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STABILITY OF BOVINE MILK EXOSOMES AND THEIR EFFECT ON PROLIFERATION OF RAW 264.7 CELLS

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To the Graduate Council:

I am submitting herewith a thesis written by Svjetlana Matic entitled "STABILITY OF BOVINE MILK EXOSOMES AND THEIR EFFECT ON PROLIFERATION OF RAW 264.7 CELLS." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science.

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STABILITY OF BOVINE MILK EXOSOMES AND THEIR EFFECT ON PROLIFERATION OF RAW 264.7 CELLS

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Svjetlana Matic
May 2019

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ABSTRACT

Exosomes are extracellular vesicles involved in intercellular communication. Bovine milk exosomes stability and their biological effects on macrophages have not been extensively investigated. Hence, the objectives of this study were to characterize bovine milk exosomes and investigate their effects on RAW 264.7 macrophages under normoxia and hypoxia. Bovine milk exosomes were isolated using differential centrifugation and identified by the presence of exosomal markers (ALIX, TSG101, and CD81) and particle size. The effect of *in vitro* digestion and pH on stability of milk exosomes was investigated. The biological activity of milk exosomes in RAW 264.7 under normoxia was determined by measuring proliferation, expression of proliferation markers p53, p21, cyclin D1, CDK2 and β -catenin, cell cycle, and the protective effect of exosomes on cisplatin-induced cytotoxicity. The effect of exosomes on RAW 264.7 under hypoxia was assessed by measuring proliferation, ROS production, cytokines, cell cycle, and the expression of hypoxia marker HIF-1 α and proliferation markers p53 and p21. Exosomes were positive for exosomal markers and their size was 106.8 nm \pm 3.4. Exosomal marker TSG101 was detected after digestion and exposure to different pH values. The treatment of exosomes significantly increased proliferation in cells under normoxia and had a protective effect against cisplatin-induced apoptosis. Proliferation markers were significantly affected by exosome treatment under normoxia. Cell cycle analysis in normoxia showed that exosome treatment reduced the percentage of pre-apoptotic cells while arresting the cells in G2/M phase. Hypoxic conditions reduced the viability of cells by 27% while exosome treatment significantly increased the cell

viability. Generation of ROS under hypoxia was significantly reduced in the cells treated with exosomes. Hypoxia alone did not affect TNF- α , but exosome treatment significantly increased TNF- α in a dose-dependent manner under hypoxia. Cell cycle analysis showed that hypoxia alone arrested cells in the G0/G1 phase whereas exosome treatment reduced the percentage of cells in this phase. The results demonstrated that bovine milk exosomes isolated from commercially available milk are stable and affect macrophage proliferation under normoxia and hypoxia indicating the potential role of exosomes in the immune system. Further research is needed to understand milk exosomes' role in human health.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Literature review

1.1.1 Exosomes

Exosomes are extracellular vesicles involved in cell-to-cell communication ¹. Initially, they were considered a waste product of the cells, but this common belief was dismissed in 1983 when two papers reported that small vesicles around 50nm in size were released from maturing blood reticulocytes ^{1,2}. Since the discovery of exosomes released from reticulocytes, their secretion has been well documented in many types of cells and body fluids ^{3,4}. It is important to mention that there are other types of extracellular vesicle including ectosomes and apoptotic bodies ⁵. Even though there is still some confusion about how to differentiate between different types of the extracellular vesicles, it is a well-accepted notion that exosomes originate from multivesicular bodies (MVB) while ectosomes originate from plasma membranes ⁶. Apoptotic bodies, on the other hand, are made during the processes of programmed-cell death called apoptosis. The size among different types of extracellular vesicles is also another important characteristic that helps differentiate between them. Exosomes are considered to be smallest among them with the size from 50-150nm while ectosomes, also called microvesicles, size ranges from 50nm-1000nm ³. Apoptotic bodies are considerably larger ranging from 800-5000nm ⁷. Thus, the particle size distribution is an important parameter in exosomes isolation and identification.

1.1.2 Generation and release of exosomes

As mentioned previously, exosomes originate from multivesicular bodies. One of the most commonly used definitions of generation of exosomes is that they are made by inward budding of endosomes⁸. It starts with the formation of an early endosome which matures into a late endosome and formation of MVB. During this time, endosomal membrane invaginates to make intraluminal vesicles (ILV) that will be released as exosomes upon the endosome fusion with the cell surface⁹. For the generation of ILVs and consequently exosomes, there are two mechanisms known: endosomal sorting complexes required for transport (ESCRT) dependent and ESCRT independent¹⁰. In the ESCRT dependent mechanism, the ESCRT complex is recruited to the endosome by transmembrane proteins that have been tagged with ubiquitin⁵. It is important to mention that tumor susceptibility gene 101 (TSG101) is also capable of recognizing ubiquitin⁵. Moreover, when TSG101 was knocked out, the secretion of exosomes was significantly impaired suggesting its importance in exosomes generation¹¹. In ESCRT independent mechanism, ESCRT complex is not required, and the formation of exosomes and sorting of exosomal cargoes is regulated by the members of tetraspanin family CD9, CD63 CD81 and CD82³. Once exosomes are formed within MVB, the fate of MVB is to fuse with cell membrane and release exosomes out of cell or to fuse with lysosome and be degraded; however, little is known about this process¹². Once exosomes are released from the cell, the exosome uptake by other cells depends on the protein contained on the exosomes surface specifically tetraspanin family¹³.

1.1.3 Function and significance of exosomes

Exosomes are small membranous vesicles with a major role in intercellular communication. In general, exosomes contain various proteins, lipids, and nucleic acids, and their content depends mostly on the type of cells they originate from ¹². Lately, the presence and importance of exosomal RNAs have been extensively researched. Exosomes contain messenger RNA (mRNA) and micro RNA (miRNA) that can be transferred to the recipient cell and deliver new genetic information ¹⁴. By transferring new genetic information, the protein expression of the recipient cell may be altered. For instance, when human cells were incubated with mouse exosomes, distinct mouse proteins were identified in human cells suggesting that exosomal mRNA can be translated into proteins ¹⁵. Moreover, the transfer of exosomal RNA can differentially affect the gene expression in the recipient cells ¹⁶. With possessing the ability to alter the protein and gene expression, exosomes have been implicated in numerous physiological and pathological processes ¹⁷. For example, in a physiological condition, exosomes from dendritic cells may play an important role in activating immune system while in a pathological condition, exosomes released from cancer cells may exchange their oncogenic content with other cells ¹⁷. The function and therefore their significance are closely related to the type of cells that exosomes originate from.

1.1.4 Exosomes isolation

One of the most commonly used methods for isolation of exosomes is differential ultracentrifugation ^{4,18,19}. Ultracentrifugation based method for exosomes isolation is considered to be the gold standard ²⁰. Differential centrifugation isolation method utilizes

consecutive centrifugation steps with increasing speed and time. This method is based on the principle that larger particles settle down first leaving the smaller particles in the supernatant ²¹. Due to the size of exosomes and some size overlap with other molecules and extracellular vesicles, ultracentrifugation yields exosomes that may be contaminated ²⁰. Ultracentrifugation can be accompanied by a density gradient which separates exosomes based on their size, mass, and density in a preconstructed density gradient medium ²⁰. When exosomes are isolated based on density gradient using sucrose gradient medium, they float in densities from 1.15 to 1.19 g/ml ²².

Another commonly used method of isolation of exosomes is size-based ultrafiltration ²³. The main principle of ultrafiltration and ultracentrifugation is the same, separation based on the size. Using ultrafiltration, exosomes may be isolated based on molecular weight or their size ²⁰. However, the ultrafiltration method utilizes force which may contribute to the loss of exosomes caused by rupturing their membranes ²⁴.

Exosomes precipitation is another technique used in isolation of exosomes and involves the use of polyethylene glycol (PEG) ²⁵. The principle behind this method is that PEG binds water molecules making other compounds to precipitate. Moreover, commercially available kits such as ExoQuick (System Biosciences, United States) use PEG for exosome isolation ²⁶. However, it is important to mention that this method has its disadvantages since other molecules such as protein can also precipitate making the exosome isolates contaminated ²⁷.

The presence of tetraspanins CD63, CD81, CD82, CD9 and ALIX among the other exosomal markers is the principle behind affinity-based isolation techniques ²⁵. Even though affinity-based techniques can reduce isolation time and yield higher purity of

exosomes, these techniques are expensive, inefficient and not recommended for isolation from large volumes ²⁶.

1.1.5 Detection and identification exosomes

Commonly used methods for detection of exosomes are dynamic light scattering, flow cytometry and electron microscopy ²⁸. However, dynamic light scattering favors larger molecules when in heterogenous solution while electron microscopy can alter the shape and size due to sample preparation steps ²⁹. Both transmission electron microscopy and scanning electron microscopy have been used to detect exosomes ³⁰. Regardless of the cell type, almost all exosomes are made by inward budding of endosomes; therefore, they contain proteins implicated in membrane transport and fusion (e.g., annexins, flotillins), biosynthesis of multivesicular body (e.g. ALIX and TSG101) and processes that require heat shock proteins integrins and tetraspanins (e.g. CD63, CD81) ³¹. Not surprisingly, the identification of exosomes has been heavily relying on the presence of these proteins ^{18,32}. Western blot and ELISA are commonly used techniques for detection of exosomal proteins ²⁸.

1.1.6 Bovine milk exosomes

Bovine milk is a nutrient rich-food that contains carbohydrates, lipids, proteins, minerals, and vitamins. Bovine milk and its compounds have been extensively researched over the years and their bioactivity have been well documented. Over the last 10 years, research related to bovine milk gained more interest since the discovery of exosomes. Earlier studies showed that microvesicles isolated from colostrum and mature milk were

approximately 100 nm in size with a distinct phospholipid bilayer membrane ³³. In addition, the study reported that isolated exosomal RNA from colostrum and mature milk was translated into casein proteins suggesting that exosomal mRNA was intact ³². Interestingly, bovine milk exosomes may have been isolated in 1973 when a study found that bovine milk contains plasma membrane containing vesicles ³⁴. However, researchers at the time started becoming aware of the difference between these vesicles and milk fat globule membranes (MFGM) ^{34,35}. Consequently, a study published in 2012 investigated the difference between MFGM proteome and exosome proteome ³⁶. The study found that milk exosomes have different but also more diverse proteome compared to MFGM proteome ³⁵. After establishing the procedure for isolation and characterization of exosomes, researchers investigated how *Staphylococcus aureus* mastitis affects exosome proteome ³⁷. Interestingly, the study found that the infection changed protein expression of members of heat shock proteins, protocadherin gamma family and to some extent casein proteins.

As the investigation on exosomes from the various cell was advancing and more information was known, consequently, research on bovine milk exosomes was advancing as well. In 2015, after transforming growth factor beta 1 (TGF- β 1) was discovered on the surface of cancer exosomes ³⁸, a study found that bovine milk exosomes are also enriched with TGF- β 1 which can have immunoregulatory function ¹⁹. Moreover, a study published in 2017 found that bovine milk exosomes were enriched with proteins implicated in immune responses, while mature milk exosomes were enriched with proteins associated with transport and apoptosis suggesting that composition of exosomes changes as the milk matures ³⁹.

1.1.7 Stability of milk exosomes

As a potential food ingredient, the stability of milk exosomes across different food matrices is imperative. However, the stability of bovine milk exosomes has not been extensively investigated. In a study published in 2012, the researchers found that bovine milk contains mRNA and miRNA that are very stable against acidic conditions and RNAase treatment suggesting that these RNAs could resist manufacturing processing⁴⁰. Since miRNA and mRNA may be enclosed into exosomes and thus protected, another group of researchers was investigating whether exosomes would be stable against a wide range of conditions. Interestingly, when isolated from commercially available milk, exosomes were stable against low pH, boiling and multiple freeze-thaw cycles¹⁹. However, when the study that was published later investigated whether the miRNA stability relies on the protection of exosomes, they concluded that stability of miRNA did not depend exclusively on exosomes but also other extracellular vesicles, proteins or lipids⁴¹. MiRNA is small, non-coding RNA that is able to regulate gene expression by binding to mRNA and preventing protein production⁴². The importance of exosomal miRNAs and their implication in numerous pathological and physiological processes⁴³ is what lead researchers to investigate bioactivity of bovine milk exosomes.

1.1.8 Biological activity of bovine milk exosomes

Studies on the biological activities of bovine milk exosomes are limited but are gaining interest among researchers. Initial studies on biological activities focused on understanding if these bovine milk exosomes can be taken up by different cell types. In

2013, a study found that macrovesicles isolated from bovine colostrum were taken by RAW 264.7 cells ⁴⁴. Moreover, the same study found that pretreatment with bovine milk vesicles affected the production of IL-1, IL-6, and IL-10 in LPS-treated RAW 264.7 cells suggesting that bovine milk exosomes have an immunomodulatory effect. Later in 2015, a study found that bovine milk exosomes can be taken by human macrophage cell line THP-1 ⁴⁵. Interestingly, the results demonstrated that only differentiated THP-1 cells were able to uptake bovine milk exosomes. Immunoregulatory properties of bovine milk exosomes isolated from commercially available milk were confirmed in another study ¹⁹. The study found that bovine milk exosomes contain transforming growth factor beta (TGF- β) on their surface and are able to differentiate naïve T-cells to T-helper 17 *in vitro*. The researchers suggested that the immunomodulatory effect of bovine milk exosomes on T-cell differentiation was associated with the presence of TGF- β .

The confirmation that bovine milk exosomes are taken by RAW 264.7 cells came from the study published in 2015 ⁴⁶. Not only that bovine milk exosomes were taken up by RAW 264.7 cells, but also the pretreatment with bovine milk exosomes before LPS treatment, reduced the production of TNF- α and MCP-1 in adherent splenocytes indicating that bovine milk exosomes could have some anti-inflammatory properties ⁴⁶.

The bioactive properties of bovine milk exosomes were investigated in a recently published paper where *in vitro*, human colonic goblet LS174T cells showed an increase in mucus production when treated with bovine milk exosomes accompanied by increased goblet cell-associated gene expression of TFF3 ³². In addition, *in vivo*, administration of bovine milk exosomes significantly increased the number of goblet cell in mice with necrotizing enterocolitis when compared to the untreated group and a

decrease of myeloperoxidase expression was seen in mice treated with bovine milk exosomes suggesting that bovine milk treatment prevented from the development of NEC ³². Even with a limited number of publications, there is evidence that bovine milk exosomes are biologically active, so a deeper understanding of their role in human health is imperative.

1.1.9 Macrophages: cells of innate immunity

Immune cells can broadly be divided into two categories: cells of innate immunity and cells of adaptive immunity. Macrophages, along with mast cells, dendritic cells, natural killer cells, eosinophils, basophils, and neutrophils are cells of innate immunity.

