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EXPERIMENTAL THESIS

PROTECTIVE EFFECTS ELICITED BY PLANT BASED EXTRACELLULAR VESICLES AND OXYGEN LOADED PARTICLES AGAINST RENAL DYSFUNCTION

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SUMMARY

RATIONALE OF THE STUDY:

The research aims to address the significant public health concern of renal diseases and disorders, which affect millions of individuals globally. By focusing on the protective effects elicited by plant-based extracellular vesicles (EVs) and oxygen-loaded particles (OLN) against renal dysfunction, the study seeks to contribute to the understanding of potential therapeutic targets and innovative therapies for renal disorders.

The research findings indicate that the use of plant-based extracellular vesicles (EVs) and oxygen-loaded nanoparticles, particularly chitosan and dextran, demonstrated protective effects on renal tubular cells under stress conditions such as inflammation and oxidative stress.

The investigation is motivated by the critical role of the kidneys in filtering waste from the blood, regulating electrolyte balance, and maintaining fluid homeostasis, as well as the detrimental impact of oxidative stress on kidney function.

PLANNING OF THE STUDY:

The study is structured to achieve specific objectives, such as examining the effects of orange EVs with or without dextran and chitosan coated OLN on renal tubular cells under stress conditions.

Methods

The study utilizes renal tubular cells, orange EVs, dextran and chitosan coated OLN, hydrogen peroxide, lipopolysaccharide (LPS), cytomix [tumor necrosis factor (TNF)-α + interferon (IFN)-γ + interleukin (IL)-1β], the MTT assay for cell viability assessment, and the DCFDA assay for reactive oxygen species (ROS) measurement. The experiments are performed by giving the protective agents (EVs, OLN) either before or after hydrogen peroxide, LPS and cytomix.

RESULTS:

The research findings show the effects of stress factors (hydrogen peroxide, LPS and cytomix) on cell viability and ROS release in renal tubular cells and the protective role of EVs and dextran and chitosan coated OLN. Specific concentrations of dextran and chitosan coated OLN are identified as effective for enhancing cell viability and reducing ROS release by renal tubular cells, with the highest effects observed with 24 h of dextran and chitosan coated OLN stimulation. The study also demonstrates that the combination of EVs and dextran OLN or EVs and chitosan OLN, sometimes at both 0.32 M and 0.00032 M, sometimes at either 0.32 M or 0.00032 M only, can provide protection against renal tubular damage caused by hydrogen peroxide, LPS and cytomix.

In general, it is better to give protective agents after stress because they are more effective.

CONCLUSIONS:

The conclusion of the study highlights the potential of orange EVs and dextran and chitosan coated OLN in counteracting kidney injury caused by stress factors.

Additionally, the research provides new avenues for the development of innovative therapies and personalized medicine for renal disorders. By achieving its objectives, the study increases our understanding about the potential role of orange EVs in the prevention and treatment of renal dysfunction, as well as to identify new therapeutic targets for the creation of innovative therapies for renal disorders.

INTRODUCTION

Millions of people of all ages and genders are affected by renal diseases and disorders, which have become a serious public health concern globally. The kidneys are essential organs that filter waste from the blood, control electrolyte balance, and preserve the body's general fluid homeostasis. Renal disorders, which can cause major problems and even life-threatening illnesses, can develop when these systems are compromised.

The corpus of research devoted to comprehending the underlying mechanisms of kidney disorders and creating efficient treatments has been expanding in recent years. Technology and genetics advancements have made it possible for researchers to discover novel therapeutic targets and biomarkers, creating new opportunities for personalized medicine.

1- KIDNEY

The kidney is a crucial organ in the body that keeps the balance of electrolytes and fluids. It removes wastes and extra fluid from the blood through filtration, excreting them as urine. In addition to controlling blood pressure, the kidney is essential for manufacturing hormones that promote the creation of red blood cells.[1-3]

The kidney's interior, or renal medulla, is made up of renal pyramids, which are pyramidal organelles. The loops of Henle found in these pyramids are in charge of continuing to reabsorb water and electrolytes. The collecting ducts, which carry the finished urine to the renal pelvis and then to the ureter, are likewise located in the renal medulla.[4, 5]

The kidneys' ability to function depends on the flow of blood to them. The renal artery transports oxygenated blood to the kidney, where the glomeruli filter it. The renal vein then transports the filtered blood outside of the kidney.[6]

In conclusion, the kidney's structure is crucial to its ability to manage the interior environment of the body. Together, the various kidney regions filter waste from the blood and control blood pressure and electrolyte balance.

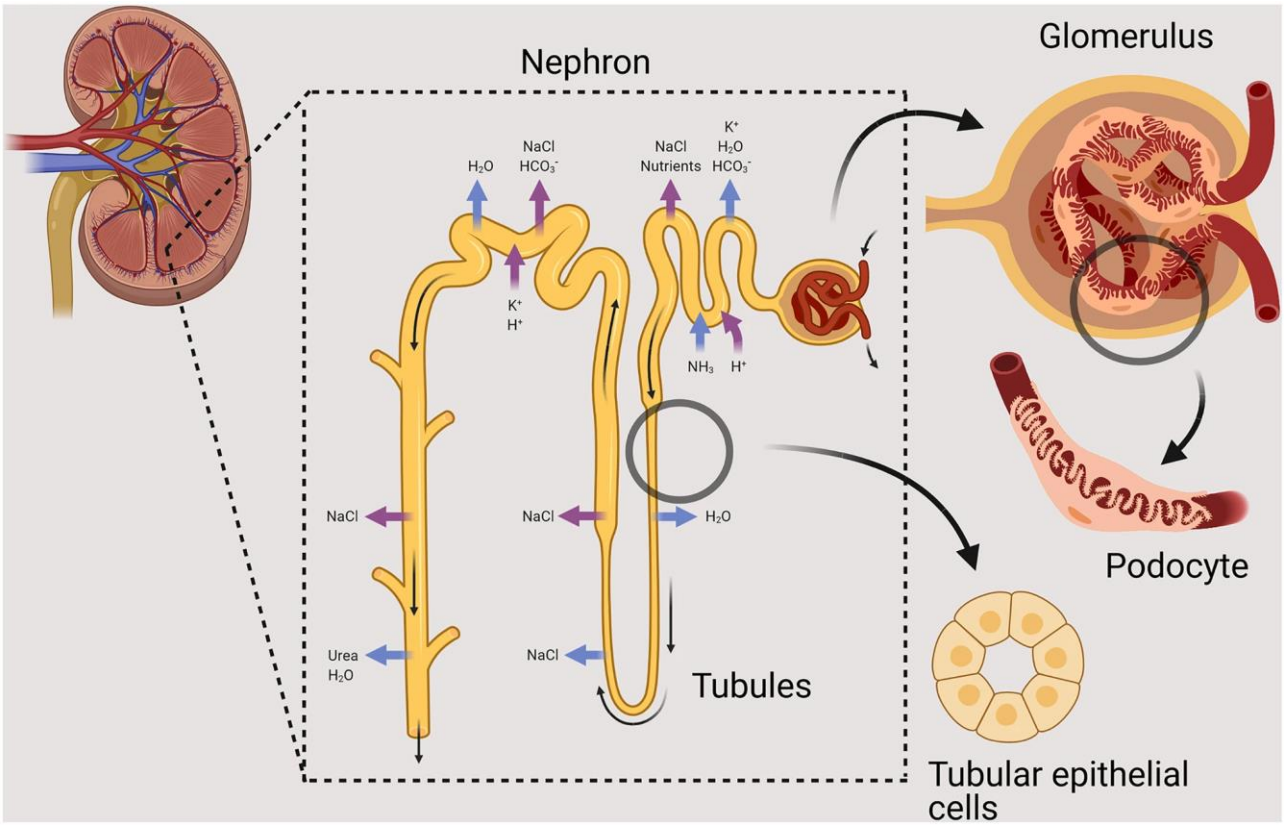


FIGURE 1. Kidney anatomy and physiology.[7]

1-1KIDNEY STRUCTURE

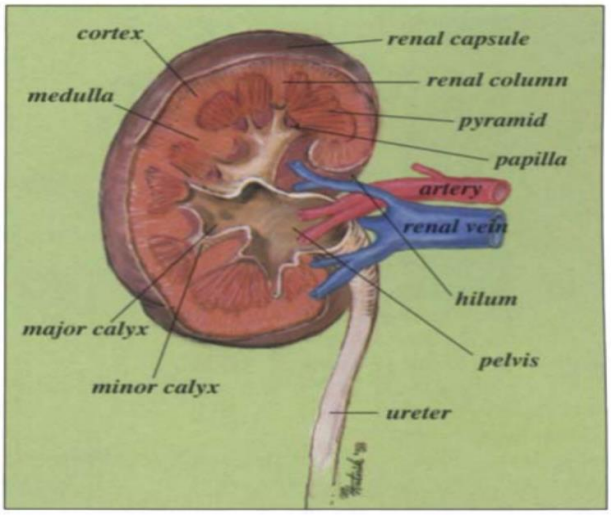


FIGURE 2. General anatomy of the kidney.[2]

Each of the several portions that make up the kidney's structure serves a distinct purpose. The two main parts of this organ are the renal cortex and renal medulla.

The renal cortex, or outer layer of the kidney, is home to the glomeruli, which are clusters of small blood vessels that serve as blood filters. The glomeruli are encircled by Bowman's capsule, which collects the filtrate before sending it to the renal tubules.

The renal tubules' jobs include reabsorbing essential nutrients and water from the filtrate and eliminating waste.[8]

1-2KIDNEY FUNCTION

The kidney not only regulates fluid and electrolyte levels, but it also generates a number of hormones necessary for bone health, the generation of red blood cells, and blood pressure control. For instance, the hormone erythropoietin promotes the creation of red blood cells, while the hormone calcitriol aids in calcium absorption and bone health maintenance.[9]

The kidney is a complicated organ that performs a variety of tasks, such as filtering waste materials, controlling blood pressure, and creating hormones that are crucial to the body's general health and function. For preserving good health and preventing ailments like hypertension, renal disease, and osteoporosis, it is essential to comprehend the kidney and its function in the body. [6]

A number of factors, including aging, illness, and lifestyle elements like nutrition and exercise, can have an impact on kidney function. Age-related declines in the kidney's capacity to filter blood and control fluid and electrolyte balance can raise our risk of developing renal disease, hypertension, and other disorders. Over time, chronic illnesses like diabetes and hypertension can also harm the kidney, resulting in diminished function and an increased chance of renal failure.[9, 10]

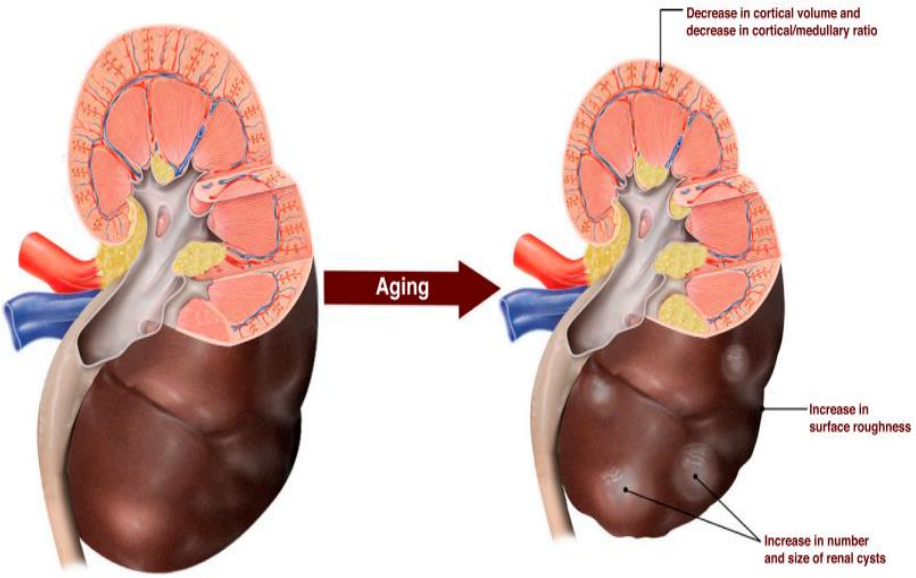


FIGURE 3. There are degenerative macro-structural changes that occur in the human kidney with even healthy aging. There is cortical volume loss, some increase in medullary volume (not shown), increase in surface roughness, increased sinus fat, and an increase in renal cysts. These findings can be attributed to underlying nephrosclerosis with nephron loss, hypertrophy of remaining tubules, and tubular diverticuli.[11]

1-3 THE ROLE OF TUBULAR CELL IN RENAL FUNCTION

Tubular cells play a critical role in renal function by facilitating essential processes involved in filtration, reabsorption, and secretion within the kidneys. The intricate network of tubular cells within the nephrons enables the regulation of electrolyte balance, acid-base homeostasis, and the excretion of waste products.[5, 12, 13]

REABSORPTION: Tubular cells actively reabsorb and transport specific molecules and ions from the renal tubules back into the bloodstream. This reabsorption process takes place in different segments of the tubules, such as the proximal tubule, loop of Henle, distal tubule, and collecting ducts. It aids in maintaining the body's electrolyte balance and fluid volume.[14, 15]

SECRETION: Tubular cells participate in the selective secretion of substances from the bloodstream into the renal tubules. This active transport process involves the secretion of various substances, including drugs, metabolites, and hydrogen ions (H+). Secretion contributes to the elimination of waste products and the regulation of acid-base balance.[13, 16-21]

ACID-BASE BALANCE: Tubular cells in the distal nephron play a crucial role in maintaining acid-base homeostasis by regulating the reabsorption and secretion of hydrogen ions (H+) and production of bicarbonate ions (HCO3-). This intricate process involves the coordination of various transporters and ion channels.[21]

ELECTROLYTE REGULATION: Tubular cells actively reabsorb or secrete various electrolytes, including sodium (Na+), potassium (K+), calcium (Ca2+), and magnesium (Mg2+). The reabsorption and secretion of these ions are vital for maintaining electrolyte balance in the body.[13, 22]

WATER BALANCE: Tubular cells also play a significant role in regulating water balance in the body. The reabsorption of water occurs in the proximal tubule, distal tubule and collecting ducts. In the distal tubule and collecting ducts this process is under the influence of antidiuretic hormone (ADH) and aquaporin channels present on the tubular cells. This process allows for the concentration or dilution of urine, depending on the body's hydration needs.[23-25]

In conclusion, tubular cells in the kidneys play vital roles in renal function. They contribute to the filtration, reabsorption, and secretion processes involved in maintaining electrolyte balance, acid-base homeostasis, waste product elimination, and water balance.

