

HEPATIC STRUCTURAL CHANGES IN HYPERVITAMINOSIS A IN ALBINO RATS

(Rattus norvegicus)

By

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A dissertation in partial fulfillment of the requirements of the Master of Science Degree in

Human Anatomy of the University of Nairobi

DECLARATION

I hereby confirm that this dissertation is my original work and has not been presented elsewhere for examination.

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DEDICATION

To the boys,

Beda and Mishael

The arrows in my quiver

&

Mercy

The queen of the house

To God be the glory

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LIST OF ABBREVIATIONS AND ACRONYMS

BMI	Body mass index
CRF	Chronic renal failure
ECM	Extracellular matrix
HSC	Hepatic stellate cells
MMPs	Matrix metalloproteinases
MVM	Multivitamins and minerals
PAS	Periodic acid Schiff staining
RAS	Renin angiotensin system
RBP	Retinol binding protein
RDI	Recommended dietary intake
SUR	Systematic uniform random sampling
TUNEL test	Terminal deoxynucleotidyl transferase nick end labelling test
UL	Upper limit of safety

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SUMMARY

Background: The storage of vitamin A and the structural effect of hypervitaminosis A on the liver demonstrate implausible spatial and temporal patterns. Even though hepatic stellate cells are uniformly distributed within the hepatic lobules, there exists a genetically predetermined zonal gradient in the pattern of storage of vitamin A in the liver. Moreover, persistent hypervitaminosis A has been known to cause hepatic stellate cells hyperplasia and liver fibrosis. The temporal sequence and the possible spontaneous reversion remain largely unexplored.

Objective: To determine the structural changes induced by persistent hypervitaminosis A in the liver and the short-term sequelae after its withdrawal

A Study design: Quasi experimental study design

Study setting: Departments of Human Anatomy, Human Pathology and Veterinary Anatomy and Physiology

Materials and methods: Ethical approval to conduct the study was obtained from the Biosafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine, University of Nairobi. A total of 45 adult albino rats were used in this study and divided into 3 groups: A, B, and C. Group A rats were given 300,000 IU/Kg of vitamin A every alternate day via subcutaneous injection for 4 weeks with half of them being followed for a further 4 weeks off treatment. Group B rats were similarly given 300,000 IU/Kg of vitamin A every alternate day via subcutaneous injection for 8 weeks. Half of the members of this group were also followed up for 4 more weeks off treatment. Group C were the control group. All rats were maintained on a normal diet and fed ad libitum. Two rats from the control group were used for baseline results. Five rats from group A were euthanized at weeks 2, 4, 6 and 8 while those from group B were euthanized at weeks 6, 8, 10 and 12. The volume of rat

livers were estimated using the Scherle method and SUR sampling used to select harvested liver segments. These were processed for histological staining using the hematoxylin and eosin, Masson's trichrome and the periodic acid Schiff stains and images taken using a digital camera. General changes in the hepatic parenchyma were noted and recorded. Stereological methods were used to estimate HSC and hepatocyte area densities. Obtained data was keyed into the SPSS for coding, tabulation and statistical analysis. Means and standard deviations were then determined. The one-way ANOVA was used to compare the hepatocyte and HSC densities for the 3 groups and changes in densities over time. The Tukey test was used as the post-hoc test to detect between which groups the significant difference lay. A *p* value <0.05 was considered significant at 95% confidence interval. Results were presented in micrographs, tables and graphs.

Results: There was a slight increase in the absolute liver volume with exposure to high dose vitamin A which continued to rise with stopping the exposure. The body mass normalized liver volume on the other hand, showed a marked reversible decline following persistent exposure to high dose vitamin A ($p=0.074$). Hepatic parenchyma distortion and hepatocyte and stellate cell vacuolation were more pronounced around the pericentral areas whereas the compensatory hyperplasia and hypertrophy of these cells seemed to emanate from the periportal areas. For both the acutely and persistently exposed groups, there was a significant temporal increase in hepatocyte density with exposure to vitamin A ($p<0.001$) followed by a significant decline when the exposure was stopped ($p<0.001$). Similarly, there was an increase in stellate cell densities ($p=0.066$) followed by a significant decline with discontinuation of exposure to vitamin A ($p=0.013$).

Conclusion: Hypervitaminosis A causes significant but reversible spatial and temporal changes in both the gross and histological aspects of the liver. Withdrawal of exposure to vitamin A in cases

of suspected toxicity would therefore be paramount in the management of both acute and chronic hypervitaminosis A.

CHAPTER ONE

1. INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Vitamin A is a fat-soluble vitamin essential for vision, growth, cellular differentiation and the integrity of the immune system (Genaro and Martini, 2004; Raoofi et al., 2010). It is also an important antioxidant that plays a vital role in various body processes including growth and metabolism. Dietary vitamin A is obtained from retinyl esters found in some vegetables and animal foods like liver, kidney, milk and fish oil (Ahluwalia et al., 2016). Fortified foods and drug supplements have also increasingly become important dietary sources of vitamin A (Murphy et al., 2007). These supplements are either taken as vitamin A capsules or as multivitamin/ mineral (MVM) supplements (National Institutes of Health State-of-the-Science Panel, 2007). However, when taken at higher doses than recommended, vitamin A has been shown to be harmful to the liver (U.S. Preventive Services Task Force, 2003). Thus, strict adherence to the recommended dietary intake is encouraged. In addition to dietary excess, other conditions associated with hypervitaminosis A include chronic renal failure and use of retinoids in the treatment of various skin conditions (Geubel et al., 1991; Verneau et al., 1984; Werb et al., 1979).

Hepatic stellate cells (HSC) are the main cells involved with the storage of vitamin A in the body (Higashi et al., 2005; Nagy et al., 1997). There exists a zonal gradient in the pattern of storage of vitamin A in the liver even though HSC are uniformly distributed within the hepatic lobules (Higashi et al., 2004; Wake and Sato, 1993; Zou et al., 1998). This pattern is genetically determined and thus varies from one animal species to another (Wake and Sato, 1993). However, few studies have described this pattern in the albino rat nor has the response of hepatocytes to hypervitaminosis A been adequately documented. Establishment of this pattern

in rats would be important as a guide to future studies on hepatic vitamin A metabolism. This is because rats are among the laboratory rodents most commonly used in medical research.

Apart from hyperplasia of HSC, the body responds to acute hypervitaminosis A by an increase in the number and the enlargement of the vacuoles found in the HSC and hepatocytes (Nagy et al., 1997). The temporal sequence of these changes however, to our knowledge, is yet to be fully documented. The possible spontaneous reversion of these changes following cessation of exposure to hypervitaminosis A remains largely unknown. Furthermore, the hepatocyte alterations in number and size accompanying the variations in the HSC hyperplasia and vacuolation have scarcely been investigated nor quantified.