Macrophages are primarily known to be professional phagocytes ⁴⁷, but their function and role in the immune response are broader ⁴⁸. Macrophages are motile cells containing various receptors on their surface that help them recognize a wide range of host and non-host ligands ⁴⁷. Macrophages are not only able to engulf pathogenic organisms but also to remove debris and dying (apoptotic) cells ^{49,50}.

Different subpopulations of macrophages have been identified based on their function and location ⁵¹. According to some sources, macrophages can be divided into two groups: tissue-resident and monocyte-derived macrophages ⁵². It was believed that tissue-resident macrophages originate from monocytes; however, there was enough evidence that tissue macrophages originate from embryonic development rather than circulating monocytes ⁵³. The function of tissue-resident macrophages is to engulf pathogens and to recruit other cells of immune system ⁵¹. In a resting state, macrophages that promote tissue repair are called M2 macrophages while

macrophages that sense a microbe become M1 macrophages ⁵⁴. Polarization toward M1 or M2 macrophages also can be achieved by the presence of different cytokines. For instance, interferon gamma (IFN- γ) is a cytokine that promotes polarization of macrophages toward M1 phenotype while interleukin 4 (IL-4) is needed to polarize macrophages to M2 phenotype ⁵⁵.

As mentioned previously, macrophages have numerous receptors which they use to sense the environment around them ⁵⁶. Receptors such as Toll-like receptors (TLR), Nod-like receptors (NLR) and scavengers are called pathogen receptors and are used to sense microbes. TLRs are membrane receptors used to sense bacterial, viral or fungal parts ⁵⁶. NLRs are similar in their function to TLRs but they are located in the cytoplasm and can recognize damage-associated molecular patterns (DAMPs) ⁵⁷. Scavenger receptors are membrane receptors and their function is to recognize bacterial parts or altered-self ⁵⁶.

One of the primary functions of macrophages is phagocytosis. When pathogen-recognition receptors such as dectin-1 on macrophages recognize pathogen-associated molecular patterns, the process can initiate phagocytosis ⁵⁸. Once the pathogen is internalized, the killing of pathogens is achieved when the phagosome fuses with a lysosome containing antimicrobial compounds ⁵⁸. It is important to mention that one of the best-studied phagocytosis processes is one that is facilitated by immunoglobulins G (IgG) when Fc γ receptors on phagocytic cells recognize pathogens coated with IgG ⁵⁹. Interestingly, even though phagocytosis was discovered more than 100 years ago, the process is not fully understood yet ⁵⁹. Similarly to the pathogen recognition process, apoptotic bodies need to be recognized in order to be phagocytosed by macrophage

cells⁵⁸. Apoptotic bodies display molecules that are not normally present outside the cells and those molecules are recognized by the phagocytes.

Another important function of macrophages is the secretion of numerous cytokines.

Cytokines are small proteins released by cells with a role in cell-to-cell communication

⁶⁰. The most important function of cytokines is to regulate inflammation, and they can be

divided into pro-inflammatory and anti-inflammatory cytokines⁶¹. Pro-inflammatory

cytokines such as TNF- α , IL-1, and IL-6 are released after the cells sense the presence

of pathogens via their receptors⁶¹. The first cytokine to be released after the indication

of immune responses is TNF- α ⁶¹. Although the function of TNF- α is complex, one of the

major roles of this cytokine is the resistance to infections⁶². IL-6 function is also

complex including reduction of available iron seen in inflammation and differentiation of

naïve CD4+ T cells⁶³. To this date, 11 members of IL-1 cytokine family have been

identified and some of them have pro-inflammatory or anti-inflammatory properties⁶⁴.

IL-1 β , as a member of IL-1 cytokine family, is a proinflammatory cytokine and its

secretion from the cell depends on the inflammasome formation⁶⁴.

The function of anti-inflammatory cytokine is to lessen inflammation and prevent tissue

damage from excess inflammatory response⁶⁵. One of the most potent anti-

inflammatory cytokines is IL-10 and its function is to inhibit the release of pro-

inflammatory cytokines⁶⁵.

1.1.10 Cell cycle and cell proliferation

Cell cycle and therefore cell proliferation is a tightly regulated process. The cell goes

through phases gap 1 (G1), synthesis (S), gap 2 (G2) and finally mitosis (M) to produce

two daughter cells. G1 phase prepare cells for DNA synthesis which happens in S phase. Similarly, G2 phase prepares cells for mitosis which happens in the M phase of the cell cycle ⁶⁶. This process is heavily controlled to prevent the division of cells if DNA is damaged. The cell has the ability to repair DNA damage; however, when DNA is too damaged to be repaired, the cell would initiate programmed cell death known as apoptosis ⁶⁷. Development of cancer is followed by uncontrolled cell proliferation caused by the cell inability to induce apoptosis when damage is too large to be repaired ⁶⁸. Factors involved in the cell cycle such as growth factors, oncogenes and anti-oncogenes play a role in the ability of cells to undergo apoptosis ⁶⁹. The central proteins that regulate the cell's progression through different phases of the cell cycle are cyclin-dependent kinases (CDKs) ⁶⁹. To become activated, CDKs need to be in the adequate phosphorylated state and in complex with cyclins ⁶⁶. Moreover, to move to the next stage of the cell cycle, the cyclin from the previous stage needs to be degraded ⁶⁶. However, tumor suppressor protein p53 can negatively regulate the cell cycle causing cell cycle arrest if DNA damage is present ⁷⁰. Activation of p53 leads to activation of p21 which forms a complex with CDKs. The formation of a complex between p21 and CDK2 or CDK4 will inactivate CDKs thus preventing progression from G1 to S phase ⁷¹. One of the commonly used methods to analyze cell cycle is by flow cytometry using a fluorescence dye that binds DNA. The principle behind cell cycle analysis is that the amount of fluorescence is proportional to the amount of DNA ⁷².

1.1.11 Hypoxia

Hypoxia is a phenomenon characterized by low oxygen concentrations that are not enough to maintain cellular function ⁷³. In aerobic organisms, oxygen serves as the final electron acceptor in the process of adenosine triphosphate (ATP) formation during cellular respiration. The cells need a high constant ATP/ADP ratio to maintain normal function hence oxygen depletion lead to reduced cell viability ⁷⁴. Cells sense hypoxia by different mechanisms and one of the ways is through mitochondria which is a primary site of oxygen consumption ⁷⁵. It is important to say that hypoxia can be acute and chronic. While acute hypoxia can cause a disturbance in ion homeostasis, chronic hypoxia can alter gene expression ⁷³. One of the first proteins that is activated in hypoxia is hypoxia-inducible factor family (HIF) which comprises of three different members HIF-1, HIF-2 and HIF-3 ⁷³. HIF-1 is the most ubiquitous and contains oxygen sensitive alpha (α) unit that is destabilized in the presence of oxygen ⁷⁶. HIF-1 α is degraded in normoxia, thus hypoxia stabilizes HIF-1 α which can dimerize with HIF-1 β . Once stabilized, HIF-1 can translocate into the nucleus and bind to targeted genes altering their expression ^{73,77}. Hypoxia can play an important role in cell proliferation, differentiation, energy metabolism, acidosis, reactive oxygen species (ROS) formation and apoptosis ⁷³.

Swelling, heat, redness, and pain are four signs of inflammation. It is known that reduced oxygen concentrations are seen in inflamed tissue mostly due to pressure caused by swelling ⁷⁸. Since hypoxia is associated with danger, the condition may result in macrophages polarizing to a pro-inflammatory phenotype ⁷⁹. It is documented that the production of nitric oxide (NO) and TNF- α in macrophage cells is HIF-1 α -dependent ⁸⁰.

Also, a previous published study demonstrated that hypoxia resulted in significant ROS generation and reduced cell viability in murine macrophage cell line RAW 264.7⁸¹. ROS can damage DNA, proteins, and lipids but also have a beneficial effect in the cells of innate immunity since it is involved in the direct killing of pathogens⁸².

1.1.12 Macrophages and chemotherapeutic drugs

Cancer is the second leading cause of death in the United States⁸³. Chemotherapeutic drugs are one of the available methods to treat cancer. The basic principle of chemotherapeutic drugs is that it targets the cells that are dividing. By doing that, chemotherapeutic drugs do not distinguish between healthy and cancer cell which can cause apoptosis in healthy cells. For instance, when RAW 264.7 cells were treated with doxorubicin, a chemotherapeutic drug, there was a significant decrease in cell viability. However, when RAW 264.7 cells were treated with doxorubicin and bacterial lipopolysaccharide (LPS), LPS prevented doxorubicin-induced apoptosis⁸⁴. Developing new drugs that will protect the healthy cell from killing while targeting cancer cells is still a challenge⁸⁵.

1.2 Hypothesis and objectives

Hypothesis of this study are:

1. Exosomes are stable against *in vitro* digestion and changes in pH.
2. Bovine milk exosomes will affect RAW 264.7 cells proliferation by affecting cell cycle.

Objectives of this study are:

1. To isolate and characterize exosomes from commercially available bovine milk.
2. To investigate the effect of bovine milk exosomes in RAW 264.7 cell under hypoxia and normoxia.

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**CHAPTER 2: BOVINE MILK EXOSOMES AFFECT PROLIFERATION
AND PROTECT MACROPHAGES AGAINST CISPLATIN-INDUCED
CYTOTOXICITY**

This chapter has been submitted to a food science and technology journal as:

Title: Bovine milk exosomes affect proliferation and protect macrophages against cisplatin-induced cytotoxicity

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Svjetlana Matic: performance of experiments and writing of manuscript,

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2.1 Abstract

Exosomes are extracellular vesicles involved in intercellular communication. The objectives were to characterize bovine milk exosomes and determine its effect on the proliferation of RAW 264.7 macrophages. Bovine milk exosomes were isolated using differential centrifugation and identified by the presence of exosomal markers (ALIX, TSG101, and CD81) and the particle size. The effect of *in vitro* simulated gastrointestinal digestion and different pH on stability of milk exosomes was investigated. The biological activity of milk exosomes in RAW 264.7 was conducted by measuring proliferation, expression of proliferation markers p53, p21, cyclin D1, CDK2 and β -catenin and analysis of cell cycle. Moreover, the protective effect of exosomes on cisplatin-induced cytotoxicity in macrophages was investigated. Exosomes were positive on exosomal markers and their size was $106.8 \text{ nm} \pm 3.4$. Exosomal marker

TSG101 was detected after digestion and exposure to different pH values. Proliferation markers were affected by exosome treatment. Cell cycle analysis showed that exosomes treatment reduced the percentage of pre-apoptotic cells while arresting the cells in G2/M phase. Exosomes had a protective effect against cisplatin-induced apoptosis in macrophages. The results demonstrated that bovine milk exosomes isolated from commercially available milk are stable and affected macrophage proliferation indicating its potential role in the immune system. Further research is needed to understand its role in human health.

2.2 Introduction

Bovine milk is a nutrient-rich food containing lipids, proteins, minerals, and vitamins that are needed for the growth and development of the calf. It contains immunoglobulins, hormones, growth factors, cytokines, enzymes and bioactive peptides ¹. The immunoregulatory effect of bovine milk peptides in humans has been well documented ². An animal study suggests that oral administration of bovine lactoferrin and lactoperoxidase may attenuate pneumonia in mice infected with influenza virus ³. The results from multiple clinical trials suggest that bovine lactoferrin may reduce the incidence of sepsis in neonates ⁴. Furthermore, pretreatment with bovine milk may prevent acid and alcohol-induced gastric ulcer development in mice ⁵. Clearly, the consumption of milk has numerous potential benefits in human health.

Exosomes are extracellular vesicle (30-120nm) and their synthesis and secretion have been documented in the most types of cell ⁶. Exosomal cargo heterogeneity depends on the type of the cell and may contain nucleic acids, enzymes, lipids and proteins ⁷.

Exosomes play an important role not only in cell-to-cell communication via transfer of the exosomal cargo from donor to recipient cell but also angiogenesis, cell survival, inflammation and immune response ^{8,9}.

Recently, food-sourced exosomes have been identified from different plants including ginger, carrots, grapefruit, and grape and they can alter the gene expression in the recipient cells ¹⁰. Moreover, isolation and identification of exosomes have been reported in human and commercially available bovine milk ^{11,12}. Human milk exosomes are stable against *in vitro* digestion and are taken by human intestinal cells *in vitro* ¹¹. In another study, oral administration of extracellular vesicles isolated from commercially available milk lessened arthritis in mice ¹².

Previous studies have revealed that bovine milk exosomes contain miRNA and messenger RNA ^{13,14}. In the previously mentioned study, a very small difference in microRNA expression between exosomes isolated from raw and commercially available milk may suggest that heat treatment (pasteurization) does not affect exosome bioavailability ¹⁴. Micro-RNA has been implicated in numerous physiological and pathological processes including cancer ¹⁵. The US Centers for Disease Control and Prevention predicts that in the near future, cancer will be the leading cause of death in the United States. Chemotherapy is one of the available treatments for cancer; however, it does not distinguish between healthy and cancer cells. Protecting the healthy cells from the cytotoxic effect of chemotherapeutic drugs is still a major problem in cancer treatment ¹⁶.

Bioactivity of bovine milk exosomes and their potential use as a functional food ingredient is still unknown. Therefore, the objective of this study was to isolate,

characterize and investigate the effect of pH and *in vitro* simulated gastrointestinal (GI) digestion on the stability of exosomes. Also, the biological activity of bovine milk exosomes and its effect on murine macrophage cell line RAW 264.7 with and without the chemotherapeutic drug cisplatin was investigated.

2.3 Materials and methods

2.3.1 Materials

Murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Manassas, VA). Growth media DMEM 1X was purchased from Corning Inc. (Corning, NY). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA). Primary antibodies (PDCD 61P (ALIX) 12422-1-AP, TSG 101 14497-1-AP, CD81 18250-1-AP, CDK2 60312-1-Ig, β -catenin 51067-2-AP, p53 10442-1-AP, p21 60214-1-Ig, cyclin D1 60186-1-Ig, and GAPDH 60004-1-Ig) were purchased from Proteintech (Rosemont, IL). Secondary antibodies (goat anti-mouse and goat anti-rabbit) were purchased from Thermo Fisher Scientific (Waltham, MA).

