‘Administration of Fluoxetine incorporated Liposomal Nanoparticles in Repeated Social Defeat Stress Animal Model for the treatment of Depression’

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I certify that this research work titled *'Administration of Fluoxetine incorporated Liposomal Nanoparticles in Repeated Social Defeat Stress Animal Model for the treatment of Depression'* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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Acknowledgments

In the name of Allah, the Beneficent, the Merciful

First, praise is to Allah, the Almighty, on whom ultimately we depend for sustenance and guidance.

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List of Abbreviations

BBB	Blood Brain Barrier
DPPC	Dipalmitoylphosphatidylcholine
EE	Encapsulation Efficiency
FST	Forced Swim Test
NPs	Nanoparticles
OFT	Open Field Test
RSDS	Repeated Social Defeat Stress
TST	Tail Suspension Test
WHO	World Health Organization

Abstract

Depression is categorized as one of the most prevalent psychological mood disorder affecting more than 350 million people worldwide. Headache, shortness of breath, stomach disturbances and general physical tension are the common symptoms of depression as reported by the National Alliance of Mental Health. This ailment needs to be addressed with utmost attention as it leads to loss of work productivity thus resulting in a tremendous economic burden. Antidepressants like Fluoxetine work by altering chemicals called Neurotransmitters namely serotonin, dopamine and norepinephrine that are primarily involved in regulating and alleviating mood. In spite of the availability of large number of drugs, majority of patients are resistant to the current modes of treatment. The blood-brain barrier (BBB) poses a significant obstacle for the transportation of beneficial therapeutic entities to the nervous system. The tight junctions that are present within the endothelial cells of the blood-brain barrier restrict the passage of drugs. In recent years, nanoparticles are receiving significant limelight owing to their small size and efficient brain targeting activity, making them highly suitable to cross the BBB while carrying the drug molecules intact which were otherwise incapable of permeation. In this study, Fluoxetine loaded liposomal nanoparticles were developed to transport the drug across the BBB to the central nervous system with much greater efficiency. For testing the drug-delivery efficiency, mice model of depression namely ‘ Repeated social defeat stress’ was designed in order to induce depression like symptoms in mice and liposomes carrying entrapped antidepressant drug molecules were introduced via intravenous route of administration thereby rendering drug loaded nanoparticles to be a better and improved treatment method.

Chapter 1

1. Introduction

1.1. Depression

Major depressive disorder (MDD) or depression is a widespread debilitating psychological mood disorder affecting approximately 300 million people worldwide that is roughly equivalent to 4.4% of the global population. It is estimated to have a lifetime prevalence of between 10 and 20% with suicide being a major risk which numbers to about 800 000 suicides per year. Depression is often comorbid with a range of anxiety disorders making it the third leading cause of disease burden thereby exerting a significant socioeconomic burden globally (Organization, 2017). It is characterized by feeling of despair, guilt, low self-esteem, negative evaluation of events, loss of appetite leading to weight loss, inability to focus and concentrate and sleep disturbances. Depression is one of the common mental health disorder that does not have a specific criterion of symptoms to be recognized but negatively affects a persons' mood, way of thinking and behavior. According to WHO symptoms of depression can be described as sadness, loss of interest in life, being miserable, or suicidal thoughts.

1.2. Depression Statistics in the U.S

Depression is the most prevalent mental illness affecting people in the US. In 2017, approximately 17.3 million adults that are of 18 years of age or above suffered from no less than one episode of major depressive disorder which represent 7.1% of all American adults. It is reported to have high prevalence among females (8.7%) as compared to a prevalence of 5.3% in males. The prevalence of depression in adults within the age group of 18-25 was found to be highest (13.1%) compared to other age groups (Health, 2019).

1.3. Depression Statistics in Pakistan

There is a very high prevalence of major depressive disorder among the developing countries. The prominent culprits are poverty, illiteracy, unemployment, homelessness, lack of mental health awareness and low mental health literacy. People tend to stick to traditional methods of treatment

rather than recognizing the severity of this ailment and seeking proper healthcare guidance. More than 20 million Pakistanis suffer from major depressive disorders that accounts for almost 10% of the total population (Nisar et al., 2019). Moreover, Pakistan has a remarkably low psychiatrist ratio compared to the number of affected people in the world. As claimed by a report of the WHO, in Pakistan only 400 psychiatrists and an astonishingly low number of five psychiatric hospitals exist to cater a vast population that is exceeding 180 million people (Organization, 2009).

1.4. Pathophysiology of Depression

It is well known that transmission of nerve impulses throughout the body involve a series of highly regulated pre and post synaptic events that form the basis of plasticity, memory and learning in the complex central nervous system. The nervous transmission begins with the synthesis of chemical messengers called the neurotransmitters that are then stored in the secretory vesicles which are then released when required into the synaptic cleft followed by fusion with the post synaptic neuron and transmitting the signal to the next neuron. However, this process does not end here. As mentioned earlier, signal transduction being a well-regulated process prevents the under-development or over-accumulation of these neurotransmitters in the area surrounding the synapse and hence a feedback mechanism comes into action to prevent such events that would otherwise lead to dysfunctioning of the CNS. A similar kind of dysfunctional behavior occurs in case of depression as explained by the Monoamine hypothesis that is the most accepted theory explaining the pathophysiology of major depressive disorder.

1.4.1. The Monoamine Hypothesis

The monoamine hypothesis is the most widely accepted hypothesis that was developed about 30 years ago to explain the mechanism by which depression results. This hypothesis suggests that depression develops from the lessened availability of the monoamine neurotransmitters namely serotonin or 5-HT, dopamine and norepinephrine as explained in figure 1. The deficiency of these neurotransmitters results from the degrading activity of the monoamine oxidases enzymes in the synaptic cleft which are found in high concentration in the depressed individuals. Continuous actions of these oxidases lead to a significantly low availability of the biogenic amines that in turn decrease the neurotransmission ultimately leading to depression (Jesulola et al., 2018).

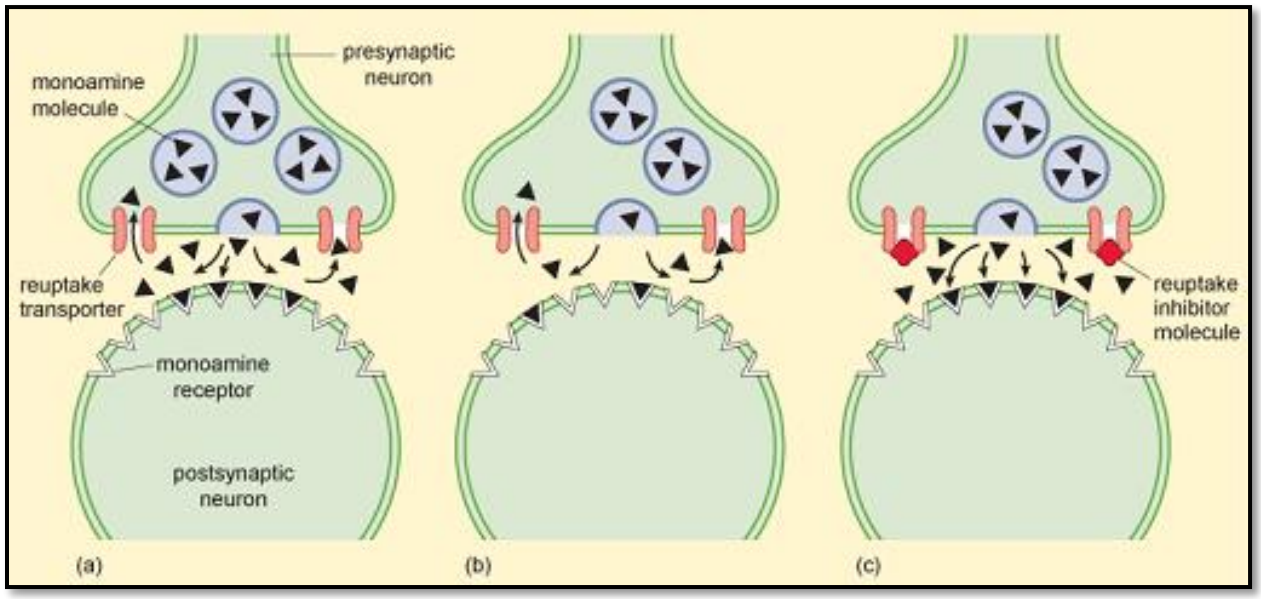

Figure 1: Diagrammatic representation of mechanism of depression. (a) normal neurotransmission of monoamine neurotransmitters; (b) in case of depression, fewer monoamine neurotransmitters are available to bind to the post-synaptic neuron as they are taken up by the reuptake transporters; (c) treatment with reuptake inhibitors increases availability of the neurotransmitters thereby regulating the normal neurotransmission.

1.5. Symptoms of Depression

Depression affects people from all walks of life belonging to all age groups. For a person to be diagnosed as a depressed individual, a minimum of five prominent stress symptoms should be present and among them feeling extremely sad or showing an excessive lack of interest or pleasure in the activities that were normally pleasing should be observed. The other distinguished and noteworthy symptoms that are linked with depression comprise of anxiety, excessive and inappropriate guilt, restlessness, changes in appetite, sleep related issues on almost daily basis such as difficulty sleeping or sleeping way too much), retardation, decreased energy and prolonged fatigue, feelings of worthlessness, recurring thoughts of death and suicidal thinking, difficulty in anger management, diminished ability to think, concentrate and make decisions. These symptoms may persist for a period of weeks or way longer and result in a significant change in a person's

behavior. Social, professional, educational and other important everyday functioning is negatively impacted. All these events result from low self-esteem, physical or sexual abuse, alcohol or drug use, loss of a friend or loved one, as a side-effect of medication or a family history of depression (ADAA, 2019).

1.6. Treatment of Depression

The precise mechanism by which depression develops and its pathophysiology is still not entirely understood owing to the heterogeneous and complex nature of this disease. It results from alteration in the signaling of chemical messengers called neurotransmitters in the brain. Several treatment regimens are available to cure this disorder depending upon its severity. Healthcare providers offer psychological treatments such as cognitive behavioral therapy or behavioral activation or in majority of cases antidepressant suppositories such as selective serotonin reuptake inhibitors (SSRIs), Monoamine oxidase inhibitors (MAOIs) and tricyclic antidepressants (TCSs). Antidepressants work by altering the signaling of mood-associated neurotransmitters namely dopamine, norepinephrine and serotonin in the central nervous system thereby alleviating our mood and making us feel better.

1.7. Antidepressants

Most currently available antidepressants work by targeting the monoamine neurotransmitters. They work by increasing the concentration of these neurotransmitters in the area surrounding the synaptic cleft and regulate the normal process of neurotransmission. Several classes of antidepressant drugs are now available each having their own mechanism of action. The tricyclic antidepressants (TCAs) such as amitriptyline prescribed to treat severe melancholic depression is a effective inhibitor of norepinephrine and serotonin in adrenergic and serotonergic neurons. The Selective serotonin reuptake inhibitors (SSRIs) are the most excessively prescribed treatment medications for major depressive disorders work by increasing the availability of serotonin in the synaptic region by blocking the serotonin reuptake transporter. Mirtazapine, a novel antidepressant blocks the α-2 adrenoceptors present on the noradrenergic neurons as well as 5-HT_{2A} and 5-HT_{2C} receptors thereby increasing the norepinephrine and dopamine release in cortical regions (Harmer et al., 2017).

1.8. Blood Brain Barrier (BBB) – the major obstacle

The brain and central nervous system is protected by a complex system of barriers that shield it from the surrounding environment. The blood brain barrier consists of a continuous endothelial membrane that carefully controls passage of substances to and fro the CNS. The striking morphological features that significantly contribute to the selective permeation of the BBB are tight junctions present in the endothelial cells and their surrounding processes of the glial cell. Chemicals substances that circulate in the blood are bound to pass through the capillaries endothelial cell membrane as well as the glial cell membrane in order to gain entrance to the cerebrospinal fluid cushioning the brain (Sweeney et al., 2018).

In spite of the availability of vast variety of antidepressant drugs, a segment of patients are resistant to the conventional mode of treatment. Moreover, currently available drugs exhibit considerable adverse side effects having delayed onset of action resulting in poor patient compliance. One of the reason is the selective permeation capacity of the blood brain barrier (BBB). It is made up of continuous non fenestrated vessels that carefully regulate passage of ions, chemicals and cells to and from the membrane thus regulating homeostasis resulting in protection of the CNS from pathogens, toxins, injury and disease. This restraining nature of the BBB poses to be a major obstacle for drug delivery to the CNS (Daneman and Prat, 2015).

