8. ANIMAL STUDIES

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8.1 Introduction

Lipid based formulations have been reported to enhance bioavailability through intestinal lipid transport system after oral administration. Hence, LBDDS were prepared with the hypothesis that; by their virtues of lipidic nature and nanometric particle size range, they would be able to increase bioavailable fraction and thereby reduce the dose required to exhibit the therapeutic activity and thus decrease the toxicity of the drugs [1]. Evaluation of bioavailability and pharmacodynamic activity are useful to confirm the extent to which the objectives have been achieved. To deduce the exact mechanism of absorption of the formulated lipid-based drug delivery systems, *in vivo* pharmacokinetics were performed in rodents [2].

8.2 Animals

The *in vivo* studies were performed on male Sprague-Dawley rats (200-300 g) and female swiss albino mice (30-45 g) procured from Pretox Research Centre, Surat, India. The protocol for the study was duly approved by Institutional Animal Ethics Committee, Faculty of Pharmacy, The M. S. University of Baroda, Vadodara, vide protocol approval no: MSU/IAEC/2016-17/1627. All experimental procedures were carried out as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines released by Ministry of Environment, Forests and Climate Change, Govt of India. The animals were housed in polypropylene cages under laboratory conditions of controlled environment of temperature 25 ± 2 °C, 60 ± 5% RH and 12 h dark/light cycle. Three animals per cage were fed *ad libitum* with animal feed allowing free access to drinking water. After acclimatization, the animals were randomly allocated to groups having six animals in each group for pharmacokinetic and pharmacodynamic study.

8.3 Pharmacokinetic study in Rats

8.3.1 Iloperidone

For ILO SMEDDS and ILO Niosomes, pharmacokinetic profile was compared against ILO suspension. To deduce the mechanism of absorption of SMEDDS, the rats were pretreated with intraperitoneally administered cycloheximide (3.0 mg/kg) dissolved in saline followed by oral administration of ILO SMEDDS using oral feeding cannula.

Animal Dose calculation [3]

Animal Equivalent dose was calculated using the following formula:

$$AED\ (mg/kg) = HED\ (mg/kg) * \frac{Human\ Km}{Animal\ Km}$$ ….. equation 8.1

Where,

AED = animal equivalent dose

HED = human equivalent dose

For Iloperidone, HED = 12 mg/60 kg = 0.2 mg/kg

Km Ratio for human to rat dose conversion = 6.2

Hence, AED for Rats = 1.23 mg/kg

The average weight of rats was considered 250 g. So, the orally administered dose of ILO was found to be 0.308 mg.

8.3.2 Vardenafil HCl trihydrate

For VDN SMEDDS and VDN Niosomes, pharmacokinetic profile was compared against VDN suspension.

Animal Dose calculation [3]

As per equation 8.1,

For VDN, HED = 10 mg/60 kg = 0.16 mg/kg

Km Ratio for human to rat dose conversion = 6.2

Hence, AED for Rats = 1.027 mg/kg

The average weight of rats was considered 250 g. So, the orally administered dose of ILO was found to be 0.256 mg.

8.3.3 Animal grouping

The animals were randomly allocated to each group as shown in table 8.1. Each group contained six animals.

Table 8.1 Groups of animals (**Rats**) for pharmacokinetic study

Groups (n=6)	Samples administered
Group 1	ILO Suspension (oral)
Group 2	ILO SMEDDS (oral)
Group 3	ILO SMEDDS (oral) + Cycloheximide (intraperitoneal)
Group 4	ILO Niosomes (oral)
Group 5	VDN Suspension (oral)
Group 6	T-20 VDN SMEDDS (oral)
Group 7	C-EL VDN SMEDDS (oral)
Group 8	VDN Niosomes (oral)

8.3.4 Procedure

For pharmacokinetics, drug and formulations were administered to rats as grouped in table 8.1 to obtain plasma concentration – time profile. The animals randomly divided in the four groups were abstained from food overnight before beginning of experiment. To block lymphatic absorption of the drug, the rats were pre-treated with 3.0 mg/kg of cycloheximide dissolved in saline [4]. Cycloheximide was administered via intra-peritoneal route using 1 mL syringe with 25G needle. After oral administration using oral gavage, blood samples were withdrawn from the retro-orbital plexus using heparinized capillary at 1, 1.5, 2, 4, 8 and 12 h. The blood samples collected in pre-heparinized tubes were centrifuged at 3600 rpm, 4ºC for 10 min (Remi Centrifuge, India) to separate plasma. The plasma samples were stored at -70 ºC until analysis by LC-MS as described in analytical methods.

[Cycloheximide blocks chylomicron absorption pathway of lipids. Hence, for this study only one group was allocated to verify the absorption pathway of lipid- based formulations.]

8.3.5 Data Analysis

The pharmacokinetic parameters were calculated using Kinetica software (Thermo Scientific™, Thermofisher.com). The maximum concentration (Cmax) and time to reach maximum concentration (Tmax) were obtained from the plasma concentration-time profile.

The area under the curve (AUC) was calculated by the trapezoidal rule [5]. The relative bioavailability was calculated using following equation 8.2.

$$Relative\ Bioavailability = \frac{[AUC]_a/dose_a}{[AUC]_b/dose_b}\ X\ 100\ \%$$ …. Equation 8.2

8.4 Pharmacodynamic study for Schizophrenia

Animal Model: MK-801 induced Psychosis in Mice

Pharmacologic animal models of schizophrenia are based on our current understanding of the alterations in various neurotransmitter systems. Several different hypotheses that aim to explain the pathophysiology of schizophrenia have emerged. Theoretically, the N-methyl D-aspartate receptor (NMDAR) hypofunction hypothesis provides the unique possibility to integrate nearly all distinct pieces of evidence from schizophrenia research regarding the involvement of different neurotransmitter systems [6]. The link to the dopamine hypothesis is demonstrated by the finding that dopamine receptor expression is altered in mice after treatment with the irreversible NMDAR-antagonist MK801. Hence, an NMDAR-deficiency might give rise to the secondary dopamine dysfunction in schizophrenia. So, for pharmacodynamic study of the developed formulations, MK-801 induced psychosis model was used [7].

8.4.1 Animal Grouping

For the pharmacodynamic study, female mice were randomly allocated to each group as shown in table 8.2. Each group contained six animals each.

Table 8.2 Groups of animals (**Mice**) for pharmacodynamic study

Groups (n=6)		Samples administered
1	Normal control	0.9% Saline
2	Disease control	0.3 mg/kg MK 801 i.p.
3	Drug suspension	1.23 mg/kg Iloperidone p.o. + 0.3 mg/kg MK 801 i.p.
4	SMEDDS formulation	1.23 mg/kg Iloperidone p.o. + 0.3 mg/kg MK 801 i.p.
5	Niosomes formulation	1.23 mg/kg Iloperidone p.o. + 0.3 mg/kg MK 801 i.p.

8.4.2 Procedure

Group 1 received saline i.p. Groups 2 – 5 were given MK-801 for 14 days. MK 801, also known as dizocilpine maleate, dissolved in saline, was injected intraperitoneally at a dose of 0.3 mg/kg. The symptoms were assessed using forced swim test and rotarod apparatus on the 1st, 6th, 13th, 14th & 15th days of the drug treatments.

Other than these behavioral parameters, biochemical estimation was also carried out using brain homogenate. For this, at the end of the 14 days treatment, animals were sacrificed by overdose of ether and cardiac reperfusion was performed with phosphate buffer saline (PBS). The perfused brains were isolated and divided in two halves. The homogenate was prepared in phosphate buffer by adding 10% of PBS to the weight of brain (i.e. 300 mg brain – add 3 mL PBS). Homogenates were centrifuged at 6000 rpm for 15 minutes at 8 °C. Supernatant was used for tissue nitrite level determination [8] . Similarly, for UPLC-MS based dopamine level detection the brain homogenates were prepared in 0.17M perchloric acid instead of PBS [9].

8.4.3 Parameters

1. Rota-Rod Test

The Rota-Rod Test can be used to assay the motor abilities of rodents [10]. It requires rodents to balance on a rotating cylinder, the speed of which can be altered. The animal is placed on the roller lane of the rotarod and the timer is started. When the animal drops safely into its own lane, the time latency to fall (minutes and seconds) and rotation speed are automatically recorded.

Procedure:

The animals were placed on roller lanes 1, 3 and 5 (i.e. leaving an empty lane between two animals). The animals were allowed to walk on the forward direction while keeping their balance on the rotating rod. The rod was initially rotating at 4 rpm constant speed to allow positioning of all the animals in their respective lanes. After balancing at 4 rpm constant speed for 60 sec training time, the rpm was increased for another 3 minutes for test. The rpm at which animal fell down was recorded. It consisted of three trials separated by 10 min inter-trial intervals.

2. Forced Swim Test

The forced swim test was used to assess muscle coordination and rigidity [11]. For this, cylindrical transparent swim tank (height 46 cm, diameter 20 cm) was filled with water to a depth exceeding the length of the mice including tail.

Procedure:

On the 1st day, each mouse was placed in a transparent glass cylinder containing water at 25 °C and was forced to swim for 5 min. The water depth of 30 cm allowed the mice to swim or float without their hind limbs touching the bottom of the tank. The immobility time was measured. The mice were removed and allowed to dry in a heated enclosure (32 °C) before being returned to their home cages after the 5 min swim in water.

After 6 days of training, the animals were divided and randomly allocated to 5 groups and forced swim test was performed to measure average immobility time.

3. Estimation of Brain Nitrite Level

Nitrite & nitrate both are nitrogen oxide (NO) metabolites. Nitrite (NO_2^-) is the result of auto oxidation of NO and can be considered an index of normal NO bioavailability. Hence, measuring nitrite in a sample gives idea about NO production [12].

Griess assay:

It detects presence of organic nitrite. Nitrite is detected and analyzed by the formation of a red pink color upon treatment of a NO_2^- containing sample with the Griess reagent. When sulphanilamide is added, the nitrite forms a diazonium salt. To this, addition of azo dye (NEDA) leads to development of pink color. The color intensity was measured at 540 nm using microtiter plate reader (680-XR, Bio-Rad, France) [12].