Hepatic changes seen in persistent hypervitaminosis A include transformation of HSC into myofibroblasts. This has been known to lead to increased collagen deposition and thus per-portal fibrosis and central vein sclerosis (Castaño et al., 2006; Moreira, 2007; Nollevaux et al., 2006; Pinzani, 1995). However, the duration over which the transformation of HSC occurs, the accompanying increase in hepatic collagen fiber deposition and the short term sequelae of these changes following the withdrawal of vitamin A is barely known. This study therefore aimed at determining the structural changes induced by persistent hypervitaminosis A in the liver and the short-term sequelae following discontinued administration of the vitamin.

1.2 LITERATURE REVIEW

1.2.1 Normal Biology and Sources of Vitamin A

Vitamin A is a fat-soluble vitamin essential for vision, growth, cellular differentiation and the integrity of the immune system (Genaro and Martini, 2004; Raoofi et al., 2010). It is an antioxidant that is important for reproductive and neurological processes. It is known to inhibit lipid peroxidation and plays a vital role in preventing retinopathy in premature infants (Mowry et al., 2015). The recommended dietary intake (RDI) for vitamin A is 700 μg and 600 μg for both men and female respectively (Johnson-Davis et al., 2009). Its best dietary sources include liver (3000-15000 μg retinol/100g) and fish liver oils. Vegetable sources of vitamin A include carrots (2000-7000 μg retinol/100g), dark-green leafy vegetables such as spinach (2000-3000 μg retinol/100g), and green leaf lettuce (1000-4000 μg retinol/100g) (Genaro and Martini, 2004). Enhanced consumption of some of these foods may however lead to inadvertent vitamin A toxicity. Furthermore, the sources of dietary vitamin A are varied which makes it difficult to account for exact daily vitamin A intake for individuals (Castaño et al., 2006).

Another increasingly important source of vitamin A is supplemental capsules and multivitamin and mineral (MVM) supplements (Myhre et al., 2003; National Institutes of Health State-of-the-Science Panel, 2007; Ribaya-Mercado and Blumberg, 2007). Intake of these supplements has been promoted by the belief that they are vital for maintaining good health and for the prevention of chronic diseases (Radimer et al., 2004; Yetley, 2007). This may lead to an increased risk of excessive vitamin A intake and toxicity in countries where vitamin A supplements and fortified foods are readily available (Penniston and Tanumihardjo, 2006a). The established tolerable upper limit of safety (UL) for vitamin A is 2800 to 3000 $\mu\text{g}/\text{day}$ for adult men and women (Genaro and Martini, 2004; Institute of Medicine (US) Panel on Micronutrients, 2001). The risk of exceeding this limit is real as previous studies have shown that there is an increased use of MVM supplements in populations whose dietary content of

vitamin A would be otherwise adequate (Ahluwalia et al., 2016; Myhre et al., 2003). Furthermore, the prevalence of taking excessive amounts of vitamin A in such populations has been reported at 10 -15% (Murphy et al., 2007).

1.2.2 Metabolism of Vitamin A

Various compounds are biologically active as vitamin A including retinol, carotenoids such as α -carotene and β -carotene and cryptoxanthine. Once ingested, they are all absorbed but most efficiently in the proximal jejunum in a dose dependent manner (Blomhoff et al., 1991; Borel et al., 2005). At physiological amounts, retinol is transported across the intestinal cell membranes by a carrier-mediated process (Borel et al., 2005). At high concentrations however, vitamin A diffuses into intestinal cells through a micellar phase. Thus, the oral bioavailability of retinol remains high even in dietary excess (Paula et al., 2006) increasing further the risk of vitamin A toxicity.

The absorbed retinol and β -carotene are converted into esters that are incorporated into chylomicrons. These are then transported in lymph and into the systemic circulation. After being converted into chylomicron remnants by lipoprotein lipase, they are then taken up in the liver by hepatocytes (Blomhoff et al., 1991). When hepatic vitamin A reserves are low, part of the absorbed vitamin A is released into blood as a 1:1 complex with plasma retinol-binding protein (RBP) while the rest is stored in hepatocytes as retinyl esters. Of the vitamin A released by hepatocytes into circulation, approximately 80% is recirculated back to the liver (Friedman, 2010). This underscores the liver as the primary organ in the metabolism of vitamin A. The role of hepatocytes in both acute and persistent hypervitaminosis A has however not been conclusively established.

When hepatic vitamin A reserves are adequate, much of the absorbed vitamin A is transferred to HSC where it is similarly stored as retinyl esters. In well-nourished individuals, the hepatic storage efficiency for ingested vitamin A is over 50% and hepatic stores account for over 90%

of the total-body pool (Paula et al., 2006; Puche et al., 2013). This is unlike what is observed in undernourished persons where the hepatic storage efficiency is poor and other organs like the kidneys store between 10% and 50% of the total-body reserve of the vitamin (Nagy et al., 1997).

1.2.3. Causes of Hypervitaminosis A

The desire to increase nutrient intake and/or improve one's health and prevent chronic diseases has seen the increase in the use of multivitamin (MVM) supplements (Radimer et al., 2004; Yetley, 2007) with the prevalence of MVM use ranging between 8% - 48% (Briefel et al., 2006; Eichenberger Gilmore et al., 2005; Lam et al., 2006; Manson and Bassuk, 2018; Murphy, 2010; Murphy et al., 2007; Sebastian et al., 2007). This prevalence increases with age and is despite the fact that many of the MVM supplement users do not actually need to take them since they tend to have higher micronutrient intakes from their diets compared to nonusers (Butte et al., 2010; Hamishehkar et al., 2016; Manson and Bassuk, 2018; Radimer et al., 2004; Rock, 2007). The consumption of these high-dose dietary supplements may therefore inadvertently predispose one to either clinical or sub-clinical hypervitaminosis A (Lam et al., 2006; Murphy et al., 2007). For instance, it has been found that the use of vitamin A and folic acid supplements to treat their deficiencies tended to push their intakes over their upper intake levels (Murphy et al., 2007) with the prevalence of potentially excessive intake of vitamin A by MVM ranging between 10% to 15% (Murphy, 2010). This is because even though the body has a high capacity of storing vitamin A, it has a limited ability to rapidly dispose of excess retinol (Graham-Maar et al., 2006; Penniston and Tanumihardjo, 2006). Thus, over supplementation may result in acute and/or chronic excess of vitamin in the body.

Vitamin A, because of its ability to act as an antioxidant, has been used in the prophylaxis and treatment of photo aging, some skin cancers and other skin disorders (Elias, 1988; Keller and Fenske, 1998). However, following such therapeutic uses of retinoic acid, several cases of

vitamin A induced hepatotoxicity have been reported (Geubel et al., 1991; Levine et al., 2003). This has been known to occur even in the absence of other clinical signs. Majority of these skin conditions develop with age and so do hepatic vitamin A stores. Thus, the use of retinoic acid precursors coupled with the age-related increases in hepatic vitamin A stores among elderly patients may predispose them to chronic hypervitaminosis A.