1-4 KIDNEY DYSFUNCTION AND ITS DISEASE RENAL DYSFUNCTION

Chronic kidney disease (CKD) is a disorder that worsens with time and causes the kidneys to lose their capacity to function properly. Urinary tract infections, kidney

cancer, and kidney stones are further disorders that can harm the kidneys. Numerous symptoms, including discomfort, fever, and trouble peeing, can be brought on by these illnesses.[26-28]

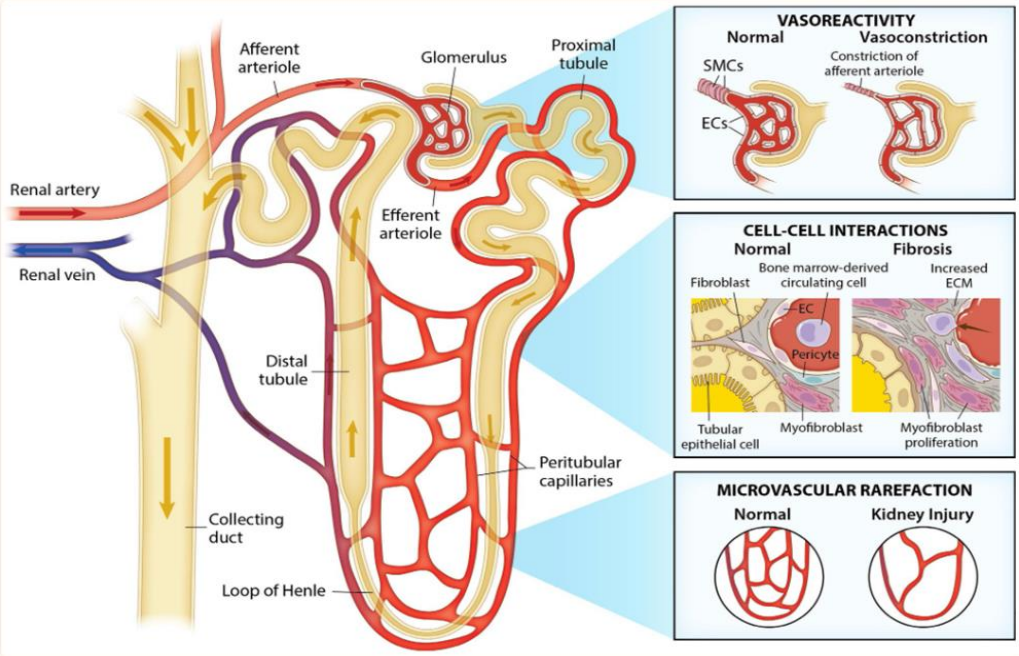


FIGURE 4. Kidney disease is associated with microvascular dysfunction characterized by altered vasoreactivity, cell coordinated fibrosis, and vessel rarefaction. These processes involve multiple cell types and are interconnected. Investigation of the involved mechanisms and overlaps between remodeling processes serve to advance the understanding of kidney disease and inspire the development of new therapies.[29]

Kidney dysfunction, commonly referred to as kidney disease, is a disorder that impairs the kidneys' ability to operate. Waste and fluids can accumulate in the body when the kidneys are not working correctly, which can cause major health issues.[27, 30, 31]

Renal dysfunction can take many different forms, such as end-stage renal disease (ESRD), CKD, and acute kidney injury (AKI). AKI is a quick start of renal impairment that is frequently brought on by a medical emergency such dehydration, a serious infection, or drug toxicity. It is common for underlying medical disorders like diabetes or high blood pressure to contribute to CKD, which is a gradual reduction in kidney function over time. When kidney function is irreversibly lost, a patient has ESRD and must receive kidney dialysis or a transplant to survive.[28, 31, 32]

In conclusion, renal dysfunction is a severe ailment that can have a negative effect on one's health in general and their quality of life in particular. To improve patients' outcomes and avoid additional problems, renal impairment must be identified and treated early. For those who are at risk for renal dysfunction, regular monitoring and

testing of kidney function are crucial, and prompt management can help the disease's progression being slowed. [27, 30]

1-5 THE ROLE OF OXIDATIVE STRESS IN KIDNEY DISEASE

Oxidative stress, which refers to an imbalance between the production of reactive oxygen species (ROS) and the ability of the body's antioxidant defenses to neutralize them, plays a significant role in the development and progression of kidney disease.

Role of oxidative stress in CKD: oxidative stress is considered a key contributor to the pathogenesis of CKD. It can lead to renal inflammation, fibrosis, and cellular damage, ultimately leading to the decline in kidney function. Oxidative stress in CKD can result from various sources, including mitochondrial dysfunction, inflammation, and activation of the renin-angiotensin-aldosterone system (RAAS).[32-36]

Oxidative stress in diabetic nephropathy: diabetic nephropathy is a common complication of diabetes and is characterized by progressive kidney damage. Oxidative stress is a major contributor to the development and progression of diabetic nephropathy. Increased glucose levels, mitochondrial dysfunction, and advanced glycation end-products (AGEs) contribute to the generation of ROS and subsequent renal injury.[34, 37-40]

Oxidative stress in AKI: AKI is characterized by a sudden decline in kidney function. Oxidative stress plays a crucial role in the pathophysiology of AKI, leading to tissue injury and impaired renal function. Ischemia-reperfusion injury, inflammation, and activation of pro-oxidant enzymes contribute to the generation of ROS in AKI.[32, 34, 41-44]

Oxidative stress and renal fibrosis: renal fibrosis is a common pathological process observed in various kidney diseases. Oxidative stress contributes to the activation of profibrotic pathways, such as transforming growth factor-beta (TGF-β) signaling, leading to excessive extracellular matrix deposition and tissue fibrosis.[32, 45-48]

Kidney transplantation is a life-saving procedure performed for patients with ESRD. Despite significant advancements in surgical techniques and immunosuppressive therapies, various factors can contribute to the development of complications post-transplantation. One such factor is oxidative stress, which has emerged as a crucial player in the pathophysiology of kidney transplantation.[49]

During kidney transplantation, the donor organ undergoes a period of ischemia (lack of blood supply) followed by reperfusion (restoration of blood flow) upon transplantation. This process can trigger the generation of ROS, leading to oxidative stress. IRI-induced oxidative stress is associated with tissue damage, inflammation, and impaired graft function. Increased production of ROS during IRI can activate various pathways, such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein

kinases (MAPKs), which contribute to inflammatory responses and cellular injury.[50, 51]

Acute rejection is a common complication following kidney transplantation, primarily mediated by immune responses against the transplanted organ. Oxidative stress has been implicated in the development and progression of acute rejection. ROS can modulate immune cell activation, cytokine production, and T-cell responses, thereby exacerbating the alloimmune response and promoting tissue injury. Additionally, oxidative stress can impair the function of endothelial cells, further contributing to graft dysfunction.[52]

Chronic allograft dysfunction, characterized by a gradual decline in renal function over time, is a major cause of graft failure. Oxidative stress has been implicated in the pathogenesis of chronic allograft dysfunction. Prolonged exposure to oxidative stress can lead to DNA damage, lipid peroxidation, and protein oxidation within the renal tissue, promoting fibrosis, vascular abnormalities, and tubular atrophy. These structural alterations eventually compromise graft function and contribute to long-term graft failure.[51, 53]

Given the detrimental effects of oxidative stress on kidney transplantation outcomes, there is a growing interest in developing therapeutic strategies to mitigate its impact.

In conclusion, oxidative stress plays a significant role in the development and progression of kidney diseases such as chronic kidney disease, diabetic nephropathy, acute kidney injury, and renal fibrosis. Numerous research papers have provided insights into the mechanisms by which oxidative stress contributes to renal injury. Additionally, studies have investigated the potential of antioxidant strategies and therapeutic targeting of oxidative stress pathways as promising approaches to mitigate kidney damage and improve renal function.

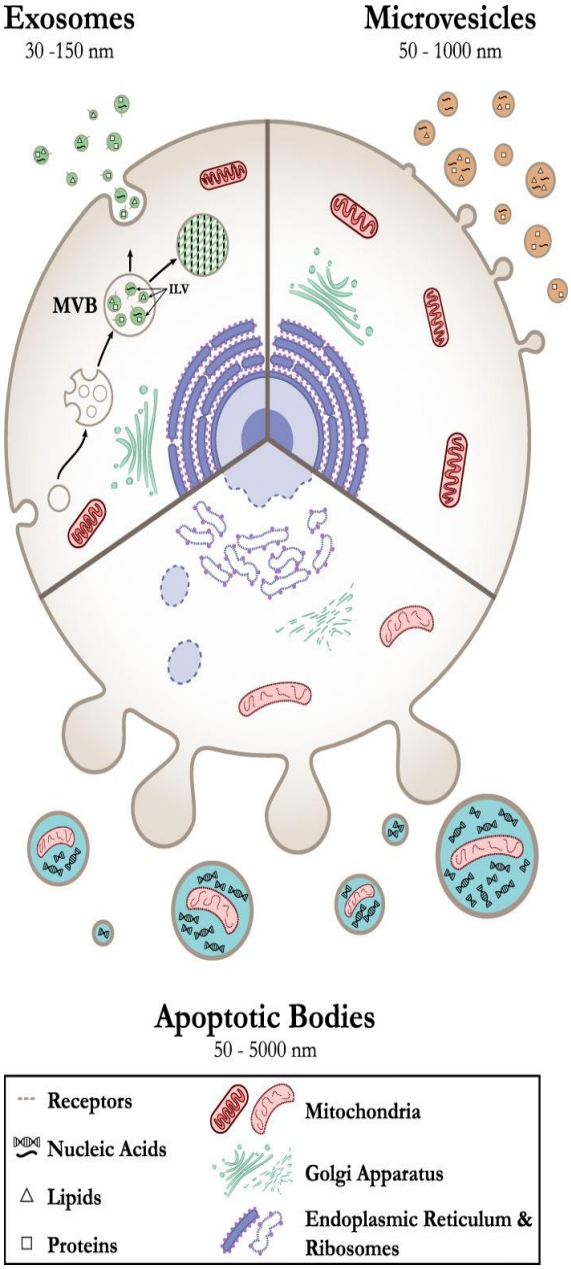
2- THERAPEUTIC APPROACH FOR RENAL DYSFUNCTION

Therapeutic targeting of oxidative stress in kidney disease: several experimental studies have explored the therapeutic potential of targeting oxidative stress pathways in kidney disease. These approaches include the use of antioxidant compounds, inhibitors of pro-oxidant enzymes, and modulation of redox-sensitive signaling pathways to attenuate renal injury and preserve kidney function.[54]

Therapeutic approaches for renal dysfunction involve various strategies aimed at preserving kidney function, managing underlying conditions, and preventing disease progression, such as extra cellular vesicle and oxygen-loaded nanoparticles.