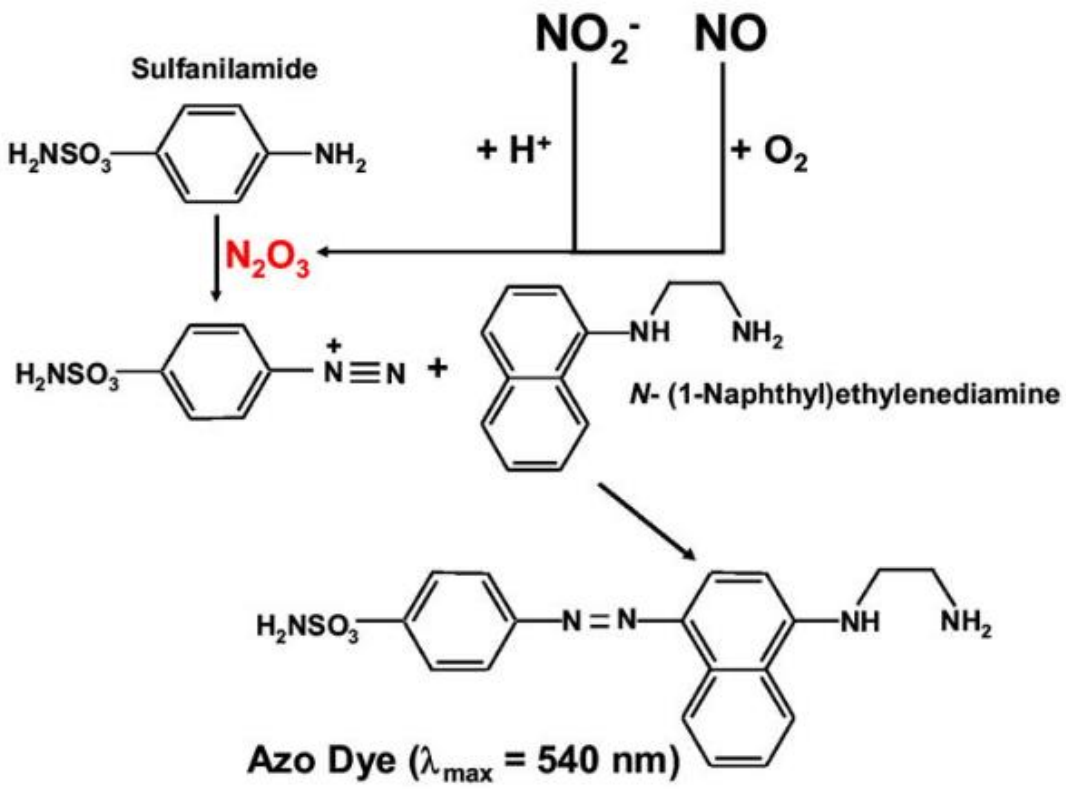

Figure 8.1 Griess reaction to detect nitrite level

Reagents:

Sodium nitrite, Tris- HCL buffer and Griess reagent (1% w/v of sulphanilamide and 0.1% w/v of naphtylethelenediamine dihydrochloride dissolved in 2.5% v/v of ortho phosphoric acid.)

Procedure:

To 1ml of brain tissue homogenate, 1 ml of Griess reagent was added and incubated for 15 minutes at 37°C. The absorbance was measured at 540 nm against the Griess reagent blank. Sodium nitrite solution was used as the standard control.

8.5 Pharmacodynamic study for Erectile dysfunction

Animal Model: Streptozotocin induced Diabetes Mellitus in Rats [13]

Diabetes mellitus is associated with both macrovascular and microvascular complications. Diabetes has been associated with sexual dysfunction in men. Diabetes is an established risk factor for sexual dysfunction in men; a threefold increased risk of erectile dysfunction (ED)

was documented in diabetic compared with nondiabetic men. So, in current research work Streptozotocin (STZ) was administered via i.p. route in order to render the rat diabetic

8.5.1 Animal Grouping

For the pharmacodynamic study, the male rats were randomly allocated to 5 groups as shown in table 8.3. Each group contained six animals.

Table 8.3 Groups of animals (**Rats**) for pharmacodynamic study

Groups (n=6)		Samples administered
1	Normal control	0.9% Saline
2	Disease control	60 mg/kg STZ i.p.
3	VDN suspension	60 mg/kg STZ i.p. + 1.027 mg/kg Vardenafil p.o.
4	VDN SMEDDS	60 mg/kg STZ i.p. + 1.027 mg/kg Vardenafil p.o.
5	VDN Niosomes	60 mg/kg STZ i.p. + 1.027 mg/kg Vardenafil p.o.

8.5.2 Procedure

Adult male rats were confirmed for normal erection by the transsexual activity with female rats. These rats were divided in different groups. Group 1 received saline (normal control). Whereas, group 2 to 5 rats were rendered diabetic by administering a single dose of STZ (60 mg/kg) dissolved in 0.1 M ice cold citrate buffer (pH 4.5) via i.p. route. 24 hours after the injection, the rats were administered 10 % w/v glucose solution orally to avoid hypoglycemia. The rats then were fasted overnight and the blood was withdrawn from retro-orbital plexus next morning from the rats. After separating plasma from the blood, fasting plasma glucose levels were estimated using commercially available kit working on the principle of GOD-POD method (Enzopak®, Reckon diagnostics P. Ltd.) [14]. The principle of the assay is: glucose oxidase (GOD) oxidizes glucose to gluconic acid and hydrogen peroxide. This hydrogen peroxide in the presence of peroxidase (POD) couples with phenol and 4-aminoantipyrine(4-AAP) to form colored quinoneimine dye, the absorbance of which is measured at 505 nm using UV-Vis Spectrophotometer - 1800 (Shimadzu, Japan).

$$\text{Glucose} + O_2 + H_2O \rightarrow \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4-AAP} \rightarrow \text{Quinoneimine} + H_2O$$

After this, the dosing treatment of drugs and formulation to groups 3 to 5 was continued for 8 weeks. At the end of the experiment (after 8 weeks) after blood collection, the rats were sacrificed and the penis and testes were stored at −70 °C until further analysis.

8.5.3 Parameters

1. Analysis of serum parameters

1.1 Estimation of serum testosterone levels in diabetic rats

Estimation of testosterone was carried out according to ChemiLuminescent ImmunoAssay (CLIA) principle using ADVIA Centaur® (Siemens Healthcare, India) at Supratech Micropath laboratory and research institute Pvt. Ltd., Ahmedabad.

1.2 Estimation of serum nitric oxide

The assay was carried out by adding 100 µL of the serum & 100 µL of the Griess reagent in a 96 well culture plate. The mixture sample was then incubated for 30 minutes and the absorption was measured at 570 nm in a UV-Visible spectrophotometer. The amount of nitrite was determined by comparison of unknown with a $NaNO_2$ standard control sample according to equation 8.3 [12].

$$Nitrite\ concentration\ in\ test = \frac{Absorbance\ of\ test}{Absorbance\ of\ standard} \times Standard\ concentration$$

…..Equation 8.3

2. Evaluation of sperm parameters in diabetic rats

At the end of 8 weeks of study, the rats were sacrificed and the effect of the drug and formulation on sperm count, morphology and defects were studied [15]. Epididymis was separated carefully from testis and divided into 3 parts; head, body and tail. The epididymal tail from both the testes were trimmed with scissors and placed in a petri dish containing 10.0 ml of 0.1 M phosphate buffer pH 7.4. Then it was gently swirled for 10 min under 37ºC for dispersion of sperm cells. This suspension was diluted 10 times with phosphate buffer and was observed under microscope for motility, number and gross morphology. For sperm motility, an aliquot of 10µL was placed in a haemocytometer chamber and observed under a light microscope. One hundred sperm were evaluated per animal and classified into motile and non-

motile. Defective sperms (coiled sperms, tailless sperms, sperms with bent neck, mid piece and tails) were observed and percentage was calculated using the following formula:
Calculations for sperm count:

$$Live\ Sperm\ (\%) = \frac{Total\ number\ of\ live\ sperm}{Total\ number\ of\ sperms\ counted}\ X\ 100 \quad \text{…Equation 8.4}$$

$$Defective\ Sperm\ (\%) = \frac{Total\ number\ of\ defective\ sperm}{Total\ number\ of\ sperms\ counted}\ X\ 100 \ \text{…Equation 8.5}$$

3. Histopathology of testis and penile tissue in diabetic rats

At the end of the study (after 8 weeks treatment period), the animals were sacrificed by cervical dislocation. The tissue of interest i.e. testis and penile tissue were cut and fixed in 10% buffered neutral formalin solution. The tissue was embedded in molten paraffin with the help of metallic blocks, covered with flexible plastic molds and kept under freezing plates to allow the paraffin to solidify. Cross sections of the fixed tissue were cut using microtome. These sections were then stained with hematoxylin – eosin (H-E) and Masson Trichome Stain (MTS) and visualized under light microscope [16].

8.6 Results and Discussion

8.6.1 Pharmacokinetic study

A) ILOPERIDONE formulations

The plasma drug concentration vs. time profile for ILO drug suspension and its SMEDDS and Niosomes formulation is shown in figure 8.2 and corresponding pharmacokinetic parameters are shown in table 8.4.