1.9. Nanotechnology

In the past two decades, rapid advancements in the field of nanotechnology particularly nanomedicine have offered tremendous opportunities in developing novel treatment strategies for several neurological and psychiatric disorders through the production of fine nano-sized particles. Nanoparticles are considered as versatile and auspicious drug delivery systems to transport drugs into regions that are normally inaccessible like the brain, thereby possessing the ability to enable safe transport therapeutic agents however simultaneously delivering them to the targeted impaired areas. Moreover, nanoparticles can be modified to have a large surface area and multiple affinity sites enabling them to entrap large quantities of useful drugs along with site specific delivery (Dimitrijevic and Pantic, 2014). Nano formulations are well designated as viable treatment methods to enhance site specific targeting particularly across the BBB, among them liposomal nanoparticles are popular and safe. Liposomes are small micro or nano sized vesicles comprised

of one or more concentric bilayers made up of phospholipids that are separated by the aqueous compartments. Owing to their inimitable physicochemical properties, liposomes possess the ability to incorporate a variety of hydrophobic, lipophilic as well as hydrophilic therapeutic agents making them an attractive delivery vehicle (Vieira and Gamarra, 2016).

1.10. Animal Models of Depression

By definition, animals are used as model organisms to study the underlying mechanisms of development of a disease and its progression and to test the efficacy and effectiveness of a new treatment regime before directly applying to the humans (Institute, 2019). For decades humans have used animal model based studies owing to the remarkable physiological and anatomical similarities between animals and humans. Animal models of depression serve as a valuable tool to investigate depression like phenotype in the animals and to study the mechanism of antidepressants.

It is difficult to develop an animal model that entirely depicts the symptomatology of depression in humans as they lack self-consciousness, consideration and self-determination. However, depression comprises of certain endophenotypes that can be independently reproduced and investigated in the animals. For instance behavioral despair in mice can be assessed by the forced swim test as well as the open field test. Similarly anhedonia in rodents can be evaluated by the sucrose preference test.

1.11. Rationale and Objectives

In this study, Fluoxetine incorporated liposomal nanoparticles were developed for the treatment of depression. The proposed structure of nanoparticles can be seen in figure 2. Depression was induced in animals following the 'Repeated Social Defeat Stress RSDS' mice model of depression. The aim of the present study was to develop an alternative treatment method that is comparatively more effective with fewer side effects and a rapid onset of action.

Chapter 2

2. Literature Review

2.1. Fluoxetine Hydrochloride (Prozac)

Prozac or Fluoxetine hydrochloride is the most extensively prescribed antidepressant drug that is used for the treatment of major depressive disorder. It works by altering the concentration of certain chemicals in the brain that are maybe unbalanced in patients suffering from depression, anxiety, panic disorders and obsessive-compulsive diseases. Fluoxetine Hydrochloride is the generic name of this antidepressant whereas the common brand names include Prozac, Sarafem, Foxetin, Lovan, Fluctin, Fludac, Fontex, Ladose and Prodep. Several studies have revealed that fluoxetine hydrochloride possesses significant anti-inflammatory, antioxidant and anti-apoptotic properties (Caiaffo et al., 2016). The molecular formula of the drug is 3-(p-trifluoromethylphenoxy)-N-methyl-3-phenylpropylamine HCl. This drug was introduced by Eli Lilly in 1972 and was used for medicinal purposes in 1986. Figure 2 shows the two dimensional structure and three dimensional conformer of the drug fluoxetine hydrochloride.

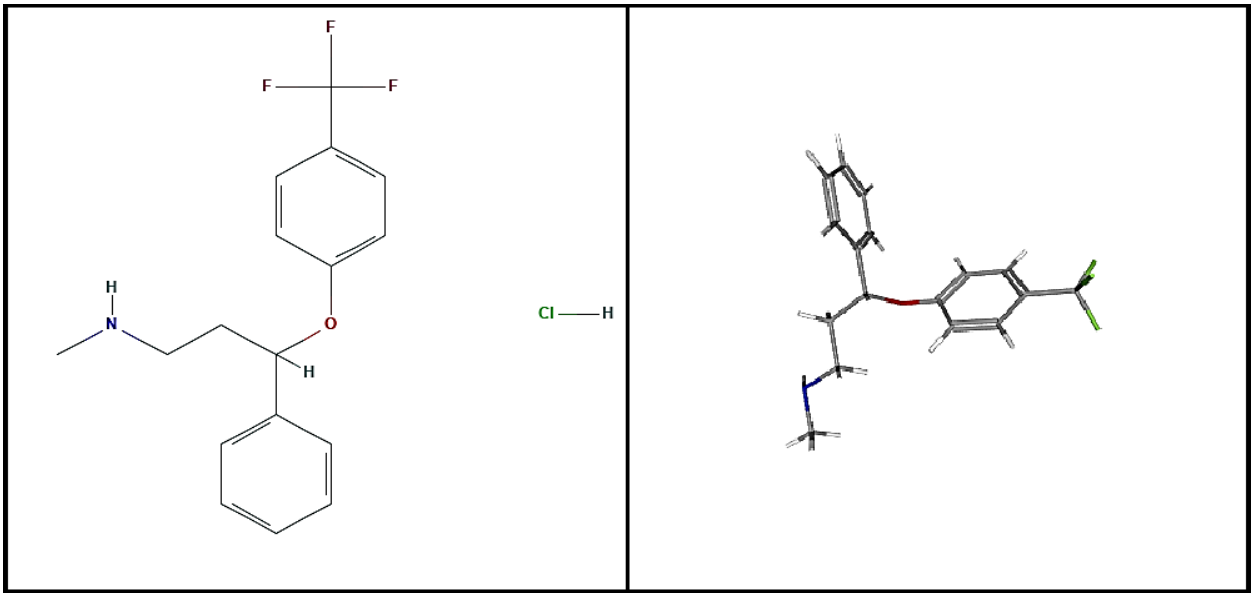

Figure 2: Adapted from (Information). 2D structure and 3D conformer of Fluoxetine Hydrochloride.

2.1.1. Drug Mechanism of Action

Fluoxetine belong to the class of drugs identified as 'selective serotonin reuptake inhibitor' that work by increasing the level of serotonin in the brain. Other common drugs included in this group are paroxetine, fluvoxamine and escitalopram. Serotonin is a neurotransmitter that is often referred to as the feel good hormone or the happy chemical as it plays a part in the happiness and wellbeing of depressed individuals. The scientific name for serotonin is 5-hydroxytryptamine, or 5-HT. Scant levels of serotonin are linked with depression (McIntosh, 2018). Fluoxetine Hydrochloride is a potent and selective inhibitor of serotonin uptake in the presynaptic neuron of the central nervous system. It is not concerned with the concentration of the other mood-associated neurotransmitters namely dopamine or norepinephrine. In depressed individuals who had received 40 to 60 mg of fluoxetine per day for a interval of 6 weeks, the concentrations of the metabolites of serotonin (5-HIAA), dopamine (HVA), and norepinephrine (HMPG) in the cerebrospinal fluid were reduced by 46%, 14%, and 18%, respectively. As a result of the long term administration of fluoxetine, the 5-HT 1 receptors are downregulated and desensitized thereby enhancing the serotonergic neurotransmission. Fluoxetine is not known to directly interact with the postsynaptic 5-HT or serotonin receptors, the muscarinic-cholinergic receptors, the alpha-adrenergic receptors or the histaminergic receptors.(Blardi et al., 2005).

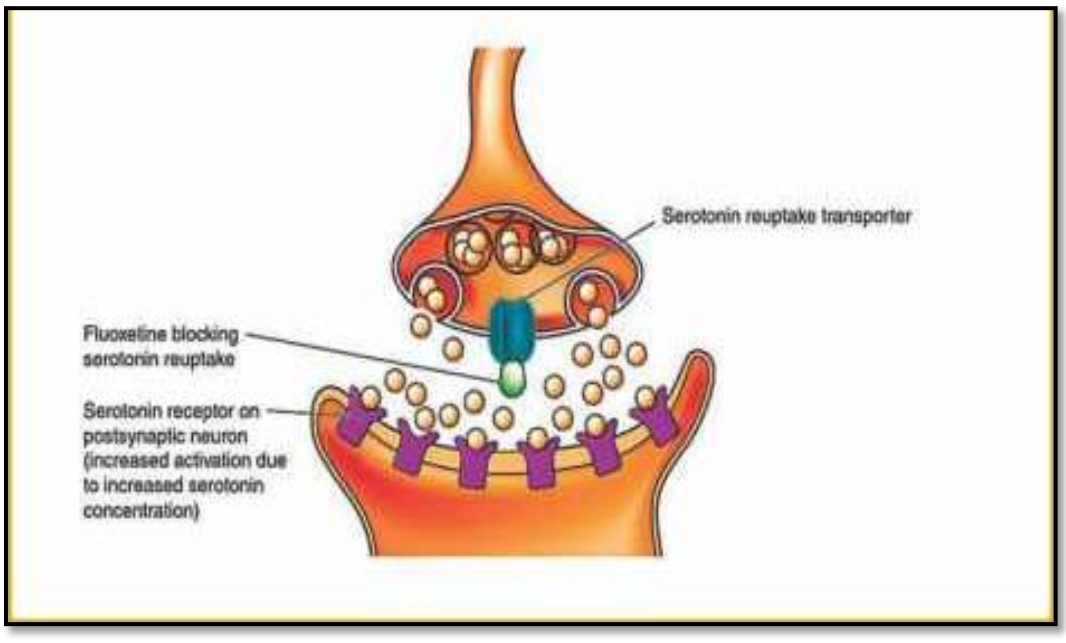

Figure 3 Adapted from (Times, 2019). How fluoxetine blocks the reuptake of serotonin in the presynaptic neuron.

2.1.2. Contraindications and limitations

Like all medicines and drugs, fluoxetine hydrochloride is associated with certain side effects. It is contraindicated in patients with renal failure and sensitivity issues. The most common side effects of fluoxetine include dehydrated mouth, headache, increased sweating, nausea, diarrhea, fatigued, feeling nervous, restlessness, sleepy or insomnia. SSRI antidepressant drugs including fluoxetine increases the risk of bleeding episodes. When used in combination with aspirin, warfarin, anti-coagulants and nonsteroidal anti-inflammatory drugs (e.g. ibuprofen, naproxen), the risk of bleeding is further enhanced. People may encounter severe symptoms such as bleeding gums, nose bleed, or in extreme cases gastrointestinal bleeding which can be life threatening.

One of the rare adverse effect associated with the use of fluoxetine is low level of sodium in the blood associated with symptoms like headache, feebleness and difficulty in focusing and remembering. Serotonin syndrome indicated by symptoms that include trembling, diarrhea, misperception of people and events, severe muscle tightness, illness, seizures and eventually death are among the rarely reported fluoxetine side effects (Brambilla et al., 2005).

2.2. Need of an efficient Fluoxetine carrier

Fluoxetine hydrochloride or Prozac is the most widely prescribed antidepressant drug worldwide. It is the only member of SSRI that has been declared safe for use in children 8 years of age and older. In spite of the wide variety of advantages this drug offers, there is still a segment of patients that are resistant to the current mode of treatment using fluoxetine owing to the few drawbacks one has to encounter following treatment with this drug. Fluoxetine hydrochloride has a delayed onset of action. The onset of action on average is within a week; however, individual responses vary greatly and a complete response may not be observed until a period of 8 to 12 weeks subsequently after the initiation of treatment (2019). Moreover, this drug has a comparatively long half-life of 1-3 days after acute treatment and 4-6 days following chronic administration as well as non-linear pharmacokinetics that make dose titration a difficult task. The elimination half-life of its active metabolite, norfluoxetine, ranges from approximately 4-16 days following both acute and chronic administration (FDA, 2017).