2.3.2 Isolation of exosomes

Exosomes were isolated from commercially available fat-free milk (Great Value brand) by differential centrifugation under sterile conditions following previously described protocol with some modifications¹⁷. Briefly, milk was centrifuged at 13,000 x g at 4 °C for 30 min in 250 mL centrifuge bottles using SS-34 rotor and SORVALL® RC-5B PLUS centrifuge. The whey was collected, transferred into polycarbonate 26.3 mL Beckman centrifuge tubes, and centrifuged at 100,000 x g at 4 °C for 60 min in Type 50.2 Ti rotor

using Optima XL-80K Ultracentrifuge (Beckman Coulter, Inc.). The final supernatant was collected, transferred to 26.3 mL centrifuge tubes and centrifuged at 135,000 x g at 4 °C for 90 min. The supernatant was discarded, and the pellet was washed with PBS twice. The exosome pellet was collected and resuspended in PBS. Isolated exosomes were sterile filtered through 0.22 µm filters. The protein concentration of exosomes was adjusted to 6 mg/mL and stored at -80 °C until further use.

2.3.3 Characterization of milk exosomes

The protein concentration of exosomes was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL U.S.A). Exosomes were diluted to 1 mg/mL concentration for particle size and zeta potential analysis. The size and zeta potential were measured by Zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Protein profile of exosomes was assessed by SDS-PAGE and exosomal protein markers tumor susceptibility gene 101 (TSG101), cluster of differentiation 81 (CD81), and programmed cell death 6-interacting protein (PDCD 6IP-ALIX) were detected by western blot analysis described below.

2.3.4 Effect of pH on exosomes stability

Exosomes were diluted to 1 mg/mL concentration using PBS. Then, 10 mL of diluted exosomes was adjusted to different pH values (2, 3, 4, 5, 6, 8, 9, 10, 11, and 12) using hydrochloric acid (HCl) and sodium hydroxide (NaOH) accordingly. pH-adjusted samples were stirred at 550 rpm for 20 min at room temperature (RT). After stirring, the samples were centrifuged at 10,000 x g for 10 min at 4 °C. The supernatant was

collected, and the protein concentration of the supernatant was determined. The particle size and zeta potential of pH-adjusted samples were measured. Protein profile and the presence of exosomal markers TSG101, CD81 and ALIX was assessed by SDS-PAGE and western blot analysis, respectively.

2.3.5 Pepsin-pancreatin digestion of exosomes

Pepsin- pancreatin digestion was performed following previously reported procedure ¹⁸. Two milliliters of approximately 6 mg/mL exosomes were mixed with 8 mL PBS and the pH of the sample was adjusted to 2.0 using HCl. Pepsin was added at a 1:10 enzyme:protein ratio. Pepsin digestion was carried out for 60 min in a shaking water bath at 37 °C. To inactive pepsin, pH was adjusted to 7.5 using sodium hydroxide. A 5 mL aliquot was taken and centrifuged at 10,000 x g for 10 min to remove any large particles. The supernatant was collected and used for further analysis. Pancreatin and bile were added to the remaining pepsin samples at a 1:10 enzyme: protein ratio, and the pancreatin digestion was carried out for 60 min in a shaking water bath at 37 °C. Pancreatin was inactivated by heating the samples at 75 °C for 20 min. Pancreatin samples were centrifuged at 10,000 x g for 10 min at 4 °C, and the supernatant was collected. Pepsin and pancreatin samples were analyzed for protein content, particle size, and zeta potential. Protein profile and the presence of TSG101, CD81, and ALIX was assessed by SDS-PAGE and western blot, respectively.

2.3.6 SDS-PAGE and western blot detection of TSG 101, CD 81 and ALIX

Isolated exosomes, pH-adjusted, and digested samples were mixed with an equal volume of 2x Laemmli sample buffer (Bio-Rad Laboratories, Inc.) containing 5% β -mercaptoethanol and heated in boiling water for 5 minutes. An equal amount of protein (for pH-adjusted samples) and an equal volume (for digested samples) of samples were loaded into Mini-Protean® TGX™ gel (Bio-Rad Laboratories, Inc.) and run in Bio-Rad Tris/Glycine/SDS buffer at 200 volts for 35 minutes. The gel was washed with deionized (DI) water 3 times (5 min each), and 50 mL of Bio-Safe™ Coomassie G-250 stain was added and gently shaken for 1 hour. The gel was rinsed with DI water for 30 min. For western blot analysis, after SDS-PAGE, the gel was equilibrated in blotting buffer for 15 min. The proteins were transferred onto an Amersham™ Hybond™ 0.45 μ m polyvinylidene difluoride membrane (PVDF) at 110 volts for 60 min at 4 °C. The PVDF membrane was blocked with 5% non-fat dry milk in Tris-Buffered Saline -0.01% Tween 20 (TBST) for 1 h at room temperature. After blocking, the membrane was washed in TBST three times each time for 5 min. The membrane was incubated with primary antibodies against TSG101, CD81 and ALIX (Proteintech Group, Inc. IL, USA) at 1:1000 dilution overnight at 4 °C. After washing, secondary anti-rabbit antibodies at 1:5000 dilutions were incubated for 1.5 h at room temperature. The membrane was washed, saturated with Bio-Rad Clarity™ Western ECL Substrate and imaged by a C-Digit blot scanner (Li-Cor Biosciences, U.S.).

2.3.7 Cell culture and cell proliferation assay

Murine macrophages RAW 264.7 were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin and at 37 °C in 5% CO₂ until confluency. Briefly, 2 X 10⁵ cells/well were seeded into a 6-well plate in the total volume adjusted to 2 mL and incubated overnight to attach at 37 °C in 5% CO₂. After overnight incubation, the cells were treated with different exosomes concentration ranging from 30 to 250 µg/mL for 24 h. The cells were harvested according to the protocol described below. For proliferation assay, 5 X 10³ cells/well were seeded into a 96-well plate in the total volume 200 µL and incubated overnight to attach at 37 °C in 5% CO₂. Then, the cells were treated with different exosomes concentration (15 to 250 µg/mL) with or without cisplatin (5 µM) for 24h. After the treatment, the growth media was replaced with 100 µL fresh plain growth media containing 10% MTS reagent and incubated for 3 h under the same conditions as described above. The absorbance was read at 490 nm and the percent of viable cells was calculated with respect to untreated cells.

2.3.8 Whole cell lysate preparation

After the cells were treated, 6-well plates were placed on ice and the growth media was removed. The cells were washed twice with ice-cold PBS to remove cell debris. After washing, 100 µL radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% protease inhibitor complex was added to each well and incubated for 5 min. Cells were harvested by scraping and transferred to 1.5 mL microfuge tubes and vortexed at 14,000 x g for 10 minutes at 4 °C. The supernatant was collected, and protein concentration was measured using Bradford reagent and BSA as the standard. The

supernatant was mixed with Laemmli buffer containing 5% β -mercaptoethanol in equal volumes and boiled for 5 min. The samples were stored at -80 °C until use.

2.3.9 Western blot for proliferation proteins p21, p53, β -catenin, cyclin D1 and CDK 2

Approximately, 10 μ g of the protein of the cell lysate was loaded in 8-16% SurePAGE (GenScript) gels. The gel electrophoresis was run in Tris-MOPS-SDS (GenScript) running buffer at 140 volts for 50 min. Proteins were transferred into the Trans-Blot[®] Turbo[™] PVDF membrane (Bio-Rad) using Trans-Blot[®]Turbo[™] Transfer System (Bio-Rad). After the transfer, the membrane was blocked with 5% non-fat dry milk in TBST for 1 h at room temperature. After blocking, the membrane was washed in TBST three times each time for 5 min. The membrane was incubated with primary antibodies against cyclin-dependent kinase inhibitor 1 (p21), cyclin-dependent kinase 2 (CDK 2) and β -catenin at dilution 1:1000, tumor protein (p53) at dilution 1:3000, and cyclin D1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at dilution 1:5000 overnight at 4 °C. After incubation, the membrane was washed as described above and secondary antibodies (anti-rabbit for p53 and β -catenin and anti-mouse for p21, cyclin D, CDK 2 and GAPDH) at dilution 1:5000 were incubated for 1.5 h at room temperature. Finally, the membrane was washed, saturated with Bio-Rad Clarity[™] Western ECL Substrate and imaged by a C-Digit blot scanner (Li-Cor Biosciences, U.S.).

2.3.10 Cell-cycle analysis by flow cytometry

The cells were fixed and stained following the previously described protocol ¹⁹. Briefly, the cells were seeded in 6-well plates and treated with exosomes (60 and 250 µg/mL) and with cisplatin and exosomes (60 µg/mL) following above described protocol. After treatment, the plates were placed on ice, and the media was removed. The cells were washed with ice-cold PBS twice. Two hundred µL of growth media was added to each well and the cells were scraped. Additional 800 µL of growth media was added to each well and cells were transferred to 1.5 mL microfuge tubes. The cells were centrifuged at 1000 x g for 5 min at 4 °C. After centrifugation, the supernatant was removed, and the cells were resuspended in 60 µL PBS. Then, 70% ethanol was added to the cell suspension drop by drop with constant vortexing. The cells were left overnight at 4 °C for fixation. After fixation, cells were centrifuged at 300 x g at room temperature, the supernatant was removed and 1 mL staining mixture containing propidium iodide and RNase was added. The cells were vortexed and incubated for 1 h at 37 °C protected from light. Cell cycle analysis was performed using MACSQuant flow cytometer and data analyzed with MACSQuantify software (Miltenyi Biotec, Somerville MA).

2.3.11 Statistical analysis

All experiments were performed in at least three independent replicates. All results are expressed as mean±SD or mean±SEM. Analysis of variance (ANOVA) with Tukey's test was done to determine a significant difference (p<0.05) between different treatment groups using Minitab statistical software (Minitab Inc., Pennsylvania, USA).

2.4 Results and discussion

2.4.1 Characterization and identification of bovine milk exosomes from commercially available milk

Bovine milk exosomes were isolated from commercially available milk using differential centrifugation technique. Milk exosomes were characterized by particles size distribution and surface zeta potential by dynamic light scattering. Fig 2.1A (all figures are located in the appendix) shows the average particle size of bovine exosomes was $106.8\text{nm} \pm 3.427$ with a polydispersity index (PDI) of 0.227 ± 0.019 consistent with previously reported values ¹⁷. The average zeta potential was $-9.88\text{mV} \pm 0.706$ (Fig 2.1B). Zeta potential is one of the important parameters that influence nanoparticle stability ²⁰. Zeta potential in this range suggests short time particle stability while zeta potential in the range from -5 mV to $+5\text{ mV}$ would indicate a fast aggregation ²⁰. For further characterization and identification, exosome lysates were prepared and analyzed using western blot for the presence of exosomal markers. As shown in Fig 2.1C, milk exosomes were positive for ALIX, TSG101, and CD81. Despite the cell type, almost all exosomes have endosomal origin; therefore, they contain proteins implicated in membrane transport and fusion (e.g., annexins, flotillins), biosynthesis of multivesicular body (e.g. ALIX and TSG101) and processes that require heat shock proteins integrins and tetraspanins (e.g. CD63, CD 81) ⁶. Consequently, exosomes identification has been relying heavily on the presence of exosomal proteins such as Alix, TSG101, and proteins of tetraspanin family ^{17,21}.

2.4.2 Bovine milk exosomes are stable against pH changes

The effect of pH on milk exosomes has not been studied well. There is a limited number of research that have been done on the exosome's stability and quality as affected by different pH. As a potential functional food ingredient, the stability of milk exosomes over a range of pH is of crucial importance for food application. As seen in Fig 2.2A, exosomes solubility is dependent on the pH of the solution. At pH 4, milk exosomes are the least soluble. The isoelectric point of milk casein is 4.6 which may suggest that some proteins may be present in the exosome's solution and precipitate at pH close to isoelectric point of casein. Proteins are least soluble around and at isoelectric point, and as pH changes away from the isoelectric point, protein solubility increases. Bovine milk exosomes are most soluble at pH 7.4 which is close to milk pH. In a previously published letter, the results showed that when exosomes were exposed to pH 4 and pH 10, exosomes concentration decreased ²². Further, Fig 2.2B shows that particle size of exosomes is also dependent on the pH of the solution. The particle size of exosomes is smallest at pH 5 ($91.96\text{nm} \pm 4.267$) but the most uniform particle size is at pH 4 with PDI of 0.093 ± 0.017 . This would confirm that larger particles precipitate at pH 4 point leaving in the solution smaller particles with similar size including exosomes. Largest PDI (0.305 ± 0.01) is at pH 6 showing that different size populations are present at this pH condition and it may suggest that at this point, the solution would not be stable. Zeta potential is an important parameter that influences the stability of nanoparticles. In Fig 2.2C, it can be seen that surface zeta potential is also dependent on solution pH. As pH decrease, zeta potential increase and vice versa. This can be explained that proteins at lower pH are protonated making the surface more positively charged ²³. Zeta potential

reaches zero value at pH between 3 and 4. At this value, exosomes are least soluble, and the solution would not be stable. Exosomal protein TSG101 was detected in all different pH exosome solutions (Fig 2.2D). At these protein concentrations, ALIX and CD 81 were not detected (not shown). A reduction in TSG101 signal can be seen at pH 12. Clearly, extremely high pH values decrease exosomes quantity which is consistent with reported results ²². However, even though soluble protein concentration at pH 12 remains almost not changed when compared to exosomes at pH 7.4, TSG101 concentration decrease. This suggests that proteins other than exosomal marker TSG101 may be present in isolated exosomes and these proteins are highly soluble at higher pH.

2.4.3 Bovine milk exosomes resist pepsin-pancreatin hydrolysis

The application of milk exosomes as a potential functional food ingredient depends on the ability of exosomes to survive human digestion and to reach intestines and circulation in an intact form. Exosome size slightly increased ($123.2\text{nm} \pm 3.394$) after subjecting exosomes to pepsin like-conditions with a wider particle size range (Fig 2.3A). Moreover, particle size decreased ($113.5\text{nm} \pm 0.167$) when pepsin-digested exosomes were subjected to pancreatin part of digestion, but still, the particle size was larger than the size of non-digested exosomes (Fig 2.3A). This may be explained by structural changes in exosomes exposed to simulated pepsin digestion. However, after pepsin-pancreatin hydrolysis, the particle size range was narrower suggesting that some large particles have been partially hydrolyzed at this point. The protein concentration of pepsin and pancreatin like solution remained unaffected (data are not

shown). The effect of *in vitro* digestion on milk exosomes stability has not been extensively researched. Recently, the results of a study suggested that milk exosomes isolation was not affected by subjecting milk to *in vitro* digestion prior to the isolation and there was no difference in exosomes size, morphology and miRNA content when compared to exosomes isolated from undigested milk ²⁴. Moreover, the same study found that milk exosomes were able to pass human intestinal epithelium *in vitro* using Caco-2 cell culture suggesting that milk exosomes may be able to reach the blood circulation.

