3 = 19.47\ \mu m^3$. The volume of a single vesicle (v) is estimated as $v = \pi d_v^3/6 = \pi (0.441)^3 = 4.49 \times 10^{-2}\ \mu m^3$. If all lost erythrocyte volume was converted to vesicles, the number of vesicles produced during the erythrocyte's lifespan would be equal to $n = L/v = 19.47\ \mu m^3 / 4.49 \times 10^{-2}\ \mu m^3 = 434$.

Subject	Condition	Method	t, ^{o}C	C, μL^{-1}	d, μm	Reference
Rat	Live	Light microscopy	36.7	1.4×10^{6}	0.436	Present work
Rat	Storage, 16 days	Light microscopy	4	163	0.3-0.6	(Giuliani et al., 2000)
Human	Storage, 5 days	Flow cytometry	4	3,370	<1	(Rubin et al., 2008)
Human	Storage, 50 days	Flow cytometry	4	64,850	<1	(Rubin et al., 2008)
Human	Fixed	Flow cytometry	37	170	0.5	(Willekens et al., 2008)
Human	Centrifuged	Flow cytometry	37	28	ND	(Berckmans et al., 2001)
Human	Centrifuged	Flow cytometry	37	192	ND	(Shet et al., 2003)

ND-not determined

Table 3. Vesicle concentration (C) and diameter (d) in rat and human blood at different conditions.

If the surface area of a single vesicle, $s=\pi d_s^2 =\pi (0.438)^2=0.602$ μm^2, then the total area (S) of 434 vesicles would be equal to 434×0.602 μm^2=261 μm^2. This value is much higher than the total area of the erythrocyte (103 μm^2). During a lifespan, rat erythrocytes reduce their surface area by 20% (Willekens, 2010), which amounts to the area loss $L_s=0.2\times103$ μm^2=20.6 μm^2. Comparing the estimated total area S=261 μm^2 to the experimental loss L_s= 20.6 μm^2, one can conclude that only 20.6/261≈8% of the lost area is converted to vesicles. On average, rat red blood cells shed one vesicle every two days during their lifespan. This means that 92% of the rat erythrocyte is lost not by vesiculation but by another unknown mechanism.

In spite of the much higher erythrocyte vesicle concentration measured in this work compared to that found with isolated vesicles we have come to the same conclusion that vesiculation alone cannot provide for the removal of all hemoglobin lost during the erythrocyte lifespan (Willekens, 2010).

During observation and video recording of fresh samples of live blood, direct and prolong contacts between echinocytes and white cells are frequently accounted (data not shown).. In contrast, white cells were not observed in contact with intact erythrocytes. When white cells approach erythrocytes, the cells repulse and scatter. We speculate that during such contacts with echinocytes, peripherally located membrane buds are directly scavenged by white cells. The facts of the selective interaction of white cell with echinocytes and the avoidance of erythrocytes are substantiated by the difference in surface electric charges. It has been demonstrated that negative surface electrical charge of aging red blood cells is reduced (Huang et al., 2011). White blood cells have a negative surface charge (Gallin, 1980), therefore the reduction of the aging erythrocyte charge reduces repulsive forces and promotes adhesiveness.

Light microscopy of live cells and vesicles

The introduction of no-label high resolution light microscopy provided a new opportunity for live cell analysis, including capability of visualizing and recording of cell events at the submicron scale. In the newly developed illumination system, the object is illuminated by the focused hollow cone of light (instead of beam of light). The effect of the annular illumination promotes the narrowing of the central spot of diffraction pattern and an increase of the intensity of the diffraction fringes. The similar effect of annular aperture is known and used in telescopes (Born and Wolf, 1999) to increase the resolution. A better than 90 nm resolution of the Richardson plate (Richardson, 1988) line and picture patterns has been achieved (Vainrub et al., 2006). The high resolution microscopic illumination system was commercialized and it is known as CytoViva and it is broadly used in live cell and nanoparticle analysis.

The chief advantages of high resolution light microscopy utilized in this work compared to scanning electron microscopy lies in the ability to image live cells in real time at a lower cost.