2.3. Liposomes

By definition, liposomes are circular or spherical bodies whose major building blocks are the phospholipid molecules that are enclosing a water droplet. The name 'liposome' comes from the Greek words: 'Lipos' that means fat and 'Soma' that signifies the body. Liposomes were discovered for the first time in 1960s by British hematologist Dr. Alec D Bangham who worked at the Babraham Institute, University of Cambridge. These liposomes consisted of single (unilamellar) or several concentric (multilamellar) phospholipid bilayers that were encapsulating a water filled compartment (Bangham and Horne, 1964). A variety of drugs can be encapsulated inside the liposomes and they can thus be used to transport drugs for the treatment of cancer and various other ailments. Liposomal membranes are typically composed of phospholipids which are molecules having a head group and a tail group. As the head is hydrophilic in nature owing to its composition, it is attracted to water and the tail region that is made of long hydrocarbon chain is hydrophobic in nature and thus it is repelled by water. In the biological system, phospholipids are a natural component of steady membranes that are usually composed of two layers or a bilayer. On encounter with an aqueous environment, their heads being attracted to water align in a manner to form a surface that is facing the water. The hydrophobic tails repel water and thus organize to form a surface that does not face the water molecules. In a cellular system, the membranes are organized in a manner that a layer of head regions interacts with the outside of the cell as is attracted towards the surrounding aqueous environment and a second layer of heads lines towards the inside of the cell and is attracted by the aqueous environment in the inside the cell. The bilayer structures are called liposomes and the monolayer structures are called micelles.

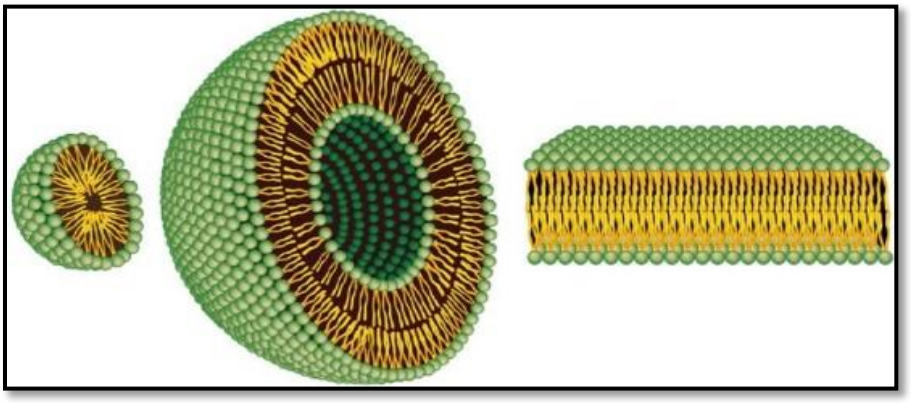

Figure 4: Adapted from (Bozzuto and Molinari, 2015). Structure of micelle (left), liposome (center) and lipid bilayer (right).

2.4. Liposomal Nanoparticles (LNPs)

Nanoparticles are an exciting system that can be used for brain targeted drug delivery as they allow us a number of possibilities to modulate and modify them resulting in particles having varied shape, size, hydrophilicity and hydrophobicity, surface coating, their surface chemistry and the net charge. A systematic control of these features can augment the ability of nanoparticles in improving the stability of the therapeutic agent in the circulation, enabling us to have a control over the rate of drug release into the preferred target site and to enhance the BBB penetration efficacy to a greater extent (Saraiva et al., 2016). Liposomal nanoparticles have garnered significant attention among various other delivery systems as they are the most translational and extensively explored drug-carriers for therapeutic interventions because of their exceptional performance in vivo and the ability to incorporate and protect a variety hydrophilic and hydrophobic modalities in an efficient and effective manner. They are also reported to elongate the circulation lifetime of various medications (Choi et al., 2015). Nanoliposomes are more advantageous as they offer more surface area and possess the ability to enhance solubility, provide a more controlled release, increase drug's bioavailability, and enable a more precise targeting of the encapsulated material compared with simple liposomes (Mozafari, 2010). Phospholipids and cholesterol are the main structural components of liposomal nanoparticles as well as the natural membranes that are found in the living system.

2.4.1. Phospholipids

Phospholipids are the key morphological constituent of the biological membranes as well as the liposomal nanoparticles. Phospholipids are amphiphilic molecules as they possess both hydrophilic and hydrophobic characteristics. There are two classes of natural phospholipids - Phosphodiglycerides and Sphingolipids. Among the most common phospholipids is the phosphatidylcholine (PC) molecule. Phosphatidylcholine molecules are insoluble in water and when they encounter an aqueous environment, they align themselves in a way forming planar bilayer sheets thereby minimizing the unfavorable and unwanted interaction between the aqueous phase and the long hydrocarbon chain region. Phospholipids including glycerol are the most commonly employed lipid components that are used broadly for the production of liposomal nanoparticles and signify more than half of the weight of lipids in the biological membranes.

2.4.2. Cholesterol

In addition to phospholipids, liposomes comprise of other molecules in their structure as well. These structural molecules are known as sterols. Sterols are an important constituent of the biological membranes and incorporation of sterols in the bilayers greatly modifies the properties of the liposomal nanoparticles (Luo et al., 2016). Among sterols, cholesterol is the most commonly utilized sterol in the synthesis of the nanoliposomal vesicles. Cholesterol moderates the fluidity of the lipid bilayer and prevents the crystallization of the acyl chains of phospholipids. It also imparts steric hindrance and decreases the permeability of lipid membranes to different solutes. The major reason for incorporating cholesterol in liposomal structures is the increased stability of the vesicles via the above mentioned mechanisms (Daraee et al., 2016).

2.4.3. Proposed structure of Fluoxetine loaded DPPC nanoparticles

The proposed model of fluoxetine hydrochloride incorporated liposomal nanoparticles can be seen in figure 6. It can be noticed that our drug being hydrophilic will be attracted towards the aqueous environment and thus will be encapsulated in the core region of the liposomal formulation and the cholesterol molecules that impart rigidity will be entrapped in the bilayer structure of the phospholipids owing to their hydrophobic nature.

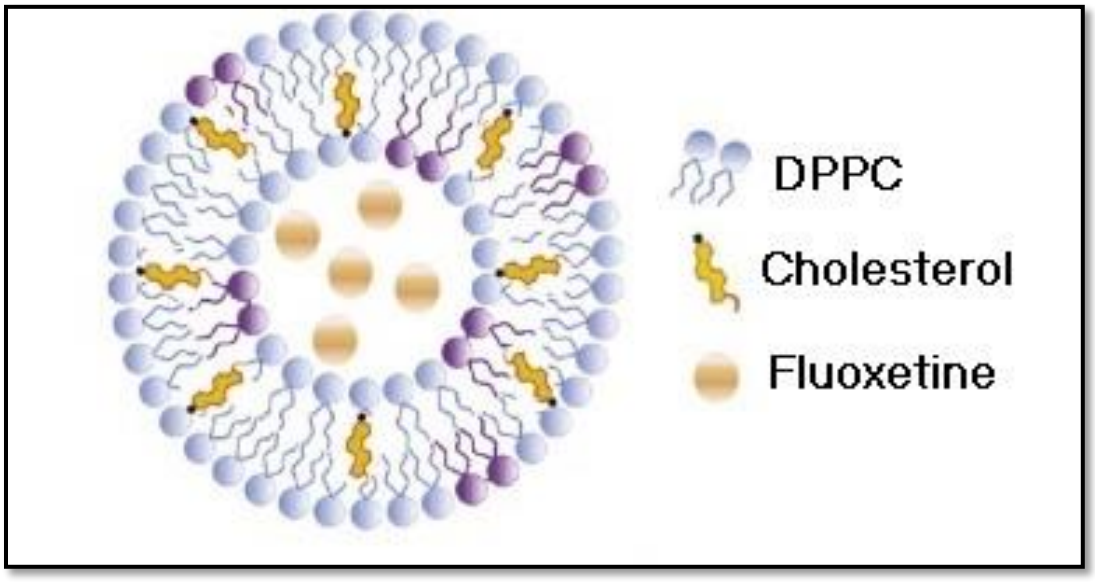

Figure 5: Proposed structure of Fluoxetine loaded DPPC liposomal nanoparticles. The polar drug will be encapsulated in the core whereas non-polar cholesterol molecules will be embedded in the bilayer.

2.5. Repeated Social Defeat Stress (RSDS) Animal model of Depression

Depression is fatal mental ailment that affect almost every individual once in the lifetime. This disease is responsive for tremendous economic loss and thus needs to be addressed with gravity. In psychiatric research and development, the major challenge is the development of animal models of depression that offers significant predictive, face and constructive validity. To improve our knowledge and to comprehend the underlying mechanism of this disease, animal models are required that can effectively mimic the symptomology of depression in humans. Repeated exposure to social stressors generates robust depression-like phenotypes in mice such as anhedonia (lack of pleasure), changes in weight, sleep disturbances, social avoidance and increased anxiety-like behavior. Another attractive feature of RSDS model is that the depression like behavior induced via this method can be reversed by antidepressant treatment thus allowing efficacy comparisons between different therapeutic interventions.

2.5.1. Validity of RSDS model

A major hindrance in the investigation and analysis of stress-related diseases is the low availability of validated rodent models that can determine the underlying mechanism of disease progression. The repeated social defeat stress protocol confirms to the four main forms of validity that are offered by any animal model:

(i) Construct or etiologic validity: Construct validity refers to the degree to which a model is reproducible in terms of the disease state in humans. Various studies have vouched in favor of this model for offering constructive validity.

(ii) Face validity: Face validity explains how accurately the behavioral and neuropathological phenotypes that are observed in humans have been reproduced in animal subjects. Results of various studies using RSDS model suggest the development of depression like phenotypes thereby confirming its face validity.

(iii) Discriminative validity: This form of validity implies how well this model can distinguish between various behavioral domains like depression and anxiety. RSDS produces a population of

susceptible animals that can be clearly discriminated from the animals exhibiting just anxiety-like behavior.

(iv) Predictive validity: Predictive validity suggests how far and to what extent can the treatment be predicted in the human patients as observed in the animal subjects.

The repeated social defeat stress thus serves as an efficient animal model for governing the mechanism of stress induction and to investigate and contrast the different modes of treatment available (Golden et al., 2011).

Chapter 3

3. Materials and Methods

3.1. Fabrication of Fluoxetine loaded Liposomal Nanoparticles

3.1.1. Materials

All chemicals were purchased from Sigma-Aldrich (USA), unless stated otherwise. Following chemicals were used for the synthesis of Fluoxetine loaded liposomal nanoparticles: Fluoxetine hydrochloride, Absolute Ethanol, Dipalmitolyphosphatadylcholine (DPPC), Milli-Q water and cholesterol.

3.1.2. Methodology

Fluoxetine loaded liposomal nanoparticles were fabricated by using modified ethanol injection method (Chorachoo et al., 2013). Fluoxetine hydrochloride was dissolved in enough amount absolute ethanol to attain a final concentration of 0.1mg/ml. For the preparation of the lipid phase at a concentration of 100 µmo*l*/ml, Dipalmitolyphosphatadylcholine (DPPC) and cholesterol were dissolved in 10ml ethanol in a ratio of 4:0.75. 500µl of fluoxetine from added in the above formed lipid suspension from its solution. The mixture was then subjected to sonication for 45 min. 10ml of Milli-Q water was placed in water bath that was previously set at a temperature of 60^{o}C along with sonicated lipid and drug suspension. After warming up to the temperature of 60^{o}C, the water phase was emulsified with the lipid phase and the resulting suspension was shaken in water bath for 15 minutes to allow even mixing and phase inversion. The resultant mixture was then transferred into a round bottle flask which was then connected to a rotary evaporator (Eyela Rotary Vacuum Evaporator N-100 series, Japan) to evaporate ethanol. Subsequently, the cloudy suspension of fluoxetine incorporated liposomal nanoparticles was then poured into a glass vial, sealed, and put in storage till further use.

3.2. Characterization of Fluoxetine loaded Liposomal Nanoparticles

Characterization of Fluoxetine loaded liposomal nanoparticles was carried out in order to determine and evaluate their size, net charge on their surface, their aggregation, drug encapsulation

and release kinetics with the purpose of ensuring they are of the right size and nature to be used for the treatment of depression in mice models.

3.2.1. Measurement of Particle size and distribution

Fluoxetine loaded Liposomal nanoparticles were examined under a scanning electron microscope (SEM, JSM 6490A, Japan) to determine their morphology. Glass slides bearing drug loaded nanoparticles were coated with gold (30nm) to make them conductive for SEM analysis. The scanning electron micrograph also explains the physical distribution of the nanoparticles in terms of their overlapping if any. Particular emphasis was placed on confirmation of the spherical morphology of the nanoparticles.