Pepsin-pancreatin hydrolysis did not affect zeta potential. However, this may be explained by non-significant changes in solution pH. After digesting exosomes with pepsin at pH 2, pepsin digestion was stopped with adjusting pH to neutral conditions (~7.5). Pepsin-pancreatin digestion was performed at the same neutral pH condition with no change in zeta potential suggesting that the pH of solution is one of the important factors that influences zeta potential. Pepsin-pancreatin digestion did reduce the signal of exosomal markers (Fig 2.3C). After the pepsin digestion, TSG101 and CD81 were detected with CD81 signal being lower than that one of TSG 101. Furthermore, after pepsin-pancreatin digestion, only TSG101 was detected. Clearly, there was a reduction in TSG101 signal when compared to the non-digested and pepsin-digested samples. The presence of TSG101 suggests that exosomes survive *in vitro* digestion.

Following *in vitro* digestion and effect of pH, exosome proteins profile was investigated by SDS-PAGE (Fig 2.3D). The most apparent bands and reduction of the same bands in hydrolyzed and exosomes exposed to low pH are seen between 25 kDa and 37kDa

which corresponds to the molecular weight of casein proteins ²⁵. Also, a slight reduction at pH 2, pH 3 and pH 4 can be seen at 75kDa band. In the previous study of human milk exosomes, bands in this range were suggested to be related to protein lactoferrin which is also abundant in bovine milk and has been found to have antibacterial, immunomodulatory and anti-cancer activity ^{11,26}. Even though there is a reduction in the appearance of some bands, clearly some proteins are still present even after complete pepsin-pancreatin digestion suggesting that exosomes are resistant to simulated digestive conditions.

2.4.4 Bovine milk exosomes affect proliferation and have a protective effect on cisplatin treated RAW 264.7 cells

Macrophages are cells of the immune system involved in the first line of defense during infection ²⁷. In Fig 2.4A, treatment with bovine milk exosomes increases proliferation of RAW 264.7 cells in a dose-dependent manner. Treatment with 15, 30, and 60 µg/mL bovine exosomes significantly increased proliferation when compared to untreated macrophages. At 250 µg/mL, macrophage proliferation was significantly increased by 49.7% when compared to untreated cells. Interestingly, the proliferation of RAW 264.7 was not accompanied by activation of the cells since nitric oxide, and proinflammatory cytokines (e.g. TNF- α , IL-6) were not produced (data not shown). The effect of different compounds on inflammatory responses and activation of RAW 264.7 have been widely researched. Mostly, these research papers focus on compounds that are capable of activating RAW 264.7 while inducing nitric oxide and cytokine production (pro-inflammatory) or alleviating inflammatory responses (anti-inflammatory)^{28,29}. Our results

showed for the first time that food-derived compounds, such as bovine milk exosomes, can influence macrophage proliferation.

Further, when cells were co-treated with exosomes and cisplatin (5 μ M), treatment with exosomes increases macrophage cell survival indicating a protective effect of bovine milk exosomes against cisplatin-induced cytotoxicity as shown in Fig 2.4B. At a concentration of 250 μ g/mL exosomes, the cell survival of 95.3% was significantly increased when compared to the survival of 81.2% when cells were treated with cisplatin only. The proliferation of macrophages as well as the potential cytotoxic effect of chemotherapeutic drugs such as cisplatin is not well studied. Cisplatin is a chemotherapeutic drug that binds to DNA and prevents its repair causing cells to go to apoptosis³⁰. However, cisplatin, as majority of other chemotherapeutic drugs, is not a selective agent meaning it does not distinguish between healthy and cancer cells. Protective effect of bovine milk exosomes may contribute to a better understanding of how to protect normal cells from the cytotoxic effect of chemotherapeutic drugs.

2.4.5 Bovine milk exosomes affect proliferation markers (proteins) in RAW 264. 7 cells

Proliferation markers such as p21 and p53 play an important role not only in cell proliferation and cell cycle but also in the immune response in macrophage cells^{31,32}.

Fig 2.5 shows a dose-dependent effect of bovine milk exosomes treatment on proliferation markers in RAW 264.7 cells. In our study, exosomes treatment with 60 and 250 μ g/mL significantly increased β -catenin expression by 42.5% and 40.8%, respectively, when compared to untreated macrophages. β -catenin is a protein involved

in multiple functions including development, tissue homeostasis, cell renewal, adhesion and cell signaling³³. Recently, the role of β -catenin in infections and immune cells was investigated. In a couple of studies, the role of β -catenin in the immune system cells susceptibility to different bacterial and viral agents was investigated^{34,35}. In the above-mentioned study, overexpression of β -catenin in *Pseudomonas aeruginosa* infected RAW 264.7 cells promoted bacterial elimination with no effect on NO and ROS production³⁴. In addition, investigators found that an increased level of β -catenin in monocytes when compared to monocyte-derived macrophages suppressed HIV replication suggesting that β -catenin plays an important role in HIV replication³⁵. Bovine milk exosomes increased p53 expression in RAW 264.7 in a dose-dependent manner with 250 $\mu\text{g/mL}$ exosome treatment significantly increasing p53 expression by 2-fold when compared to negative control. The p53 protein has a vital role in cell proliferation by repairing DNA and inducing apoptosis when DNA damage is too serious to be repaired³⁶. Also, a frequent p53 mutation in cancer cells makes this protein as a potential target in normal cells that would prevent them from the killing effect of chemotherapeutic drugs¹⁶. Increased expression of p53 protects normal cells from the cytotoxic effect of chemotherapeutic drugs¹⁶ is consistent with our observation that bovine milk exosomes protected macrophages against cisplatin-induced cytotoxicity. Moreover, in one study, transient overexpression of p53 in RAW 264.7 significantly reduced *Listeria monocytogenes* invasion when compared to control cells. *In vivo* results from the same study confirmed that knocking out p53 expression accelerated and increased lethality in mice³².

Bovine milk exosomes also significantly affected p21 expression (Fig 2.5). At a concentration of 250 µg/mL, p21 expression was significantly increased by 66.8%. p21 is transcriptionally regulated by p53 and it has numerous functions in the cell cycle and apoptosis. For instance, overexpression of p21 in several cell type can protect cells from apoptosis³⁷. Also, in a previous study, the role of p21 in LPS-stimulated peritoneal macrophage was investigated and the results showed that deletion of p21 was accompanied by an increase in the production of pro-inflammatory cytokines TNF-α and IL-1β³¹. While CDK-2 expression levels in RAW 264.7 cells were not significantly affected by exosomes treatment (p=0.08), cyclin D1 expression was significantly affected. It can be seen that bovine milk exosomes treatment reduced the expression of cyclin D1 in a dose-dependent manner where treatment with 60 µg/mL and 250 µg/mL reduced the expression by 36.2% and 51.8% respectively when compared to untreated cells. Cyclin D1 is one of the regulators of the cell cycle progression from G₁ to S phase and its quick degradation is required for cells to move from G₁ to S phase³⁸. Also, in a previous study, the results showed that Bac1.2F5 macrophages treated with IFN-γ showed an increased level of cyclin D1 resulting in blocking macrophage proliferation³⁹. A decrease in the expression of cyclin D1 upon treatment of exosomes may play an important role in the proliferation of macrophages making cells progress to the next phase in the cell cycle.

2.4.6 Milk bovine exosomes affect the cell cycle of RAW 264.7 with and without cisplatin

Fig 2.6 shows the effect of bovine milk exosomes on RAW 264.7 proliferation cell cycle with or without cisplatin treatment. Exosomes treatment affected the proliferation of the cells in a dose-dependent manner. In the absence of cisplatin (Fig 2.6A), the number of apoptotic cells when macrophages were treated with exosomes was significantly reduced by 37.9% and 53.35% when cell treated with 60µg/mL and 250 µg/mL respectively although there was no significant difference between the two exosomes concentrations. Also, the reduction of cells by 10.7% can be seen in the G0/G1 phase of the cell cycle when cells are treated with 250 µg/mL exosomes. The exosomes treatment arrest cells in S and G2/M phase. In the S phase, 250 µg/mL exosomes treatment increased the number of cells by 16.0%, while in G2/M phase the cell number increased by 47.9%. To our knowledge, there have been only very few research conducted on RAW 264.7 cell cycle. One of the papers was investigating how *Salmonella* infection affects the cell cycle in RAW 264.7 ⁴⁰. In a study of the effect of riboflavin deprivation in RAW 264.7, when cells were deprived of riboflavin, the results showed a decrease in the S and G2/M phase ⁴¹. On the other hand, another study reported that cypermethrin treatment increased the percentage of the cells in G1; therefore, inducing death by arresting cells in this phase ⁴². Our results indicate that an increase in proliferation is accompanied by an increase in S and G2/M phase. When RAW 264.7 cells were treated with exosomes and cisplatin (5 µM), exosomes treatment (60 µg/mL) reduced the number of pre-apoptotic cells by 17.4% when compared to the cells treated with cisplatin only (Fig 2.6B). Cisplatin treatment arrests

the cells in pre-apoptotic phase. Moreover, exosomes treatment in the presence of cisplatin reduced the number of cells in the G0/G1 phase by 35.9% when compared to the cells treated with cisplatin only. Arrest in G2/M phase was seen in the cells treated with exosomes and cisplatin with an increase in the number of cells by 28.5%.

2.5 Conclusion

Our study found that commercially available milk exosomes resist *in vitro* digestion and pH change. Moreover, this is the first report on how bovine milk exosomes affected proliferation of macrophages, an important component of our immune system. Bovine milk exosomes also protected macrophages against cisplatin-induced cytotoxicity by affecting the expression of proteins involved in cell cycle and proliferation. Further research is needed to better understand the role of bovine milk exosomes and their effect on human health.

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Appendix

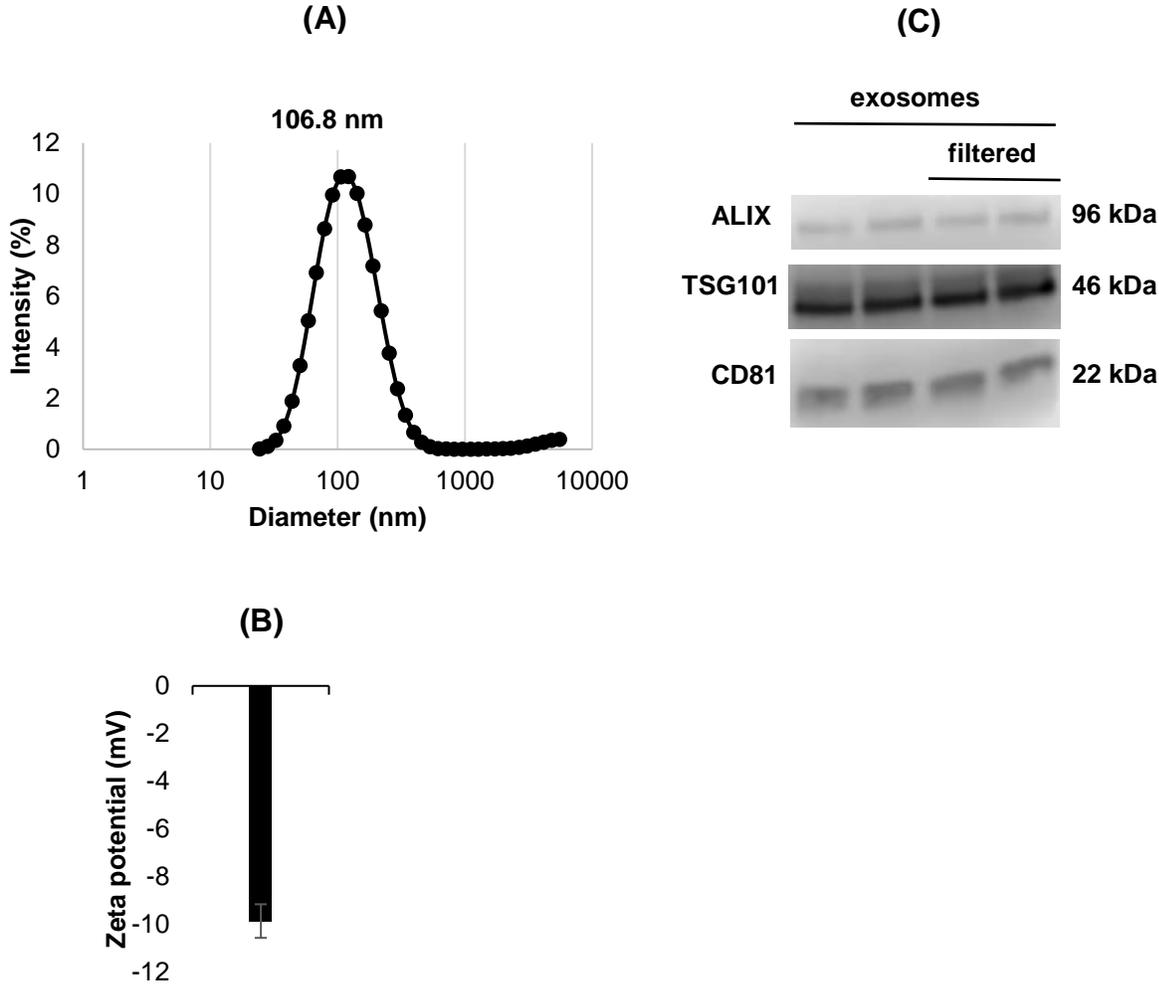


Figure 2.1. Characterization of milk exosomes by particle size distribution (A) and zeta potential (B). Identification of milk exosomes by western blot analysis on exosomal markers ALIX, TSG101, and CD81 (C).