Even though there is no need for vitamin A supplementation in chronic renal failure (CRF) (Handelman and Levin, 2011; Doireau et al., 1996), it is important to ensure that the RDI for the vitamin is adhered to. This is because there is an increased risk of vitamin A toxicity in CRF with several documented cases in literature (Beijer and Planken, 2001; Doireau et al., 1996; Hammoud et al., 2014; Lipkin and Lenssen, 2008; Ono, 1984; Safi et al., 2014). The risk is occasioned by an increase in serum RBP levels that results from the decreased filtration of low molecular weight proteins in renal dysfunction. This then leads to derangements in the metabolism of vitamin A (Axelsson et al., 2009; Domingos et al., 2016) exposing CRF patients to the adverse effects of chronic hypervitaminosis A.

1.2.4 General Hepatic Parenchymal Changes Associated with Hypervitaminosis A

The myofibroblast-like phenotype acquired by activated HSC enables them to synthesize and secrete various ECM proteins which accounts for the fibrotic changes seen in hypervitaminosis A (Moreira, 2007; Pinzani, 1995). The main ECM proteins secreted are fibril-forming collagens particularly types I and III (Hautekeete and Geerts, 1997; Inuzuka et al., 1990). ECM deposition in the space of Disse leads to the disruption of the normal microanatomy of the liver sinusoids leading to their capillarization. This impairs the exchange of materials from systemic circulation into the hepatocytes and vice versa. These myofibroblasts are also contractile contributing to vascular distortion and increased vascular resistance seen in liver disease (Soon and Yee, 2008). Activated HSC also protrude into the sinusoidal lumen which leads to sinusoidal congestion (Le Bail et al., 1990). This may result in non-cirrhotic portal

hypertension and fibrosis. How these changes in collagen fiber deposition secondary to chronic hypervitaminosis A occur over time and the accompanying inflammatory response has however not been established conclusively.

1.2.5 Hepatic Stellate Cells and Hypervitaminosis A

Vitamin A in the liver is mainly stored by HSC (Higashi et al., 2005; Nagy et al., 1997). These fat-storing hepatic lipocytes, also known as Ito cells, are perisinusoidal cells that store the vitamin A in characteristic lipid droplets (Hautekeete and Geerts, 1997; Moreira, 2007; Pinzani, 1995). Other than production of growth factors, prostaglandins and other bioactive substances (Brandão et al., 2006; Gressner and Weiskirchen, 2006), HSC also synthesize hepatic ECM, a function well amplified in liver injury (Moreira, 2007; Puche et al., 2013; Bataller and Brenner, 2005). Other functions of HSC include: vasoregulation; preservation of hepatocyte mass; drug metabolism and detoxification and control of normal development of the liver (Puche et al., 2013).

Hepatic stellate cells under normal physiological conditions store up to 80% of the total body vitamin A pool (Higashi et al., 2005; Nagy et al., 1997). However, there exists inter-species variation in the ability of hepatic vitamin A storage with arctic animals having a bigger capacity compared to other animals (Higashi et al., 2004; Senoo et al., 2012). Even though the distribution of HSC in most animals is uniform across the liver lobules, there is a zonal gradient in the pattern of storage of vitamin A which varies from one animal species to another (Higashi et al., 2004; Wake and Sato, 1993; Zou et al., 1998). This pattern, according to Wake and Sato, 1993, is genetically determined. In arctic foxes and polar bears for instance, the maximum storage capacity of Vitamin A occurs midway between the portal and central veins and then dips symmetrically towards these vessels (Higashi et al., 2004). In the porcine liver on the other hand, the vitamin A storing capacity was found to be greater in HSC located nearer to the peripheral zone compared to those near the central zone (Wake and Sato, 1993; Zou et al.,

1998). This zonal distribution pattern in the vitamin A storage capacity of HSC has however been scarcely investigated in rats. Since rats are among the most common laboratory rodents used in medical research, establishment of this pattern would serve as an important reference to future studies on hepatic vitamin A metabolism.

Wake in 1971 showed a dose depended increase in the number of HSC after exposure to vitamin A in rabbits and rats over a period of 4 weeks. In a later study, he noted that the HSC in rats with hypervitaminosis A were located in the peripheral, intermediate and central zones of the hepatic lobules unlike in the controls where the HSC were limited to the peripheral zone (K. Wake, 1974). These changes were most pronounced at doses of 300,000 IU/Kg or higher. Apart from the documented increase in the number and size of the cytoplasmic vacuoles in the HSC, Nagy et al., 1997 further found that this increase in vacuolar size and stellate cell number was not limited to the liver but was also observed in lungs and intestines albeit to varying degrees. These studies were however limited to between 18 days and 4 weeks respectively (Nagy et al., 1997; Wake, 1971). The changes thus reported are in keeping with features of acute liver injury (Pereira et al., 2009). There are hardly any studies detailing the temporal sequence of the HSC changes seen in chronic hypervitaminosis A. Furthermore, hardly any studies have been done investigating the reversibility of the hepatic changes seen in acute and chronic hypervitaminosis A in rats. Moreover, the hepatocyte alterations in number and size accompanying the variations in the HSC hyperplasia and vacuolation have scarcely been investigated nor quantified.

Chronic vitamin A toxicity in humans has been shown to cause HSC hypertrophy and hyperplasia with subsequent transformation into myofibroblast-like cells (Castaño et al., 2006; Geubel et al., 1991a; Mounajjed et al., 2014; Nollevaux et al., 2006). Such changes result in loss of normal retinoid-storing capacity (Moreira, 2007). The transformed HSC have a high fibrogenetic capacity and are characterized by a reduction in the number of lipid droplets and

acquisition of extensive rough endoplasmic reticulum and prominent Golgi apparatus (Pereira et al., 2009; Inuzuka et al., 1990). In rats however, the duration over which HSC transform into myofibroblasts following long term exposure to hypervitaminosis A has not yet been established. Similarly, the spontaneous resolution of this HSC transformation is yet to be ascertained.

1.3 STUDY JUSTIFICATION AND SIGNIFICANCE

1.3.1 Study Justification

With increase in clinical use of over the counter vitamin A supplements and the therapeutic use of retinoic acid derivatives in the management of dermatologic conditions (Keller and Fenske, 1998; Levine et al., 2003; Rocha et al., 2011; Sheth et al., 2008), the risk of hypervitaminosis A is heightened. In addition, the high prevalence of chronic renal disease in sub-Saharan Africa (Stanifer et al., 2014; Naicker, 2010) and which alters the filtration of low molecular weight proteins including RBP may predispose these patients to vitamin A toxicity (Beijer and Planken, 2001; Hammoud et al., 2014).