2-1 EXTRACELLULAR VESICLES (EVs)

In Figure 5, the types and generation of EVs are shown



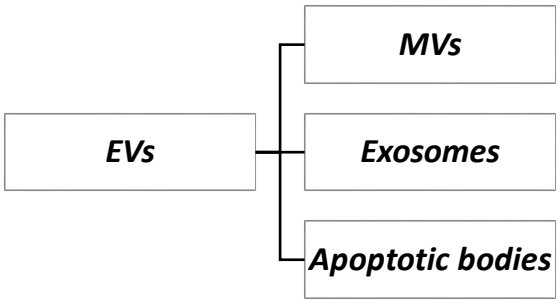


FIGURE 5. Biogenesis of various forms of EVs from a eukaryotic cell. Exosomes are generated through multivesicular bodies (MVB) and intraluminal vesicles (ILV) formation whereas microvesicles/microparticles and apoptotic bodies are vesicles generated through blebbing of plasma membrane.[55]

Exosomes (30–150 nm), microvesicles (50–1000 nm), apoptotic bodies (800–5000 nm), and oncosomes (1–10 mm) are common subgroups of EVs that are characterized according to size (Figure 6).[56-58]

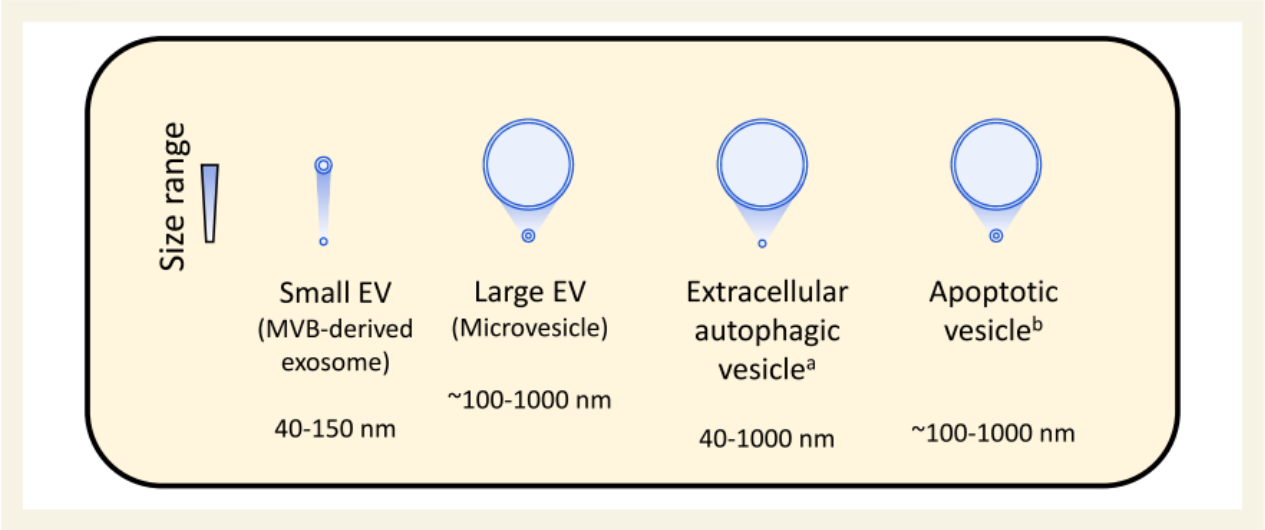


FIGURE 6. The typical size range of the major lipid-bilayer EVs up to 1000 nm diameter. As reported by Jeppesen et al. the size of apoptotic vesicles/ bodies can range up to 5 μm in diameter. Please be aware that the diameter of EVs depends on the detection method used.[56]

EVs, which act as biologically active cargo carriers naturally, have a distinct micro/nanostructure, bioactive composition, and recognizable morphology. They also possess an array of fascinating physical, chemical, and biochemical properties that have shown promise in the treatment of a variety of diseases. Numerous different cell types are capable of producing them.[58, 59]

By transporting active components like nucleic acids and proteins, they operate as a conduit for intercellular communication. The function of recipient cells is influenced by the cargo of EVs, which allows them to be employed for therapeutic and diagnostic reasons. The cargo of EVs reflects the physiological condition of the cells from which they originate. EVs have performed well in preclinical tests, but it is still difficult to deliver cargo precisely and in a controlled manner to the intended location.[60]

The quantity and stability of EVs in any type of biofluid, including blood, urine, saliva, and breast milk, makes them excellent candidates for use as disease biomarkers. These vesicles, which are coated by phospholipids and released by a number of mammalian cells, also travel throughout the whole body to act as long-distance intercellular communication tools. Due to the fact that both tumor cells and the cells around them release EVs, EVs in noninvasive biofluid samples like urine are anticipated to be particularly useful as biomarkers for longitudinal cancer surveillance. [29, 61, 62]

Since EVs are produced by several cell types during both healthy cellular activities and pathological situations, they can serve as biomarkers for the diagnosis and prognosis of various illnesses. Exosomes can directly fuse with the membrane of the target cell to release their contents into the cytosol, or the cargo can be ingested by the recipient cell primarily by endocytosis. In part in response to the miRNAs present, the cargo delivery modifies and reroutes the biological functions of the recipient cell, controlling post-transcriptional gene expression, differentiation, proliferation, and cell-to-cell communication by repressing or degrading their target mRNAs.[61, 63]

The way that EVs moderate their effects is what makes them particularly intriguing. Both MVs and exosomes have been found to carry a wide range of bioactive cargo, including cell surface, cytosolic, and nuclear proteins as well as RNA transcripts, micro-RNAs (miRNAs), and even DNA fragments. It has been demonstrated that both kinds of EVs contain a wide range of proteins, including cell surface receptors, cytosolic signaling proteins, transcription factors, metabolic enzymes, extracellular matrix proteins, and RNA binding proteins. EVs also include RNA transcripts, miRNAs, and pieces of genomic DNA in addition to proteins.[57, 58, 64]

When originally identified, EVs, previously known as "platelet dust", were described as subcellular components of platelets in healthy plasma and serum. Later, the release of plasma membrane vesicles by a mechanism called exocytosis in activated neutrophils was reported. Microvesicles have historically been mostly investigated for their function in blood coagulation.

In addition to lipids, carbohydrates, and genetic material like mRNA and miRNAs, EVs can transport membrane-derived receptors, proteins such as cytokines and chemokines and proteins involved in cellular signaling and/or migration.[57, 59, 65]

The parent cell, the surrounding microenvironment, and the events that led up to their release all affect what they contain. The phenotypic of the target cell may change as a result of the transfer of these chemicals to recipient cells.[65]

Recently, techniques that are often used to separate EVs from mammalian resources have been applied to tissues, organs, and juices of edible plant species, such as ginger and carrot, grape, and orange fruits, to isolate EV-like vesicles.[59]

The vesicle populations seen in these isolates are extremely diverse in size, shape, and origin, according to studies. It's intriguing that edible plant-derived vesicles have good biocompatibility, a high rate of cellular internalization, and serve as a useful source of a number of bioactive chemicals that have the potential to be used for nutraceutical and therapeutic reasons.[56]

2-2 PLANT EVs

Organisms of all life forms can secrete EVs into their surrounding environment; they serve as important communication tools between cells and between cells and the environment and participate in a variety of physiological processes. According to new evidence, plant EVs play an important role in the regulation of transboundary molecules with interacting organisms. In addition to carrying signaling molecules (nucleic acids, proteins, metabolic wastes, etc.) to mediate cellular communication, plant cells EVs themselves can also function as functional molecules in the cellular microenvironment across cell boundaries. [66, 67]

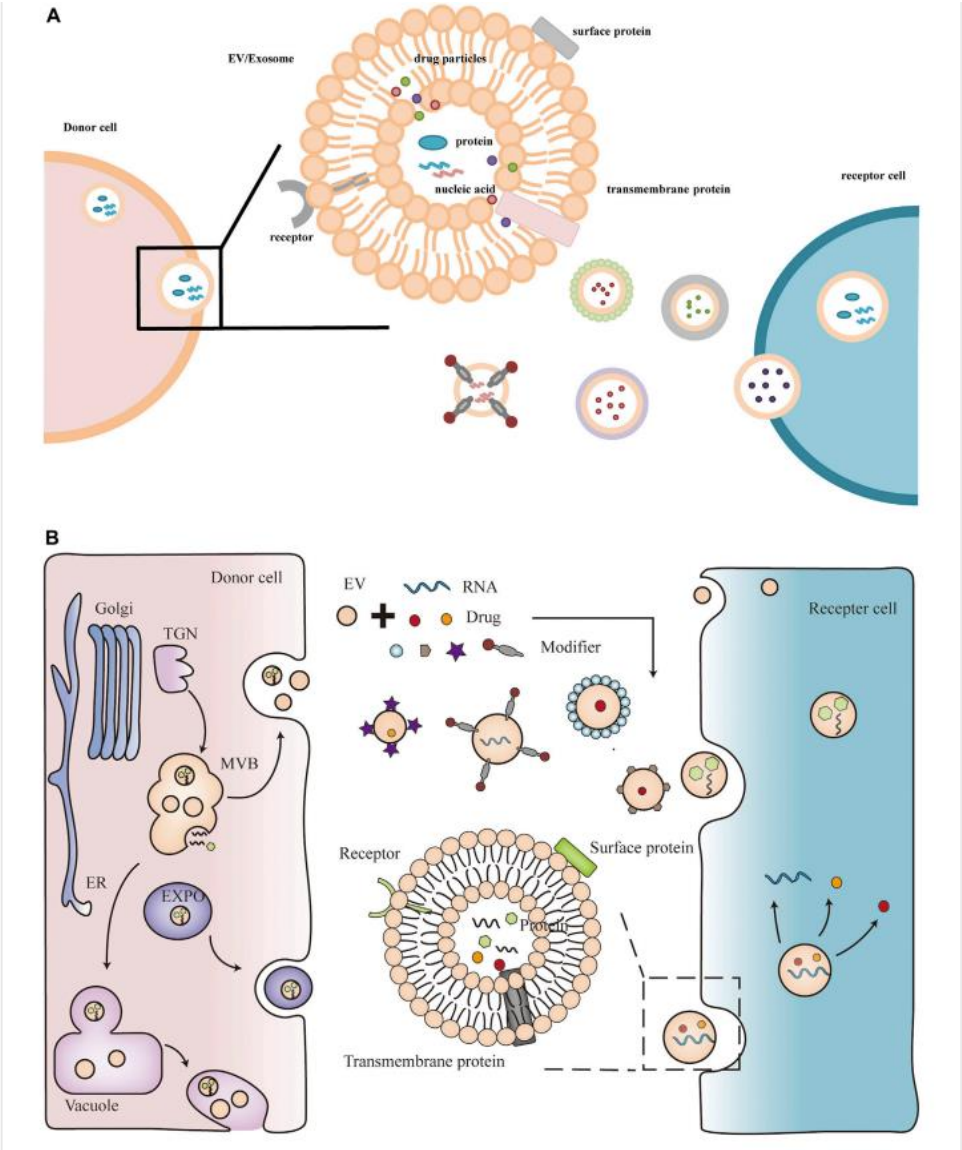


FIGURE 7.Plant EVs contain active components such as nucleic acids and proteins. Plant EVs can also be modified as delivery vehicles for therapeutics such as miRNAs and drugs.[68]

Similar to mammalian cells, there is growing evidence that plants also generate EVs that are involved in various functions.[66]

Plant EVs have attracted attention for their potential roles in human health and disease. For example, EVs extracted from edible fruits and vegetables have antioxidant functions, and strawberry- and blueberry-derived exosome-like nanoparticles prevent oxidative stress in human mesenchymal stromal cells and endothelial cells. [69]

Plant EVs contain a large amount of proteins, lipids, and miRNAs, which can act as cell messengers to transfer these biologically active substances from in vitro to in vivo, then to the lesion tissue, and finally to cells. Thus, plant EVs can mediate specific transboundary cellular or tissue responses.[70]

Orange EVs, tiny membrane-bound objects that orange cells release into the extracellular space, have drawn more attention in recent years due to their possible health advantages. Numerous bioactive substances, including phenolic acids, flavonoids, and carotenoids as well as RNA and proteins, have been shown to be present in these EVs.[71]

Orange EVs are categorized as EVs, just as exosomes and other EVs that may be discovered in other bodily fluids. With a diameter ranging from 50 to 500 nanometers, they are microscopic, spherical particles. Signals between cells and tissues are carried by these vesicles, which have been proven to be important in intercellular communication. Orange EVs have, furthermore, been suggested as a possible delivery route for bioactive substances, such as nutraceuticals and medicinal medicines. Inflammation, oxidative stress, and apoptosis are just a few of the cellular processes that these vesicles have been found to affect in studies. Orange EVs, for instance, have been demonstrated to decrease the synthesis of pro-inflammatory cytokines in macrophages and to lessen oxidative stress in endothelial cells.[72]

Orange EVs have also been found to have a preventive impact against a number of chronic illnesses, such as diabetes and cardiovascular disease. Orange EVs, for instance, have been demonstrated to increase insulin sensitivity and lower blood glucose levels in animal models of diabetes.