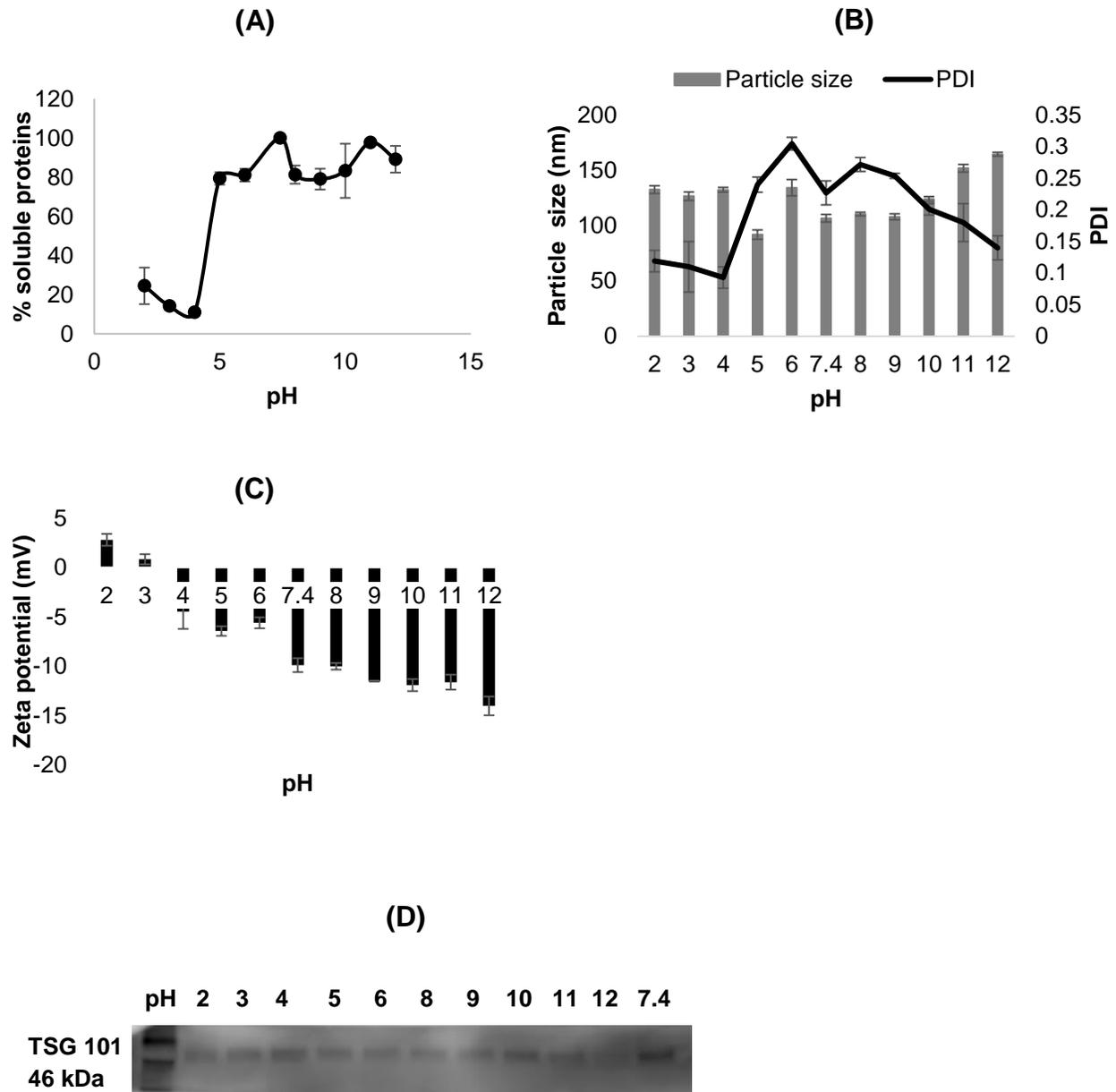


Figure 2.2. Stability of exosomes at different pH values presented as solubility of milk exosome proteins over a range of pH values (A), particle size distribution with PDI (B), surface zeta potential (C) and detection of exosomal protein TSG101 (D). Results are presented as mean \pm SD (A, B, and C).

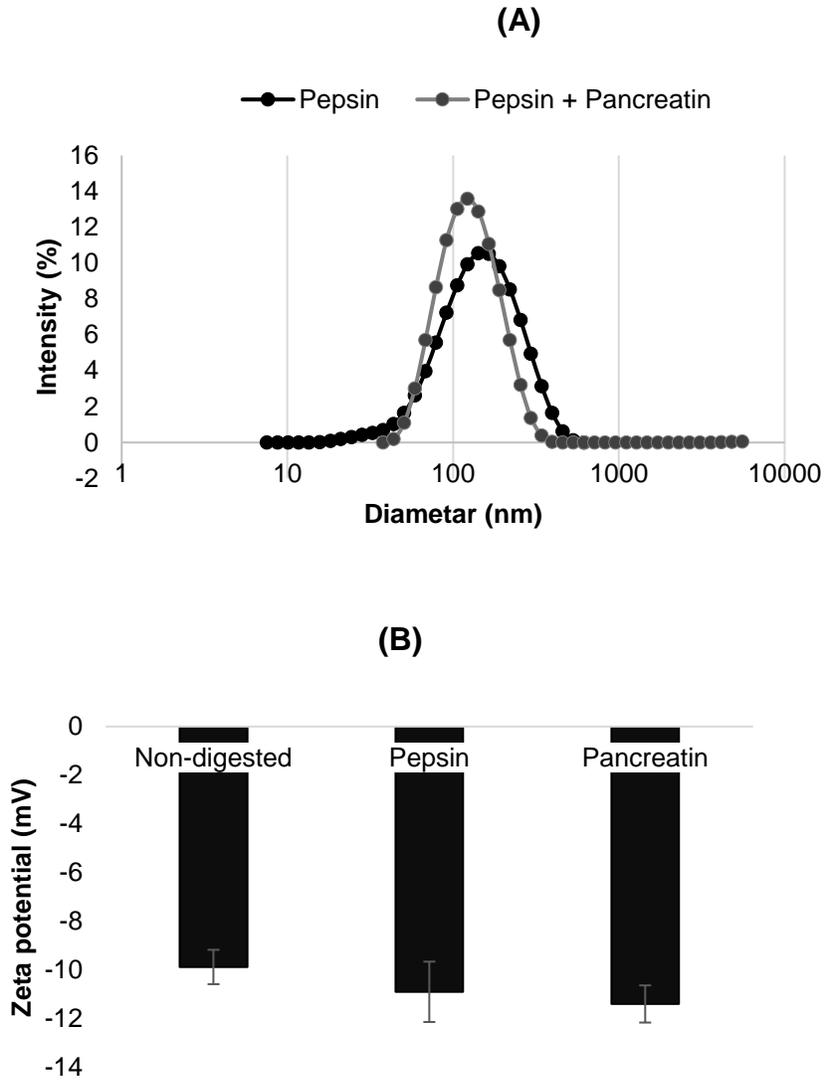


Figure 2.3. Effect of simulated pepsin-pancreatin digestion on size (A), and zeta potential of milk exosomes (B), the presence of exosomal proteins ALIX, TSG101 and CD81 (C), and the effect of pH and pepsin-pancreatin hydrolysis on the protein profile using SDS-PAGE (D). Results are presented as mean \pm SD (B).

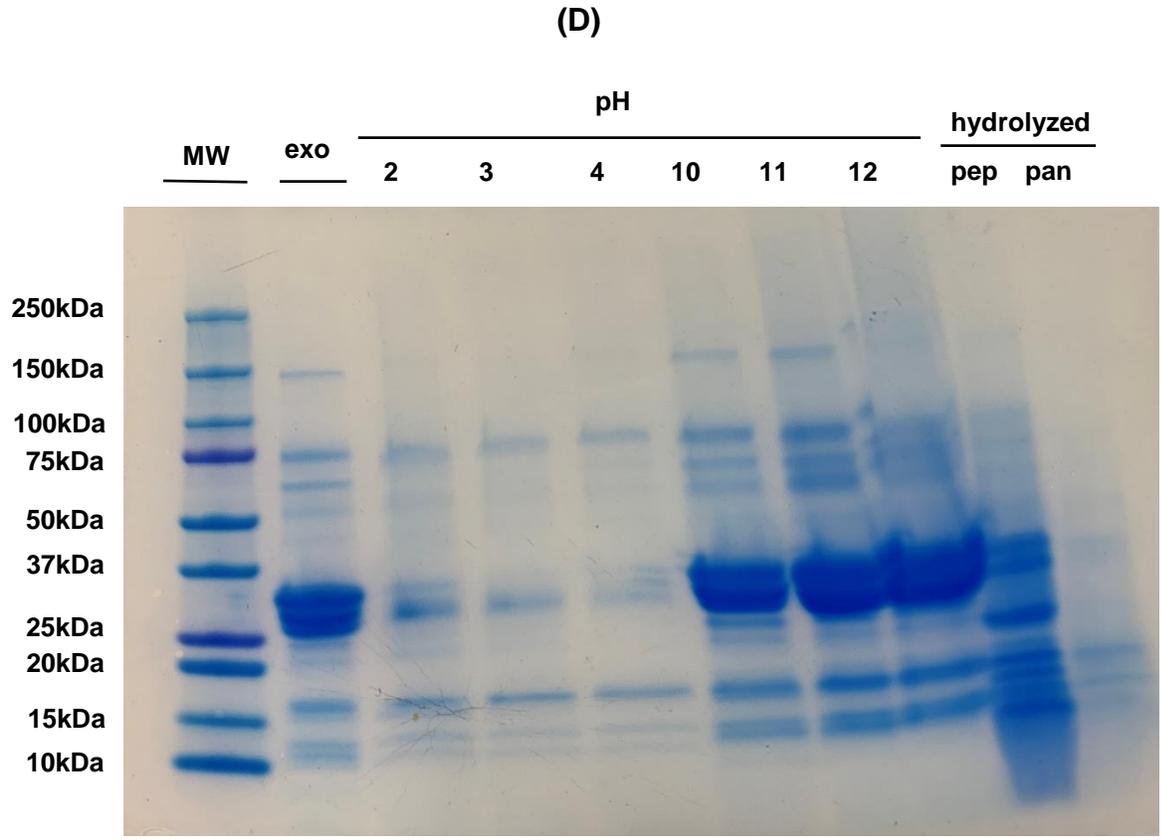
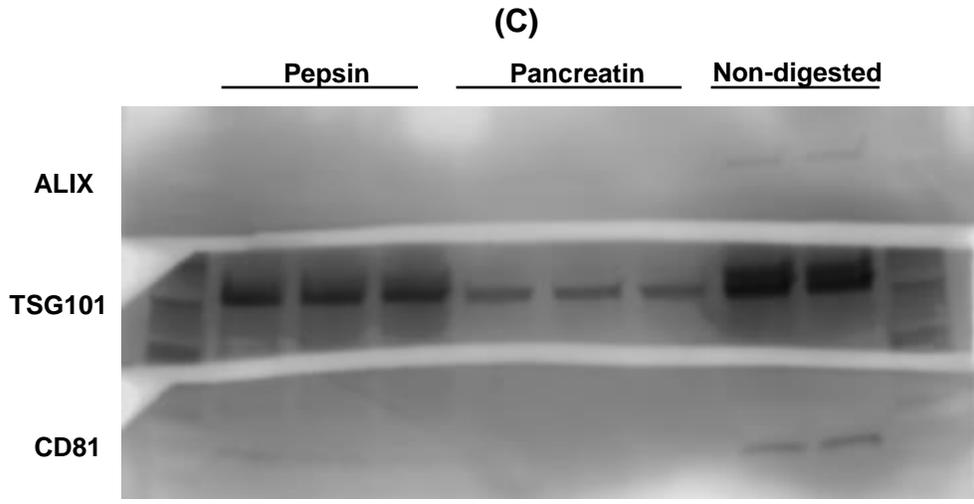


Figure 2.3 continued. Effect of simulated pepsin-pancreatin digestion on size (A), and zeta potential of milk exosomes (B), the presence of exosomal proteins ALIX, TSG101 and CD81 (C), and the effect of pH and pepsin-pancreatin hydrolysis on the protein profile using SDS-PAGE (D). Results are presented as mean \pm SD (B).

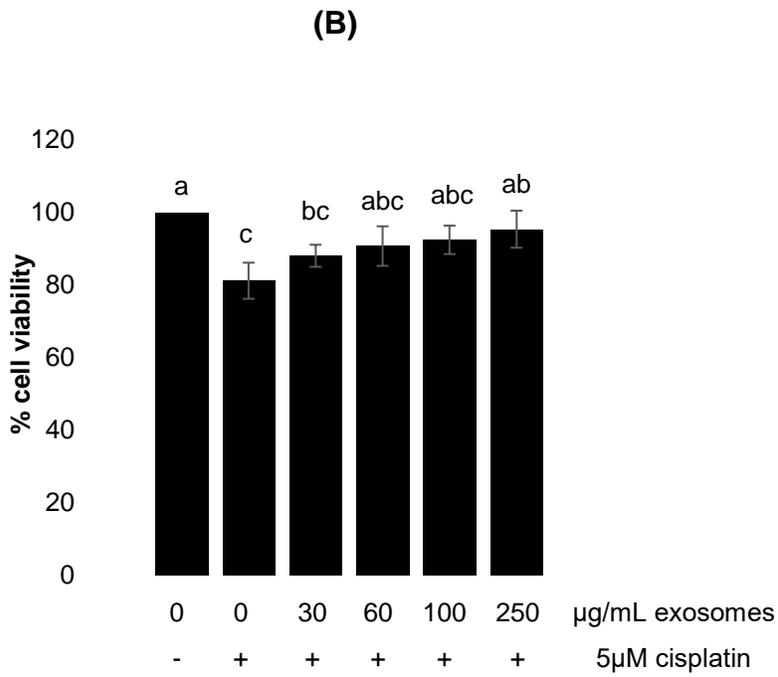
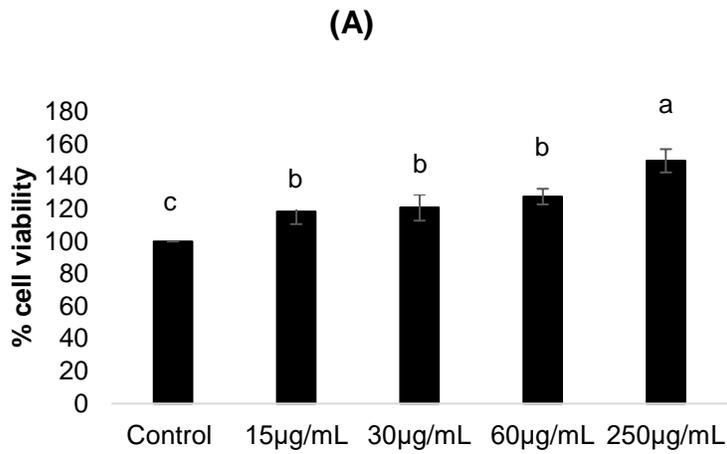


Figure 2.4. Effect of milk exosomes on RAW 264.7 proliferation without (A) and with cisplatin (B). Results are presented as mean \pm SD. Means that do not share a letter are significantly different ($p < 0.05$).