3.2.2. Surface charge, Zeta potential and Polydispersity index (PDI)

The value of zeta potential basically explains the net charge on the nanoparticles in terms of the magnitude of attraction or repulsion between them as well as their stability. Zeta potential, the hydrodynamic diameter as well as the polydispersity index (PDI) of Fluoxetine loaded Liposomal nanoparticles was determined by dynamic light scattering technique (Zetasizer-Nano Malvern (Germany)).

3.2.3. Encapsulation Efficiency

The supernatant of nanoparticles formulation was collected after centrifugation were and filtered by passing through a polycarbonate membrane filter and the quantity of drug present in it was determined by using the UV spectrophotometer. The standardize calibration curve of different millimolar concentrations of drug dissolved in ethanol was developed and a graph of concentration along with absorbance at 263nm was plotted for this reason. The total amount of drug present in supernatant was then subtracted from the initial amount of drug that was added for the preparation of fluoxetine loaded liposomal nanoparticles and the encapsulation efficiency was calculated using the formula:

$$\text{E.E (\%)} = \frac{\text{Total Drug added} - \text{Free nonentrapped drug}}{\text{Total Drug added}} \text{ x } 100$$

3.2.4. Drug Loading Capacity

Loading capacity explains out of the total weight of the nanoparticles, how much of the weight is defined by the entrapped drug. After the preparation of nanoparticles, they were centrifuged at 4500rpm in single column filter at 30 ^{o}C for 2.5 hours. Nanoparticle filtrate was stored. Column was again refilled by PBS solution and centrifuged at 4500rpm at 30 ^{o}C for 2 hours. PBS filtrate was stored at 4^{o}C. Column was again filled with distilled water and centrifuged at 4500rpm at 30^{o}C. 500µl of column's nanoparticles were put in pre-weighed eppendorf and weighed again. The drug loading capacity was calculated by using the formula:

$$\text{LC (\%)} = \frac{Entrapped\ drug}{Nanoparticles\ weight}\ x\ 100$$

3.2.5. Drug Release Kinetics

Drug release was monitored for up to 48 hours by mixing together same volumes of phosphate buffer solution and nanoparticles in different falcons. After every 30 minutes each falcon containing the nanoparticles and drug suspension was centrifuged and its UV was taken. This process was continued for 48 hours with 30 minutes difference. At 263nm absorbance was located and a graph was plotted exhibiting the drug release kinetics.

3.3. Induction of Depression using Repeated Social Defeat Stress (RSDS) model in Mice

Animal models, particularly the rodents' models of depression are extensively studied as an important tool to recreate or mimic the symptoms of major depressive disorder in humans. Exposure to different stressors is a very popular approach used to model depression like behavior in mice and rats. Continuous exposure to stressful events is one of the major predisposing factors leading to the development of mood disorders and in spite of the fact that various crucial symptoms of depression like feeling of worthlessness, guilt and suicidal thinking cannot be modeled in animals, these models are proven to have high construct and predictive validities.

3.3.1. Animals

20 Female Balb/c mice aged between 6-7 weeks and weight 24-28g and 10 female 12 weeks old Wister rats were purchased from National Institute of Health (NIH) Islamabad. The animals were made into groups randomly and subjected to an acclimatization period for about 2 weeks with free supply of clean water and food as per the requirement. Mice were kept in Standard home cages (42 × 26 × 18 cm). Home cages were filled with fresh sawdust that was replaced after every 3 days and a consistent 9:15 h light/dark cycle was kept. Temperature was maintained at 27°C ±2°C with humidity 50% ±5%. All the animals were closely monitored and observed. Several studies have demonstrated that exposure to chronic stress reduces food intake in animals thereby resulting in weight loss. For this purpose, weights of mice were recorded after every three days throughout the depression and treatment timeline.

3.3.2. Grouping of mice

Mice were divided into four groups with five mice per group. This grouping was based on the mode of treatment that the mice will undergo.

Sr. no	Groups	Size	Label
1.	**Group.01**	5	RSD-1
2.	**Group.02**	5	RSD-2
3.	**Group.03**	5	Control
4.	**Group.04**	5	Simple Depression

Table 1: Grouping of Mice.

3.3.3. RSDS Protocol

In this study Repeated Social Defeat Stress (RSDS) protocol was performed according to the previously described protocol with a few modifications (Golden et al., 2011). The subjects were

allocated into different groups as described above. Mice in both stress groups, also known as intruders were subjected to defeat by larger and aggressive rat termed as resident, which was previously tested for its aggressive behavior.

For the purpose of training of the residents, their confrontation with an unacquainted conspecific female mice was performed by introducing into each resident's home cage for a period of 3 sessions per week, during the one week acclimatization period until the resident presented stability in its aggressive behavior.

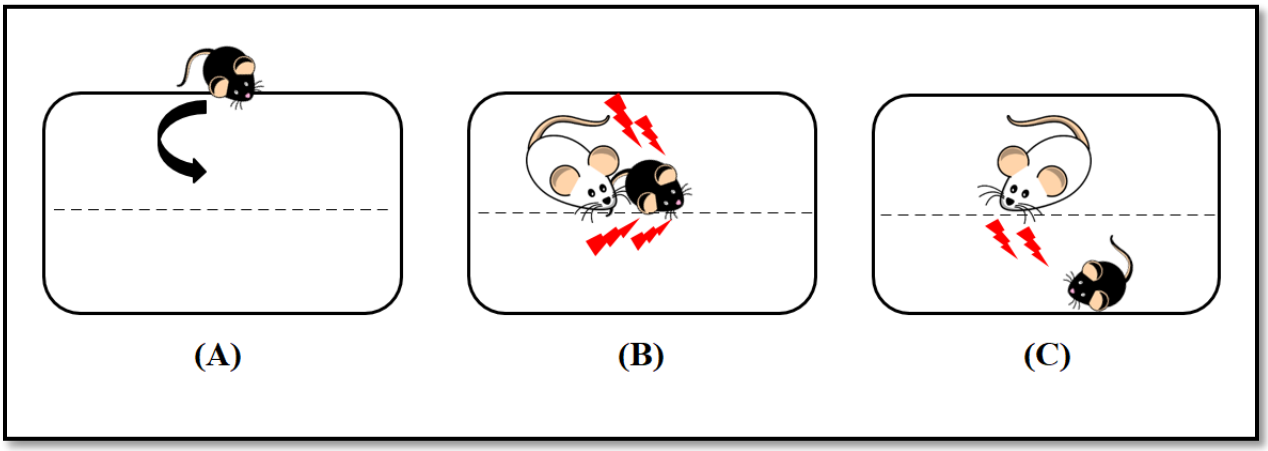

Figure 6: Development of Animal Model of Social Defeat. A Wister rat is housed in one side of perforated divider of the cage. (a) Balb-C mouse introduced in the same compartment. (b) Social conflict (10 minutes). Wister rat will attack balb-c mouse and establish dominance. (c) Balb-C housed in the other side of perforated divider for remainder 24hr period. This sequence of physical and physiological stress is repeated for 10 days to induce depression symptoms in BALB-C mice.

Figure 6 is a figurative representation of the repeated social defeat stress model. One episode of social defeat lasted for 10 minutes: in the initiation phase, the intruder was placed into the home cage compartment of the aggressive resident. The aggressive defeat session normally lasted up to five minutes but can be subjected to interruption earlier if the intruder exhibited a submissive position for a duration of consecutive 4 seconds or else any sign of severe injury. After the defeat session was over, aggressor and intruder were then separated by the same perforated Plexiglas wall and the defeated animal was subjected to physiological and sensorial contact with the aggressive resident for the remaining 24 hours period until the occurrence time of the next defeat, with unlimited access to feed and water, as described previously by Golden et al.

The defeated mice were alternated on daily basis, so that each day a new and unfamiliar aggressor established dominance over the intruder with the sole purpose of avoiding any sort of habituation. The Control mice and the Simple Depression group mice were kept in isolation and undisturbed in their home cages and were just being handled every other day for the purpose of weighing. At the termination of the 10-days long stress protocol, all of the subjects were kept back in their respective home cages.

3.3.4. Behavioral Tests

For the purpose of analysis of depression, standard behavioral tests were performed twice throughout the experiment. First round of tests were conducted before the treatment in order to access the stress level of mice confirming the successful induction of depression like symptoms in mice. The second round of tests were performed right after the treatment to investigate and analyze the effectiveness of the administered therapeutic agents.

(i) Forced Swim Test (FST) procedure: All mice were subjected to forced swim test using the standard protocol that has been described previously (Can et al., 2012a). For this purpose, a large glass tank was filled with clean water at room temperature. All the mice were placed in water tank one by one and video recording was conducted simultaneously. A recording of 6 minutes was saved for the purpose of analysis. Later on their behavior was analyzed by software and results were generated.

(ii) Tail Suspension Test (TST) procedure: All the mice were subjected to the tail suspension test using standard protocol (Can et al., 2012b). For this purpose, tail of each mice was wrapped in an adhesive tape and attached to table surface in a manner that their bodies were hung towards the ground. A video recording of 6 minutes was saved for the purpose of analysis. Later on their behavior was analyzed by software and results were generated.

(iii) Open Field Test (OFT) procedure: All the mice were subjected to the open field test using standard protocol (Seibenhener and Wooten, 2015). A large cubic cardboard box was used for this purpose. Mice belonging to each group were placed inside the box keeping the box uncovered from top and their exploratory activity was recorded through a video recorder. Later on their exploratory activity was analyzed by software and results were generated.

3.3.5. Drug Dosage and Route of Administration

For the purpose of treatment of depression, following doses were used. The drugs were administered via the intravenous route of administration through the tail of mice using a standard 1ml syringe for a period of 2 weeks (Table 2).

Sr.no	Groups	Treatment	Dosage
1.	**RSD-1**	Fluoxetine loaded liposomal nanoparticles	500µg/kg
2.	**RSD-2**	Simple Fluoxetine	10mg/kg
3.	**Control**	-	-
4.	**Simple Depression**	-	-

Table 2: Mice groups and drug dosage.

3.3.6. Euthanization of mice and sample storage

After induction and treatment, mice were euthanized using standard surgical procedure by abdominal incision. After euthanization their brain, heart, liver, spleen, and kidneys were stored in a 10% formalin solution for histological analysis.

3.3.7. Histological Examination with H&E staining

Slides of brain, heart, liver, spleen and kidney of H&E stain were prepared and organ embedded paraffin plates were obtained from Ali pathology lab, Islamabad. Slides were examined under a Labomed LB-200 Binocular Biological Microscope. Images were captured with magnification 100x using Pixel Pro software for a Labomed biological microscope.

Chapter 4

4. Results and Discussion

4.1. Characterization of Fluoxetine loaded Liposomal Nanoparticles

Characterization of Fluoxetine loaded liposomal nanoparticles was performed for the purpose of evaluation of our study in terms of determination and investigation of their size, net charge on their surface, their aggregation, drug encapsulation and release kinetics with the purpose of ensuring they are of the right size and nature to be used for the treatment of depression in mice model. The goal of the present research was to generate drug loaded liposomal nanoparticles that can penetrate the fine membranous obstacles surrounding the brain. Brain targeting is not a simple task as our brain is surrounded by structures having minute fenestrations that allow only selectively sized particles to pass through. Following the production of fluoxetine loaded liposomal nanoparticles, characterization was performed in order to ensure that the drug has been successfully entrapped within the nanoparticles and that the drug loaded particles possess the right properties and morphological features to be used for brain targeted delivery for the effective and efficient treatment of depression.

4.1.1. Particles size and distribution

Scanning electron microscopy enables us to understand the physical properties of materials and particles at nanoscale. The properties of liposomal nanoparticles can be effectively tuned via engineering their particular shapes and sizes so that it can be governed which particles are optimally favorable for the treatment of depression in the given circumstances. The results of SEM (figure 7) reflect that the formulated fluoxetine loaded liposomal nanoparticles possess well defined boundaries with distinct spherical shape. It can be seen that the nanoparticles have a mean diameter in the range of 190 nm. This result implies that this formulation consisting of fluoxetine, DPPC (100µM/ml) and cholesterol in a ratio (4:0.75) generates well non aggregated fine nano sized particles that fall in the range that is permitted to gain entry into the central nervous system via the protective selectively permeable blood brain barrier (BBB).