1.3.2 Study Significance

The treatment of vitamin A intoxication, besides other adjunctive therapies, involves withdrawal of exposure to the vitamin (van Dam, 1989). This is despite the acknowledgment that the body has a very limited ability to get rid of excess retinol despite its vast storage capacity (Graham-Maar et al., 2006; Penniston and Tanumihardjo, 2006). Hardly any previous research work has documented the progression and the reversal of the vitamin A induced hepatic changes yet multiple reports have documented the clinical sequelae of hypervitaminosis A. This has been shown to range from the complete resolution of the signs and symptoms to even death secondary to severe complications of vitamin A induced hepatotoxicity (Cheruvattath et al., 2006; Lam et al., 2006; Sheth et al., 2008). The findings of this study are

therefore useful in describing the structural changes seen in liver disease in the face of persistent hypervitaminosis A.

STUDY QUESTION:

What are the hepatic structural changes in hypervitaminosis A in the albino rat (*Rattus norvegicus*)?

HYPOTHESES:

H₀: Hypervitaminosis A does not alter the structure of the liver

H₁: Hypervitaminosis A alters the structure of the liver

OBJECTIVES

Broad objective:

To determine the structural changes induced by persistent hypervitaminosis A in the liver and the short-term sequelae after its withdrawal

Specific objectives:

1. To determine the zonal gradient pattern of vitamin A storage in a rat
2. To determine the morphologic changes in the hepatic tissues of control and experimental rats over a period of 8 weeks
3. To compare the morphologic changes in the hepatic tissues of control and experimental rats over a period of 8 weeks

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 STUDY DESIGN

Quasi experimental study design

2.2 MATERIALS

2.2.1 Ethical Considerations in Animal Handling

Ethical approval to conduct the study was obtained from the Biosafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine, University of Nairobi. Animals were handled while fully awake to minimize stress to them and were held by the skin over their back. Injection of the vitamin A dose was via small hypodermic needles (gauge 25) and only the exact volume of the vitamin solution was injected (150 microliter). To prevent infections, the injection site was swabbed with surgical spirit prior to injection. The needles were disposed after every single use. Injection sites were alternated every day to minimize pain at the injection site.

2.2.2 Study Setting

The study was conducted in the University of Nairobi. Study animals were obtained from the Zoology Department and were housed in the animal house belonging to the Department of Veterinary Anatomy and Physiology. Histological processing of collected specimens and stereological analysis of images was done at the Departments of Human Pathology and Human Anatomy.

2.2.3 Choice of Rats

Previous studies have used rats to illustrate hepatic fibrosis with an emphasis on the role of HSC (Bataller et al., 2005; French et al., 1988). In both rodents and humans, hepatocytes and HSC play the same functions in the metabolism and storage of retinoid and in liver fibrosis. The only documented differences between human and rat stellate cells have been in the

expression of intermediate filaments and other cellular markers (Hautekeete and Geerts, 1997). However, the current study did not focus on these differences and therefore rats were the most suitable for our study model. Furthermore, various studies have made attempts to correlate human and rat ages giving approximate translatable ages and exposure times for the rat that mirror human experiences (Andreollo et al., 2012; Sengupta, 2013).

2.2.4 Sample Size Calculation

Sample size was calculated using the formula below (Charan and Biswas, 2013).

$$n = \frac{2[Z_{(1-\alpha/2)} + Z_{(1-\beta)}]^2}{\Delta^2}$$

Where:

n= sample size

$Z_{(1-\alpha/2)}$ = Standard normal variate for level of significance (1.96 for significance level of 0.05)

$Z_{(1-\beta)}$ = Standard normal variate for power (for 80% power it is 0.84.)

Δ (effect size) = $\frac{\text{smallest meaningful difference}}{\text{Standard deviation}}$

Yi et al., 2012, reported a standard deviation of 0.87% in the mouse liver for mean fibrosis score. Using a statistical significance of 0.05, a power of 80% and a smallest meaningful difference being 1.4%, the sample size was calculated as follows:

$$\Delta \text{ (effect size)} = \frac{\text{smallest meaningful difference}}{\text{Standard deviation}} = 1.4 / 0.87 = 1.61$$

$$n = \frac{2(1.96 + 0.84)^2}{1.61 * 1.61} = 6.1 \approx 6$$

Every cohort therefore, contained 6 animals. Since there were 3 groups in the study, a total of 45 rats were used in the study (as described in the sampling and tissue harvesting subsections below). The rats were obtained from the animal house belonging to the Department of Biochemistry, University of Nairobi.

2.2.5 Selection and Exclusion Criteria

Only adult male rats were used in this study. This is because estrogen has been shown to affect the progression of hepatic fibrosis (Shimizu et al., 1999). Any rats that had gross malformations observed at the time of commencement of the study were excluded.

2.2.6 Sampling Technique

The rats were divided into 3 groups: A, B, and C. Group A rats were given high dose vitamin A (300,000 IU/Kg) every alternate day via subcutaneous injection for 4 weeks with half of them being followed for a further 4 weeks without the high dose vitamin A treatment. They represented the acutely exposed group. Group B rats were similarly given high dose vitamin A (300,000 IU/Kg) every alternate day via subcutaneous injection for 8 weeks. Half of the members of this group were then followed up for 4 more weeks without being injected with the high dose of vitamin A. This group represented the rats subjected to persistent hypervitaminosis A. Group C were the control group (5 rats) and received subcutaneous injection of sterile normal saline every alternate day over the duration of the study. All rats were maintained on a normal diet and fed ad libitum. Two rats from the control group were used for baseline results while one each was sacrificed at weeks 4, 8 and 12 (Table 2.1).

2.2.7 Handling of Animals

Rats were handled with the help and supervision of qualified animal house attendants of the Department of Veterinary Anatomy and Physiology. They were kept in standard and well labeled cages measuring 109cm by 69cm by 77.5cm. Each cage contained 6 animals. These cages

were floored with wood shavings which were replaced every two days. The rats were placed under a normal 12 hours' light/dark diurnal cycle and provided with standard rat pellets and water ad libitum.

Table 2.1: Summary of animals sacrificed at particular weeks

Week at Sacrifice	Animals sacrificed		
	Group A	Group B	Group C (Controls)
Week 2	5		2 (Baseline)
Week 4	5		1
Week 6	5	5	
Week 8	5	5	1
Week 10		5	
Week 12		5	1

2.2.8 Occupational Precautions

The animals were handled carefully to avoid defensive scratching and biting. For restraint, they were removed from their cages by grasping the base of their tails using bite resistant gloves. We did not encounter any incidence of defensive biting by the rats. Administration of halothane anesthesia was done in an air tight glass jar in a well-ventilated room to minimize the side effects of halothane. Once used, sharps were disposed in safety disposal containers. Protective gloves were used to handle chemicals such as xylene during processing of harvested tissues and staining procedures.

2.2.9 Administration of Vitamin A

Rats in both groups A and B were given 300,000 IU/Kg of vitamin A palmitate (Aquosol A by Pfizer Inc.) every alternate day via subcutaneous injection for 4 and 8 weeks respectively.

All the study animals were closely monitored for their feeding and sleeping patterns and for their temperament.