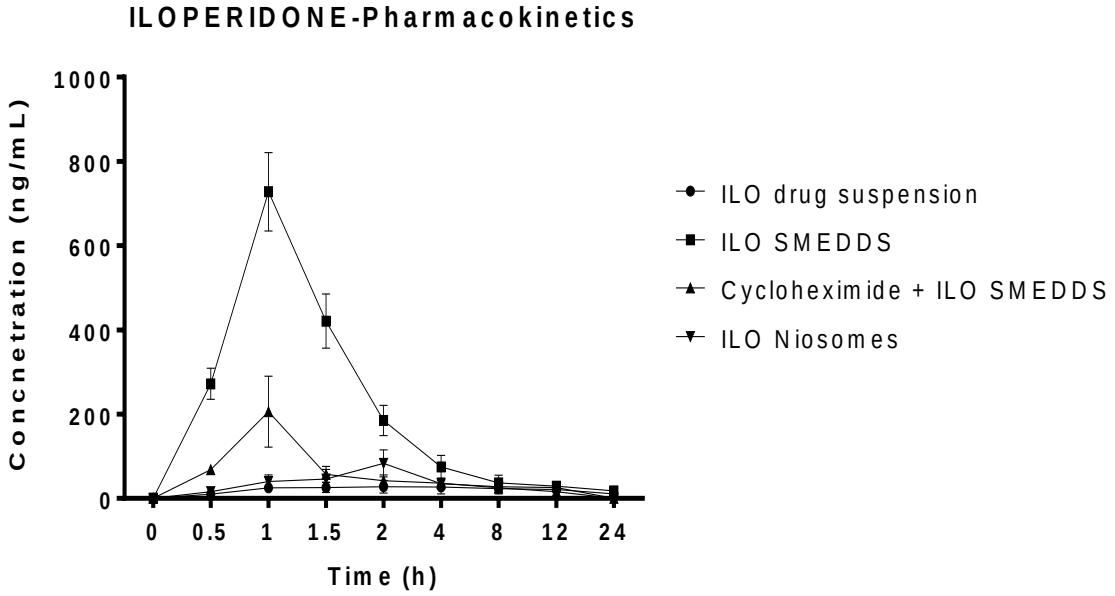

Figure 8.2 Concentration of ILO in plasma after oral administration of formulations

Table 8.4 Pharmacokinetic Parameters for ILO delivered orally as suspension, SMEDDS and Niosomes

Parameters	ILO Suspension	ILO SMEDDS	Cycloheximide (i.p.) + ILO SMEDDS (oral)	ILO Niosomes
C_{max} (ng/mL)	28±07	728±53	206±49	83±24
T_{max} (h)	2.50±1.22	0.91±0.37	1.16±0.51	2.16±0.93
$t_{1/2}$ (h)	15.32±2.10	15.82±1.98	6.838±0.42	18.654±2.17
$AUC_{0\rightarrow t}$ (µg/L*h)	751±163	2015±485	607±176	1098±349
F_{rel}	-	2.68	0.80	1.46
p value		0.0204	0.0262	0.0237

Less absorption of the pure drug can be attributable to slow and incomplete dissolution of this BCS class II drug in the GIT. Whereas for ILO SMEDDS and Niosomes formulations, high C_{max} was observed. AUC for ILO SMEDDS was around 3 times higher than drug suspension. This might be due to dual advantage of increased concentration in plasma and decreased clearance from plasma.

Extent of absorption of drug was significantly increased as evident from F_{rel} for ILO SMEDDS. Compared to drug suspension, ILO SMEDDS enhanced bioavailability by 2.63 folds (p value = 0.0204 indicated significant difference). To evaluate the mechanism of absorption, when SMEDDS was orally administered to cycloheximide treated rats, there was significant decrease in AUC value (p value = 0.0262). F_{rel} value decreased to 0.808 from the 2.63 folds. Hence, it can be concluded that the prepared SMEDDS increased the bioavailability of ILO via lymphatic pathway [4]. This increase in bioavailability would eventually result in an escalation in the intensity of therapeutic effect of ILO.

Comparing the bioavailability of Niosomes with pure drug suspension, niosomes were able to increase bioavailability by 1.46 folds (p value = 0.0237). This might be attributed to colloidal particulate nature of niosomes [17].

Because dissolution is the rate-limiting step in the absorption of a Biopharmaceutics Classification System class II drug, even a small increase in dissolution rate can lead to a significant increase in oral absorption. Improved absorption and higher plasma drug concentrations from niosomal formulation may be due to the surfactant acting as penetration enhancer. Additionally, lipophilic nature, small vesicular size and higher dissolution rate compared to ILO suspension may have contributed to increase in its bioavailability [18].

B) VARDENAFIL formulations

The plasma drug concentration vs. time profile for VDN drug suspension and its SMEDDS and Niosomes formulation is shown in figure 8.4. and the pharmacokinetic parameters for VDN drug and its formulations are shown in table 8.5.

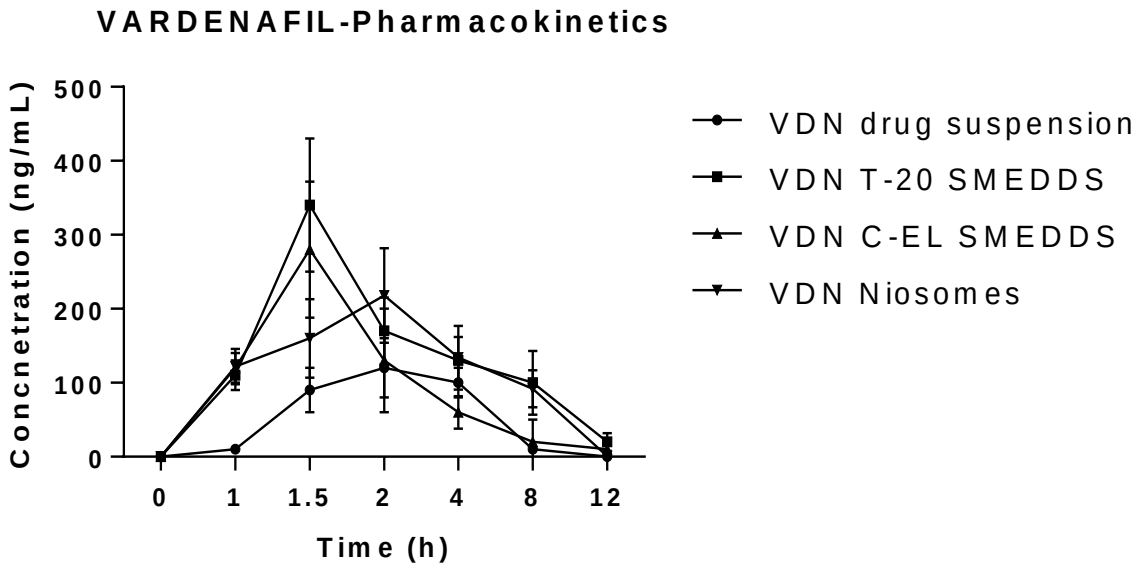

Figure 8.3 Concentration of VDN in plasma after oral administration of formulations

Table 8.5 Pharmacokinetic Parameters for VDN delivered orally as suspension, SMEDDS and Niosomes

Parameters	VDN Drug Suspension	VDN T-20 SMEDDS	VDN C-EL SMEDDS	VDN Niosomes
C_{max} (ng/mL)	120±25	342±38	277±41	218±24
T_{max} (h)	2.33±0.81	1.41±0.37	1.58±0.37	1.83±0.25
$t_{1/2}$ (h)	1.43±0.63	3.11±0.28	2.67±0.54	1.37±0.25
$AUC_{0\rightarrow t}$ (µg/L*h)	471.57±63.72	1358.88±131.50	650.38±72.62	1103.51±89.72
F_{rel}	-	2.88	1.37	2.34
p value		<0.0001	0.0009	<0.0001

The results are clearly indicative that solubilization of drug in oil could overcome the absorption barrier. As the oils used herein increased lipoprotein synthesis and consequent lymphatic absorption, high bioavailability from both the SMEDDS may be attributed to lymphatic transport through transcellular pathway [19]. The increased bioavailability can also be accredited to M cell-mediated transport in Peyer's patch [20].

While comparing both the SMEDDS, it was noticed that there was prominent difference in bioavailability. The primary rate-limiting barrier for drug absorption and diffusion is of intestinal epithelial cell. High content of surfactants in SMEDDS could increase the permeability by disturbing the cell membrane as surfactant molecules are capable of partitioning into the cell membrane where they can form polar defects in the lipid bilayer. At high surfactant concentrations in the cell membrane, surfactant–surfactant contact happens, and the membrane can be dissolved into surfactant–membrane mixed micelles. It ought to be noticed that the surfactant with best enhancement ability requires both hydrophilic and lipophilic domains reaching a balance with intermediate values of HLB. The structural characteristics impart both lipophilic and hydrophilic properties to the surfactant, allowing it to partition between lipid and protein domains of the bilayer. Surfactant molecules have a reversible effect on the opening of tight junction; surfactants may interact with the polar head groups of the lipid bilayers, modifying hydrogen bonding and ionic forces between these groups. It may also embed itself between the lipophilic tails of the bilayers, resulting in a disruption of the lipid-packing arrangement [21].

SMEDDS formulations imparted a higher $AUC_{0\rightarrow t}$ and enhanced the oral bioavailability of VDN (~1.4 fold for VDN C-EL SMEDDS and ~2.8 folds for VDN T-20 SMEDDS). This may be due to increased solubility and prevention of precipitation of drug in git environment provided by encapsulation of drug in oil droplets. The enhanced permeation across intestine and direct uptake by enterocytes resulted into bypass of first pass metabolism which led to increased absorption and bioavailability for VDN SMEDDS as compared to pure VDN suspension.

Unambiguously, the capacity to enhance the permeation of the drug of these formulations is purely based on the nature of the surfactant and specifically on the chemical structure of the surfactant used [22]. While comparing the formulations, the one containing the polysorbates T-20 surfactant enhanced permeation of the drug more than C-EL. It can be owing to the ability of the emulsifying dispersion to interact with the intestinal surface. Surfactants with medium

chain were able to interact well with the intestinal cells layer and increase the transport of the drug as in the case of T-20. On the other hand, C-EL showed poor permeation ability for the drug because of its bulky nature which retarded the actual interaction with the cell surface. Thus, from the above results it can be concluded that judicial selection of the SMEDDS excipients are need for better *in vivo* performance of the formulation.

Compared to drug suspension, niosomal formulation of VDN increased the relative bioavailability by 2.34 folds. Several mechanisms either alone or in combination might have contributed to the increased bioavailability of VDN from niosomes [23]:

a) The aqueous solubility of VDN was significantly improved by incorporation into niosomes.
b) The surfactants may have contributed to an increase in the permeability of the intestinal membrane, increased membrane fluidity or disrupted tight junctions or improved the affinity between lipid particles and the intestinal membrane.
c) The particle size was also a key factor for increased bioavailability. Smaller sized particles may have been efficiently uptaken in the intestine, particularly in the lymphoid sections, where they can bypass the liver first pass metabolism and increase bioavailability.
d) Increased effective surface of nanosized niosomal formulation promote diffusion of drug and therefore prevalence of higher concentration gradient might have resulted in increased rate of absorption.

Thus, it can be concluded from the data that niosomes are a suitable carrier for the oral bioavailability enhancement of VDN, a poorly water-soluble drug undergoing extensive first pass metabolism.

8.6.2 Pharmacodynamic study for Schizophrenia

Negative symptoms of schizophrenia are insufficiently treated by current antipsychotics. Hence, the main objective of the pharmacodynamic study in mice was to evaluate effect of ILO and its formulations; SMEDDS and niosomes, in MK 801 induced Schizophrenia which is a well-established model for negative symptoms.