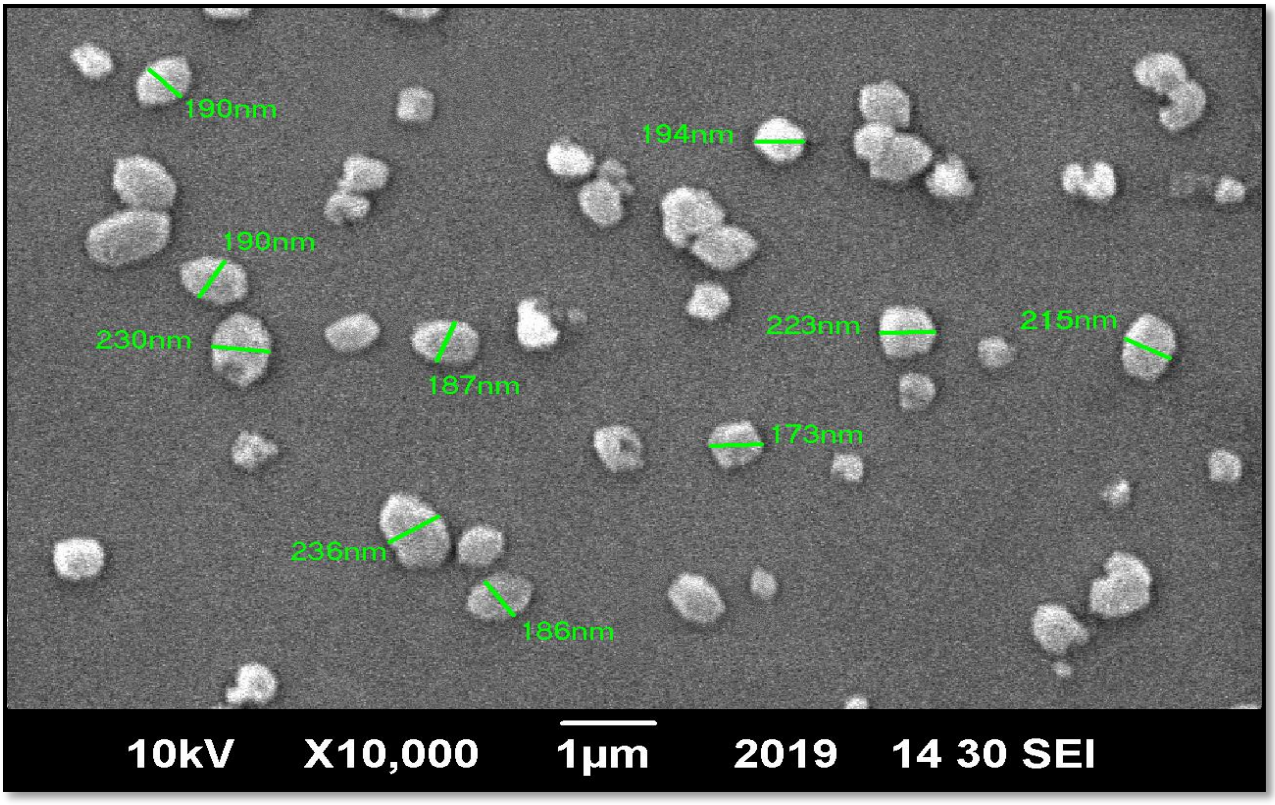

Figure 7: Scanning Electron Micrograph shows well dispersed nanoparticles in a size range of 170-250nm.

4.1.2. Surface charge, Zeta potential and Polydispersity index (PDI)

The value of zeta potential was recorded to be -19mV (figure 8). The average particle size also known as the hydrodynamic diameter of fluoxetine loaded liposomal nanoparticles was 187.2 nm with a polydispersity index of 0.245 (figure 9). The value of zeta potential defines the charge on the surface of the nanoparticles and has a marked affect the stability of particles present in the suspension that is a result of the electrostatic attraction and repulsion between the nanoparticles. Moreover, it also determines how the particles will interact with each other in-vivo. For charged nanoparticles, the magnitude of interactions between the particles will be more as the zeta potential value increases thereby resulting in the formation of stable nanoparticles with a highly uniform size distribution. Low PDI indicates formation of uniform nanoparticles with minimum aggregation.

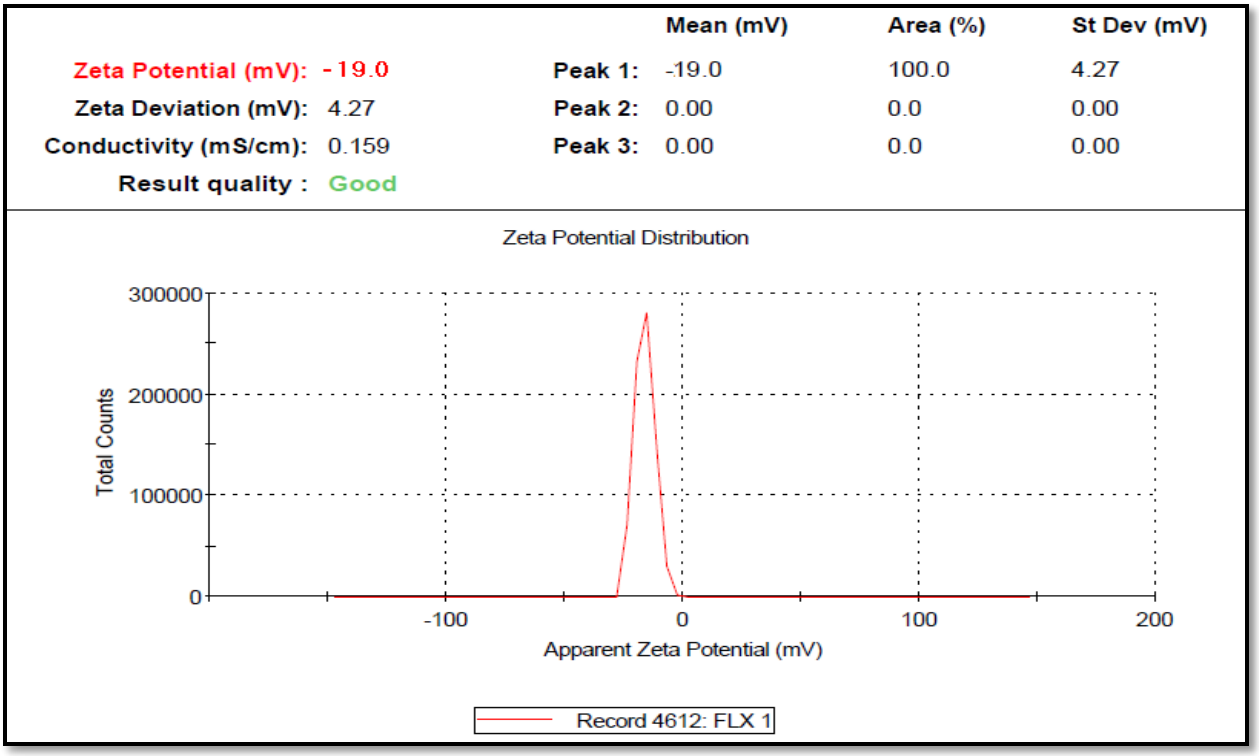

Figure 8: Zeta potential plot of fluoxetine loaded liposomal suspension indicates a liposome net charge of -19mV.

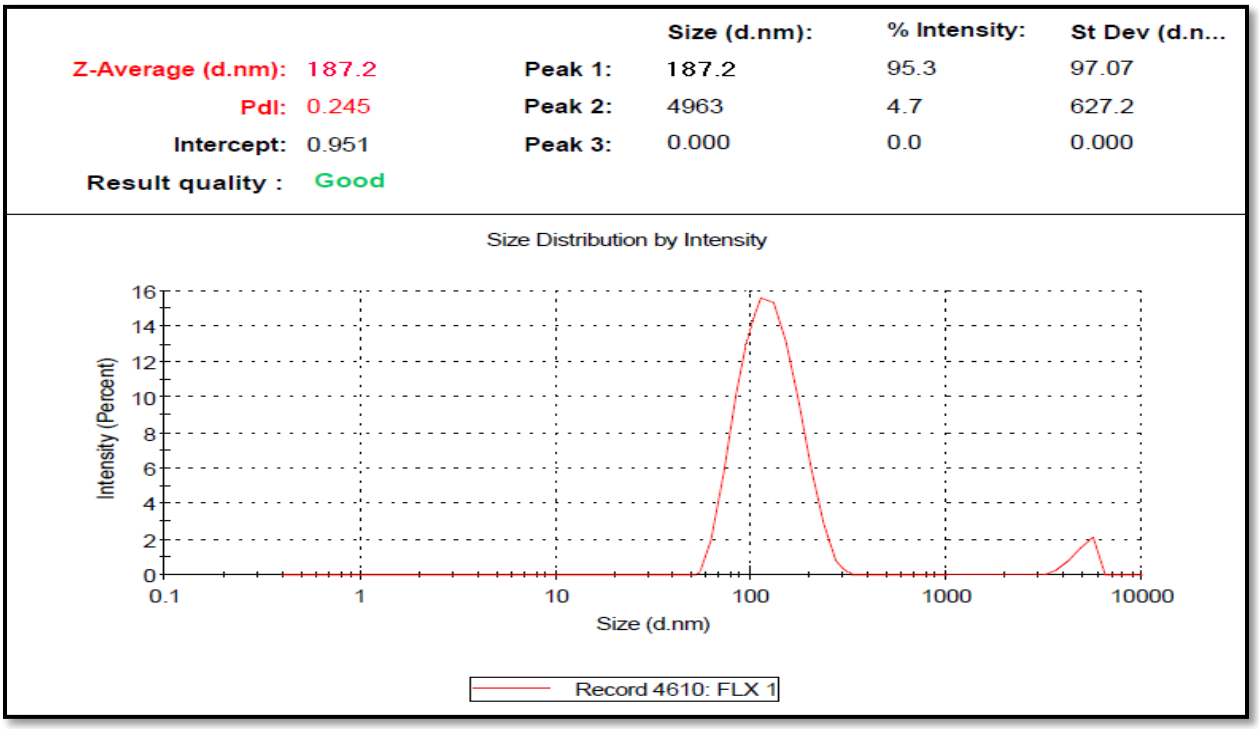

Figure 9: Particle size distribution profile and polydispersity index (PDI) of fluoxetine loaded DPPC liposomes.

4.1.3. Encapsulation Efficiency (E.E)

The encapsulation of a drug within a liposomal nanoparticle depends to a much greater extent on the lipid composition of the nanoformulation. This in turn governs the availability, cost, safety, and ease of utilization of the drug loaded nanoparticles (Haeri et al., 2014). By calculating from formula, encapsulation efficiency was estimated to be 69.1%. This implies that out of the total drug used, 69.1% of fluoxetine was successfully entrapped inside our liposomal nanoparticles and can be thus made available in the cellular environment for the treatment of depression in mice. This also reflects on the effectiveness of the protocol used for nanoparticles fabrication as a value of 69.1% is a good score implying the overall positive result in favor of this method.

4.1.4. Drug Loading Capacity (L.C)

Loading capacity is the amount of drug loaded per unit weight of the nanoparticle, which indicates that out of the total mass of the nanoparticles, how much in percentage of the mass is that of our drug. Using the formula, the drug loading efficiency was calculated to be 34%. It means that 34% of the nanoparticle weight is that of fluoxetine, implying that sufficient quantity of our drug has been successfully entrapped inside the nanoparticles and that they can be utilized for the purpose of treatment in the lining system.

4.1.5. Drug Release Kinetics

Since the release kinetics of Fluoxetine loaded liposomal nanoparticles at the desired site are of a great importance for the development of an ideal brain-targeted drug delivery system for the treatment of depression, in-vitro release kinetics studies were executed. The analysis of drug release profile of nanoparticles was carried out using UV spectrophotometer using fluoxetine loaded liposomal nanoparticle solution as control over a duration of 48h. The results were then tabulated and a cumulative drug release graph was generated as shown in figure 10. This graph shows a sustained release of fluoxetine hydrochloride from the liposomal nanoparticles for an entire 48 h period and it can be observed that after almost 5-6 hours, all the drug was released in the system.