2.3 TISSUE HARVESTING AND PROCESSING

2.3.1 Tissue harvesting

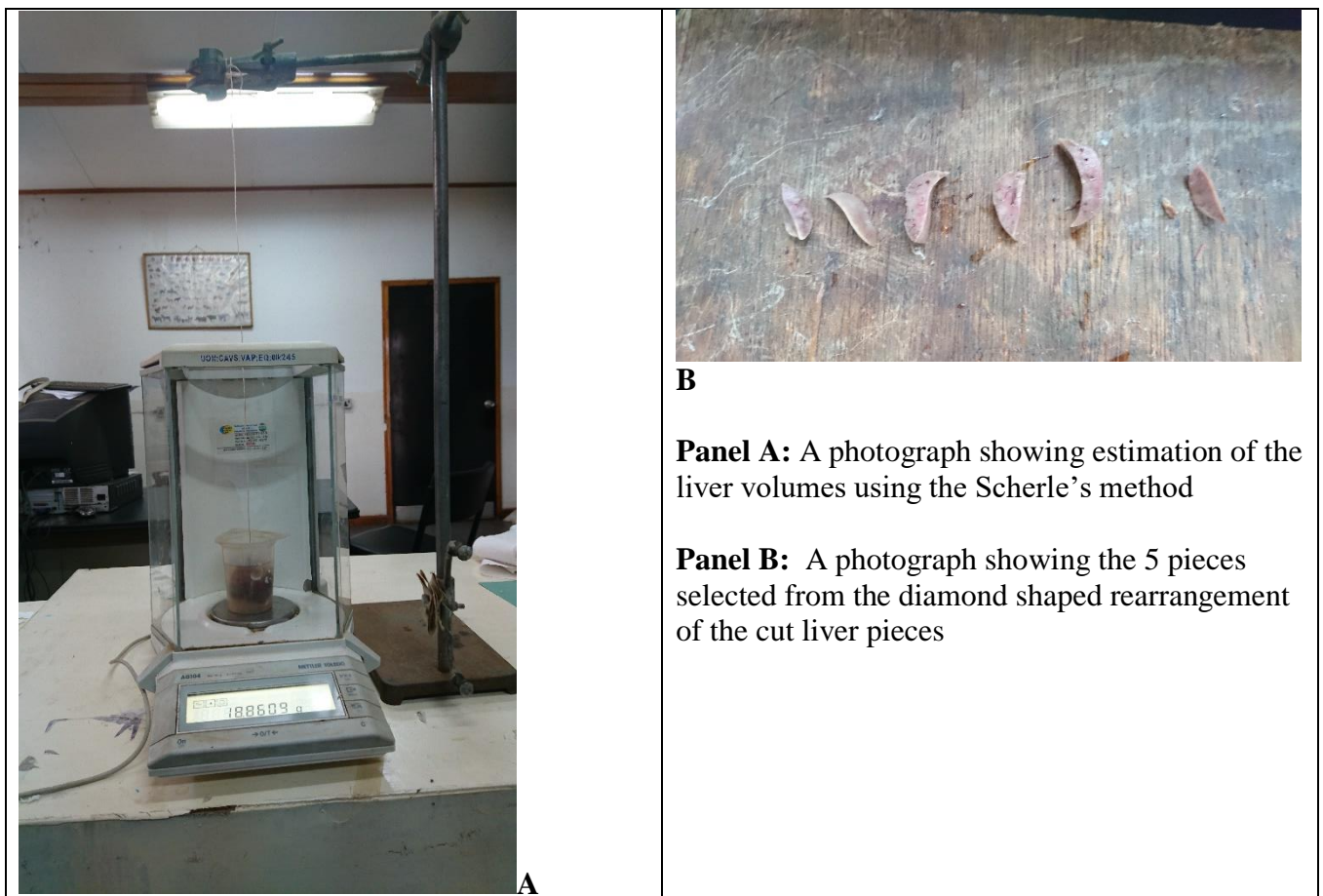
All rats were weighed at the onset of the study prior to the onset of administration of vitamin A and just before euthanasia was done. Five rats for every week from group A were euthanized at weeks 2, 4, 6 and 8 while those from group B (5 rats for each week) were euthanized at weeks 6, 8, 10 and 12. Control rats were on the other hand sacrificed at baseline (2), weeks 4, 8 and 12. Euthanasia was performed using gaseous halothane (1-3%) soaked in cotton wool inside an airtight glass jar and was ascertained when the rats showed absent pupillary light reflex and exhibited minimal response to pain. Following this, a midline body incision was made, and formal saline infused via the trans-cardiac method while there was still some cardiac activity to aid in the flushing out of the blood. Perfusion was stopped only when rigor mortis was complete. The liver was then harvested *en masse* and stored in formal saline. Its volume was measured thrice using the Scherles's method of water displacement and an average of the 3 readings recorded (Figure 2.1A). Body mass-normalized liver volume (BM-NLV) was calculated by dividing the average volume of the liver (cm³) by the respective weight of the animal (g) and multiplying the result by 100. All the remaining carcasses were incinerated at the department of Veterinary Anatomy and Physiology. The harvested livers were then studied for pattern of storage of vitamin A in the liver, general parenchymal changes and hepatocyte and HSC density.

2.3.2 Sampling of Liver Segments for histological Processing

The systematic uniform random (SUR) sampling method, as previously described (Gundersen, 2002; Nyengaard, 1999), was employed to get the liver segments for histological processing. To achieve this, a whole liver was sliced across the various lobes into 16 equal segments. The

segments were then rearranged into a diamond shape with smaller pieces arranged uniformly on either side of the largest piece of liver (Burity et al., 2004; Junatas et al., 2017; Marcos et al., 2012). Following this, the second piece was selected and thereafter every third piece was picked. A total of 5 pieces per liver were thus picked for histological processing (Figure 2.1 B).

Figure 2.1: Photograph showing Scherle's method of liver volume estimation (A) and selection of liver segments for histological processing (B)



2.3.3. Tissue processing

The selected liver segments were fixed in 10% buffered formal saline for at least 24 hrs. This was followed by dehydration in increasing strengths of alcohol beginning with 70% up to absolute alcohol at one-hour intervals. The tissues were then cleared in toluene before being infiltrated with wax in a memmert oven for 24 hours. Thereafter they were embedded in paraffin wax and then left to cool. The embedded tissues were then blocked in tissue

cassettes before being serially cut into 7 µm thick sections using a microtome (Leica® Model SM2400, Leica Microsystems, Nussloch GmbH, Germany). The sections were then picked on a glass slide and dried in an oven for 12 hours. Thereafter, staining was done using Masson's trichrome for collagen fibers, periodic acid Schiff (PAS) for vitamin A storage vacuoles HSC while Hematoxylin and Eosin Stain was used to study the general architecture of the liver.