1. **Rota-Rod Test**

On day 1, the fall-off time in model control (201.83±57.25 sec) was found to be not significantly different from normal control (219.46±37.95 sec). The fall-of time on 6^{th}, 13^{th}, 14^{th} and 15^{th} days in model control were found to be 150.78±17.45, 94.45±13.27, 131.57±10.8 and 100.57±28.8 sec respectively. Whereas in normal control, values were 210.64±20.46, 163.57±13.2, 162±11.9 and 183±25.5 sec respectively. On day 15, fall off time in model control (100.57±18.8 sec) remained significantly lower than normal control animals (183.57±25.5 sec). After treatment with ILO and its formulations SMEDDS and Niosomes, there was significant increase in the fall-off time duration. The values of fall off time on days 1, 6, 13, 14 and 15 in ILO drug suspension were found to be 236.32±23.27, 160.72±18.4, 135.32±12.0, 176.2±19.3, and 120.39±10.6 seconds respectively whereas in ILO SMEDDS treatment group, the values were 198.93±18.9, 192.98±16.61, 236.39±42.9, 210.38±53.29 and 236.83±32.51 sec respectively. Similarly, for ILO niosomes also, there was significant increase in fall-off time (p-value < 0.05) (Figure 8.5).

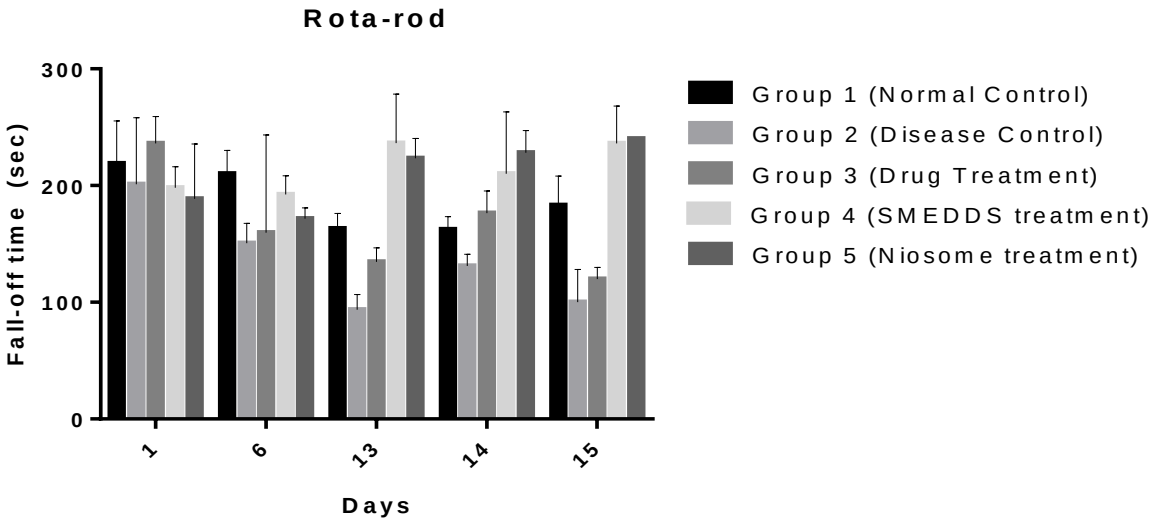

Figure 8.4 Effect of ILO and its formulations on fall off time in Rota-rod test in MK 801 induced psychosis in mice

[Data were analyzed using GraphPad Prism version 6. Data represent mean ± SEM. (n=6). Values are statistically evaluated using t-test. Significant values were compared with model control group ($p<0.05$).]

It is reported that the behavioral stimulant effects of NMDA receptor antagonists are mediated through dopaminergic activity [24]. Hence, the effects of ILO and its formulations on negative symptoms induced by MK801 (NMDA receptor antagonist) were studied in mice. When motor coordination and balance in mice were tested with the rotarod test, model control mice showed lack of motor coordination. Results demonstrated that ILO and its formulations treated mice showed significant improvement in latencies to fall as compared to drug suspension. When groups were compared on different days, lack of motor coordination was more profound in model control and drug suspension treated rats whereas improvement was observed with formulation treated groups, indicating that ILO and its formulations were effective in controlling the negative symptoms of schizophrenia. This proves that behavioral abnormalities induced by NMDA receptor antagonist administration were prevented with the antipsychotic drug - Iloperidone.

2. Forced swim test (FST)

On day 1, the immobility time in disease model control (98.33±29.32secs) mice were found to be not significantly different from normal control (86.33±26.81secs). MK 801 i.p. administration resulted in significant increase as compared with normal control starting from 6th day and continued till the end 15th day.

The immobility time 6th, 13th, 14th, 15th days in model control were found to be 125.33±32.89; 160.66±36.11; 145.33±28.60; 155.66±32.68 respectively whereas in normal control, values were 100.33±36.89; 83.33±24.51; 97.66±27.81; 78.66±21.75 respectively. On day 15, immobility time in model control (155.66±32.68secs) remained significantly ($P<0.001$) elevated than normal control animals (78.66±21.75 secs).

Treatment with ILO significantly decreased immobility time as compared to model control. The values of immobility time on days 1, 6, 13, 14 and 15 in ILO drug treatment were found to be 98.33±22.51; 110.33±24.75; 117.66±26.51; 102.33±32.51 and 118.33±20.54; seconds respectively whereas in ILO SMEDDS treatment, values were 84.66±22.03; 79.33±32.16; 85.66±20.75; 83.33±26.51; 84.33±28.83 respectively. Similarly, treatment with ILO niosomes significantly attenuated the rise in immobility time as compared to model control. The values of days 1, 6, 13, 14, 15 were found to be 92.33±25.37; 101.66±32.51; 86.33±34.54; 82.66±28.54; 80.33±26.54 seconds.

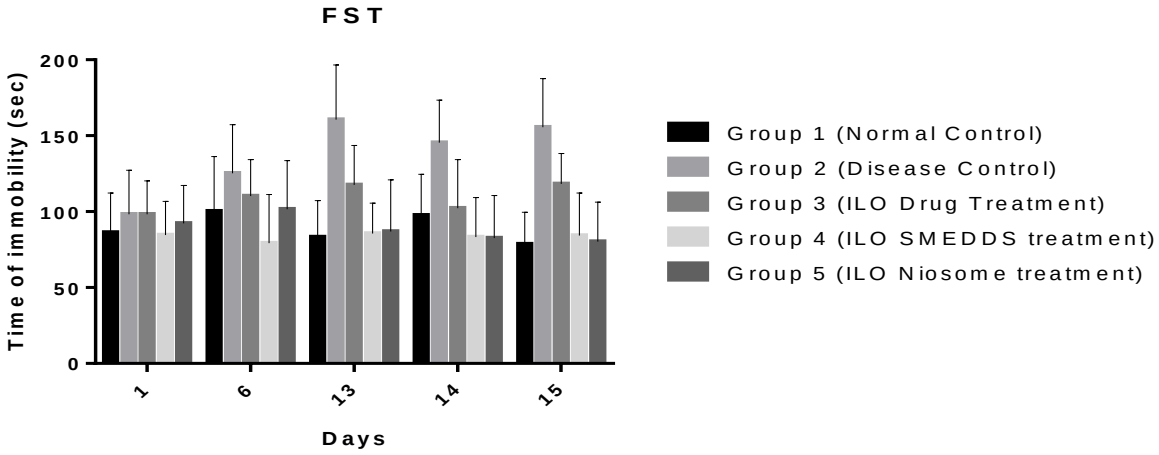

Figure 8.5 Effect of ILO and its formulations on immobility time in forced swim test in MK 801 induced psychosis in mice

The chronic forced swim test is an interesting approach to mimic depression-like negative symptoms of schizophrenia. Mice that were forced to swim in a restricted area from which they could not escape, rapidly ceased attempts to escape and adopted a characteristic immobile posture, which was readily identified and timed. Immobility was taken as depression-related negative symptom in the schizophrenic mice [25].

From the results, it was observed that drug and its formulations, SMEDDS and Niosomes were able to decrease the immobility time compared to MK-801 treated disease control group. This is due to antagonism at 5-HT_2A receptors in the mesocortical pathway by ILO which consequently increased dopamine transmission in the prefrontal cortex which led to increased effectiveness against negative symptoms [26]. Moreover, ILO has more pronounced effect on 5HT2A blockade due to higher affinity for 5HT2A (Ki <10 nM) than D2 receptor (Ki = 1 -100 nM) [27]. This action, along with moderate blockade effect on α2c receptor contributes to mood and cognition effect of animals.

Comparison between drug and its formulations shows increased efficacy of SMEDDS formulation to treat schizophrenia (p-value <0.005). This might be due to increased concentration of drug at target site due to increased bioavailability [20]. Increased plasma concentration might have led to increased concentration in brain as well.

3. Estimation of Brain Nitrite Level

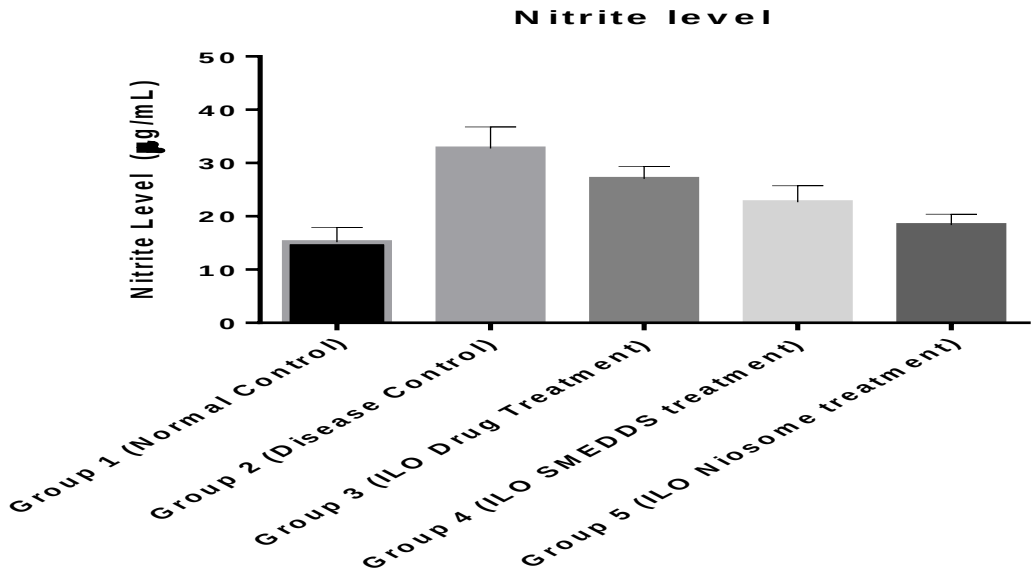

Figure 8.6 Effect of ILO and its formulations on Brain Nitrite level in MK 801 induced psychosis in mice

Disease model control animals showed significant increase in brain nitrite levels (32.71±4.05 µg/mL) as compared to normal control brain nitrite levels (15.14±2.74 µg/mL). ILO drug treatment significantly prevented this increase in brain nitrite levels (27.02±2.34 µg/mL). Interestingly, the effect was more pronounced after treatment with SMEDDS and Niosomes (22.65±3.08 and 18.37±2.04 µg/mL respectively) due to higher plasma concentration than drug suspension treatment. Comparing the effectiveness of SMEDDS and niosomes formulation in decreasing reactive nitrogen species (RNS), niosomes were found to be more effective than SMEDDS (p-value – 0.0184).