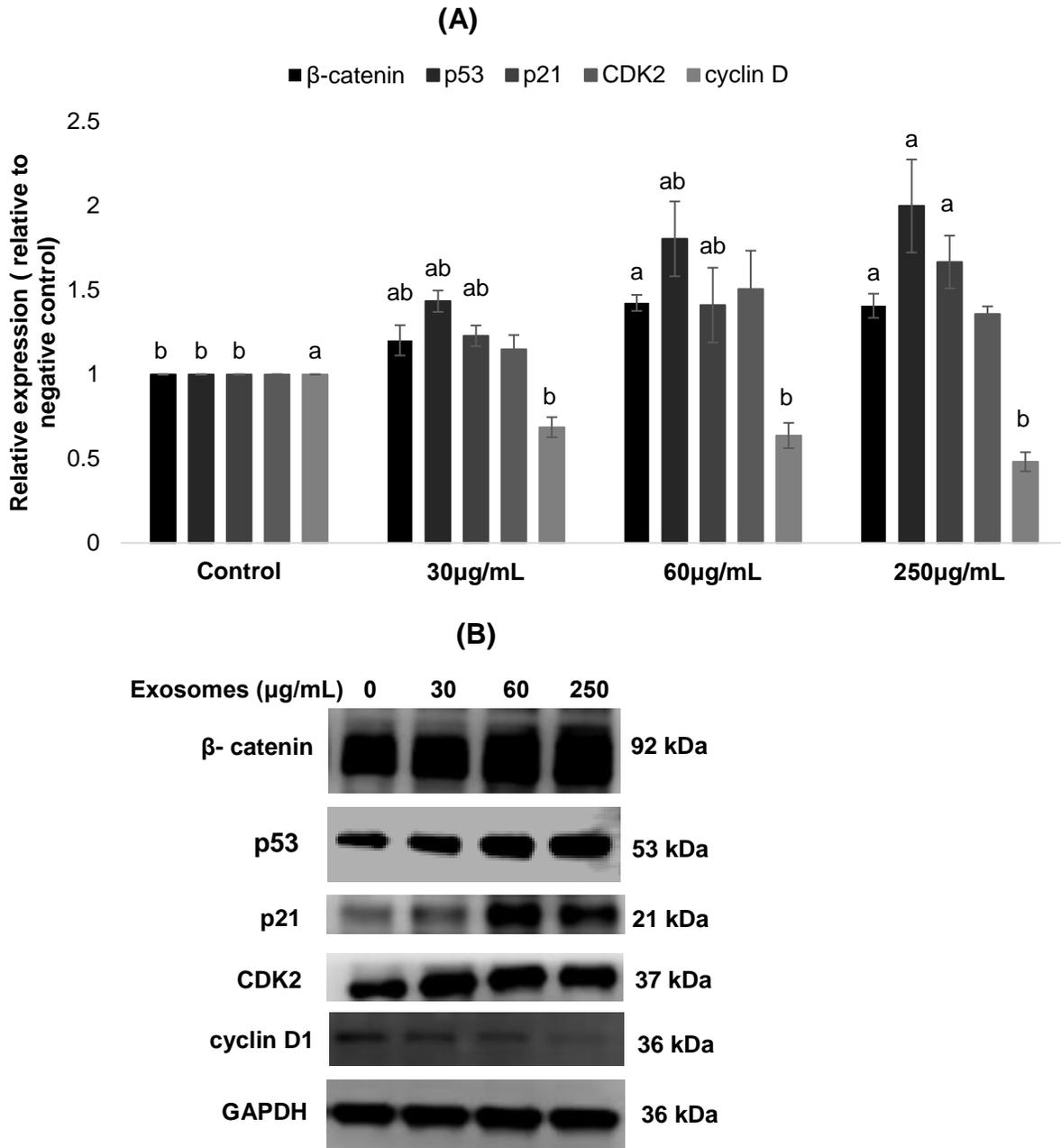


Figure 2.5. Effect of milk exosomes on RAW 264.7 expression of proliferation proteins β -catenin, p53, p21, CDK2 and cyclin D1 (A). Western blot on proliferation markers β -catenin, p53, p21, CDK2 and cyclin D1. GAPDH shown present housekeeping protein (B). Results are presented as mean \pm SE (A). Means that do not share a letter are significantly different ($p < 0.05$).

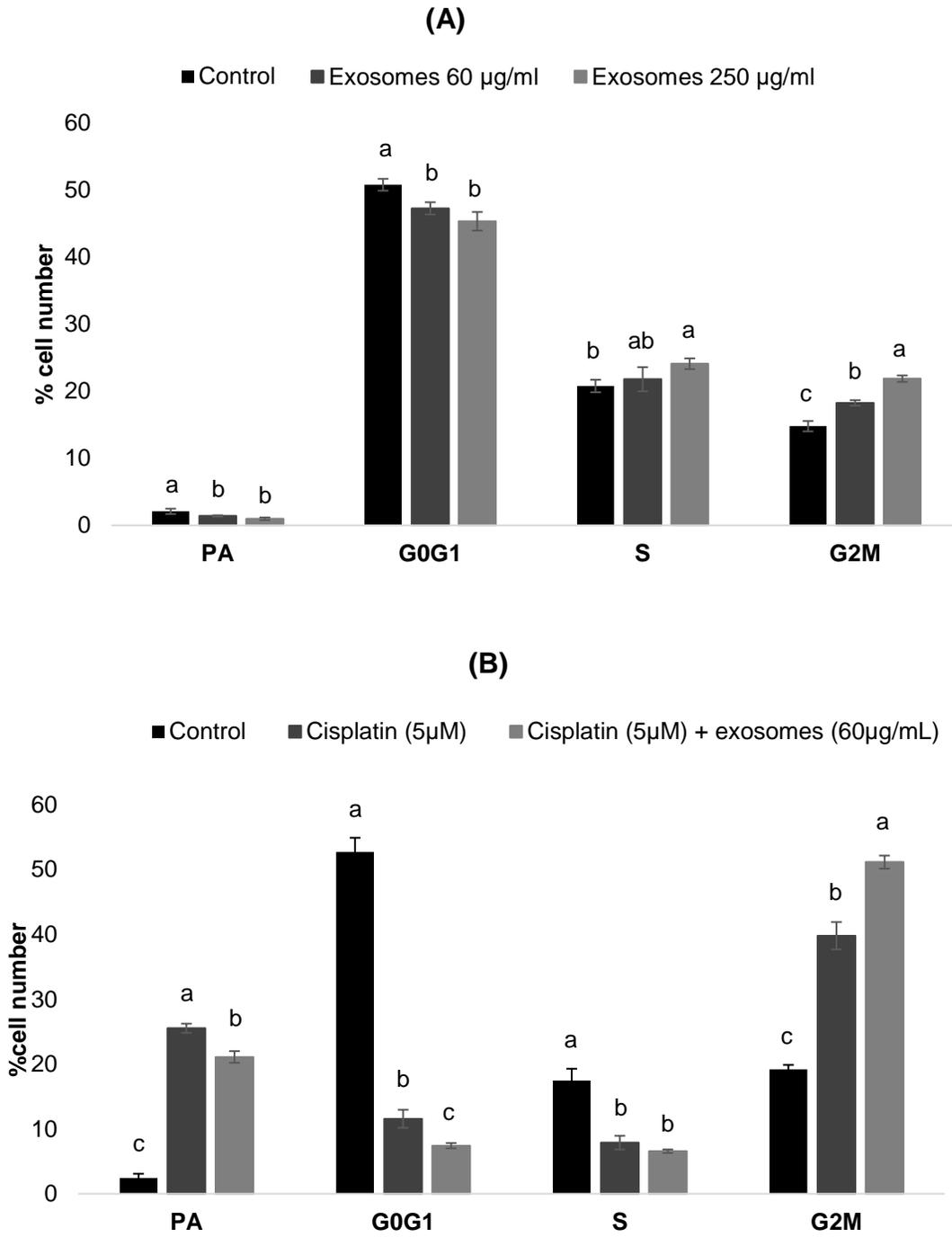


Figure 2.6. Cell-cycle of RAW 264.7 cell treated with bovine milk exosomes without (A) and with cisplatin (B). Results are presented as mean \pm SD (A). Means that do not share a letter are significantly different ($p < 0.05$).

**CHAPTER 3: THE EFFECT OF BOVINE MILK EXOSOMES ON THE
PROLIFERATION OF RAW 264.7 CELLS UNDER HYPOXIA**

3.1 Abstract

Exosomes are extracellular vesicles implicated in cell-to-cell communication and their secretion has been documented in almost all types of cells. The objective of this study was to isolate exosomes from commercially available milk and investigate its effect in RAW 264.7 cells under hypoxia. Exosomes were isolated from commercially available skim milk using differential centrifugation and their presence was identified by particle size and exosomal markers TSG101, CD81, and ALIX. The effect of bovine milk exosomes on RAW 264.7 cells under hypoxia was investigated by assessing proliferation, cytokine and ROS production, and cell cycle. Moreover, the hypoxia marker HIF-1 α and proliferation markers p53 and p21 were assessed by western blot. The average size of exosomes was 152.2 nm \pm 4.25 measured by dynamic light scattering method. Exosomes treatment increased the cell viability under hypoxia when compared to untreated cells. ROS production was significantly reduced with exosomes treatment ($p < 0.05$). The production of TNF- α was not affected by hypoxia alone but increased in a dose-dependent manner in cells treated with exosomes under hypoxic condition. Hypoxia arrested cells in the G0/G1 phase whereas exosome treatment reduced the cell in this phase. However, there was no effect on the expression of HIF-1 α , p53, and p21. Our study found that bovine milk exosomes affect the proliferation of RAW 264.7 cell under hypoxia and are able to reverse the adverse effects of hypoxia on cell viability.

3.2 Introduction

Hypoxia is a condition that is characterized by low oxygen concentrations caused by the low blood supply to the body's tissue. Not all forms of hypoxia are deleterious to body tissue. In some cells and tissue, such as intestinal epithelial cells, low oxygen concentrations are considered physiological, hence the term physiological hypoxia ¹. However, when an environment of low oxygen causes the impaired function of the cells and tissues, hypoxia can become pathological. Often, the reduction of blood supply and consequent low oxygen concentrations can be seen in the inflamed and injured tissue. This is due to the inability of blood vesicles to grow and supply oxygen to the growing affected tissue caused by infiltration of the cells ². Consequently, hypoxia may cause immune cell impairment and dysfunction.

Macrophages are the cells of the innate immune system and their primary function is phagocytosis. They exist in our body in two different forms: tissue-resident and monocyte-derived macrophages ³. When a pathogen gets sensed, one of the functions of the tissue-resident macrophage is to start initiating inflammatory response ⁴. In an environment of low oxygen tension, which is often seen in inflammation, macrophages upregulate some genes in order to perform their function in hypoxic environment.

Hypoxia-inducible factor family is one of the genes that is upregulated in low oxygen conditions and upregulation of HIF-1 α is implicated in hypoxia-induced apoptosis ^{5,6}.

Furthermore, when macrophage cells were infected with *Escherichia coli* and *Staphylococcus aureus* and exposed to hypoxic conditions, the ability of macrophages to eliminate these two pathogens was significantly reduced even though these pathogens are readily killed by macrophages under normoxic conditions ⁷.

Consequently, hypoxia and its effect on the cells of the immune system have been extensively investigated and alternative ways such as the application of bioactive food compounds in lessening the effects of hypoxia have been a novel approach.

Exosomes are extracellular vesicles with sizes ranging from 50-150nm⁸. Their synthesis and secretion have been well documented in almost all types of cells⁹. Lately, the presence and isolation of exosomes from different foods including milk and edible plants and their effect on human health have been investigated^{10,11}. It has been documented that bovine milk exosomes contain miRNAs that are resistant to *in vitro* digestion and are taken up by the human macrophage cell line THP-1^{12,13}. Moreover, in a recently published study, bovine milk exosome treatment prevented the development of necrotizing enterocolitis *in vivo*¹⁴.

The effect of food derived exosomes on hypoxia-induced changes has not been extensively investigated. Moreover, a recently published study found that bovine milk exosomes increased the proliferation of intestinal epithelial cells *in vitro* under hypoxia¹⁵. Therefore, the objective of this study was to isolate bovine milk exosomes from commercially available milk and investigate its effect on the proliferation of murine-like macrophages RAW 264.7 under hypoxic conditions.

3.3 Materials and methods

3.3.1 Materials

Murine macrophage RAW 264.7 cell line was purchased from American Type Culture Collection (Manassas, VA). Growth media DMEM 1X was purchased from Corning Inc. (Corning, NY). Fetal bovine serum (FBS) was purchased from Life Technologies

(Carlsbad, CA). Primary antibodies (p53 10442-1-AP, p21 60214-1-Ig, HIF-1 α 20960-1-AP, and GAPDH 60004-1-Ig) were purchased from Proteintech (Rosemont, IL). Secondary antibodies (goat anti-mouse and goat anti-rabbit) were purchased from Thermo Fisher Scientific (Waltham, MA).

3.3.2 Isolation and characterization of bovine milk exosomes and particle size analysis

Exosomes were isolated from commercially available fat-free milk by differential centrifugation under sterile conditions following previously described protocol with some modifications¹⁶. Briefly, milk was centrifuged at 13,000 x g at 4 °C for 30 min in 250-mL centrifuge bottles using SS-34 rotor and SORVALL® RC-5B PLUS centrifuge. The whey was collected, transferred into polycarbonate 26.3-mL Beckman centrifuge tubes, and centrifuged at 100,000 x g at 4 °C for 60 min in Type 50.2 Ti rotor using Optima XL-80K Ultracentrifuge (Beckman Coulter, Inc.). The final supernatant was collected, transferred to 26.3-mL centrifuge tubes and centrifuged at 135,000 x g at 4 °C for 90 min. The supernatant was discarded, and the pellet was washed with PBS twice. The exosome pellet was collected and resuspended in PBS. The protein concentration of exosomes was determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL U.S.A). Exosomes were diluted to 1 mg/mL concentration for particle size and zeta potential analysis. The size and zeta potential were measured by Zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Exosomal marker TSG101, CD81, and ALIX were determined by western blot. Isolated exosomes were sterile filtered through 0.22 μ m filters and stored at -80 °C until further use.

3.3.3 Cell culture and cell proliferation assay

Murine macrophages RAW 264.7 were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin and at 37 °C in 5% CO₂ until confluency. For proliferation assay, 5 X 10³ cells/well were seeded into a 96-well plate in the total volume of 200 µL and incubated overnight to attach at 37 °C in 5% CO₂. Then, the cells were treated with different exosomes concentration (0, 100 and 200 µg/mL) and placed in a hypoxia chamber for 24 h. The composition of the gas in the hypoxia chamber was 94% N₂, 5% CO₂ and 1% O₂. Untreated cells were incubated under normoxia. After the treatment, the growth media was replaced with 100 µL fresh plain growth media containing 10% MTS (Promesa, Madison WI) reagent and incubated for 3 h under normoxia. The absorbance was read at 490 nm and the percent of viable cells was calculated with respect to untreated cells under normoxia.