This technique is especially important for free moving vesicles because it allows their discrimination from background. The present work on vesicles shed by erythrocytes is consistent with our previous publications describing structural and biochemical properties of erythrocyte vesicles produced at elevated temperatures in human, rabbit, and dog blood (Samoylov et al., 2005; Vodyanoy et al., 2007)

In this work, vesicle concentrations in freshly drawn rat blood samples at basal conditions were found to be at the level of 10^6 vesicles/μL. This concentration is higher than those found in the literature (Table 3). This discrepancy is explained by two facts: (1) the capability of high resolution microscopy used in this work to account for vesicles smaller than 0.4 μm, while the most traditional detection technique, flow cytometry, has a detection threshold in the range of 0.4-0.5 μm. (2) The vesicles in this work were observed and recorded in the live blood just a few minutes after it was drawn, whereas the most conventional techniques for examining blood vesicles include preparation procedures with essential differences in anticoagulants, filtration, centrifugation and flow cytometry. These differences frequently result in significant data variations (Dey-Hazra et al., 2010; Hind et al., 2010; Huica et al., 2011). Most of the erythrocyte vesicles range between 0.1 and 1 μm (Hind et al., 2010). The cumulative frequency analysis of vesicle diameters showed that 80% of all vesicles found in blood are smaller than 0.45 μm (Fig. 1 c). It is therefore concluded that majority of vesicles are lost during preparation and detection in many conventional methods.

Conclusions

High resolution light microscopy of live blood cells is an appropriate technique for imaging heat induced structural erythrocyte transformations and vesicle release.

Vesicle release accounts for only a small part of erythrocyte surface reduction during its lifespan. It is suggested that a large part of the aged erythrocyte membrane is removed by direct contacts with white cells.

Exposure to heat results in elevation of body temperature, and in turn increases the number of erythrocyte released vesicles. These changes reduce erythrocyte stability. Therefore, the number of vesicles increases due to environmental heat has a diagnostic value of heat stress level.

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6. Conclusions

This work examined the effects of heat stress on the structure and integrity of the gastrointestinal barrier, as well as the effects of heat on the rate of erythrocyte vesiculation. Further, the ability of a *B. subtilis* probiotic to reduce or prevent the effects of heat stress was also evaluated. The following conclusions can be drawn from this work:

1. Gut microflora homeostasis is critical to maintaining gastrointestinal mucosal barrier function. There is strong evidence to support the influence of stress on depression of intestinal microflora and, as a result, on the overall immune resistance of the organism.
2. Modulating the intestinal flora might offer a novel and non-invasive therapeutic approach for promoting well-being by prevention and treatment the adverse effects of stress in the gut.
3. Effectiveness of *Bacillus subtilis* probiotic strain for prevention of heat stress related complications has been characterized in rats. It has been demonstrated that pre-treatment of rats with probiotic bacteria resulted in the prevention of microbial translocation from the gut into mesenteric lymph nodes and liver.
4. Heat stressed animals devoid of probiotic treatment had a higher level of lipopolysaccharides (LPS) in the blood. By comparison, animals in the probiotic group, subjected to high temperature did not reveal increases of LPS level. Cytokine IL-10

concentration was substantially elevated in stressed animals with no probiotic pretreatment.

5. Increases of the temperature of the blood for the duration of heat stress leads to an elevation of the shedding of erythrocyte membrane vesicles. This increase of temperature from 36.7±0.3 to 40.3±0.4 ^{o}C leads to a significant increase of the number of vesicles in the blood. At a temperature of 37 ^{o}C, mean vesicle concentrations observed in rat blood was $(1.4\pm0.2)\times10^{6}$ vesicles/μL. Whereas, following exposure to heat, a concentration of $(3.8\pm0.3)\times10^{6}$ vesicles/μL was observed in the group of animals without probiotic treatment. Treatment with probiotics prior to heat stress prevented vesiculation of erythrocytes in animals.
6. The results have demonstrated high effectiveness of *B. subtilis* probiotics in the prevention of heat stress related complications in rats.
7. The images of human erythrocytes and vesicles have been examined by a light microscopy system with spatial resolution of better than 90 nm. The samples have been analyzed in a natural environment and required no freezing, dehydration, staining, shadowing, marking or any other modification. Temperature elevation resulted in substantial concentration increases of structurally transformed erythrocytes (echinocytes) and vesicles in the blood. The process of vesicles separating from spiculated erythrocytes has been video recorded in real time.
8. It was estimated that 80% of all vesicles found in rat blood are smaller than 0.45 μm.
9. The increase in the number of vesicles associated with elevated temperatures may be indicative of the heat stress level and serve as diagnostic test of erythrocyte stability and heat resistance and adaptation.

10. Microscopic examination of ideal intestinal cross sections showed that rats exposed to high heat stress with probiotics experienced shrinkage of overall villi height of approximately 50% and a reduction of the mucosal boundary of approximately 30%. Whereas, rats exposed to high heat with probiotics did not experience villi height or mucosal boundary reduction.

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