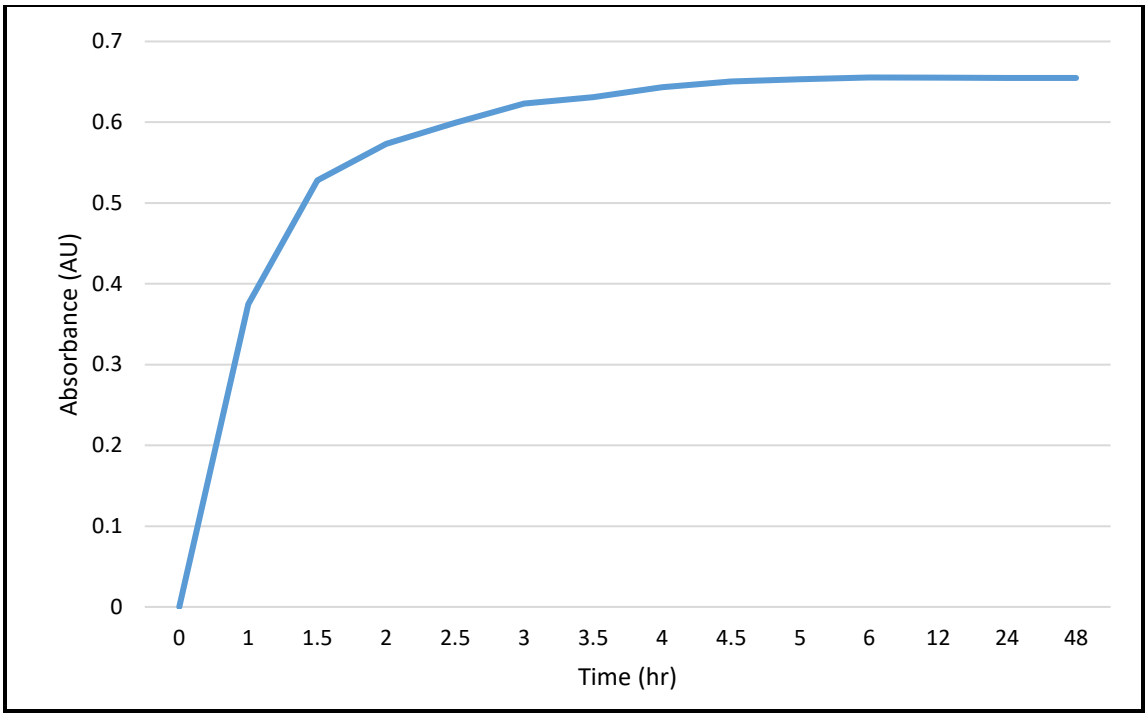

Figure 10: Drug release graph of Fluoxetine loaded liposomal nanoparticles. After 5-6hrs all drug is released in the system.

4.2. Induction of Depression using Repeated Social Defeat Stress (RSDS) model in Mice

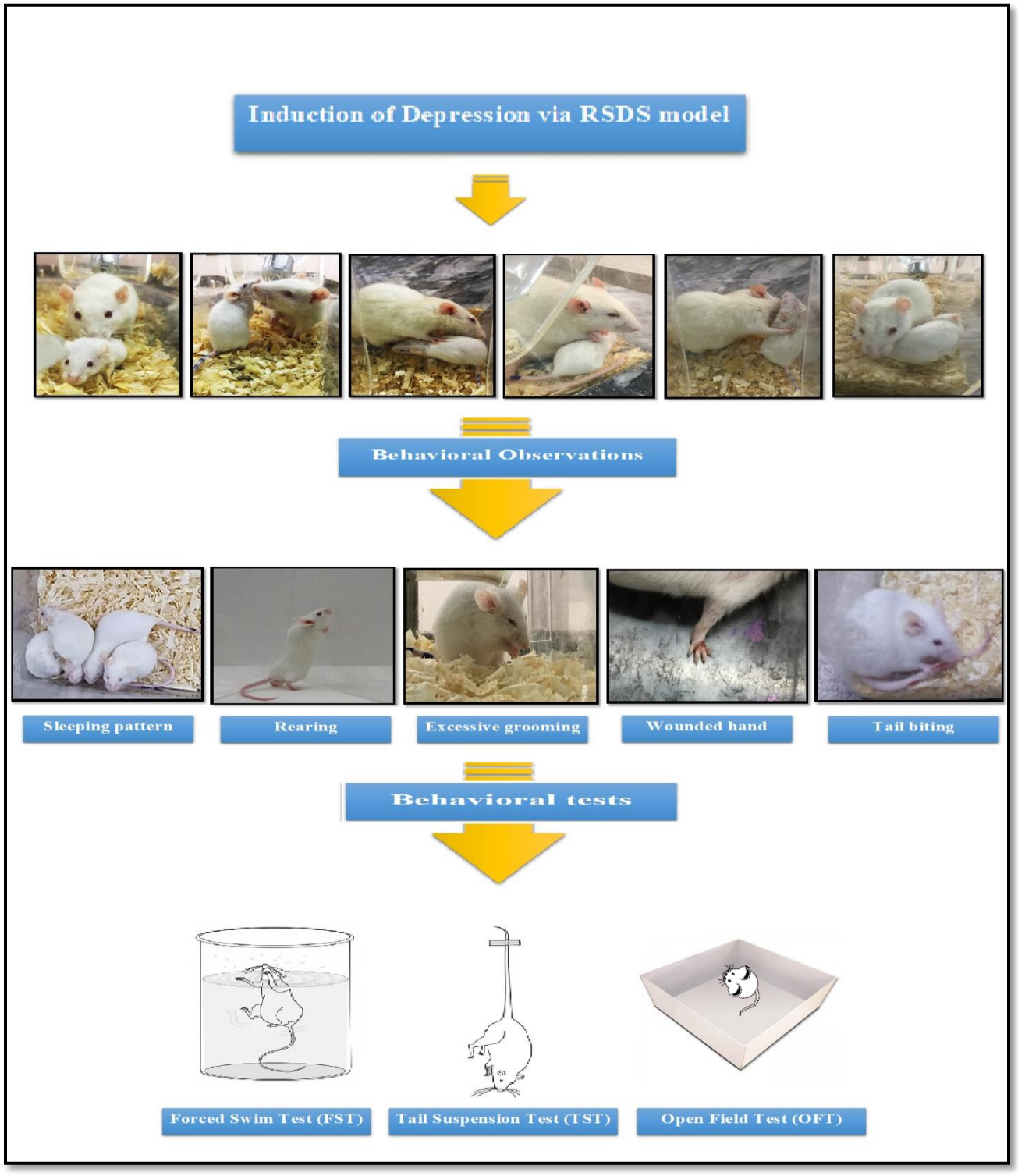

Figure 11: The Repeated Social Defeat Stress (RSDS) model for induction of Depression in mice.

Following the 10 days long repeated social defeat stress protocol, animals were closely monitored for any unusual symptoms that can be linked to depression before being subjected to the behavioral tests (figure 10). Once introduced into their home cages, the mice preferred to avoid contact with cage mates and preferred to stay and sleep alone around the corner zones. Moreover an increase in rearing activity, increased grooming and increased exploratory activity were also observed thereby reflection anxiety and depression-like behavior in mice.

4.2.1. Weight Analysis

An overall trend of decrease in body weight which is a consequence of stress and depression was observed after the induction of depression through RSDS model. Following the treatment of depression using both simple fluoxetine and fluoxetine loaded nanoparticles an increment in mice weight was seen thereby indicating a positive treatment response.

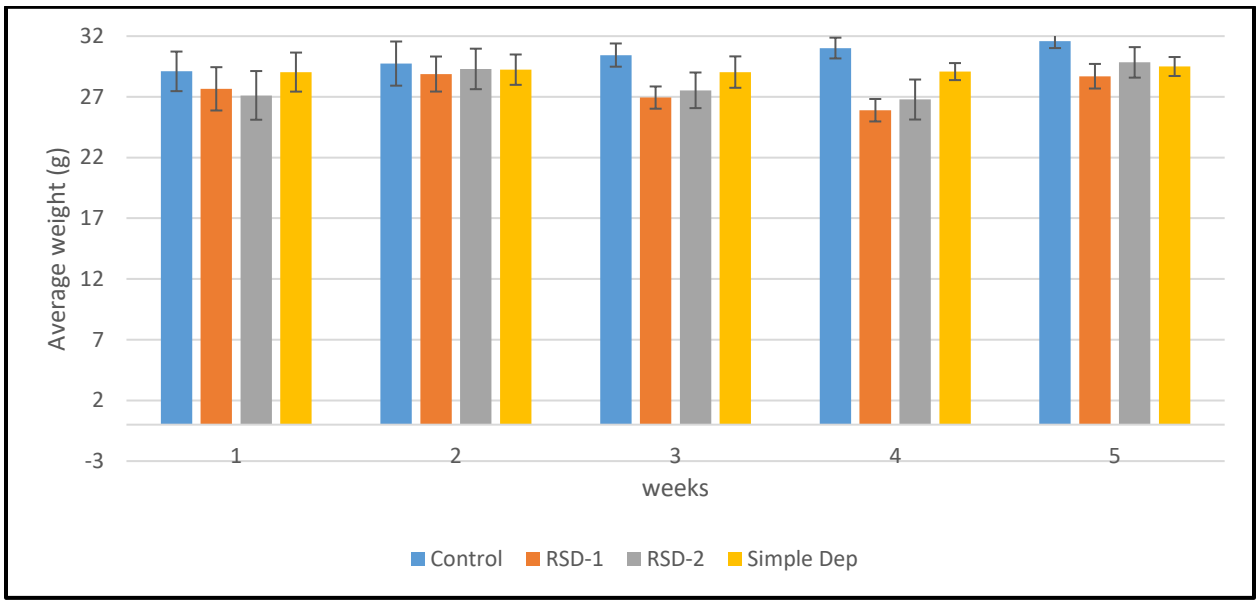

Figure 12: Weights graph of mice subjected to Repeated Social Defeat Stress (RSDS).

4.2.2. Behavioral Tests and Treatment Results

Behavioral tests were conducted for two purposes, firstly to confirm the successful induction of depression-like symptoms in mice after undergoing the repeated social defeat stress protocol and secondly, to confirm the reversal of depression like symptoms in mice after following the one-week long treatment regime. Results of the behavioral tests are explained below individually.

(i) Forced Swim Test (FST): The forced swim test is a well-established test for accessing the depressive state of a mice. When a mice is subjected to the forced swim test, a recording of 6 minutes is made out of which the last four minutes are analyzed as during the initial two minutes mice are very active and behave aggressively and the effects of treatment can be better observed and investigated during the last two minutes. For the purpose of analysis, the immobility time of each mice is noted. Greater the immobility time, more depressed the mice will be and vice versa. From the results (figure 13) it can be seen that the depressed mice exhibited the greatest immobility time, followed by the mice treated by simple fluoxetine and the once treated by fluoxetine loaded liposomal nanoparticles compared to the control. This implies a positive treatment response with the nanoparticles treated mice showing the best outcome among the other groups.