As a result of their shown anti-cancer effects, researchers are looking at how orange EVs may be used in medicine delivery and formulations for cosmetic products. They represent a promising tool for disease prevention and therapy due to their capacity to control immune responses and encourage tissue repair.

Overall, orange EVs have a bright future as a natural source of bioactive substances with potential medicinal and functional capabilities for a variety of uses in the food industry, medication delivery, and disease prevention and therapy.[69]

2-3 OXYGEN LOADED NANOPARTICLE (OLN)

OLN refer to tiny particles that are specifically designed to carry and deliver oxygen molecules to target tissues or organs. These nanoparticles are engineered to encapsulate or bind oxygen molecules, allowing them to be transported to areas where oxygen supply is limited or compromised.

The primary goal of OLN is to enhance oxygen delivery to specific sites in the body, particularly in situations where traditional methods of oxygenation, such as breathing air or using supplemental oxygen, may not be sufficient or feasible.[73]

Once the OLN reach the intended location, they can release oxygen through various mechanisms. This can involve the nanoparticles degrading or breaking down, allowing the oxygen to diffuse into surrounding tissues. Alternatively, the nanoparticles may be designed to respond to specific triggers, such as changes in pH or temperature, which can lead to the controlled release of oxygen.[74]

OLN have the potential to be used in a range of medical scenarios, including emergency medicine, tissue engineering, and regenerative medicine. By enhancing oxygen delivery to specific tissues, these nanoparticles can aid in tissue repair, support cell survival, and promote healing processes.[75]

Dextran and chitosan are two biocompatible polymers that have been explored for their potential role in oxygen therapy. While they have distinct properties and applications, both polymers have been studied as carriers for oxygen delivery in different contexts.

Dextran

Dextran is a polysaccharide composed of glucose units. It has excellent biocompatibility and is water-soluble, making it suitable for various biomedical applications. In oxygen therapy, dextran-based carriers can be utilized to enhance oxygen solubility and transport.[76, 77]

One approach involving dextran is the development of oxygen-carrying solutions or suspensions. Dextran-based oxygen carriers can encapsulate or bind oxygen molecules, thereby increasing the oxygen-carrying capacity of a fluid. These solutions can be infused into the bloodstream to deliver oxygen to tissues in situations where traditional means of oxygenation are insufficient.[78]

Additionally, dextran-based oxygen carriers can serve as blood substitutes, mimicking some of the oxygen-carrying properties of red blood cells. These carriers are particularly valuable in emergency medicine, where rapid oxygen delivery is crucial, such as in cases of severe blood loss or during surgical procedures.[79]

Chitosan

Chitosan is a biocompatible and biodegradable polysaccharide derived from chitin, a compound found in the shells of crustaceans. It has attracted attention in various biomedical applications due to its unique properties, including mucoadhesion, biocompatibility, and antimicrobial activity.[80]

In the context of oxygen therapy, chitosan has been explored as a scaffold material for tissue engineering and wound healing. It can be used to create three-dimensional structures or films that can encapsulate or immobilize oxygen molecules. These structures can be applied topically to wounds or incorporated into tissue engineering constructs to provide a localized oxygen supply and support tissue regeneration processes.[81, 82]

Chitosan-based nanoparticles have also been investigated for oxygen delivery. These nanoparticles can be loaded with oxygen molecules and targeted to specific sites, such as ischemic tissues or regions with low oxygen levels. By delivering oxygen directly to these areas, chitosan nanoparticles can help improve tissue oxygenation and support healing.[83]

Both dextran and chitosan offer advantages in terms of biocompatibility, biodegradability, and oxygen-carrying capabilities. However, it's important to note that the development and optimization of dextran- and chitosan-based systems for oxygen therapy are still areas of

As previously said, kidney disease is a common condition that can result in tissue hypoxia and oxidative stress. Recently, OLN-based therapies have been investigated as a potential treatment strategy for various kidney diseases, including AKI and CKD.

One study investigated the use of dextran and chitosan-coated OLN therapy in a mouse model of AKI and found that OLN treatment reduced oxidative stress and inflammation, improved kidney function, and decreased tissue damage compared to control mice. Another study investigated the use of chitosan-coated OLN therapy in a rat model of CKD and found that OLN treatment improved kidney function and reduced fibrosis compared to control rats.

The role of OLN in renal dysfunction lies in their ability to improve oxygenation in the renal tissue. Reduced oxygen supply, known as tissue hypoxia, is a common feature in many kidney diseases and can contribute to tissue damage and impaired renal function. By delivering oxygen to the renal tissue, OLN may help alleviate hypoxia, improve cellular metabolism, and potentially promote tissue repair.[84]

While research on the use of OLN in renal dysfunction is still in its early stages, some studies have shown promising results. For example, in a study published in Biomaterials, researchers developed oxygen-carrying microparticles and evaluated

their effects in a rat model of AKI. The study demonstrated that the oxygen-carrying particles improved renal oxygenation and reduced kidney injury.[75]

3-AIM OF STUDY

The purpose of this thesis is to examine any potential protection against renal dysfunction provided by orange EVs and dextran and chitosan coated OLN given alone or co-stimulation.

This research specifically intends to examine how orange EVs, with or without dextran and chitosan coated OLN, affect renal tubular cells when they are under stress, caused hydrogen peroxide, lipopolysaccharide (LPS) and cytomix.

Through the accomplishment of these objectives, this study hopes to further increase knowledge of the possible function of orange EVs in the prevention and treatment of renal dysfunction and find new therapeutic targets for the creation of innovative therapies for renal diseases.

MATERIALS AND METHODS

CULTURE OF RENAL TUBULAR CELLS

Renal tubular cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Euroclone) at 37°C with 5% CO2 in incubator.

EVs ISOLATION

EVs isolation was performed in university of Turin.

Preliminary Phase

In the first part of the study, experiments were performed in order to evaluate the proper timing of stimulation of renal tubular cells with orange EVs, and the proper timing of stimulation of renal tubular cells with hydrogen peroxide, lipopolysaccharide (LPS, 30 μM) and cytomix [tumor necrosis factor (TNF)-α + interferon (IFN)-γ + interleukin (IL)-1β] (50 ng/ml). To do this, renal tubular cells were stimulated with orange EVs (50000 particles/cell) for 30 min,12 h, 24 h, with hydrogen peroxide (200 μM) for 5, 15, 30 min, with LPS (30 μM) for 1 h, 2 h, 4 h and with cytomix (50 ng/ml), for 1 h, 2 h, 4 h. The concentration of EVs we used is the same that we used in our previous studies about the effects of EVs from HCV and subarachnoid hemorrhagic patients. [85, 86]

After each stimulations, the cells were washed with phosphate buffer saline (PBS; Sigma) and conditioned media were replaced. After that the MTT assay was performed, as previously executed.[87-90]

In addition, the renal tubular cells were treated with dextran and chitosan coated OLN in order to establish the most appropriate dose (dose–response) and timing (time–course). For the dose response study, the doses were: 0.32 M, 0.032 M, 0.0032 M, 0.00032 M. The timings of stimulation were 4 h and 24 h. After the stimulations, the cells were washed with PBS (Sigma) and conditioned media was replaced. After that the MTT assay was performed.

All the experiments were performed in triplicate and repeated at least 4 times.

Extended Phase

From the results obtained in the preliminary phase, in the extended phase we used EVs (50000 particles /cell) 24 h stimulation, two concentrations of dextran and chitosan coated OLN (0.32 M and 0.00032 M), 24 h stimulation, hydrogen peroxide (200 µM), 30 min stimulation, cytomix (50 ng/ml) and LPS (30 µM), 4 h stimulation.

In the extended phase, we examined the effects of dextran and chitosan coated OLN on cell viability and ROS release in renal tubular cells in two different experimental protocols: pre and post stimulation with hydrogen peroxide, cytomix and LPS. [87]

In the pre stimulation experiments, the renal tubular cells were treated with hydrogen peroxide, cytomix and LPS at the selected timing of stimulations, thereafter the cells were washed and stimulated with EVs (50000 particles /cell) dextran and chitosan coated OLN (two concentrations) alone or in co-stimulation. We washed with PBS and performed the MTT assay and ROS assay.[88, 91]

In the post stimulation experiments, the renal tubular cells were treated with EVs (50000 particles /cell), dextran and chitosan coated OLN (two concentrations) alone or in co-stimulation. After washing with PBS, the cells were treated with hydrogen peroxide, cytomix and LPS at the selected timing of stimulations. After washing with PBS, we performed the MTT assay and ROS assay. [88] [91]

All the experiments were performed in triplicate and repeated at least 4 times.

Cell Viability

The 10% of the MTT solution was prepared by dissolving 50 mg of the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in 10 mL of PBS (pH 7.4) and kept stored at 4 °C protected from the light. Following the stimulations of renal tubular cells (10,000 cells/well in 96 well plates), as above described, the media was removed and washed with PBS (Sigma) After the stimulation, 100 µL of the MTT solution diluted in DMEM high glucose *w/o* phenol red, supplemented with 2 mM L-glutamine, and 1% penicillin-streptomycin (P/S) were added to each well. Thereafter, the plate was incubated at 37 °C for 2 h.

Once the reaction had occurred, the supernatant was removed and the formazan crystals formed in each well were dissolved with 100 µL of dimethyl sulfoxide (DMSO; Sigma, Milan, Italy). Cell viability was finally determined by measuring the absorbance through a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer; Waltham, MA, USA) with a wavelength of 570 nm. Cell viability was calculated by setting control cells (untreated cells) as 100%.[85, 89, 92, 93]

DCFDA Assay

The oxidation of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) into 2,7-dichlorodihydrofluorescein (DCF) was used to assess ROS generation, following the manufacturer's instructions (Abcam; Cambridge, UK), and as previously performed. [85, 94-96]

After stimulation, as described for MTT, the medium was removed, and staining was performed with 10 µM H2DCFDA for 20 min at 37 °C. The fluorescence intensity of DCF was measured at an excitation and emission wavelengths of 485 and 530 nm, respectively, by using a spectrophotometer (VICTOR™ X Multilabel Plate Reader; PerkinElmer). Results were expressed as DCF fluorescence intensity, which was proportional to the amount of intracellular ROS. The data were normalized versus control cells (untreated cells).[97]

STATISTICAL ANALYSIS

The statistical analysis was performed with SPSS for Windows version 24.0. Means ± standard deviations (SD) were given as descriptive statistics. The results obtained were examined through MANN-WHITNEY U-TEST. A value of $P < 0.05$ was considered statistically significant.

RESULTS

In the preliminary phase, the experiments were conducted by using EVs (50000 particles /cell) at different timings, including 30 min, 12 h, and 24 h. Based on our observations shown in FIGURE 8, the optimal incubation time for studying the effects of orange EVs appears to be 24 h, as it closely resembles the results obtained with 30 min stimulation.