Increased reactive nitrogen species (RNS) and altered antioxidant molecules in the brain of schizophrenic subjects supports the role of nitrosative stress in the pathology of this complex disease [28]. As brain tissue has greater susceptibility factors such as low antioxidant defense system, high oxygen utilization and high levels of polyunsaturated fatty acid which may oxidize easily, nitrosative stress becomes a major pathogenic cause of schizophrenia. Severity of the

disease may depend on the activity of free radicals generated during metabolism of neurotransmitters. For example, metabolism of neurotransmitters including dopamine and glutamate generates large amounts of ROS that are known to affect synaptic plasticity and signal transduction pathways via redox-sensitive receptors, such as NMDA and D2 receptors. Indeed, continuous generation of ROS and RNS further cause neuronal cell death via glutamate mediated oxidative stress processes. Moreover, the increased levels of MDA and NO found in postmortem studies of schizophrenia brains further support the role of oxidative stress and nitrosative stress in the pathogenesis of the disease. Thus, it has been suggested that these culprits might be targets for the treatments of schizophrenia especially negative and cognitive symptoms associated with the disease. Inhibition of nitrosative and oxidative stress mechanisms is one of the novel therapeutic approaches for the reduction of neuronal damage in schizophrenia [28].

Following the treatment with ILO and its formulations, SMEDDS and Niosomes, there was decrease in nitrite levels of brain in treatment groups (group 3, 4 and 5) compared to disease control group. Comparison between drug and its formulations, the formulations showed superiority in decreasing the RNS level in the brain. This might be due to increased bioavailability of drug via lipid-based drug delivery approach.

8.6.3 Pharmacodynamic study for Erectile Dysfunction

After STZ administration via i.p. route, rats having blood glucose levels >16.6mmol/l were considered as diabetic models. These rats were used as model control (group 2) and also as test groups (group 3, 4 and 5). As described in the procedure, after 8 weeks of treatment with VDN suspension and VDN formulations, different parameters were measured for the rats.

As seen from the pharmacokinetic studies, T-20 VDN SMEDDS showed better effectiveness compared to C-EL VDN SMEDDS. Considering this fact, only T-20 VDN SMEDDS formulation was further taken into consideration for pharmacodynamic study.

Parameters:

1. Analysis of serum parameters

1.1 Estimation of serum testosterone levels in diabetic rats

Testosterone has been postulated to improve vascular function by acting as a vasodilator. Testosterone-induced vasodilation has been demonstrated in isolated animal and human blood vessels and involves the activation of rapid nongenomic signaling pathways [29].

The serum testosterone levels of the rats treated with drug suspension, SMEDDS and niosomes formulation were found to be 98.62 ± 15.92, 145.2 ± 32.42 and 132.9 ± 24.05 ng/dL respectively which were significantly higher than that of diabetic control rats (75.34 ± 12.37 ng/dL) (p-value < 0.05). However, none of the treatment groups showed testosterone levels comparable to normal control group (214.52 ± 29.76 ng/dL).

Hence, monotherapy of PDE-5 inhibitors needs supplementing with testosterone (in case of hypotestosternonemics) to treat erectile dysfunction prevailing in the diabetic patients for acquiring maximum benefit of PDE-5 treatment.

1.2 Estimation of serum nitric oxide

The serum nitric oxide levels of the rats treated with VDN suspension, SMEDDS and Niosomes formulations were found to be 194.60 ± 22.62, 252.58 ± 32.80 and 229.60 ± 22.91 μmol/L respectively which were significantly higher than that of diabetic rats (111.68 ± 15.59). The results of NO level after treatment with VDN and its formulations were comparable to normal control group (274.52 ± 18.49 μmol/L).

The release of nitric oxide (NO) from noradrenergic, noncholinergic nerve terminals and the endothelium is a major mediator of erection. NO works in the smooth muscle cell to activate a soluble guanylyl cyclase. This enzyme leads to an increase in the production of the second messenger cyclic guanosine monophosphate (cGMP). Increased cGMP concentration activates protein kinase G (PKG). The activated PKG phosphorylates multiple intracellular proteins to cause: sequestration of intracellular Ca^{2+} in the endoplasmic reticulum, inhibition of cell

membrane calcium influx channels, and opening of potassium channels with resultant myocyte hyperpolarization. The resultant decrease in intracellular calcium concentration and hyperpolarization leads to smooth muscle relaxation via what is essentially a reversal of the process for smooth muscle contraction described above [30].

The results of our study showed that the rats treated with VDN suspension and its formulations had significantly more serum nitric oxide levels than the diabetic control rats. This, in turn, will increase the response for tumescence in erectile dysfunction.

2. Evaluation of sperm parameters in diabetic rats

The sperm count of the rats treated with VDN and its formulations SMEDDS and Niosomes were found to be 8.26 ± 1.28, 10.98 ± 1.48 and 9.03 ± 1.27 million respectively which were significantly higher than that of diabetic rats (6.25 ± 0.61 million). The results of total sperm count for treatment with drug and its formulations were comparable to normal control group (12.42 ± 1.21 million). The photomicrograph of sperms for all treatment groups and controls are shown in figure 8.7.

The percentage of defective sperms of the rats treated with VDN and its formulations SMEDDS and Niosomes were found to be 24.38 ± 2.07, 16.82 ± 3.81 and 18.98 ± 3.24 % respectively which were significantly lower than that of diabetic control rats (32.12 ± 5.10 %). The results of percentage of defective sperms for treatment with drug and its formulations were comparable to normal control group (13.42 ± 1.21%).

Oligospermia and sperm motility may or may not have any effect on erectile dysfunction but they surely contribute to fertility of an individual. Reduction of sperm count in diabetic rats supports the fact that chronic diabetes induces oxidative stress in the testes thereby causing reduction in the formation of sperm cells. Presence of defective sperms indicate the production of free radicals in the testes. Treatment with PDE-5 inhibitor VDN and its formulations showed an increase in sperm count and decrease in percentage of defective sperms [31]. Although both the formulations, SMEDDS and Niosomes showed significant increment in sperm count when

compared with diabetic control group, treatment with SMEDDS showed significant improvement in sperm count. This might be due to higher bioavailability from SMEDDS formulation. Additionally, structural likeliness between Tween 20 structure and lipid A of lipopolysachharides, gives an easy access inside the cellular structure. Once inside the cell, Tween might release fatty acids that activate or inhibit Toll like receptors and NF-κB depending on the receptor, cell type or the particular fatty acid in question. Due to these anti-oxidant effects, SMEDDS might have produced more pronounced effect than niosomes.

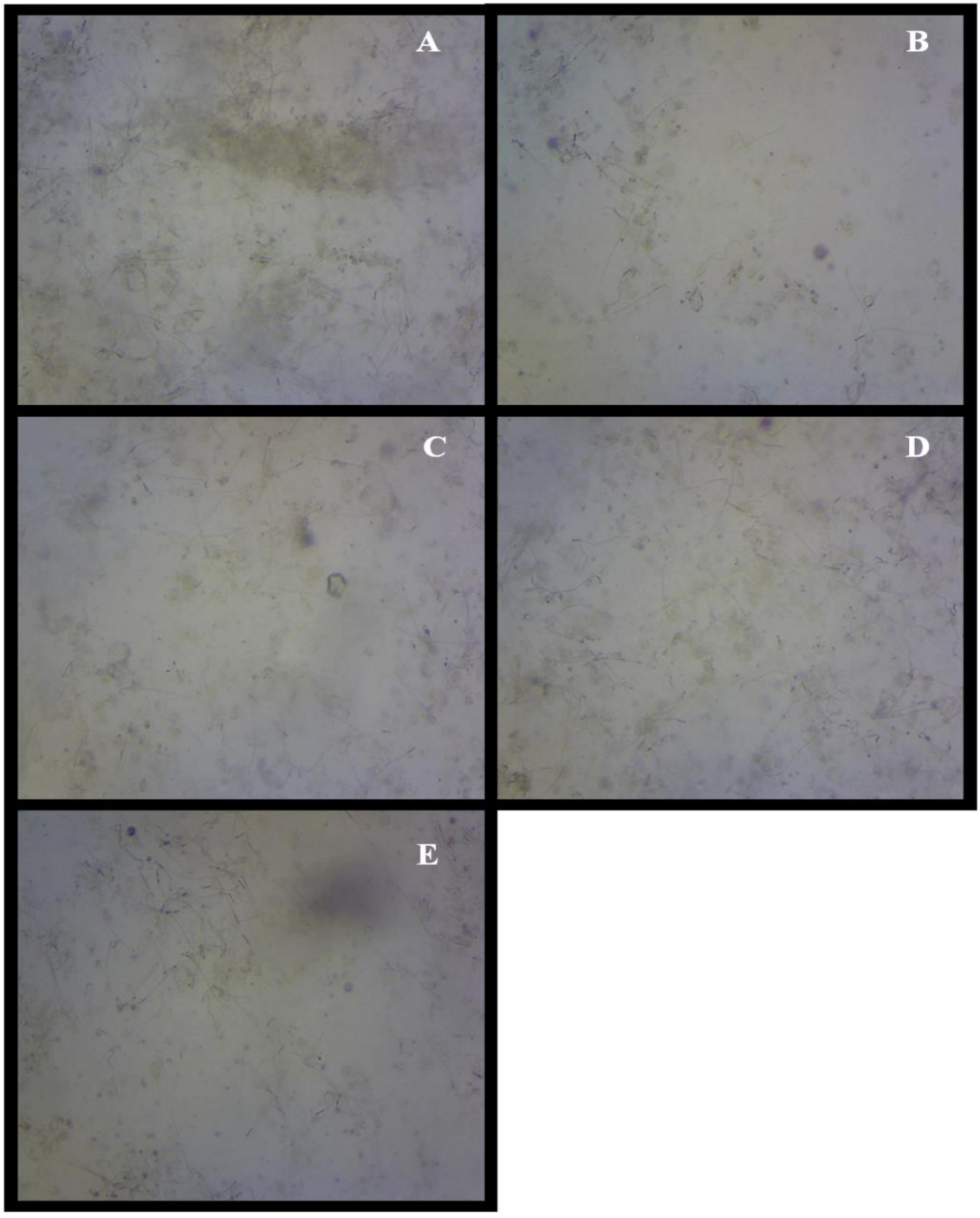

Figure 8.7: Sperm count study for VDN and its formulations

A-Normal Control, B- Disease Control, C-Drug Suspension, D-SMEDDS Formulation, E-Niosomes Formulation