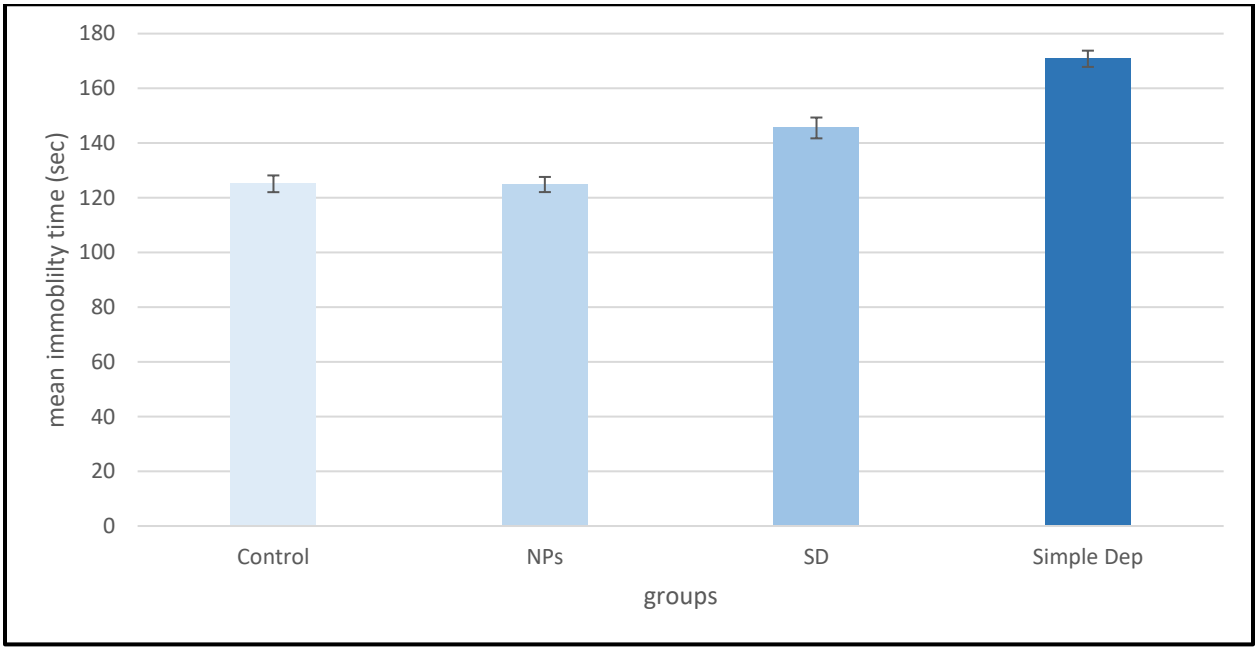

Figure 13: Forced Swim Test (FST) results of mice subjected to Repeated Social Defeat Stress (RSDS)

(ii) Tail Suspension Test (TST): Tail suspension test is an experimental test extensively used in scientific research to measure stress and anxiety in rodents. The tail climbing behavior and the overall activity of mice is noticed which depicts the stress and depression state of the mice. The results are recorded in terms of the mean immobility time of each mice. A more active mice will have greater immobility and thus low depression and anxiety like behavior and vice versa. From the results of the tail suspension test (figure 14) it can be observed that the depressed mice

exhibited the greatest immobility time, followed by the mice treated by simple fluoxetine and the once treated by fluoxetine loaded liposomal nanoparticles compared to the control. The results show a positive treatment response with the nanoparticles treated mice showing the contrasting result among the other groups.

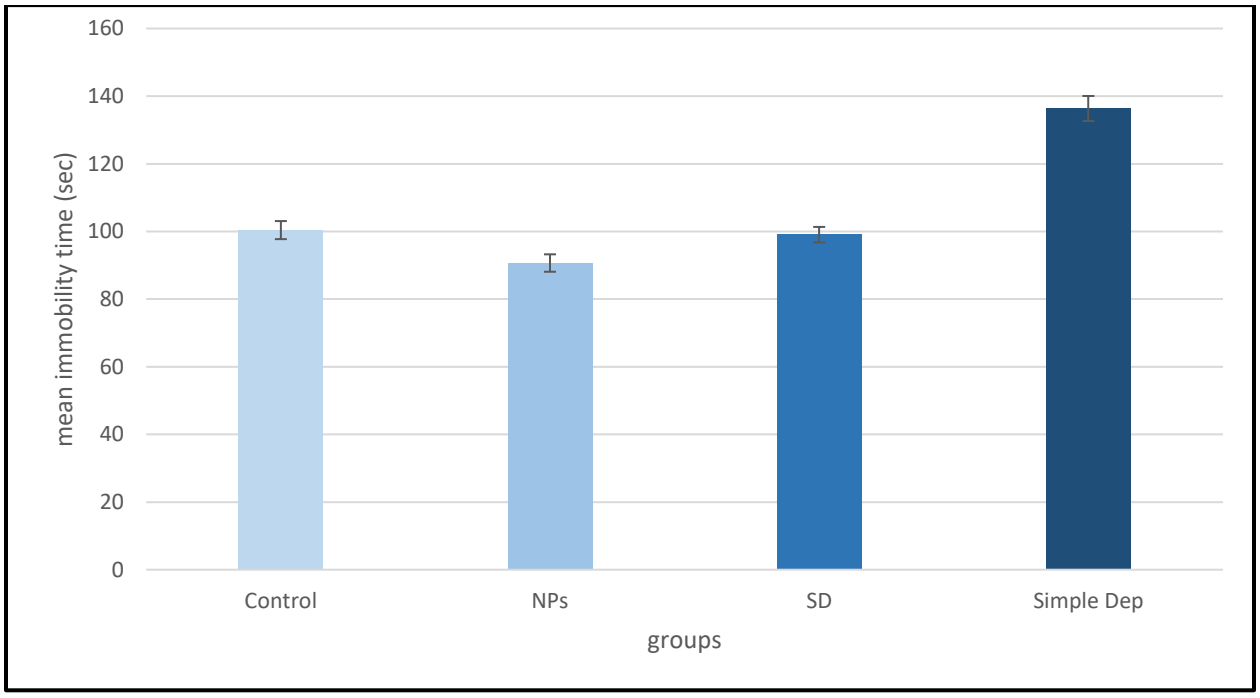

Figure 14: Tail Suspension Test (TST) results of mice subjected to Repeated Social Defeat Stress (RSDS).

(iii) Open Field Test (OFT): The open field tests is used to access the locomotor activity and willingness to explore in a novel environment thereby indicating the depression and anxiety like behavior of animals. Depressed animals show less locomotion and prefer to stay close to the walls of the arena in comparatively dark areas. When introduced into a new environment, mice tend to show anxiety like behavior irrespective of their history. A careful and focused observation shows that stressful mice spend less time in bright and exposed spaces that is the center zone compared to the sides were they assume themselves to be safe. Treatment of mice with simple fluoxetine and fluoxetine loaded liposomal nanoparticles had a noticeable effect on the anxiety behavior resulting in an increased time that was spent in the central zone compared to control and depressed mice as seen in figure 15. This indicates a positive treatment response with the nanoparticles treated mice showing the best results.

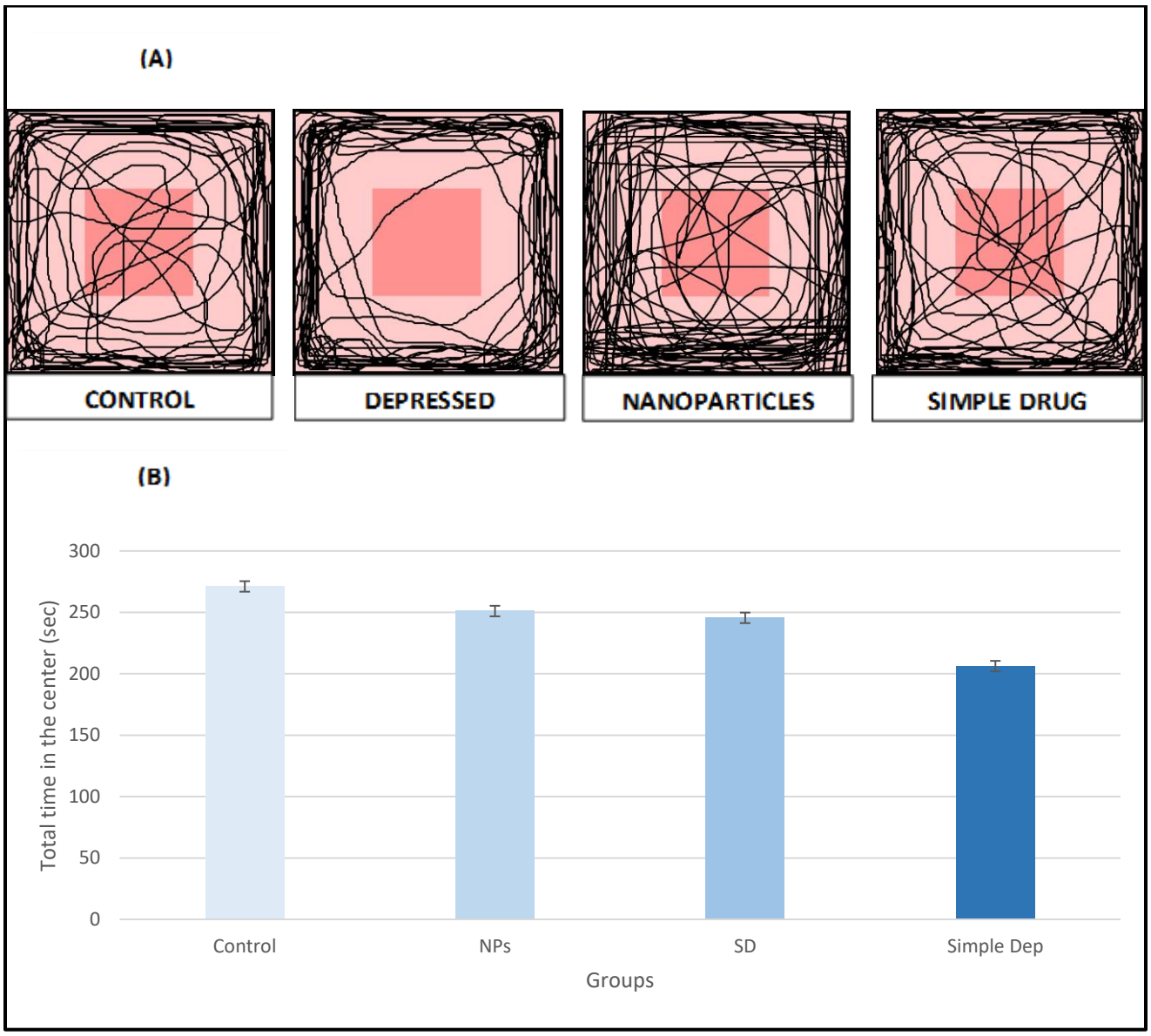

Figure 15: Open Field Test (OFT) results of mice subjected to Repeated Social Defeat Stress (RSDS). (A) Results of the mice tracking software and (B) Results in graphical format.

4.3. Histological Examination Results

4.3.1. Histology of Brain

From the histological analysis of nanoparticles and simple drug treated mice brain it can be observed that the oligodendrocytes appear to be intact as indicated by red arrows. Well defined

cell membranes and nuclei can be seen. No cell debris or other excretion products are visible thus indicating an overall sign of non-cytotoxicity.

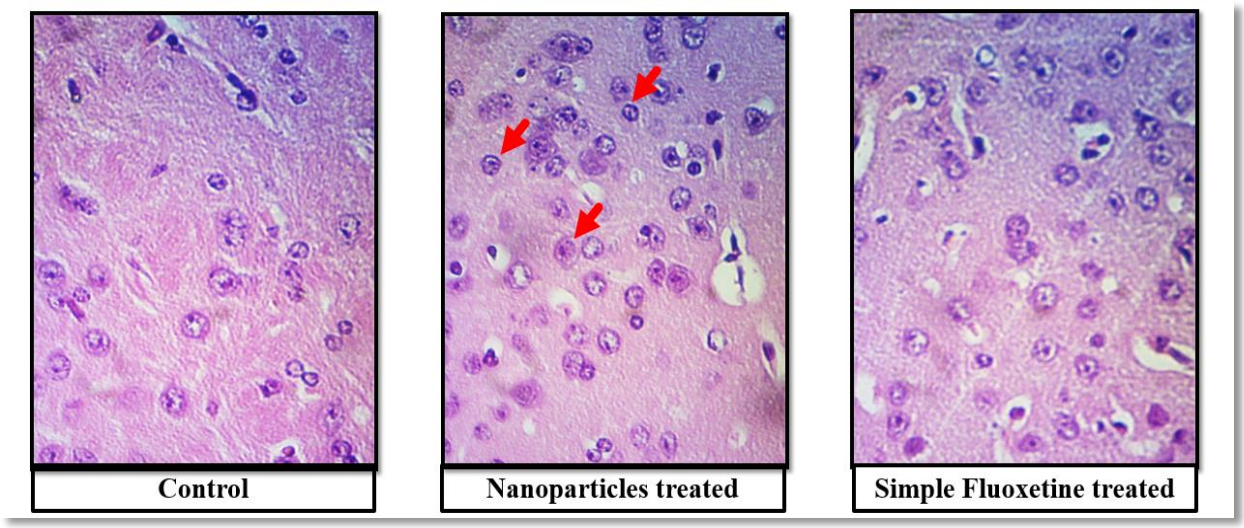

Figure 16: Histology of Brain (40x)

4.3.2. Histology of Heart

From the histological analysis of nanoparticles and simple drug treated mice heart it can be observed that the cardiomyocytes appear to be intact as indicated by red arrows. Well defined cell membranes and nuclei can be seen. No cell debris or other excretion products are visible thus indicating an overall sign of non-cytotoxicity.