2.3.4 Selection of Sections for Staining

During sectioning of a liver segment, a ribbon of 16 successive 'sections' was picked. The second 'section' was identified and picked after which every third section was also picked. Therefore, for every such ribbon, a total of six pieces were picked. This process was repeated until a total of 15 sections were selected. The selected 'sections' were then divided into 3 groups assigned X, Y and Z. Group X sections were stained with the Hematoxylin and Eosin Stain, Group Y sections were stained with Masson's trichrome stain while Group Z sections were stained with the PAS stain. This process was then repeated for each of the selected liver segments for every rat involved in the study.

2.3.5 Hematoxylin and Eosin Staining

Selected slides were dipped in Iron Hematoxylin for 15 minutes and then washed in running water for 2 minutes to remove any excess stain. This was followed by the sections being stained in 1% eosin solution for 3 minutes, followed by dehydration in ascending grades of ethanol from 70% to absolute alcohol. The sections were then cleared in two changes of xylene before being mounted for observation under a light microscope. The hepatocytes were identified as large cells arranged in cords that were separated by sinusoids. HSC on the other hand, appeared as small deeply eosinophilic cells located in the peri-sinusoidal space and frequently occurring in clusters.

2.3.6 Masson's Trichrome Staining

The slides were kept in Iron Hematoxylin for 15 minutes. They then differentiated in acid alcohol before being blued in running water for one hour. They were then dipped in Ponceau stain for 6 minutes followed by distilled water after which they were placed in mordant for 4 minutes, dipped in distilled water and then kept in light green stain for 2 minutes. This was followed by dehydrating then in ascending grades of ethanol. Afterwards, the specimen was cleared in two changes of xylene before being mounted for light microscopy. Collagen fibers stained green when sections were stained with the Masson's trichrome stain.

2.3.7 Periodic Acid Schiff (PAS) Staining

Slides were placed in Schiff reagent for 15 minutes until the sections were light pink in color. The sections were then washed in lukewarm tap water for 5 minutes until they turned dark pink in color. They were then counterstained in Mayer's hematoxylin for 1 minute. After that, the tissues were washed in tap water for 5 minutes before being dehydrated. They were then mounted using a cover slip and synthetic mounting medium. The PAS stain showed the fat vacuoles as clear spaces mainly around the nuclei of HSC.

2.4 IMAGES FOR MORPHOMETRIC ANALYSIS

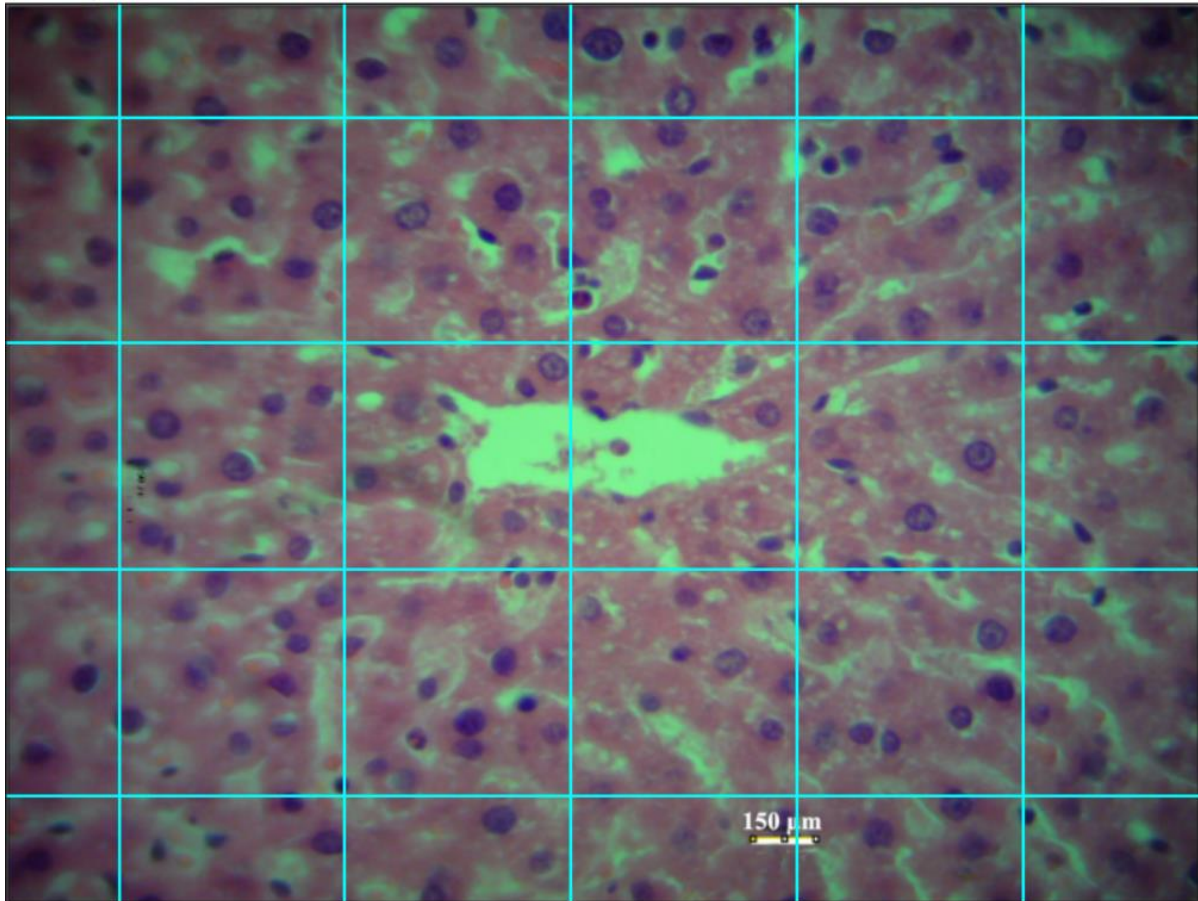
Photomicrographs of the sections were taken using a Canon Digital Camera (5 megapixels) mounted on a photomicroscope. Calibration was done at 1500 μ m, 600 μ m and 150 μ m for images taken at the magnification of X40, X100 and X400 respectively. Six images per section were taken; three within the vicinity of a central vein and three within the vicinity of a portal vein. To achieve this, the central vein nearest to the left uppermost part of the section was identified and the surrounding parenchyma photographed. The section was then moved down and to the right past two hepatic lobules where a peripheral vein was identified and the surrounding parenchyma photographed. It was then again moved up and to the right past two hepatic lobules and a central vein identified. The surrounding parenchyma was then photographed. The process was then repeated until 3 pericentral and three periportal

parenchyma photographs per section were taken. For the next section to be photographed, a portal vein instead of a central vein was initially identified and the surrounding parenchyma photographed then followed by a central vein using the routine described above. These photographs were then uploaded into Fiji Image J software (National Institutes of Health image program) for morphometric and stereological analysis as described below. Changes in the general liver histoarchitecture were observed and recorded.