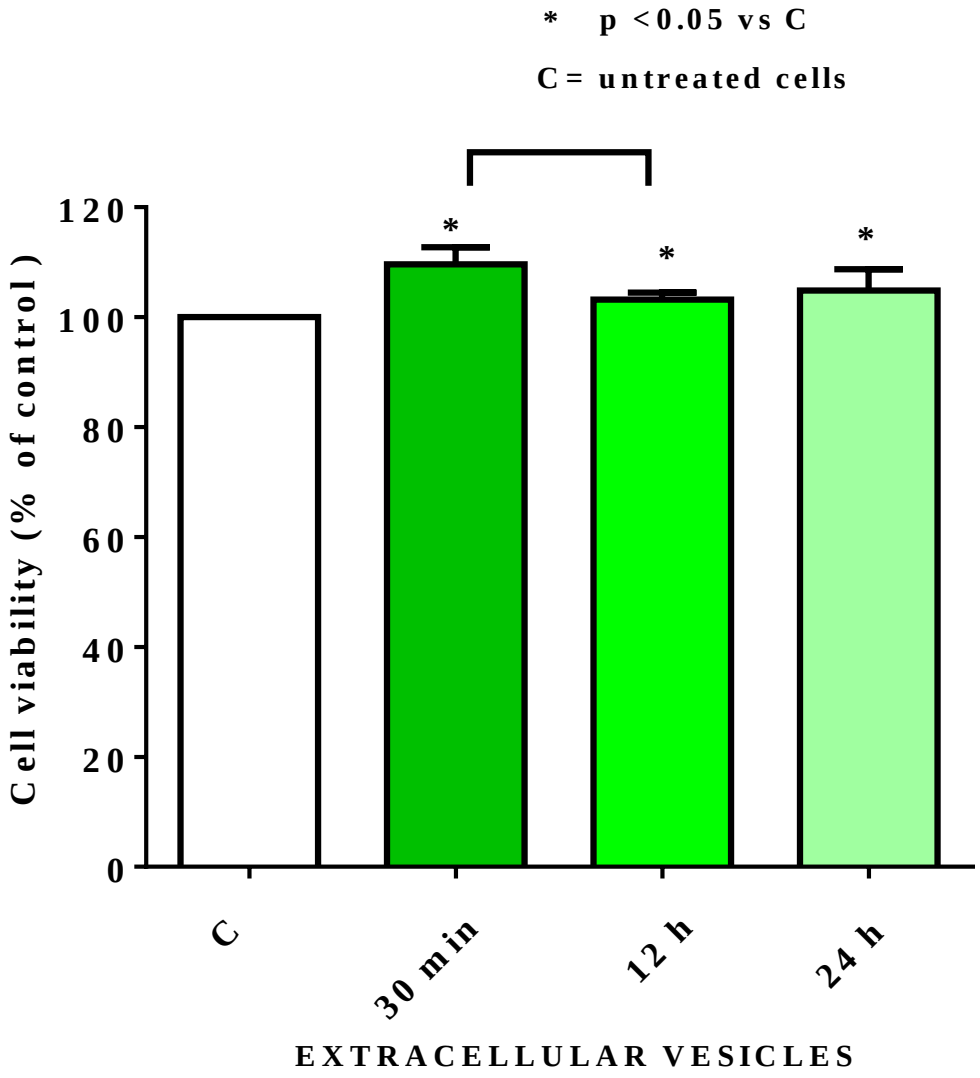


FIGURE 8. Time-course effects of orange EVs (50000 particles /cell) on cell viability of renal tubular cells. Reported data are means ± SD of repeated experiments. Square bracket indicates the significance ($p<0.05$) between the groups.

In the following experiments, we analyzed the time-dependent effects of stress factors on cell viability (FIGURE 9). Our goal was to determine the optimal exposure time for each stimulus that could induce the most significant effects on cell viability in

comparison to the control (untreated cells). As regarding hydrogen peroxide treatment, the 30-min exposure exhibited the highest impact on cell viability. Instead, 4 h stimulation with cytomix (50 ng/ml) and LPS (30 μM) caused the highest effects on cell viability. On the ground of the results obtained, we selected 30 min H202 (200 μM) and 4 h LPS and cytomix for the next experiments.

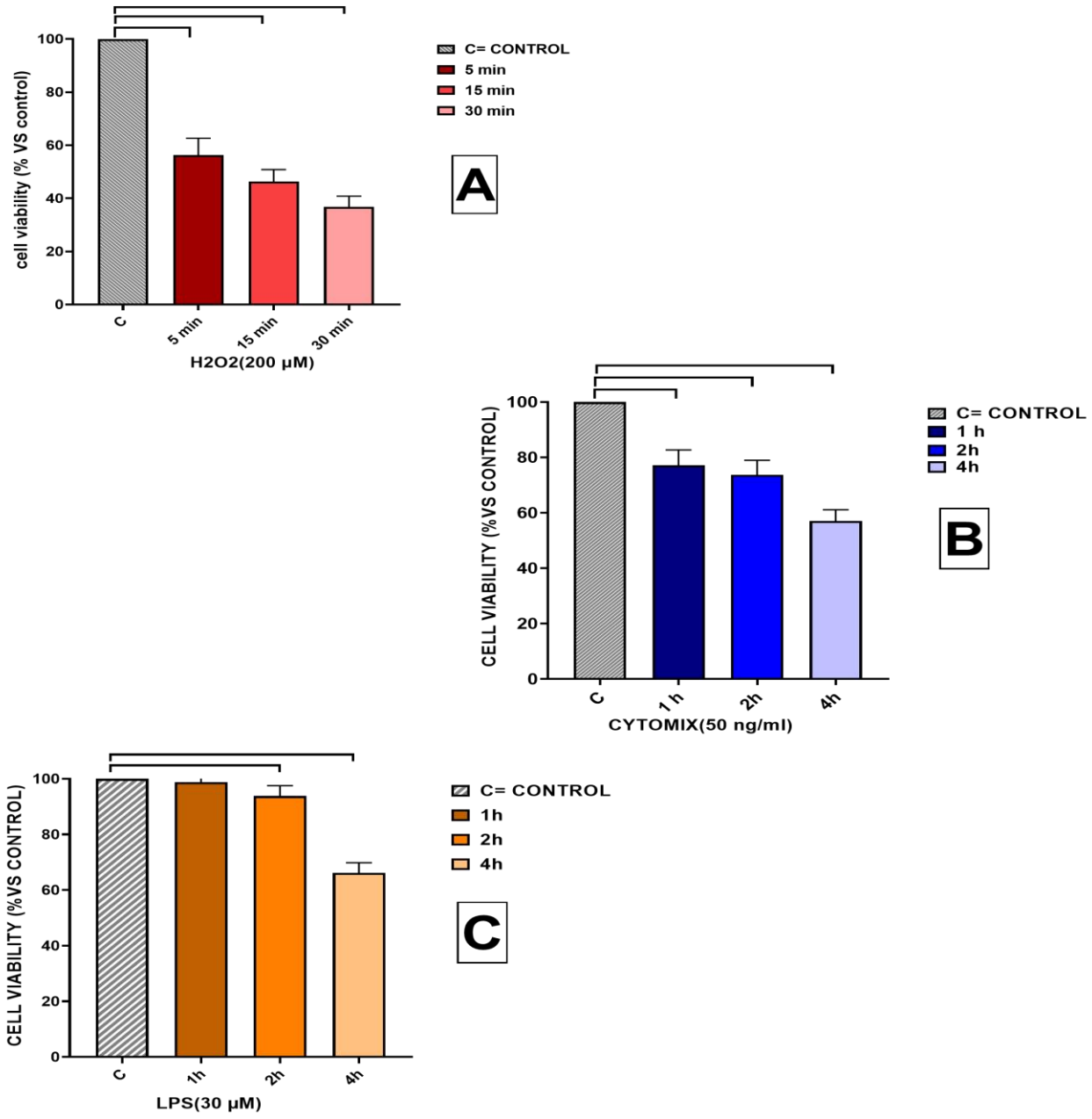


FIGURE 9. Time-course effects of stress factors. In A: hydrogen peroxide (200 μM), B: cytomix (50 ng/ml), C: lipopolysaccharide (LPS, 30μM) on cell viability of renal tubular cells. Reported data are means ± SD of repeated experiments. Square bracket indicates the significance ($p<0.05$) between the groups. C= untreated cells (control)

As shown in FIGURE 10, our study identified two dextran and chitosan coated OLN concentrations for cell viability, which were specifically 0.32 M and 0.00032 M. These two concentrations were the ones that caused, respectively, the greatest and lowest effect on cell viability. As regarding the timing, we found that with 24 h stimulation, the effects of dextran and chitosan coated OLN on cell viability were the highest.

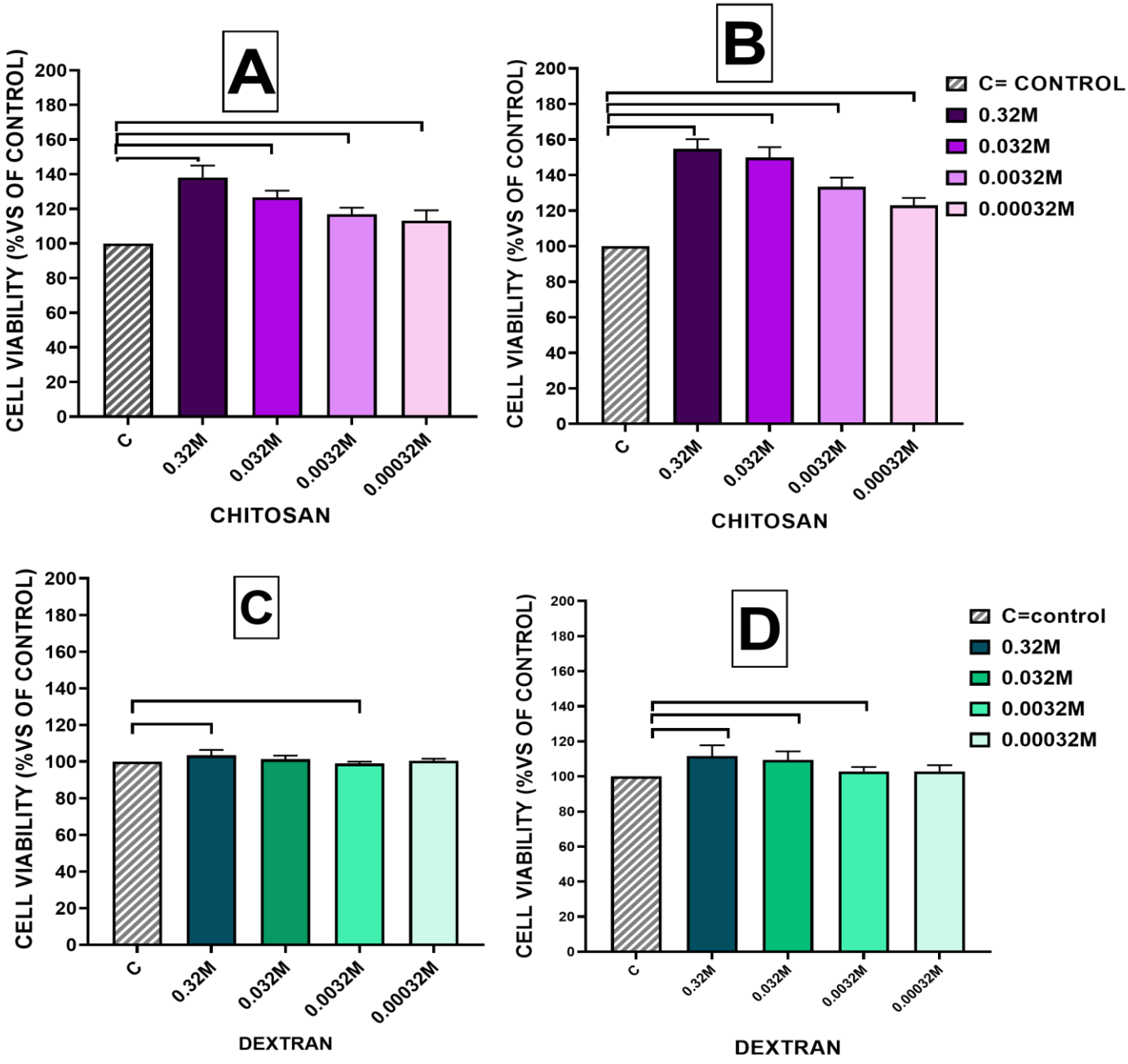


FIGURE 10. The effects of dextran and chitosan coated OLN in different concentrations and timing of stimulations on cell viability of renal tubular cells (A and C show 4 h and B and D show 24 h). Reported data are means ± SD of repeated experiments. Square bracket indicates the significance ($p<0.05$) between the groups. C= untreated cells (control).

As shown in FIGURE 11, only EVs alone were able to protect renal tubular cells against H202 when they were given either before or after it. Instead, dextran and chitosan coated OLN were able to protect renal tubular cells against H202 when they were given after it (FIGURE 11 B and D).

When we performed the co-stimulation experiments, we found that EVs were able to potentiate the effects of dextran and chitosan coated OLN, when they were both given before or after H202. However, the improvement of cell viability was higher when the protective agents were given in co-stimulation, after H202.

In addition, we found that dextran and chitosan coated OLN could potentiate the effects of EVs on cell viability when they were given either before or after H202 at both concentrations, except for dextran coated OLN, which could exert potentiation only at 0.00032 M, when it was given before H2O2.