3. Histopathology of sexual organs

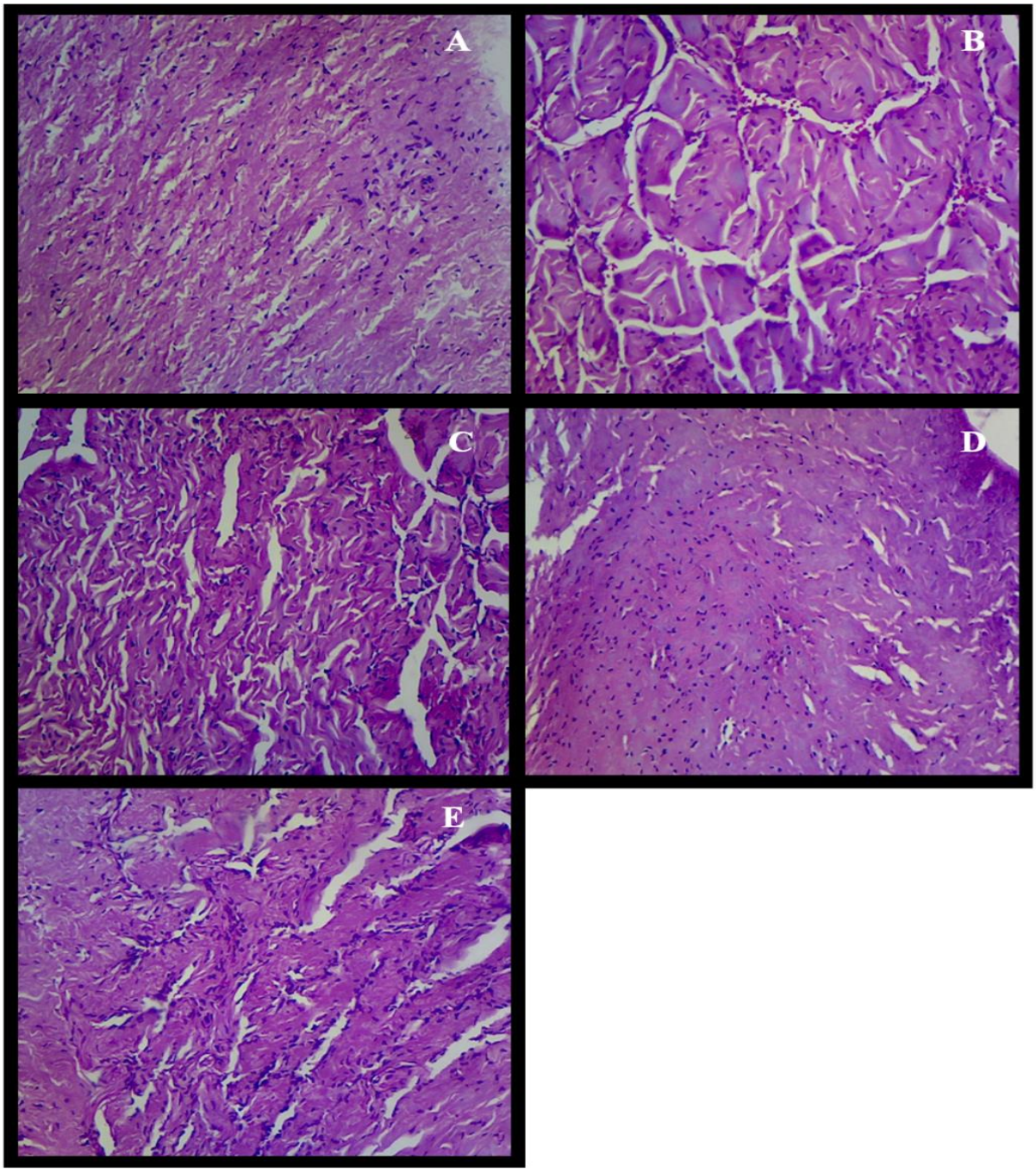

Figure 8.8: Hematoxylin – Eosin staining of Penis

A-Normal Control, B- Disease Control, C-Drug Suspension, D-SMEDDS Formulation, E-Niosomes Formulation

In the figure 8.8 of H-E staining of penile tissue, for normal control group (A), intact corpus cavernosum is seen, whereas in diabetic control group (B) the surrounding smooth muscle cells are absent. The spongy corpus cavernosum seems to be replaced by fat cells making it irresponsive to stimuli. Whereas in treatment groups (C), (D) and (E) (VDN drug, VDN SMEDDS and VDN Niosomes respectively), there is presence of somewhat intact corpus spongiosum compared to disease control (B). In all three images (C, D and E), corpus cavernosum contains cavernous spaces with vascular spaces.

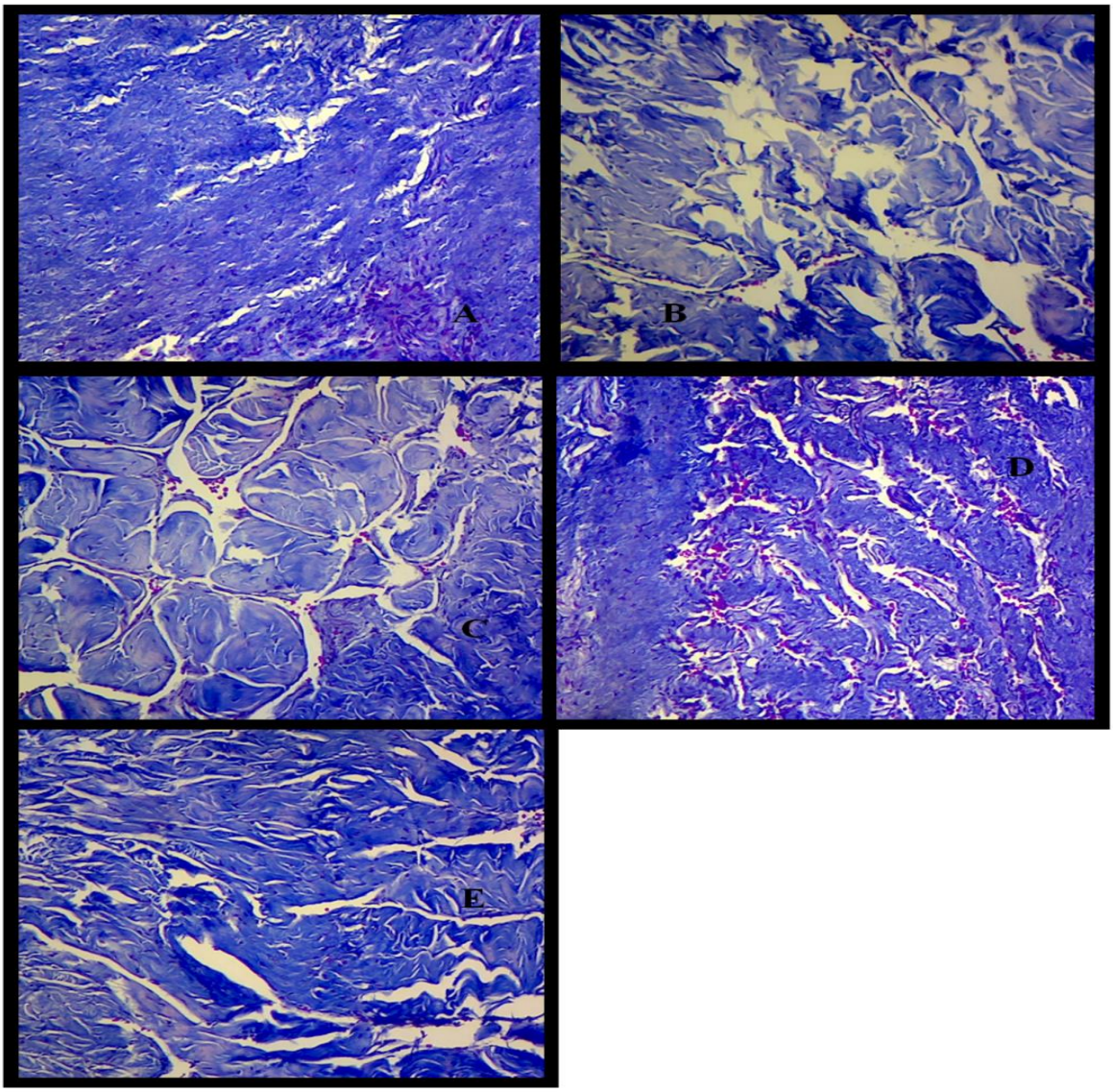

Figure 8.9: Masson Trichome Staining of Penis A-Normal Control, B- Disease Control, C-Drug Suspension, D-SMEDDS Formulation, E-Niosomes Formulation

In the Masson Trichome Staining of Penile tissue, collagen is stained blue whereas smooth muscles are stained pink-red. Compared to Normal control (A), the diabetic control (B) group shows more collagen tissue, whereas in other treatment groups (C), (D) and (E), the smooth muscles are more as can be seen from more pink-red coloration.