2.5 ESTIMATION OF HEPATOCYTE AND HSC DENSITIES

Hepatocyte and HSC area density estimation was done using the Cavalieri's principle of point counting (Mandarim-de-Lacerda, 2003). Using the Image J software, the selected histological area was analyzed using a superimposed grid on the digital image on a monitor screen as previously described (Gundersen et al., 1988). Such a grid system produces lines and point probes regularly arranged, which are used to facilitate estimation of the specific tissue densities (Mandarim-de-Lacerda, 2003). The unit area in the grid was $285,967.77\mu\text{m}^2$ and every superimposed image in the grid gave about 15-unit areas (Figure 2.2). The second unit areas for every section were selected for counting of cells and after that every third one to a total of 5-unit areas per image. Only cells that fell within the grid area and those that touched the lower and right borders were counted. The average cellular count/ μm^2 of the 5-unit areas was then used as the cell density of that image. Since every rat liver had 5 liver segments processed for histological examinations, and 5 sections per segment were stained for a particular stain, and from every section 6 images were taken, a total of 150 images per liver were analyzed for hepatocyte and HSC densities. The cellular densities were evaluated while unaware of the source of the tissue samples. All the counts were done at the 150 μm calibration and on the hematoxylin and eosin stained sections.

Figure 2.2: Image showing grids used in counting of Hepatocytes and HSC in determining their respective densities



2.6 STATISTICAL ANALYSIS

Data obtained from the stereological methods described above were keyed into the Statistical Package for Social Sciences (SPSS) software (version 21.0, Chicago, Illinois) for coding, tabulation and statistical analysis. Measurements were expressed in numbers/mm². The data was grouped into three groups as earlier described (Groups A, B and C). Normality of the data was determined using histograms and box plots. Means and the standard deviations were then determined. The one-way ANOVA was used to compare the hepatocyte and HSC densities for the 3 groups and changes in the densities over time. When a significant difference

was shown with ANOVA, the Tukey test was used as the post-hoc test to detect between which groups the significant difference lay. A *p value* <0.05 was considered significant at 95% confidence interval. Results were presented in micrographs, tables and graphs.

CHAPTER 3

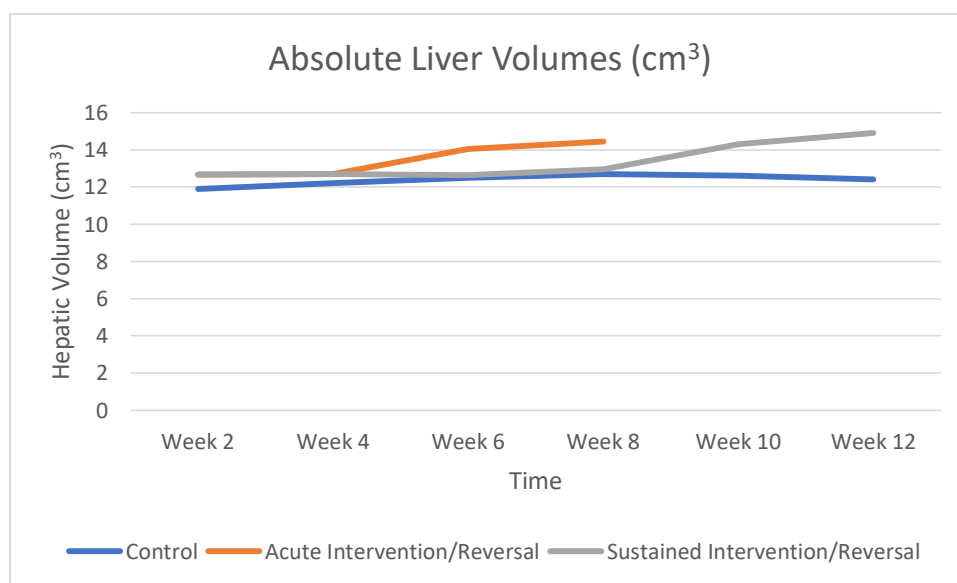
3. RESULTS

All the animals initially recruited into the study survived to the end of the experiment period. There were no discernible gross changes seen in the intervention group animals compared to the control ones. The animals gained weight appropriately and showed normal social behavior as observed on the maintenance of the expected daily animal routine.

3.1 Absolute Liver Volumes

The liver volumes of the control rats remained steady over time and ranged between 11.9 cm³ and 12.7 cm³. Acute exposure to high dose vitamin A, on the other hand, caused a slight elevation of the hepatic volume while persistent injection beyond 4 weeks caused a marginal decline. Stopping the exposure to vitamin A, for both acutely and persistently exposed animals, resulted in continued increase in the liver volumes (Figure 3.1.1). These changes were, however, not statistically significant ($p=0.895$).

Figure 3.1.1: Graph Showing the Trend of Absolute Liver Volume over Time



3.2 Body Mass -Normalized Liver Volume (BM-NLV)

While the control group had an almost constant BM-NLV over the entire study period, the BM-NLV for the animals exposed to acute hypervitaminosis A showed a gradual decline up to the 4th week before going up with stopping of injection of vitamin A to peak at week 6. It then returned to the baseline by the 8th week. On the other hand, prolonged exposure to high dose vitamin A resulted in a more acute decline of the BM-NLV between weeks 6 and 8. However, following cessation of vitamin A administration this decline reversed to near normal levels by week 12 (Figure 3.1.2). These differences were however, not statistically significant (Table 3.1).

Figure 3.1.2: Graph Showing the Trend of the Body Mass -Normalized Liver Volume (BM-NLV) over Time

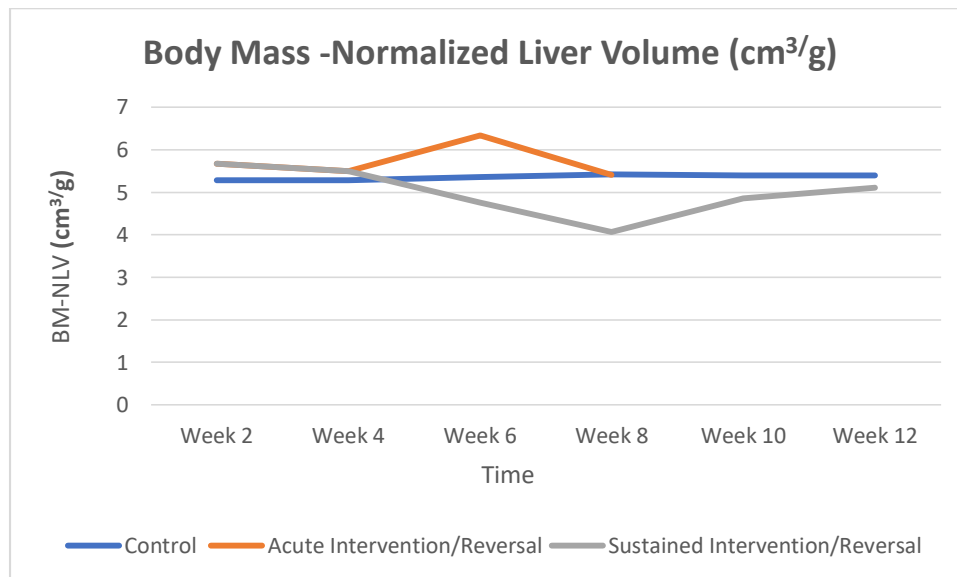


Table 3.1: Body Mass -Normalized Liver Volume (cm³/g)