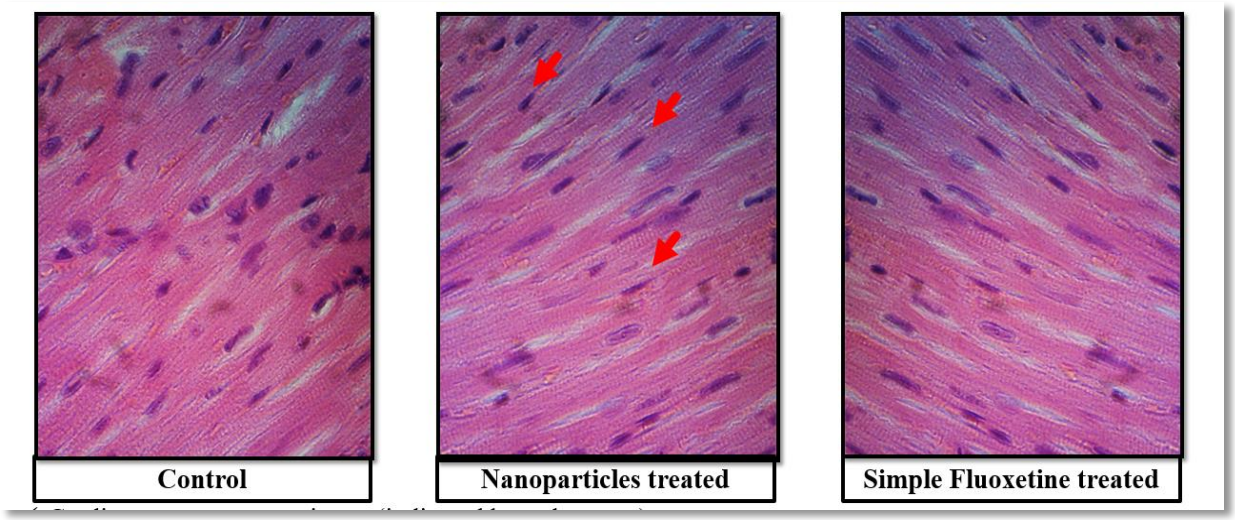

Figure 17: Histology of heart (40x)

4.3.3. Histology of Liver

From the histological analysis of nanoparticles and simple drug treated mice liver it can be observed that the hepatocytes appear to be intact as indicated by red arrows. Well defined cell membranes and nuclei can be seen. No cell debris or other excretion products are visible thus indicating an overall sign of non-cytotoxicity.

However in case of depressed mice liver, an irregular cellular morphology can be seen. Cells appear to be stretched and some crystalline inclusions can be visualized as indicated by the red arrows. This gives as an insight that depression might have some influence on the liver cells and further studies can be conducted to unfold this phenomenon.

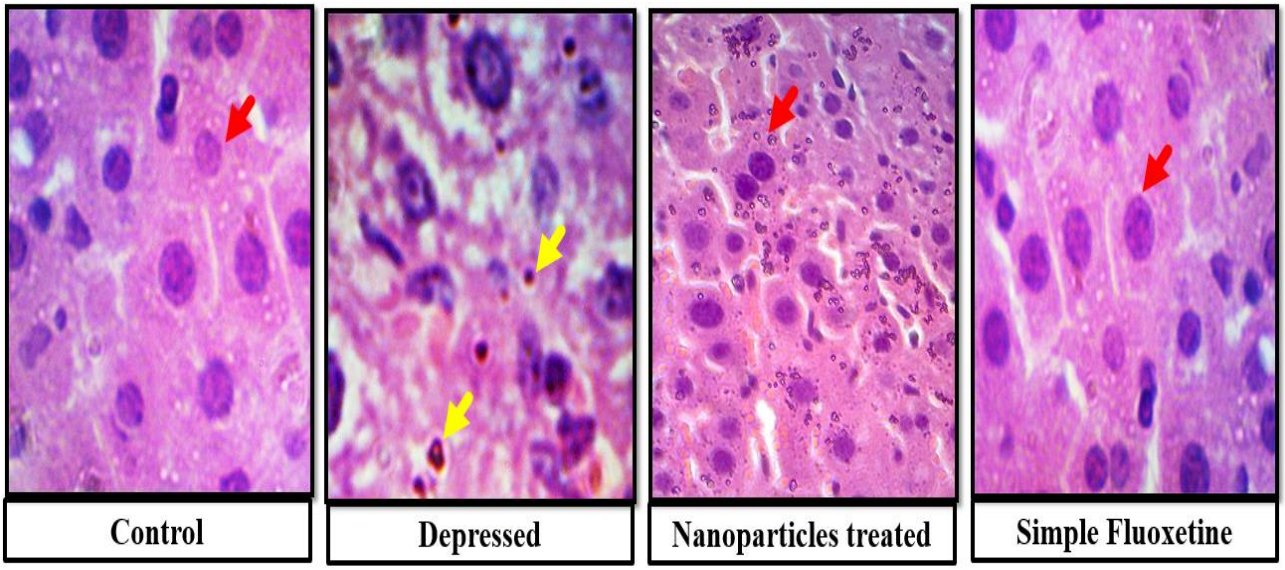

Figure 18: Histology of Liver (40x)

4.3.1. Histology of Spleen

From the histological analysis of nanoparticles and simple drug treated mice spleen it can be observed that the cells appear to be intact as indicated by red arrows. Well defined cell membranes and nuclei can be seen. No cell debris or other excretion products are visible thus indicating an overall sign of non-cytotoxicity.

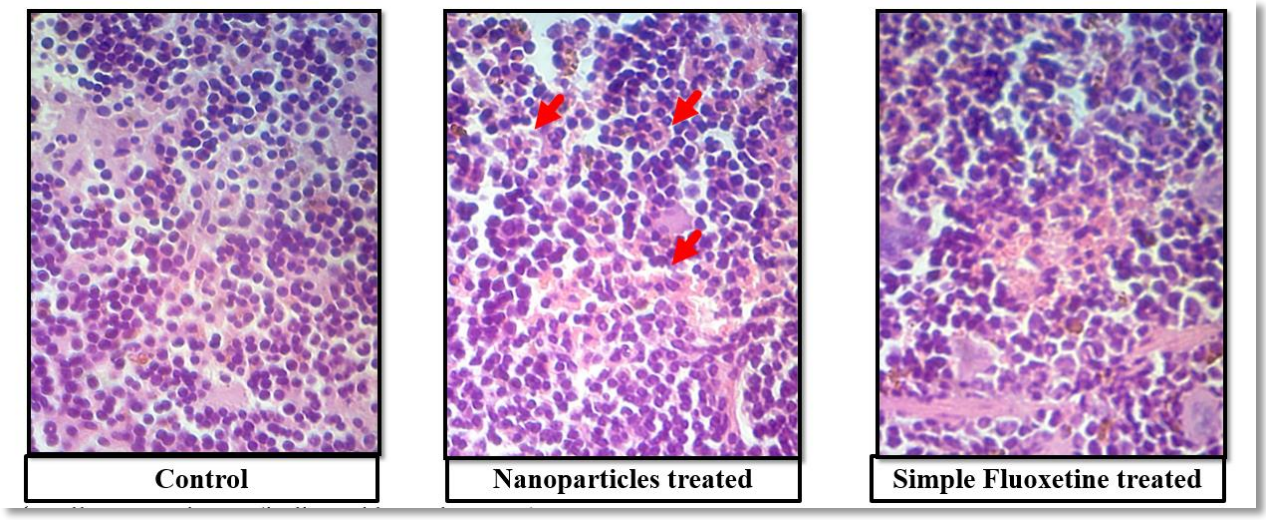

Figure 19: Histology of Spleen (40x).

4.3.1. Histology of Kidney

From the histological analysis of nanoparticles and simple drug treated mice kidney it can be observed that the cells appear to be intact as indicated by red arrows. Well defined cell membranes and nuclei can be seen. No cell debris or other excretion products are visible thus indicating an overall sign of non-cytotoxicity.

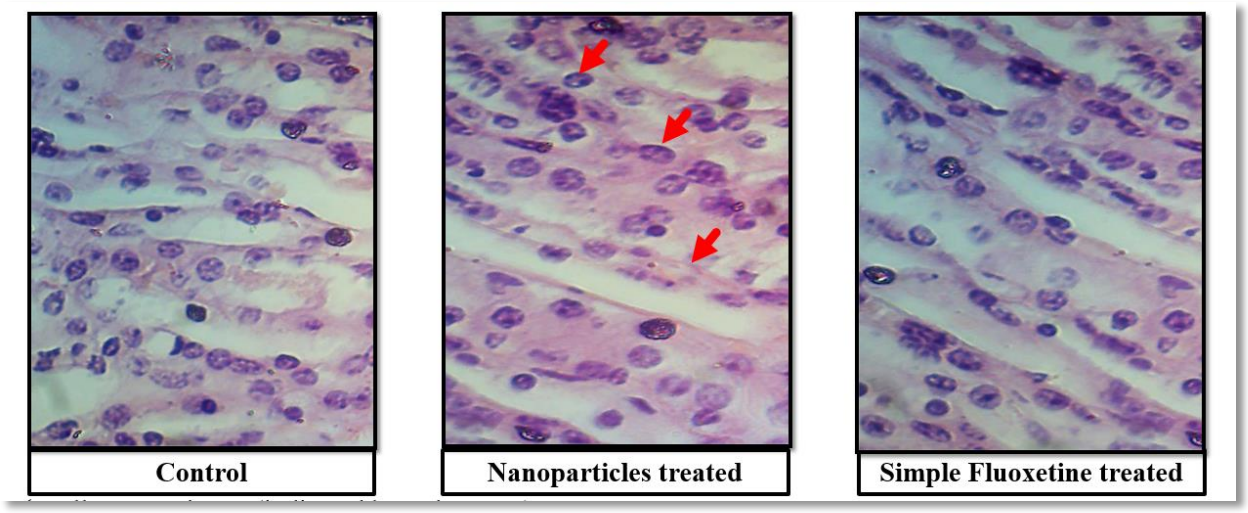

Figure 20: Histology of Kidney (40x).

Conclusion

Depression is a serious mental condition affecting almost every individual once throughout the lifetime. It is characterized by feeling of despair, guilt, low self-esteem, negative evaluation of events, loss of appetite leading to weight loss, inability to focus and concentrate and sleep disturbances. The main objective of this study was to design a nanoformulation that can serve as an efficient and effective tool for the treatment of this ailment.

Although a variety of antidepressant drugs are currently available but the selective permeability of the blood brain barrier possess a significant hurdle thereby limiting the advantages offered by these medications. It is made up of continuous non fenestrated vessels that cautiously regulate passage of ions, chemicals and cells to and from the membrane thus regulating homeostasis resulting in protection of the CNS from pathogens, toxins, injury and disease. This restraining nature of the BBB poses to be a major obstacle for drug delivery to the CNS. Nanotechnology has revolutionized the world of science and medicine enabling the development of fine nano-sized structure that can pass through any minute biological system delivering the required beneficial service.

Fluoxetine hydrochloride is the most widely prescribed antidepressant that was used in this study after being encapsulated inside DPPC liposomal nanoparticles. Liposomes were the vehicle of choice owing to their striking resemblance with the natural biological system. However, a drawback of using liposomal nanoparticles for treating depression is that they cannot be delivered via oral route of administration. The reason behind this is that they are metabolized via the first pass metabolism in the liver and thus no liposomes bearing the drug will be available in the systemic circulation ultimately reaching the brain and effecting the underlying pathways of depression. It is unlikely for a depressed individual to opt for injections as they are not in a normal state to receive medication via this route serving liposomal formulations useless. For this purpose, the drug loaded liposomal formulations should be subjected to surface coating that can enable them to overcome the first pass metabolism and thereby treat depression.

For the induction of depression, the repeated social defeat stress mice model was developed in which mice were subjected to bouts of social defeat as a result of encounter with an aggressive animal. The significance of this mice model lies in the way how effectively mimics the depression-

like symptoms in humans. The vast variety of behavioral symptoms include anhedonia i.e. loss of pleasure in activities that were otherwise very pleasing, changes in weight, anxiety like behavior and social avoidance to name a few. These are the common phenotypes exhibited by depressed individuals thereby serving as a good model for investigation of treatment of depression. Behavioral tests were employed for the confirmation of the results of the model as well as the treatment regime from which it can be concluded that fluoxetine loaded liposomal nanoparticles showed contrasting results in terms of reversal of depression-like symptoms thereby serving as an efficient and effective alternative for the treatment of depression.

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