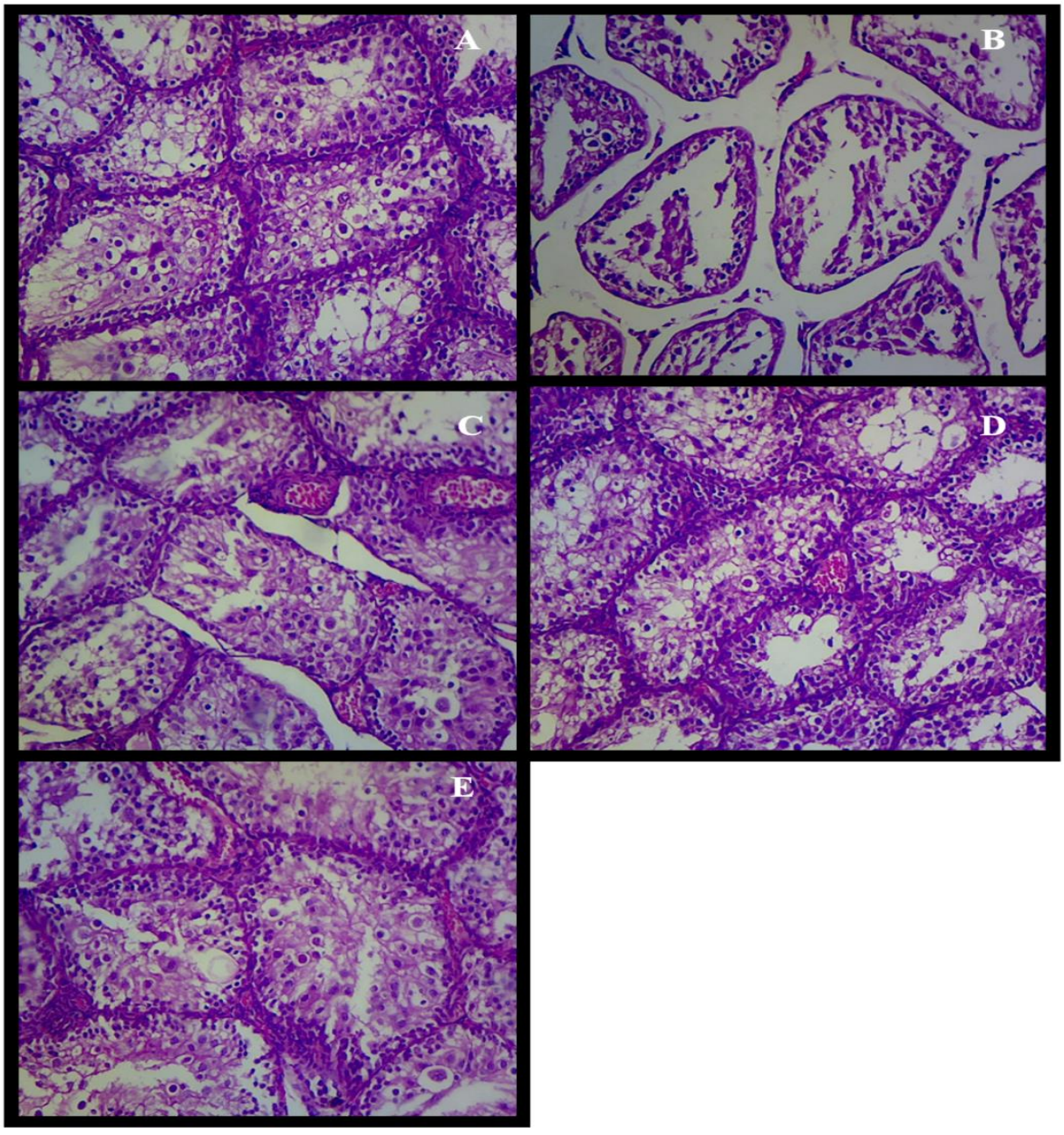

Figure 8.10 Hematoxylin – Eosin staining of Testis

A-Normal Control, B- Disease Control, C-Drug Suspension, D-SMEDDS Formulation, E-Niosomes Formulation

H-E staining of normal testis (A) shows intact architecture comprising closely packed seminiferous tubules having intact germinal epithelium and supporting sertoli cells. The spermatogonia appear adequate in number and size. The spermatocytes, spermatids and spermatozoa appear adequate. The seminiferous tubules are bounded by fibrovascular septa containing fibroblasts, collagen fibers and vascular spaces. Whereas in diabetic control (B) the testis shows distorted architecture comprising of some closely packed seminiferous tubules with distortion of germinal epithelium and loss of supporting sertoli cells. The spermatogonia, spermatocytes, spermatids and spermatozoa appear disintegrated. The seminiferous tubules are separated by fibrovascular septa containing fibroblasts, collagen fibers and vascular spaces. Whereas in sections studied from the testis of the rats treated with VDN and its formulations, testis shows partially distorted architecture comprising of few dispersed seminiferous tubules with distortion of germinal epithelium and supporting sertoli cells. The spermatogonia, spermatocytes, spermatids and spermatozoa appear decreased in VDN drug suspension treatment (C), whereas VDN SMEDDS and Niosomes treated testis (D and E) shows somewhat distorted architecture but it comprises some of closely packed seminiferous tubules. The spermatogonia and spermatocytes appear adequate. The seminiferous tubules are separated by fibrovascular septae containing fibroblasts, collagen fibers and vascular spaces.

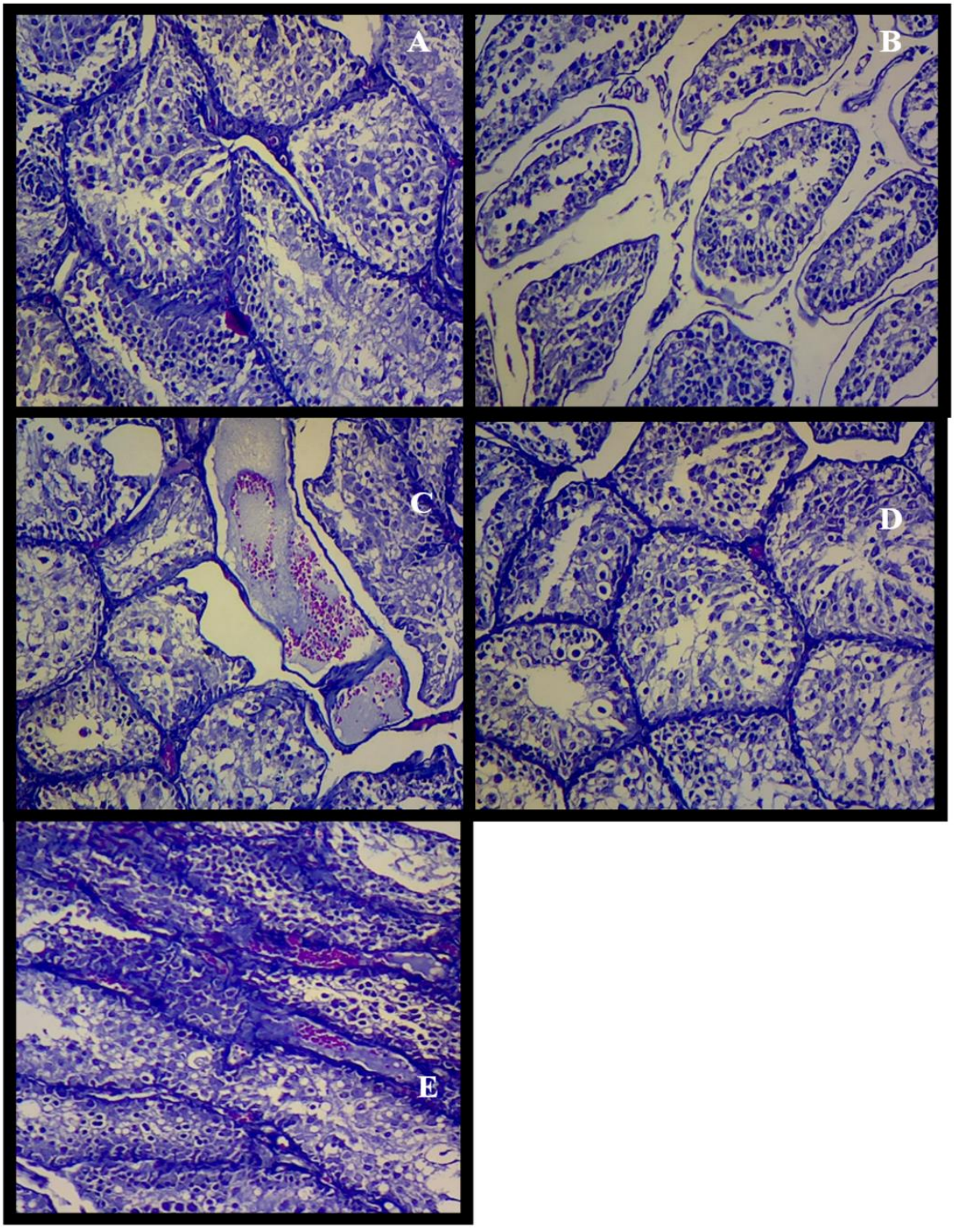

Figure 8.11 Masson Trichome Staining of Testis A-Normal Control, B- Disease Control, C-Drug Suspension, D-SMEDDS Formulation, E-Niosomes Formulation

The Masson-stained section provides a good view of the interstitial connective tissue (CT), blood vessels, and interstitial (Leydig) cells. As described for H-E staining, the normal control (A) indicated intact architecture whereas for disease control group (B), it was very distorted. However, after treatment with VDN and its formulation (C, D and E), there was remodeling of tissue which showed nearly compact structure as seen for normal. In MTS of testis, connective tissue septa and the interstitial tissue in amongst the seminiferous tubules are stained a bluish-purple hue while the seminiferous tubules are stained with reddish color.

Corpus cavernosum is a pair of sponge like tissue in the penis which contains most of the blood in the penis. This spongy tissue presses against dense tunica albuginea constricting the veins preventing the blood from leaving the penis thus maintaining sufficient length and girth of the penis for a proper erection. The histopathology study suggested that all the rats had intact corpus cavenosa with proper cavernous and vascular spaces [32].

The tunica albuginea is a fibrous tissue containing 5 % elastin & about 95 % of collagen. It is directly involved in maintaining an erection by constricting the dorsal vein of the penis, preventing the blood to leave the penis thus maintaining an erection. Corpus spongiosum is the mass of spongy tissue that surrounds the urethra in the penis. It functions by preventing the urethra from getting closed maintaining the urethra open so as to form a viable channel for ejaculation. Our histopathology reports suggested that all the rats treated with VDN and its formulations had intact tunica albuginea with surrounding smooth muscle cells.

The percentage of smooth muscle in the penile tissue determines the degree of erection in the rats. Higher the smooth muscle higher is the erectile response. Increment in the levels of collagen indicates the development of fibrosis, thus decreasing the smooth muscle content and development of erectile dysfunction [16]. The MTS of the penile tissue showed that the rats treated with VDN and its formulations had increased smooth muscle as compared to diabetic control.