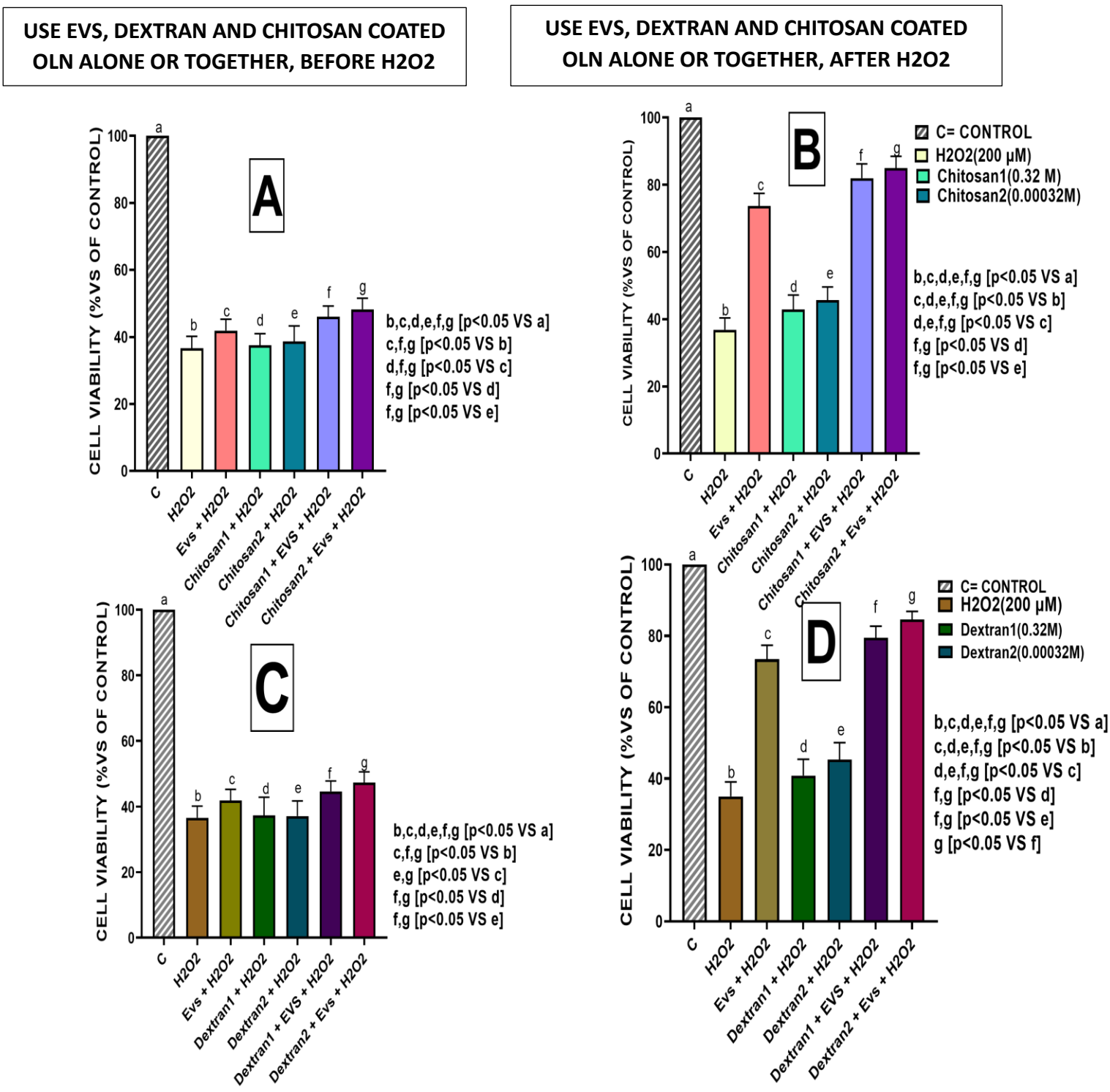


FIGURE 11. The effects of EVs, dextran and chitosan coated OLN, two concentrations, on cell viability of renal tubular cells before (A and C) and after (B and D) H2O2 stimulation. Reported data are means ± SD of repeated experiments. The significances ($p<0.05$) between the groups are reported in the figures. C= untreated cells (control).

As shown in FIGURE 12, EVs, dextran and chitosan coated OLN were able to protect renal tubular cells against cytomix, when they were given either before or after it.

When we performed the co-stimulation experiments, we found increased protective effects on cell viability exerted by dextran and chitosan coated OLN (0.32 M) plus EVs, in comparison with dextran and chitosan coated OLN alone, in the experiments performed by giving protective agents before and after cytomix (FIGURE 12). In case of the experiments performed with the protective agents given after cytomix, we found a potentiation of the effects exerted by dextran and chitosan coated OLN (both concentrations) plus EVs, in comparison with dextran and chitosan coated OLN alone (FIGURE 12).

Instead, we found that dextran and chitosan coated OLN could potentiate the effects of EVs on cell viability when they were given either before or after cytomix at both concentrations, except for dextran coated OLN, which could exert potentiation only at 0.32 M.

USE EVS, DEXTRAN AND CHITOSAN COATED OLN ALONE OR TOGETHER, BEFORE CYTOMIX

USE EVS, DEXTRAN AND CHITOSAN COATED OLN ALONE OR TOGETHER, AFTER CYTOMIX

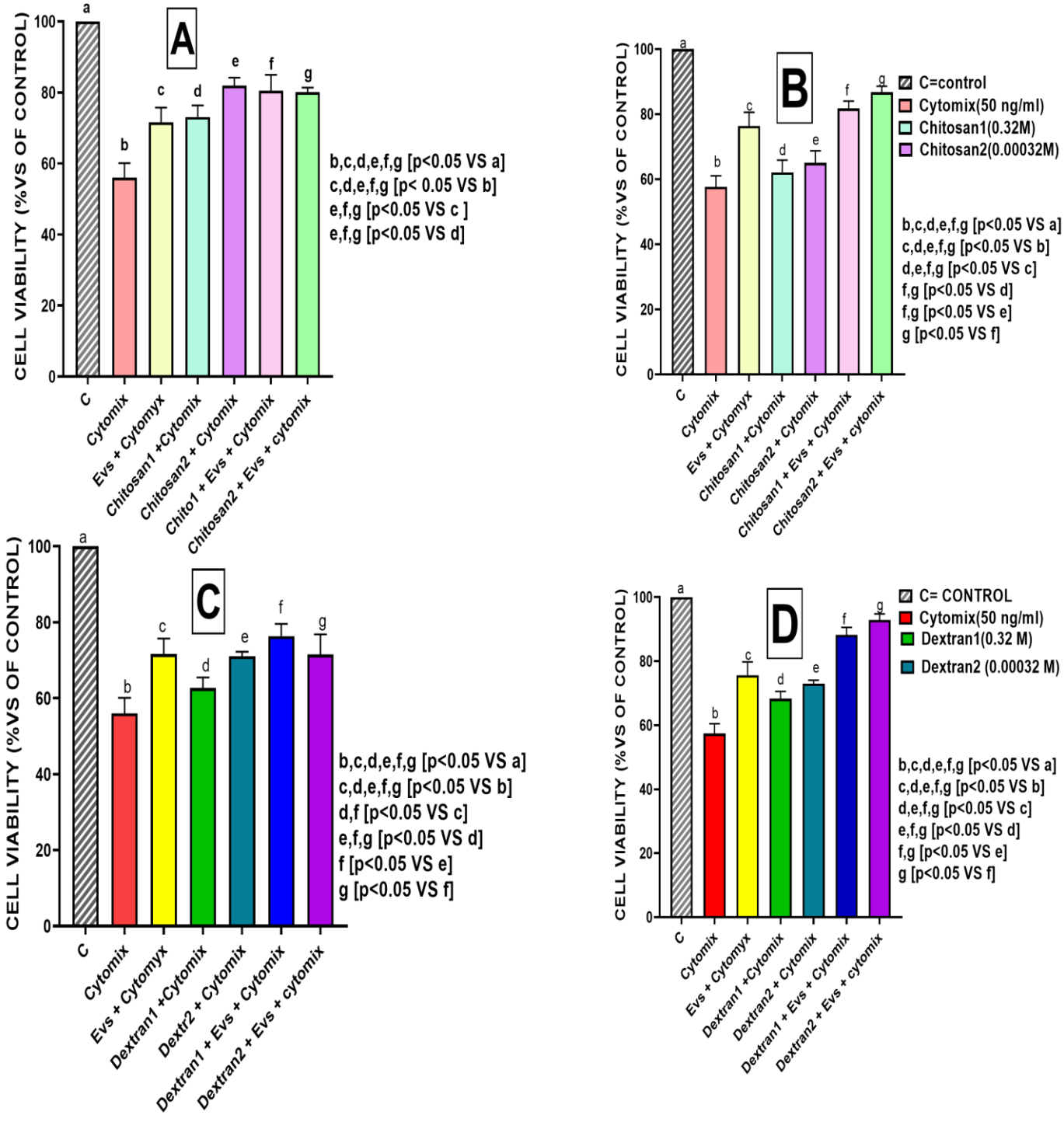


FIGURE 12. The effects of EVs and dextran and chitosan coated OLN two concentrations on cell viability of renal tubular cells, before (A and C) and after (B and D) cytomix stimulation. Reported data are means ± SD of repeated experiments. The significances ($p<0.05$) between the groups are reported in the figures. C= untreated cells (control).

As shown in FIGURE 13, EVs, dextran and chitosan coated OLN, both concentrations, were able to protect renal tubular cells against LPS, either when they were given before or after it.

When we performed the co-stimulation experiments, we found increased protective effects on cell viability exerted by dextran and chitosan coated OLN (both concentrations) plus EVs, in comparison with dextran and chitosan coated OLN alone, in the experiments performed by giving protective agents after LPS, only.

In the experiments performed by giving protective agents before LPS, 0.32 M chitosan coated OLN only, plus EVs, was able to potentiate the effects of chitosan coated OLN.

Instead, we found that dextran and chitosan coated OLN, both concentrations, could potentiate the effects of EVs on cell viability, when they were given after LPS (FIGURE 13). Instead, chitosan coated OLN only, both concentrations, could exert potentiation of EVs when the protective agents were given before LPS (FIGURE 13).

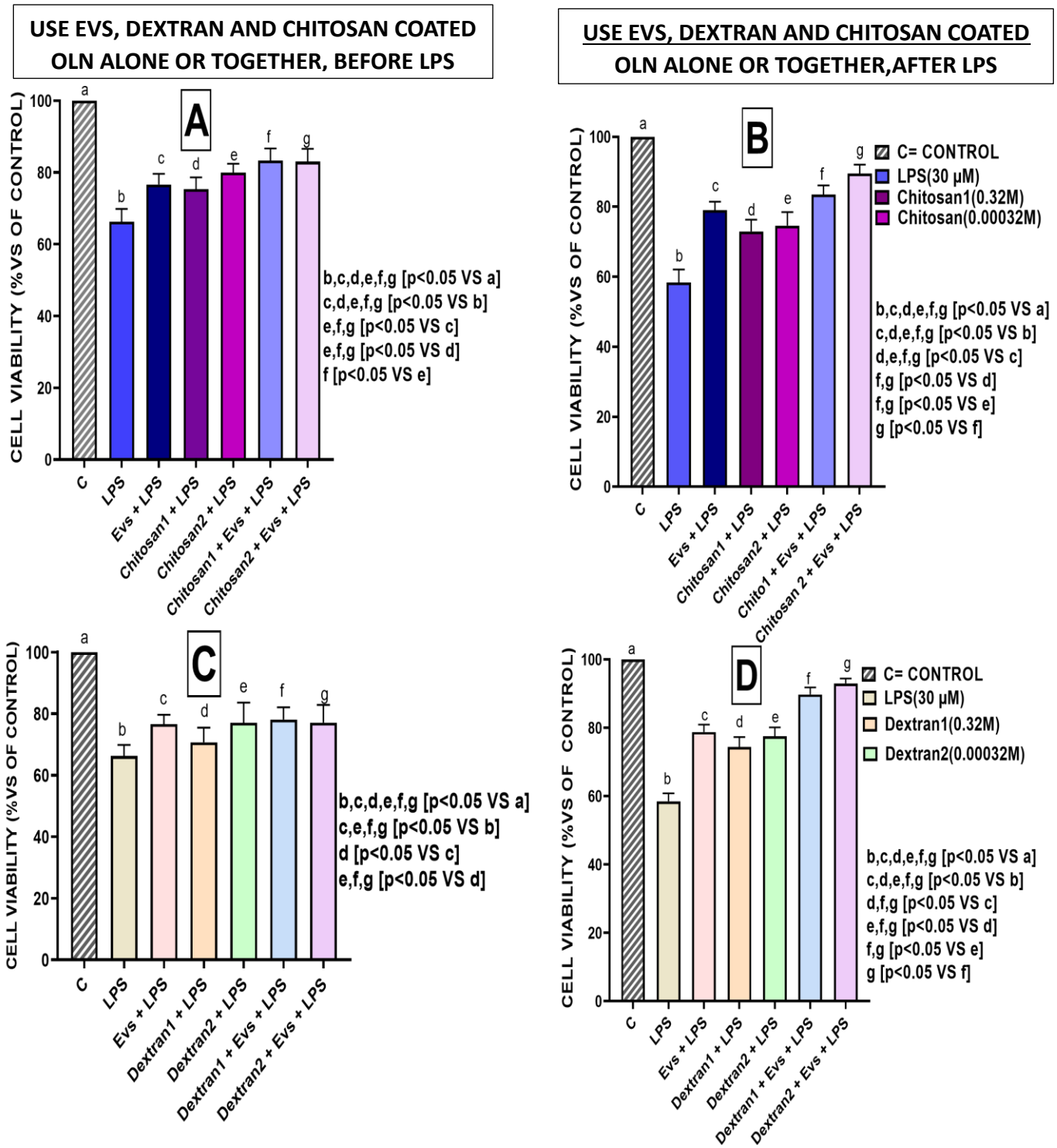


FIGURE 13. The effects of dextran and chitosan coated OLN two concentrations on cell viability of renal tubular cells, before (A and C) and after (B and D) LPS stimulation. Reported data are means ± SD of repeated experiments. The significances ($p<0.05$) between the groups are reported in the figures. C= untreated cells (control).