3.3.4 Whole cell lysate preparation and supernatant collection

Briefly, 2 X 10⁵ cells/well were seeded into a 6-well plate in the total volume adjusted to 2 mL and incubated overnight to attach at 37 °C in 5% CO₂. After overnight incubation, the cells were treated with 0, 100 and 200 µg/mL exosomes and placed in a hypoxia chamber containing 94% N₂, 5% CO₂ and 1% O₂ for 24h. Untreated cells were incubated under normoxia. After 24h of treatment, the 6-well plates were placed on ice and the growth media was collected for cytokine analysis. The cells were washed twice with ice-cold PBS to remove cell debris. After washing, 100 µL radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% protease inhibitor complex was added to each well and incubated for 5 min. Cells were harvested by scraping and transferred to 1.5

mL microfuge tubes and vortexed at 14, 000 x g for 10 min at 4 °C. The supernatant was collected, and protein concentration was measured using Bradford reagent and BSA as the standard. The supernatant was mixed with Laemmli buffer containing 5% β -mercaptoethanol in equal volumes and boiled for 5 min. The samples were stored in -80 °C until use.

3.3.5 Production of ROS by flow cytometry

Briefly, RAW 264.7 cells were seeded, treated and incubated in the 6-well plates under normoxia and hypoxia following the above-mentioned protocol. After treatment, the plates were placed on ice and the media was removed. The cells were washed with ice-cold PBS twice. Two hundred μ L of growth media was added to each well and the cells were scraped. Additional 800 μ L of growth media was added to each well and cells were transferred to 1.5 mL microfuge tubes. The cells were centrifuged at 1,000 x g for 5 min at 4 °C. After centrifugation, the supernatant was removed, and the cells were resuspended in 1 mL PBS. The cells were again centrifuged at 1,000 x g for 5 min at 4 °C and supernatant was removed. Finally, the cells were resuspended in 500 μ L PBS containing 10 μ M 2',7'-dichlorofluorescein diacetate and incubated at 37 °C for 30 min in a CO₂ incubator. The number of cells with green fluorescence was measured using the FL 1 channel of a MACSQuant flow cytometer and data was analyzed with MACSQuantify software (Miltenyi Biotec, Somerville MA).

3.3.6 Enzyme-linked immunosorbent assay (ELISA) for measurement cytokine production (IL-1 β , IL-6, IL-10 and TNF- α)

Production of IL-1 β , IL-6, IL-10 and TNF- α was measured using commercially available ELISA kits (BioLegend, San Diego CA). The cytokine detection was performed using the supernatant collected after the cell treatment with exosomes. Briefly, uncoated plates were incubated with capture antibodies overnight at 4 °C. After incubation, the plates were washed with washing buffer 4 times and blocked with assay diluent for 1 h with shaking at room temperature. After blocking, the plates were washed, and standards and samples were plated and incubated with shaking at room temperature for 2 h. The plates were washed after the sample incubation and detection antibodies were added and incubated for 1 h with shaking after which the plates were washed and the avidin-HRP solution was added and incubated for 30 min with shaking. Finally, after washing, the substrate solution was added and incubated in the dark for 15 or 20 min. The stop solution was added, and absorbance was read at 450 nm. The concentration of cytokines was calculated based on the standard curve for each cytokine.

3.3.7 Western blot for hypoxia marker HIF-1 α and proliferation proteins p21 and p53

Approximately, the 10 μ g of proteins of the cell lysate was loaded in 8-16% SurePAGE (GenScript) gels. The gel electrophoresis was run in Tris-MOPS-SDS (GenScript) running buffer at 140 volts for 50 min. Proteins were transferred into the Trans-Blot[®] Turbo[™] PVDF membrane (Bio-Rad) using Trans-Blot[®]Turbo[™] Transfer System (Bio-Rad). After the transfer, the membrane was blocked with 5% non-fat dry milk in TBST

for 1 h at room temperature. After blocking, the membrane was washed in TBST three times for 5 min each. The membrane was incubated with primary antibodies against cyclin-dependent kinase inhibitor 1 (p21) at dilution 1:1000, tumor protein (p53) at dilution 1:3000, hypoxia-inducible factor-1 α (HIF-1 α) at 1:1000 dilution and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (house-keeping protein; for normalization of data) at dilution 1:5000 overnight at 4 °C. After incubation, the membrane was washed as described above and secondary antibodies (anti-rabbit for p53 and HIF-1 α and anti-mouse for p21 and GAPDH) at dilution 1:5000 were incubated for 1.5 h at room temperature. Finally, the membrane was washed, saturated with Bio-Rad Clarity™ Western ECL Substrate and imaged by a C-Digit blot scanner (Li-Cor Biosciences, U.S.).

3.3.8 Cell-cycle analysis by flow cytometry

The cells were fixed and stained following the previously described protocol ¹⁷. Briefly, the cells were seeded in 6-well plates and treated with exosomes (0, 100 and 200 $\mu\text{g}/\text{mL}$) under hypoxia following above-described conditions. Also, untreated cells were incubated under normoxia. After treatment, the plates were placed on ice and the media was removed. The cells were washed with ice-cold PBS twice. Two hundred μL of growth media was added to each well and the cells were scraped. Additional 800 μL of growth media was added to each well and cells were transferred to 1.5 mL microfuge tubes. The cells were centrifuged at 1,000 x g for 5 min at 4 °C. After centrifugation, the supernatant was removed, and the cells were resuspended in 60 μL PBS. Then, 70% ethanol was added to the cell suspension drop by drop with constant vortexing. The

cells were left overnight at 4 °C for fixation. After fixation, cells were centrifuged at 300 x g at room temperature, the supernatant was removed and 1 mL staining mixture containing propidium iodide and RNase was added. The cells were vortexed and incubated for 1 h at 37 °C protected from light. Cell cycle analysis was performed using MACSQuant flow cytometer and data analyzed with MACSQuantify software (Miltenyi Biotec, Somerville MA).

3.3.9 Statistical analysis

All experiments were performed in at least three independent replicates. All results were expressed as mean±SD or mean±SEM. Analysis of variance (ANOVA) with Tukey's test was done to determine a significant difference ($p < 0.05$) between different treatment groups using Minitab statistical software (Minitab Inc., Pennsylvania, USA)

3.4 Results and discussion

3.4.1 Identification of bovine milk exosomes isolated from commercially available milk using particle size

Bovine milk exosomes were isolated from commercially available milk. The particle size distribution was measured using a dynamic light scattering method. As shown in Fig 3.1, the average particle size of milk exosomes was $152.2 \text{ nm} \pm 4.25$ which is consistent with the literature¹⁸. Polydispersity index (PDI) was 0.289 ± 0.024 . In the previously mentioned article, the size of bovine milk exosomes depends on the maturation stage of milk¹⁸. Mature milk has a larger particle size distribution when compared with exosomes isolated from colostrum. In addition, the amount of exosomal miRNA isolated

from commercially available milk did not significantly differ from the amount of exosomal miRNA from raw milk suggesting that standard heat treatment does not affect exosomes¹⁰. The average zeta potential of bovine milk exosomes was $-10.9 \text{ mV} \pm 0.834$ which would suggest short-term stability. Furthermore, the stability of exosomes also depends on storage conditions, pH and thaw-freeze cycles¹⁹. Consequently, the refrigeration temperatures are not recommended for long-term storage and can reduce exosomal proteins and RNA²⁰. Bovine milk exosomes isolated from commercially available milk were positive for exosomal markers ALIX, TSG101 and CD81 (Fig 3.2). The identification of exosomes has been relying on the presence of ALIX, TSG101 and CD81¹⁶.

3.4.2 Bovine milk exosomes increased the proliferation of RAW 264.7 cell under hypoxia

Macrophages are fast acting phagocytic cells of innate immune system²¹. In Fig 3.3, exosomes treatment significantly increased the proliferation of RAW 264.7 cells when compared to untreated cells under hypoxia. The cell viability decreased by 27% when untreated cells were incubated under hypoxia compared to untreated cells incubated under normal oxygen conditions. Moreover, exosomes treatment at 100 and 200 $\mu\text{g/mL}$ significantly increased the cells proliferation by 27.9% and 32.6%, respectively. The results showed that exosomes treatment reversed the anti-proliferative effect of hypoxia in macrophages and macrophage proliferation when treated with exosomes under hypoxia is the same as proliferation of macrophages in normoxia. The macrophage cells exist in two states: tissue-resident and monocyte-derived macrophages³. Since they

are the cells of innate immunity, they are the first line of defense when an infection happens. Often, in the case of inflamed and damaged tissue, the levels of oxygen may decrease which may cause a decrease in macrophage function and finally apoptosis ²². Decreased function or apoptosis can lead to a reduced ability of macrophages to fight an infection. Furthermore, to our knowledge, this is the first report on the effect of bovine milk exosomes on RAW 264.7 cell proliferation under hypoxia. In a recently published article, bovine milk exosomes and yak milk exosomes increased the cell survival of mouse small intestine epithelial cells (IEC-6) under hypoxia conditions ¹⁵. Moreover, in the same article, both yak and bovine milk exosomes were taken up by IEC-6 in both normoxic and hypoxic conditions.

3.4.3 Bovine milk exosomes reduced production of ROS in RAW 264.7 cell under hypoxic conditions

Reactive oxygen species are generated under oxidative stress. The oxidative stress may be defined as an imbalance between free radicals and antioxidants ²³. In our study, hypoxia caused a significant 4- fold ROS reduction in untreated cells when compared to the untreated cell under normoxic conditions (Figure 3.4). Furthermore, the exosome treatment significantly reduced the ROS production when compared to untreated cells in hypoxic conditions. However, there was no significant difference in ROS production between the cell treated with 100 µg/mL and 200 µg/mL exosomes. It is still controversial whether hypoxic conditions increase ROS production. Our findings are not consistent with current literature ^{24,25}. For instance, when RAW 264.7 cells were incubated in hypoxic conditions (1% O₂) for 20 h, there was a significant increase in

ROS production when compared to the cell incubated in normoxic conditions ²⁴ and when RAW 264.7 cells were grown under hypoxia (0.5% O₂) for 10 h, there was a significant increase in ROS production when compared to the cells grown under normoxia ²⁵. Consequently, differences in exposure time and oxygen concentrations may have affected the production of ROS.

Cellular hypoxia is more defined as a state of reductive stress where reducing equivalents such as NADH and FADH₂ build up if oxygen is not available ²³. Moreover, the buildup of reducing equivalents makes the electrons more available to reduce the available oxygen to superoxide. However, even if reducing equivalents are available, the formation of ROS is not possible when oxygen is not present ²⁶. It was reported that the formation of ROS depends on constant cycles of anoxia and reoxygenation ²⁶.

Another study repeated that reoxygenation increased the formation of ROS in RAW 264.7 cell with fast reoxygenation producing more ROS when compared to slow reoxygenation ²⁴. In our study, we have seen a decrease in ROS when the cells were exposed to hypoxia and it may be due to exposing the cells to hypoxia for a longer period of time where no more available oxygen was present to produce ROS. Moreover, ROS are very short-living molecules with unpaired electrons that are transformed and eliminated in the variety of cellular processes ²⁷. The reduction in ROS generation in our study may be explained by possible transformation or elimination of ROS over a prolonged period under hypoxia when reoxygenation was not present.

3.4.4 Bovine milk exosomes increased TNF- α in RAW 264.7 under hypoxia

Production of TNF- α was increased in the cells treated with exosomes under hypoxia in a dose-dependent manner (Fig 3.5). When the cells were treated with 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$ exosomes, the concentration of TNF- α was 90.8 pg/mL and 172.4 pg/mL respectively. However, there was no significant difference in the TNF- α concentration between untreated cell under hypoxia when compared to the untreated cell under normoxia. Clearly, hypoxia itself was not enough to affect TNF- α production in RAW 264.7 cells which is consistent with the literature where TNF- α was not induced in RAW 264.7 cells in hypoxic conditions even after 48 h ²⁸. TNF- α is a proinflammatory cytokine that plays an important role in host defense against pathogens ²⁹. Previously published study found that the treatment with human TNF- α increased the resistance against *Listeria* infection in *in vivo* mouse model ³⁰. Immunomodulatory effect of bovine milk exosomes in RAW 264.7 cells under hypoxia that was observed in our study may have a beneficial effect in fighting an infection in a low oxygen environment. IL-1 β , IL-6, and IL-10 were not affected by hypoxia and exosome treatment.

3.4.5 Bovine milk exosomes did not affect the expression of HIF-1 α , p21 and p53

As shown in Fig 3.6, HIF-1 α , p21, and p53 were not affected by hypoxia or exosomes treatment under hypoxia. Although a low oxygen environment is considered to be on the most important factors of HIF-1 α expressions, there is evidence that prolonged hypoxia may decrease expression of HIF-1 α ³¹. In the previously mentioned study, when the human lung adenocarcinoma cells (A549) were exposed to 0.5%O₂ for 12 h, HIF-1 α expression significantly decreased when compared to the expressions at the exposure

time of 4 h. The expression of p21 is regulated by p53³², so it is not surprising that both proteins were not affected.

3.4.6 Bovine milk exosomes affect the cell cycle of RAW 264.7 cells under hypoxia

The cell cycle of RAW 264.7 cells under hypoxia and the effect of bovine milk exosomes on the cell cycle can be seen in Fig 3.6. Our results showed that exposing RAW 264.7 cells to hypoxia caused cell arrest in the G0/G1 phase of the cell cycle when compared to the cell under normoxia. The percent number of cells in G0/G1 phase significantly decreased from 61.3% in untreated cells under hypoxia to 56.7% and 56.9% in the cell treated with 100 µg/mL and 200 µg/mL exosomes, respectively. There was no significant difference ($p < 0.05$) between untreated cells under normoxia and the cells treated with exosomes under hypoxia suggesting that bovine exosomes treatment is capable of reversing the effect of hypoxia on cell cycle of RAW 264.7 cell. The number of cells in pre-apoptotic phase did not significantly differ between different treatments. A significant reduction of cells in the S phase can be seen in untreated cells exposed to the hypoxic condition when compared to the cells exposed to normoxic conditions. Moreover, when the cells were treated with 100 µg/mL and 200 µg/mL exosomes, the percent of untreated cells under hypoxia in S phase increased from 10.9% to 15.5% and 16.9%, respectively, which was not statistically different from the number of untreated cells under normoxia in S phase. It is well documented that exposing the cells to a low oxygen environment affects the cell cycle progression. Not many studies have been done on the effect of hypoxia on RAW 264.7 cell. One of the previously published

articles found that exposing RAW 264.7 cells to hypoxia increased the number of apoptotic cells in 24 h³³. While our study did not find any difference in the number of apoptotic cells, the arrest in G0/G1 and reduction of cells in S phase was consistent with the current literature³⁴. To our knowledge, this is the first study that investigates the effect of bovine milk exosomes on the cell cycle progression in RAW 264.7 cells under hypoxia. Clearly, bovine milk exosomes were able to reverse the effect of hypoxia on the cell cycle of RAW 264.7 cells.