The improved sexual performance of the treated rats would have been due to contributive effects like increased serum testosterone levels and nitric oxide levels along with increase in smooth muscles in penile tissue and intact architecture of testes due to increased bioavailability of drug by the VDN formulations. As discussed in serum parameters estimation (section 8.6.3/1), there was increased level of testosterone (up to 145.2 ng/dL) and nitric oxide level (up to 252.58 µmol/L). Sperm parameters also indicated improved sperm count owing to Tween 20 in its composition (Section 8.6.3/2). Considering these pharmacodynamic effects along with positive histopathology results indicating increased smooth muscle level, it was concluded that there will be improved sexual performance. Comparative pharmacodynamics of VDN SMEDDS and niosomes indicated that SMEDDS formulation proved to be more effective for ED treatment. In SMEDDS formulation surfactant Tween 20 also played a major role in increasing the sperm count. Effect on tissue morphology and architecture indicated similar actions of SMEDDS and niosomes. There might be difference in acute effect of formulations on tissue architecture but herein we have performed the study for 8 weeks which showed similar effects.

REFERENCES

1. Porter, C. J. H., Pouton, C. W., Cuine, J. F., & Charman, W. N. (2008). Enhancing intestinal drug solubilisation using lipid-based delivery systems. *Advanced Drug Delivery Reviews, 60*(6), 673-691, doi:https://doi.org/10.1016/j.addr.2007.10.014.
2. Kollipara, S., & Gandhi, R. K. (2014). Pharmacokinetic aspects and in vitro–in vivo correlation potential for lipid-based formulations. *Acta Pharmaceutica Sinica. B, 4*(5), 333-349, doi:10.1016/j.apsb.2014.09.001.
3. Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers (2005). *Food and Drugs Administration*.
4. Garg, B., Katare, O. P., Beg, S., Lohan, S., & Singh, B. (2016). Systematic development of solid self-nanoemulsifying oily formulations (S-SNEOFs) for enhancing the oral bioavailability and intestinal lymphatic uptake of lopinavir. *Colloids and Surfaces B: Biointerfaces, 141,* 611-622, doi:https://doi.org/10.1016/j.colsurfb.2016.02.012.
5. Parmar, C., Parikh, K., Mundada, P., Bhavsar, D., & Sawant, K. (2018). Formulation and optimization of enteric coated bilayer tablets of mesalamine by RSM: In vitro – In vivo investigations and roentogenographic study. *Journal of Drug Delivery Science and Technology, 44*, 388-398, doi:https://doi.org/10.1016/j.jddst.2018.01.008.

6. Balu, D. T. (2016). The NMDA Receptor and Schizophrenia: From Pathophysiology to Treatment. *Advances in pharmacology (San Diego, Calif.), 76*, 351-382, doi:10.1016/bs.apha.2016.01.006.
7. Eyjolfsson, E. M., Brenner, E., Kondziella, D., & Sonnewald, U. (2006). Repeated injection of MK801: An animal model of schizophrenia? *Neurochemistry International, 48*(6), 541-546, doi:https://doi.org/10.1016/j.neuint.2005.11.019.
8. Yao, J. K., Leonard, S., & Reddy, R. D. (2004). Increased nitric oxide radicals in postmortem brain from patients with schizophrenia. *Schizophrenia bulletin, 30*, 923-934.
9. Kim, C., Speisky, M. B., & Kharouba, S. N. (1987). Rapid and sensitive method for measuring norepinephrine, dopamine, 5-hydroxytryptamine and their major metabolites in rat brain by high-performance liquid chromatography: Differential effect of probenecid, haloperidol and yohimbine on the concentrations of biogenic amines and metabolites in various regions of rat brain. *Journal of Chromatography A, 386*, 25-35.
10. Deacon, R. M. J. (2013). Measuring Motor Coordination in Mice. *Journal of Visualized Experiments : JoVE*(75), 1-8, doi:10.3791/2609.
11. Can, A., Dao, D. T., Arad, M., Terrillion, C. E., Piantadosi, S. C., & Gould, T. D. (2012). The Mouse Forced Swim Test. *Journal of Visualized Experiments : JoVE*(59), 1-5, doi:10.3791/3638.
12. Bryan, N. S., & Grisham, M. B. (2007). Methods to Detect Nitric Oxide and its Metabolites in Biological Samples. *Free radical biology & medicine, 43*(5), 645-657, doi:10.1016/j.freeradbiomed.2007.04.026.
13. Bai, Y., & An, R. (2015). Resveratrol and sildenafil synergistically improve diabetes-associated erectile dysfunction in streptozotocin-induced diabetic rats. *Life Sciences, 135*, 43-48, doi:https://doi.org/10.1016/j.lfs.2015.04.020.
14. Ambade, V. N., Sharma, Y. V., & Somani, B. L. (1998). Methods for estimation of blood glucose : A comparative evaluation. *Medical Journal, Armed Forces India, 54*(2), 131-133, doi:10.1016/S0377-1237(17)30502-6.
15. Kashif, S., Razdan, R., Jamwal, R., & Goswami, S. K. (2018). Allantoin Alleviates Male Sexual Dysfunction in Diabetic Rats through Augmenting the Level of Testosterone. *Journal of Young Pharmacists, 10*(1), 57-61, doi:10.5530/jyp.2018.10.14.
16. Goswami, S. K., Gangadarappa, S. K., Vishwanath, M., Razdan, R., Jamwal, R., Bhadri, N., et al. (2016). Antioxidant Potential and Ability of Phloroglucinol to Decrease Formation of Advanced Glycation End Products Increase Efficacy of Sildenafil in Diabetes-Induced Sexual Dysfunction of Rats. *Sexual Medicine, 4*(2), 106-114, doi:10.1016/j.esxm.2015.12.002.
17. Arzani, G., Haeri, A., Daeihamed, M., Bakhtiari-Kaboutaraki, H., & Dadashzadeh, S. (2015). Niosomal carriers enhance oral bioavailability of carvedilol: effects of bile salt-enriched vesicles and carrier surface charge. *International Journal of Nanomedicine, 10*, 4797-4813, doi:10.2147/IJN.S84703.
18. Kamboj, S., Saini, V., & Bala, S. (2014). Formulation and Characterization of Drug Loaded Nonionic Surfactant Vesicles (Niosomes) for Oral Bioavailability Enhancement. *The Scientific World Journal, 2014*, 1-8, doi:10.1155/2014/959741.
19. Mundada, V., Patel, M., & Sawant, K. (2016). Submicron Emulsions and Their Applications in Oral Delivery. *Critical Reviews™ in Therapeutic Drug Carrier Systems, 33*(3), 265-308, doi:10.1615/CritRevTherDrugCarrierSyst.2016017218.

20. Deepa, P., & Krutika, K. S. (2009). Self Micro-Emulsifying Drug Delivery System: Formulation Development and Biopharmaceutical Evaluation of Lipophilic Drugs. *Current Drug Delivery, 6*(4), 419-424, doi:http://dx.doi.org/10.2174/156720109789000519.
21. Wu, W., Wang, Y., & Que, L. (2006). Enhanced bioavailability of silymarin by self-microemulsifying drug delivery system. *European Journal of Pharmaceutics and Biopharmaceutics, 63*(3), 288-294, doi:https://doi.org/10.1016/j.ejpb.2005.12.005.
22. Bandivadekar, M., Pancholi, S., Kaul-Ghanekar, R., Choudhari, A., & Koppikar, S. (2013). Single non-ionic surfactant based self-nanoemulsifying drug delivery systems: formulation, characterization, cytotoxicity and permeability enhancement study. *Drug development and industrial pharmacy, 39*(5), 696-703, doi:10.3109/03639045.2012.687745.
23. Ong, S. G. M., Ming, L. C., Lee, K. S., & Yuen, K. H. (2016). Influence of the Encapsulation Efficiency and Size of Liposome on the Oral Bioavailability of Griseofulvin-Loaded Liposomes. *Pharmaceutics, 8*(3), 1-25, doi:10.3390/pharmaceutics8030025.
24. Rung, J. P., Carlsson, A., Rydén Markinhuhta, K., & Carlsson, M. L. (2005). (+)-MK-801 induced social withdrawal in rats; a model for negative symptoms of schizophrenia. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 29*(5), 827-832, doi:https://doi.org/10.1016/j.pnpbp.2005.03.004.
25. Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015). The Forced Swim Test as a Model of Depressive-like Behavior. *Journal of Visualized Experiments : JoVE*(97), 52587, doi:10.3791/52587.
26. Montes, A. B., & Rey, J. A. (2009). Iloperidone (Fanapt): An FDA-Approved Treatment Option for Schizophrenia. *Pharmacy and Therapeutics, 34*(11), 606-613.
27. Tonin, F. S., Wiens, A., Fernandez-Llimos, F., & Pontarolo, R. (2016). Iloperidone in the treatment of schizophrenia: an evidence-based review of its place in therapy. *Core Evidence, 11*, 49-61, doi:10.2147/CE.S114094.
28. Bitanihirwe, B. K. Y., & Woo, T.-U. W. (2011). Oxidative Stress in Schizophrenia: An Integrated Approach. *Neuroscience and biobehavioral reviews, 35*(3), 878-893, doi:10.1016/j.neubiorev.2010.10.008.
29. Lopes, R. A. M., Neves, K. B., Carneiro, F. S., & Tostes, R. C. (2012). Testosterone and Vascular Function in Aging. *Frontiers in Physiology, 3*, 1-9, doi:10.3389/fphys.2012.00089.
30. Francis, S. H., Busch, J. L., & Corbin, J. D. (2010). cGMP-Dependent Protein Kinases and cGMP Phosphodiesterases in Nitric Oxide and cGMP Action. *Pharmacological Reviews, 62*(3), 525-563, doi:10.1124/pr.110.002907.
31. Adewoyin, M., Ibrahim, M., Roszaman, R., Md Isa, M. L., Mat Alewi, N. A., Abdul Rafa, A. A., et al. (2017). Male Infertility: The Effect of Natural Antioxidants and Phytocompounds on Seminal Oxidative Stress. *Diseases, 5*(1), 1-26, doi:10.3390/diseases5010009.
32. Krause, W. K. H. (2007). Physiology of Erection. In *Drugs compromising male sexual health* (pp. 23-30). Heidelberg: Springer Science & Business Media.