The results of the experiments performed by given EVs, dextran and chitosan coated OLN before or after H202 on ROS release are described in FIGURE 14.

As shown in FIGURE 14, EVs and dextran coated OLN (both concentrations) were always able to reduce ROS release caused by H2O2. Instead, chitosan coated OLN (both concentrations) could reduce ROS release when it was given after H2O2. In fact, only 0.000032 M chitosan coated OLN could exert the protective effects against H2O2 when it was given before it.

When we performed the co-stimulation experiments, we found increased protective effects on ROS release exerted by dextran and chitosan coated OLN (both concentrations) plus EVs, in comparison with dextran and chitosan coated OLN alone, in the experiments performed by giving protective agents after cytomix, only.

In the experiments performed by giving protective agents before cytomix, 0.32M chitosan coated OLN only plus EVs was able to potentiate the effects of chitosan coated OLN.

Instead, we found that dextran and chitosan coated OLN could potentiate the effects of EVs on cell viability, when they were given either before or after cytomix at both concentrations, except for dextran coated OLN, which could exert potentiation only at 0.32 M.

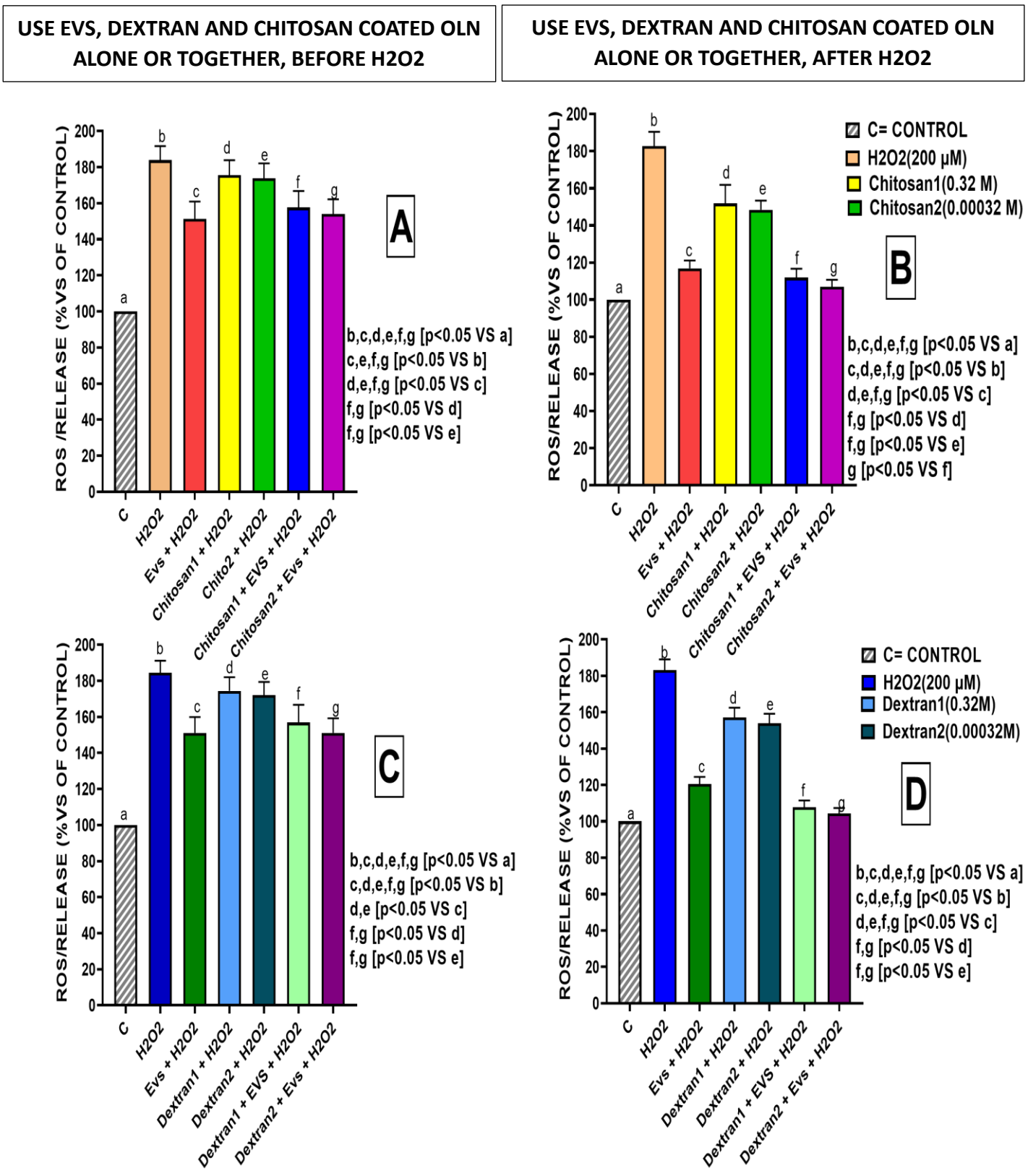


FIGURE14. ROS release by renal tubular cells treated with EVs and dextran and chitosan coated OLN two concentrations, before (A and C) and after (B and D) stimulation with H2O2. Reported data are means ± SD of repeated experiments. The significances ($p<0.05$) between the groups are reported in the figures. C= untreated cells (control).

The results of the experiments performed by given EVs, dextran and chitosan coated OLN before or after cytomix on ROS release by renal tubular cells are described in the FIGURE 15.

As shown in FIGURE 15, EVs were always able to reduce ROS release caused by cytomix. Instead, dextran and chitosan coated OLN (both concentrations) reduced the ROS release caused by cytomix when they were given after cytomix, only.

When we performed the co-stimulation experiments, we found increased protective effects on ROS release exerted by dextran coated OLN (both concentrations) plus EVs, in comparison with dextran coated OLN alone, in the experiments performed by giving protective agents before cytomix, only. As regarding chitosan coated OLN plus EVs, we observed a potentiation of the effects elicited by chitosan coated OLN (both concentrations), in the experiments performed by giving protective agents after cytomix, only (FIGURE 15). Differently, 0.000032 M chitosan coated OLN only, plus EVs, was able to increase the effects of chitosan coated OLN, when the protective agents were given before cytomix (FIGURE 15).

Instead, we found that dextran and chitosan coated OLN could never potentiate the effects of EVs on ROS release (FIGURE 15).

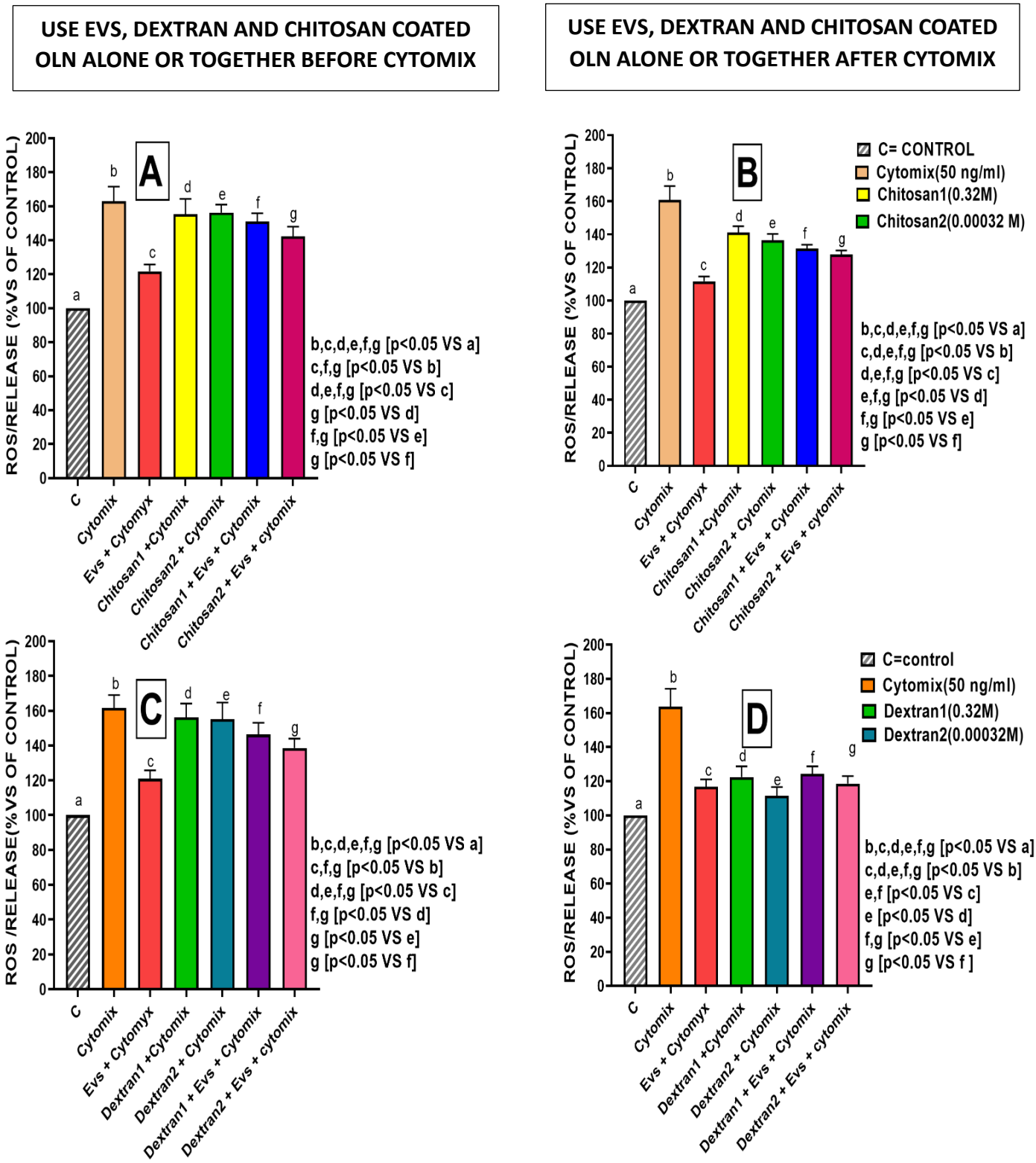


FIGURE 15. ROS release by renal tubular cells treated with EVs and dextran and chitosan coated OLN two concentrations, before (A and C) and after (B and D)) stimulation with cytomix. Reported data are means ± SD of repeated experiments. The significances ($p<0.05$) between the groups are reported in the figures. C= untreated cells (control).

The results of the experiments performed by given EVs and dextran and chitosan coated OLN before or after LPS on ROS release by renal tubular cells are described in the FIGURE 16.

As shown in FIGURE 16, EVs and dextran and chitosan coated OLN at both concentrations, were always able to reduce ROS release caused by LPS.

When we performed the co-stimulation experiments, we found increased protective effects on ROS release exerted by dextran coated OLN (both concentrations) plus EVs, in comparison with dextran coated OLN alone, in the experiments performed by giving protective agents after LPS, only (FIGURE 16).

Instead, we found that dextran and chitosan coated OLN could never potentiate the effects of EVs on ROS release (FIGURE 16).