3.5 Conclusion

Our study found that bovine milk exosomes are present in commercially available bovine milk suggesting that the pasteurization process does not destroy them. Their bioactivity was investigated in RAW 264.7 cells subjected to hypoxia, and we found that bovine milk exosomes can reverse adverse effects caused in a case of low oxygen supply which can be seen in damaged or inflamed tissue. Further investigation on a deeper understanding by which mechanism bovine milk exosomes reverse the effect of hypoxia in immune cells is needed.

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Appendix

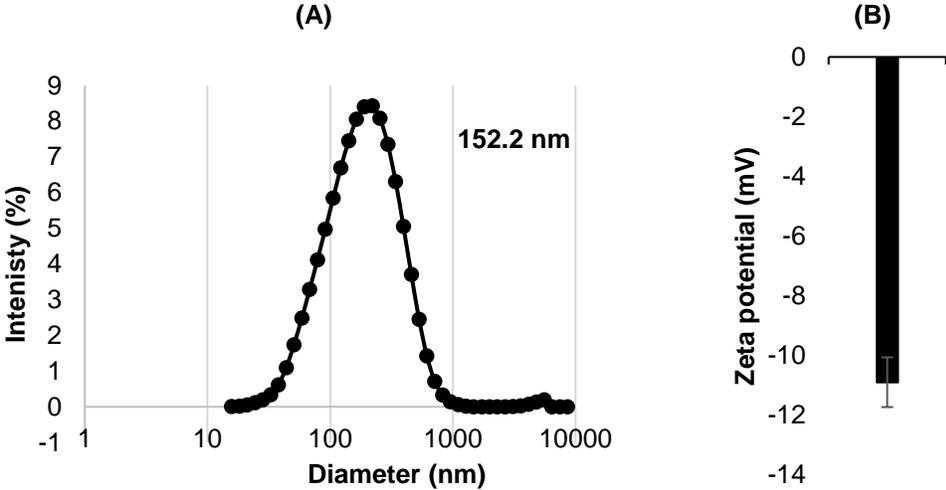


Figure 3.1. Particle size (A) and zeta potential (B) of bovine milk exosomes.

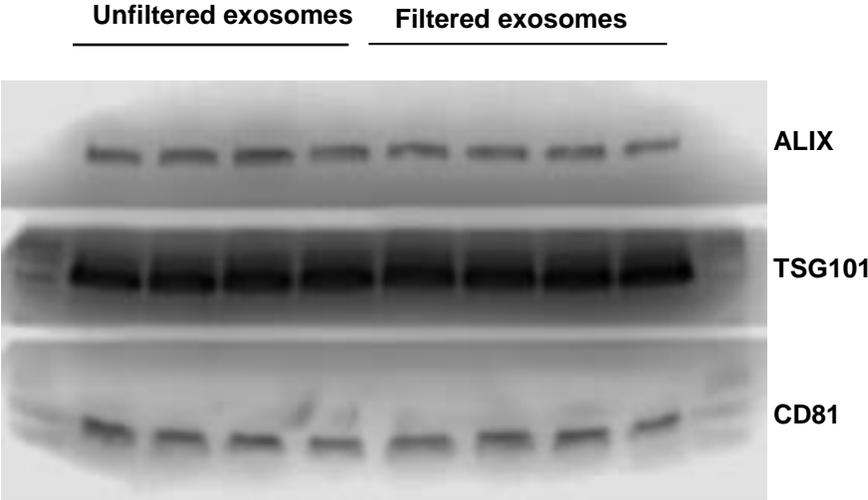


Figure 3.2. Exosomal markers ALIX, TSG101 and CD81.

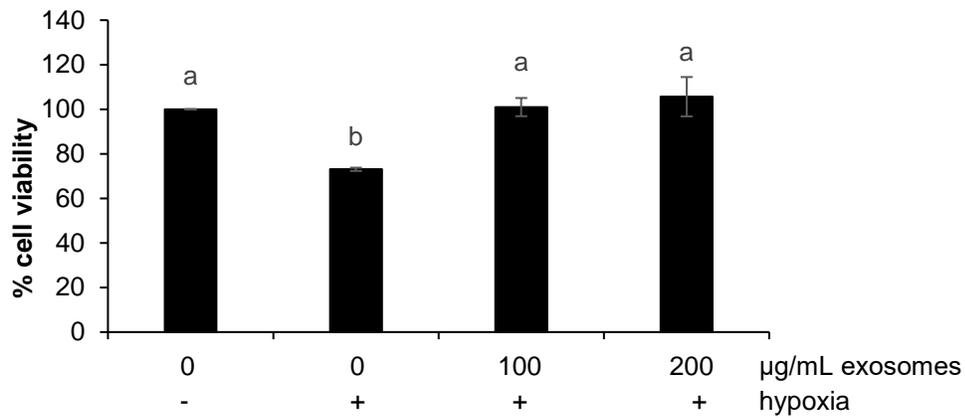


Figure 3.3. Proliferation of RAW 264.7 cell treated with bovine milk exosomes under hypoxia. Results are presented as mean \pm SD. Means that do not share a letter are significantly different ($p < 0.05$).

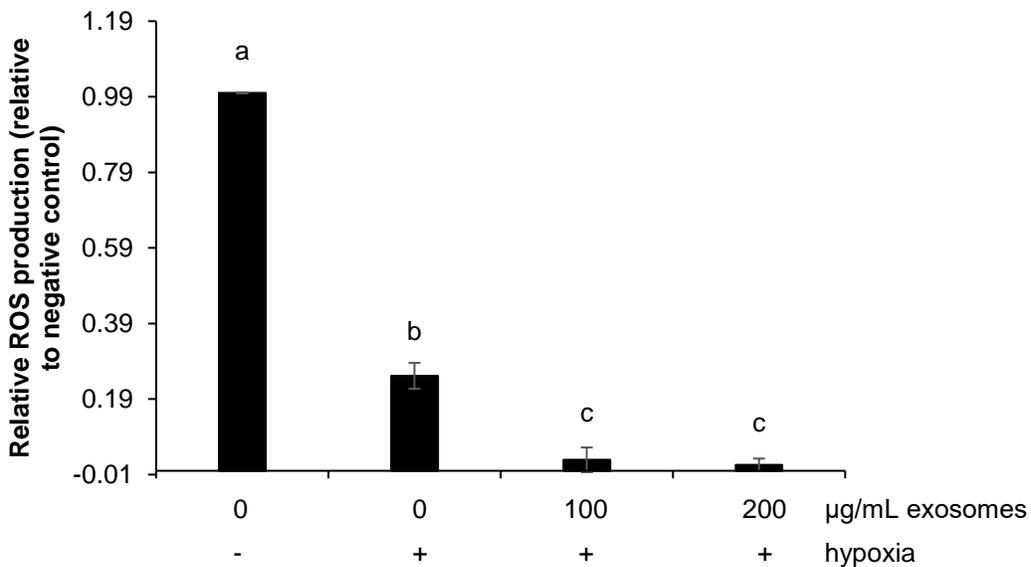


Figure 3.4. Relative production of reactive oxygen production (ROS) in RAW 264.7 cell treated with bovine milk exosomes under hypoxia. Results are presented as mean \pm SD. Means that do not share a letter are significantly different ($p < 0.05$).

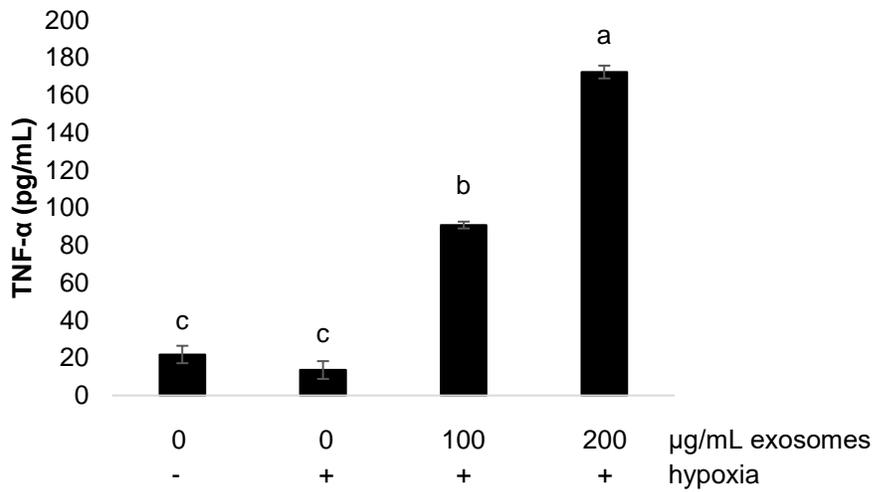


Figure 3.5. TNF- α production in RAW 264.7 cells treated with bovine milk exosomes. Results are presented as mean \pm SD. Means that do not share a letter are significantly different ($p < 0.05$).

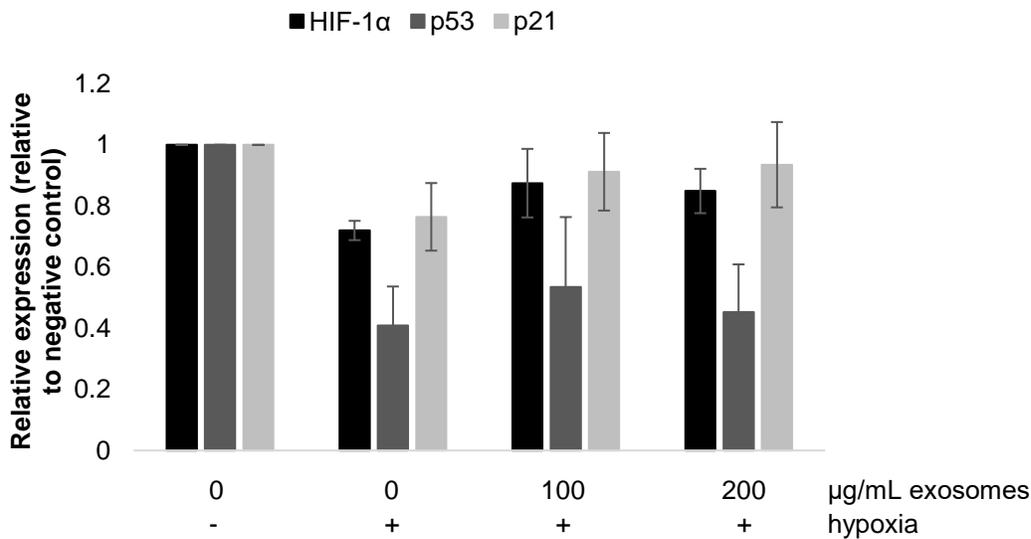


Figure 3.6. Effect of milk exosomes on RAW 264.7 expression of hypoxia protein HIF-1 α and proliferation proteins p53 and p21. Results are presented as mean \pm SE. Means that do not share a letter are significantly different ($p < 0.05$).

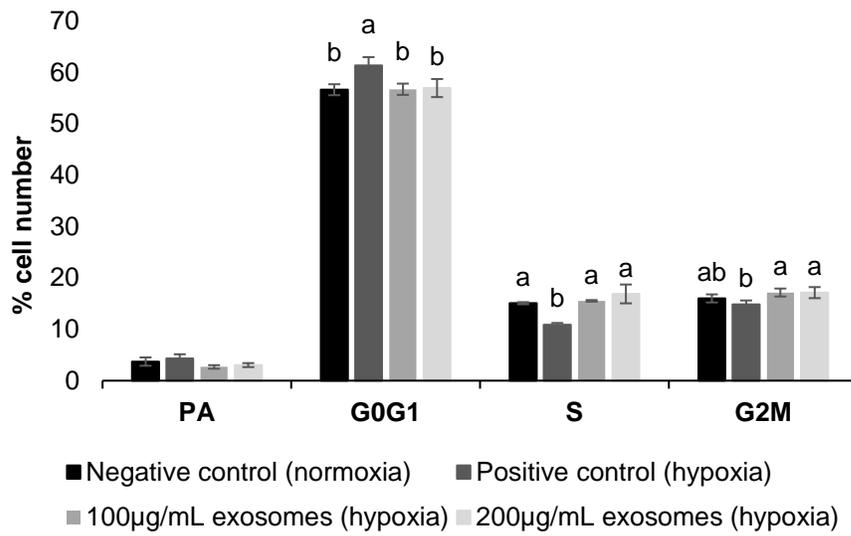


Figure 3.7. The effect of hypoxia and bovine milk treatment on the cell cycle of RAW 264.7 cells. Results are presented as mean \pm SD. Means that do not share a letter are significantly different ($p < 0.05$).

CHAPTER 4: CONCLUSION AND FUTURE WORK

It has been well-documented that the consumption of bovine milk has multiple benefits on human health. Recently, the discovery of exosomes and their presence in bovine milk contributed to a better understanding of how milk affects human health. Our study found that exosomes are present in commercially available milk suggesting that pasteurization temperatures do not destroy their integrity. The presence of exosomes after pasteurization process suggests that consuming heat-treated may not affect their biological activity but also this may be important for the potential application in the food industry. Also, our results supported the previously published findings that bovine milk exosomes are stable and can resist *in vitro* digestion suggesting that bovine milk exosomes may reach small intestines and therefore be absorbed in an intact form. Bovine milk exosomes increased the proliferation and protect RAW 264.7 cells from cisplatin-induced apoptosis in normoxia. Moreover, bovine milk exosome treatment significantly increased cells proliferation and had an immunomodulatory effect under hypoxia. Our results suggest that bovine milk exosomes could have a beneficial effect on human health so further research is needed to understand their biological activity. Bovine milk exosomes have not been extensively studied so there is still a lot unknown about the topic. Clearly, for any further research, isolation of bovine milk exosomes needs to be improved. Our study demonstrated that differential centrifugation along with filtration was not enough to remove all residual milk proteins. Application of pH or enzymes could be used to remove residual proteins before subjecting it to differential ultracentrifugation.

As a bioactive milk component, the stability of milk exosomes over time, and how the storage time affects their bioactivity and uptake should be investigated. To our knowledge, no study has been published on the effect of storage condition and time on the stability of bovine milk exosomes. Also, we demonstrated that bovine milk exosomes resisted *in vitro* digestion so the next step would be to investigate their uptake and internalization *in vivo* using animal models.

VITA

Svjetlana Matic was born and raised in Banja Luka, Bosnia and Herzegovina. She came to the United States in 2010. Sara, her daughter was born in 2011. In 2015, she enrolled at the Food Science and Technology program at the University of Tennessee.

In 2017, she received her Bachelor of Science degree in Food Science. In January 2018, she started the graduate school in Food Science at The University of Tennessee where she studied food chemistry. After receiving her MS degree, she plans to work in the food industry.