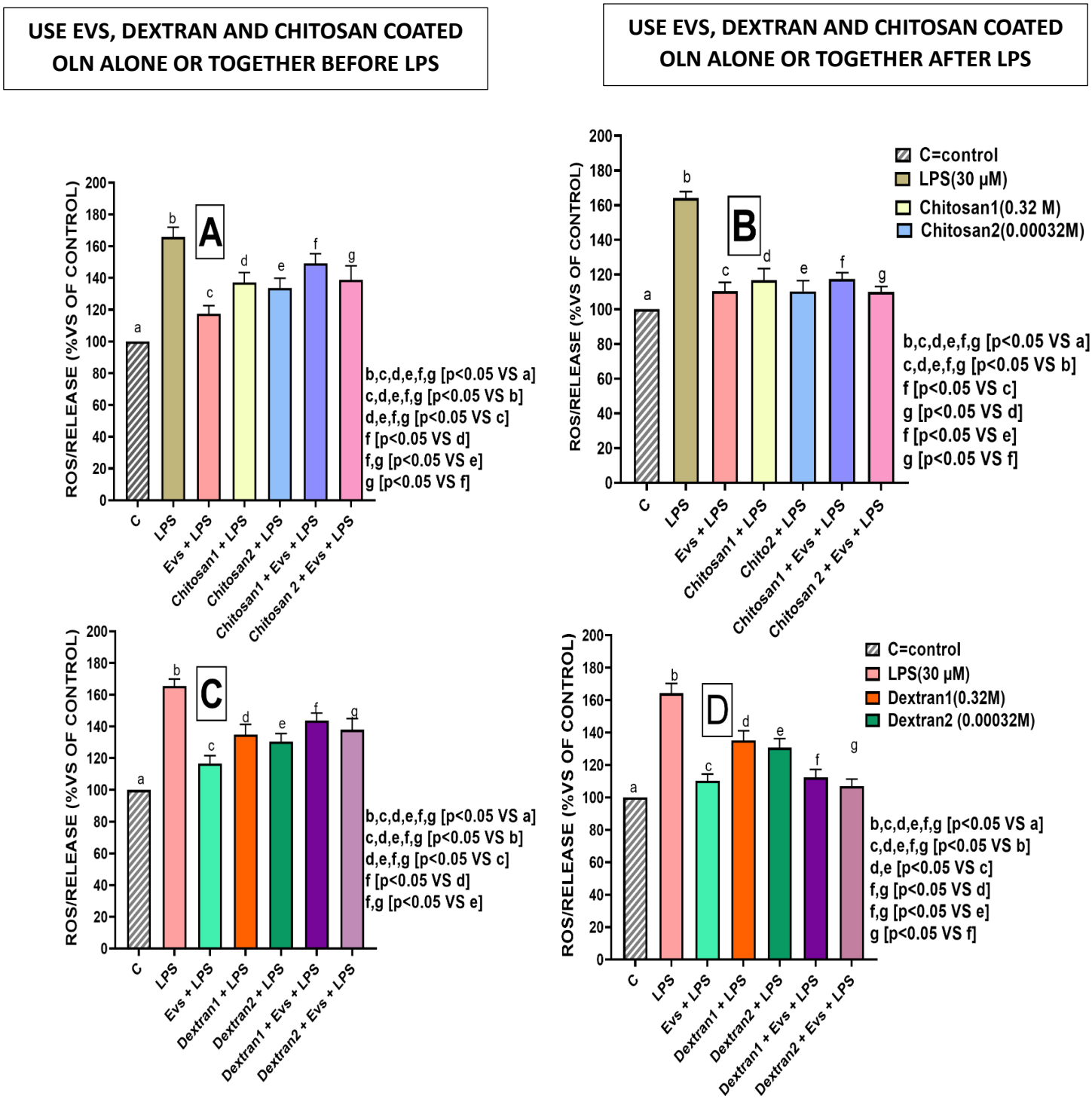


FIGURE 16. ROS release by renal tubular cells treated with EVs and dextran and chitosan coated OLN two concentrations, before (A and C) and after (B and D) stimulation with LPS. Reported data are means ± SD of repeated experiments. The significances ($p<0.05$) between the groups are reported in the figures. C= untreated cells (control).

DISCUSSION

Plant-based EVs are small membrane-bound structures released by plant cells into the extracellular space. These vesicles contain bioactive substances such as nucleic acids, proteins, and metabolic wastes, and have gained attention due to their potential therapeutic applications.[6] Studies have shown that plant-derived EVs, including orange EVs, have good biocompatibility, high rates of cellular internalization, and serve as a useful source of bioactive chemicals with potential nutraceutical and therapeutic uses. These vesicles play a crucial role in intercellular communication and have been found to mediate specific cellular responses and intercellular communication. [7]

Oxidative stress refers to an imbalance between the production of ROS and the ability of the body to detoxify these reactive intermediates or to repair the resulting damage. In the context of renal diseases, oxidative stress plays a significant role in the development and progression of conditions such as CKD, diabetic nephropathy, AKI, and renal fibrosis. It is implicated in the damage to renal cells and tissues, leading to impaired renal function.

Experimental studies have explored therapeutic strategies targeting oxidative stress pathways in kidney disease, including the use of antioxidant compounds, pro-oxidant enzyme inhibitors, and modulation of redox-sensitive signaling pathways to attenuate renal injury and preserve kidney function. Additionally, the detrimental effects of oxidative stress on kidney transplantation outcomes have led to a growing interest in developing therapeutic strategies to mitigate its impact.

Aim of this study was to examine potential protective effects against renal dysfunction caused by inflammation and oxidative stress provided by orange EVs, dextran and chitosan coated OLN, when given alone or in co-stimulation.

Dextran is a polysaccharide composed of glucose units. It has excellent biocompatibility and is water-soluble, making it suitable for various biomedical applications. In oxygen therapy, dextran-based carriers can be utilized to enhance oxygen solubility and transport.[76, 77]

Chitosan is a biocompatible and biodegradable polysaccharide derived from chitin, a compound found in the shells of crustaceans. Chitosan-based nanoparticles have been investigated for oxygen delivery. These nanoparticles can be loaded with oxygen molecules and targeted to specific sites, such as ischemic tissues or regions with low oxygen levels. By delivering oxygen directly to these areas, chitosan nanoparticles can help improve tissue oxygenation and support healing. Both dextran and chitosan offer

advantages in terms of biocompatibility, biodegradability, and oxygen-carrying capabilities. [83]

In particular, we evaluated the effects of EVs alone or in co-stimulation with dextran and chitosan coated OLN on cell viability and ROS release in renal tubular cells.
Firstly, we performed preliminary experiments on cell viability, in order to identify the optimal incubation times for orange EVs and to identify the optimal concentrations and incubation time for dextran and chitosan coated OLN. Instead, we used EVs at 50000/ml because we did not have data about their concentration; here we used the same EVs concentration adopted in previous experiments performed in HUVEC [85, 86].

In addition, preliminary experiments on cell viability were performed in order to identify the optimal incubation times for H202, cytomix, LPS. Cell viability refers to the ability of cells to survive and proliferate under specific conditions. In the context of the study, the assessment of cell viability is crucial for understanding the protective effects of EVs, dextran and chitosan coated OLN on renal tubular cells under stress conditions, such as inflammation and oxidative stress which can be mimed by hydrogen peroxide, cytomix ([tumor necrosis factor (TNF)-α + interferon (IFN)-γ + interleukin (IL)-1β]) and LPS. Those agents are widely used in various medical scenarios and experimental settings for mimicking inflammation and peroxidation.[98-102]

As regarding OLN, they are specifically designed to carry and deliver oxygen molecules to target tissues or organs, enhancing oxygen delivery to specific sites in the body where traditional methods of oxygenation may not be sufficient or feasible. Additionally, chitosan-based nanoparticles have been investigated for oxygen delivery and have shown potential in improving tissue oxygenation and supporting healing. Furthermore, dextran and chitosan offer advantages in terms of biocompatibility, biodegradability, and oxygen-carrying capabilities, making them promising for oxygen therapy and tissue engineering applications. These findings are supported by research studies such as those by Jiang et al. (2013) and Abd El-Hameed et al. (2020), which have explored the use of nanoparticles for oral chemotherapy and ameliorating diabetic nephropathy, respectively. [86-88, 91, 102, 1, 3].

Thereafter, we used the selected concentrations and timings of EVs, dextran and chitosan coated OLN to do the extended phase, which was executed not only on cell viability but also on ROS release.

The results we obtained show that EVs, dextran and chitosan coated OLN alone and co-stimulated improve cell viability and are able to counteract the effects of stress-inducing factors, in particular when they are co-stimulated.

Hence, our data highlighted the potential of EVs and dextran and chitosan coated OLN in reducing ROS release when co-stimulated with stress-inducing factors. Specifically, the study found that EVs plus dextran coated OLN, both concentrations, were able to increase the effects of dextran coated OLN alone on ROS release caused by hydrogen peroxide, cytomix and LPS. Instead, EVs plus chitosan coated OLN, both concentrations, were able to potentiate the effects of chitosan coated OLN alone on ROS release induced by cytomix, only.

It is also to note that, chitosan and dextran coated OLN were, in general, able to potentiate the protective effects elicited by EVs on cell viability. As regarding ROS release, however, the potentiation exerted by chitosan and dextran coated OLN on the effects of EVs could be observed when hydrogen peroxide, only, was used.

Our findings align with previous research, such as the work of Li et al.[103] , which reported that chitosan nanoparticles can effectively scavenge ROS and protect against oxidative stress-induced cell damage

Also, our findings align with the work of Zhang et al., which demonstrated that plant-derived EVs can enhance cell viability and reduce inflammation in a model of renal injury. [104]

Furthermore, our findings align with the work of Li et al., which reported that chitosan nanoparticles can effectively scavenge ROS and protect against oxidative stress-induced cell damage. [103]

Also Abd El-Hameed et al. [81] was able to demonstrate that polydatin-loaded chitosan nanoparticles ameliorates early diabetic nephropathy by attenuating oxidative stress and inflammatory responses in diabetic rats. Thus, those findings align with the protective effects of chitosan observed in the study, indicating its potential in addressing oxidative stress-related conditions. Additionally, Cavalli et al.[82] explored ultrasound-mediated oxygen delivery from chitosan nanobubbles, highlighting the potential of chitosan-based nanoparticles in delivering therapeutic agents and mitigating oxidative stress.

Moreover, our data on the protective effects of OLN are consistent with the work of Zeng et al. [105], which investigated a drug-free nanozyme for mitigating oxidative stress and inflammatory bowel disease.

The study's findings underscore the importance of addressing oxidative stress in the management of kidney diseases and provide insights into innovative therapeutic strategies for renal dysfunction. Numerous research papers have provided insights into the mechanisms by which oxidative stress contributes to renal injury, and studies have investigated the potential of antioxidant strategies and therapeutic targeting of oxidative stress pathways as promising approaches to mitigate kidney damage and improve renal function [1]. References supporting these findings include Wallace et al [104]Cachofeiro et al. [106], Forbes et al. [107], Mennuni et al. [108], Hosohata et al. [109], Tomsa et al. [110], Heyman et al. [111], Gorin et al. [112], Zeng et al. [113], Sugimoto et al. [114] and Wang et al. [103].

Our findings, which highlight protective effects exerted by orange EVs alone and in co-stimulation with chitosan and dextran OLN against induced renal tubular cells damage, hold significant potential for therapeutic applications in the treatment of renal dysfunction.

In particular, the study suggests that it is better to give protective agents after stressful conditions, as they are more effective in mitigating kidney injury caused by hydrogen peroxide, cytomix and LPS. This finding has important clinical implications, as it indicates the potential for these substances to counteract the effects of stress-inducing factors and provide therapeutic benefits in the context of renal dysfunction.

Once the safety and efficacy of these substances are established in preclinical studies, clinical trials could be conducted to evaluate their therapeutic potential in humans, providing a pathway for the development of innovative therapies for renal disorders.

LIMITATIONS AND FUTURE PERSPECTIVES

This study provides a novel perspective on the potential therapeutic applications of plant-based EVs and dextran and chitosan coated OLN in mitigating renal dysfunction. The research contributes to the understanding of how these substances can enhance cell viability, reduce inflammation, and protect against oxidative stress-induced cell damage. This opens up new avenues for the development of innovative treatments for renal diseases.

Despite the promising results, there are several limitations to consider.

In this study we used the same EVs concentration that was used previously in a study about HCV and about subarachnoid hemorrhage [85, 86]. Nanosight analysis and the use of MACSPLEX could increase knowledge about the size/concentrations of EVs and their cellular origin.

The experiments performed in this study were executed *in vitro*, only, by using one cell line. The use of other cell lines, such as podocytes and glomerular endothelial cells, the increase of assays (mitochondrial function, cell cycle, apoptosis) and the analysis of intracellular pathways, could increase knowledge about the issue. In addition, while the cellular models provide valuable insights, they may not fully replicate the complex physiological environment in a living organism. Conducting *in vivo* studies using animal models could provide a more realistic assessment of the therapeutic potential of plant-based EVs and dextran and chitosan coated OLN. This could help validating the findings from *in vitro* studies and provide more insights into the mechanisms of action of these substances in a living organism. In addition, long-term animal studies would be useful to evaluate their safety and efficacy over time.

Once the safety and efficacy of these substances are established in preclinical studies, clinical trials could be conducted to evaluate their therapeutic potential in humans. This could help determining the optimal dosage, assess potential side effects, and evaluate the overall effectiveness of these substances in treating renal dysfunction.

Future studies could also explore the potential synergistic effects of these substances in combination with other therapeutic agents. This could potentially enhance their effectiveness and provide more comprehensive treatment options for renal dysfunction.

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