Week	Mean	p-value	
		Against Control	Against Immediate Preceding Group
Control	5.28+/-0.23		
Week 2 Intervention	5.67+/-0.54	0.375	0.375
Week 4 Intervention	5.5+/-0.12	0.967	0.855
Week 6 Reversal	6.34+/-0.86	0.082	0.079
Week 8 Reversal	5.4+/-0.68	1	0.055
Week 6 Intervention	4.76+/-0.45	0.493	0.087
Week 8 Intervention	4.07+/-0.32	0.074	0.0754
Week 10 Reversal	4.86+/-0.19	0.253	0.098
Week 12 Reversal	5.11+/-0.67	0.432	0.785

3.3 Pattern of Vitamin A Storage

Animals subjected to high dose vitamin A had a time dependent increase in vacuolation of hepatocytes and HSC accompanied by fatty changes in the hepatic parenchyma (Figure 3.1; Figure 3.7). These changes were worse around the central vein and reduced towards the periportal area. Rebound hepatocyte and HSC hyperplasia secondary to hypervitaminosis A was noted to be more pronounced around the portal triad area and less around the central vein. This was unlike in the control group livers that had a heterogenous distribution of hepatocytes and HSC between the two zones (Figure 3.1).

Figure 3.1 – Storage Pattern of Hepatic Vitamin A

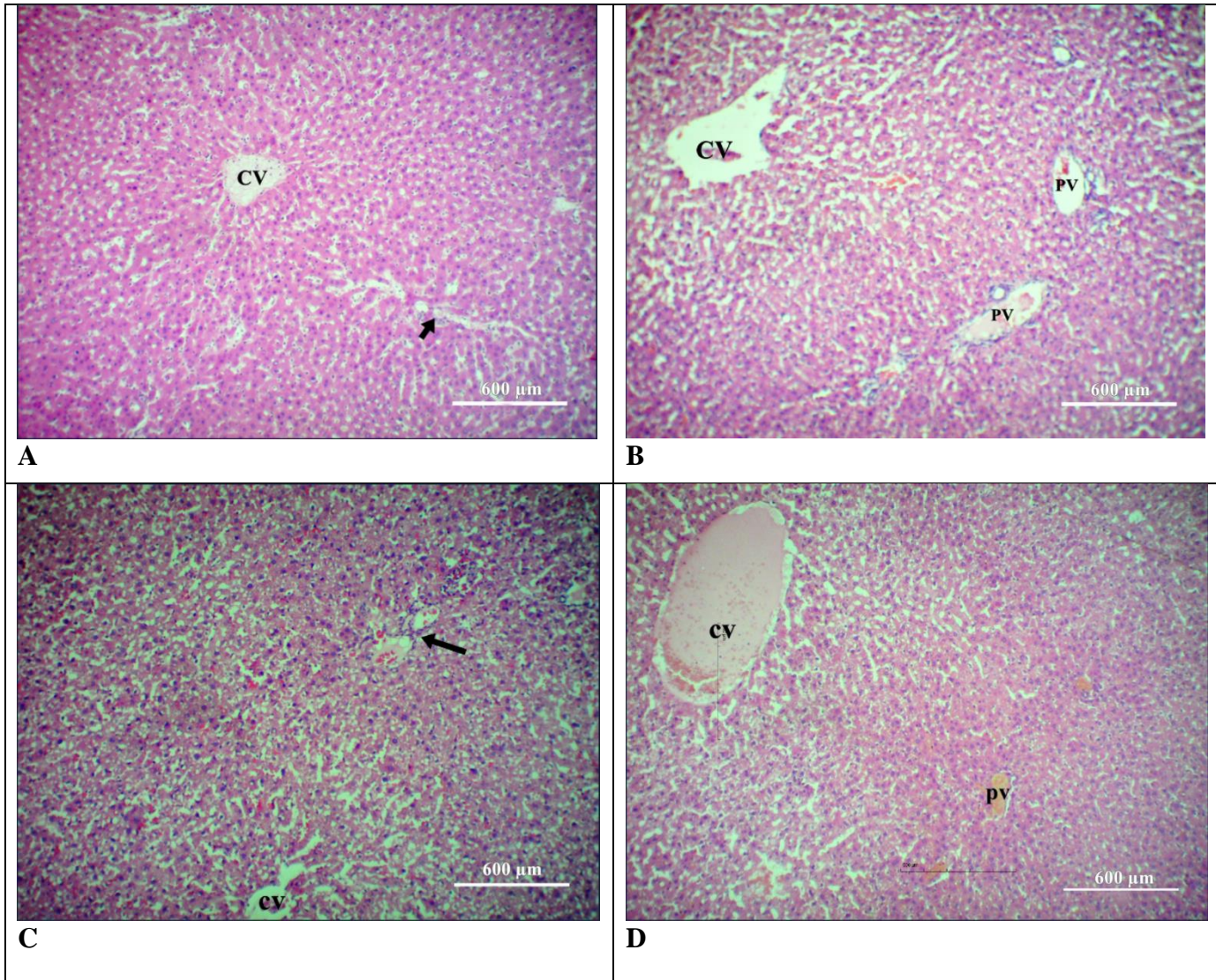
Panel A: Photomicrograph of a control rat liver showing heterogenous distribution of hepatocytes between a central vein (CV) and portal vein (PV) – pointed arrow. (H&E stain). Scale: 600 μm .

Panel B: Photomicrograph of a rat liver at week 2 on high dose vitamin A showing increased fatty changes around the ventral vein (CV) and reducing as one approaches the portal vein (PV). Note also the increased cellularity near the PV. (H&E stain). Scale: 600 μm .

Panel C: Photomicrograph of a rat liver showing at week 6 of injection showing vitamin A induced fatty changes now extending to the PV (Arrow). Note that the increased cellularity is located within the proximity of the PV. (H&E stain). Scale: 600 μm .

Panel D: Photomicrograph of a rat liver at week 8 of injection showing increased hyperplasia around a portal vein (PV) compared to a central vein (CV). The fatty changes are still more prominent around the CV compared to the PV. (H&E stain). Scale: 600 μm .

Figure 3.1: Storage Pattern of Hepatic Vitamin A



3.4 General Histoarchitectural Changes and Collagen Fiber Deposition

The hepatic parenchyma of the control group was made up of hepatic cords separated by sinusoids radiating towards a central vein. There were hardly any collagen fibers seen along the space of Disse. With exposure to high dose vitamin A, there was an observed temporal increase in collagen fiber deposition along the perisinusoidal space occasionally accompanied by bridging fibrosis. The fibrosis was most pronounced around the biliary apparatus with accompanying hypertrophy of the duct epithelium. The increase in fibrosis paralleled the hyperplasia of the HSC and the hepatocytes (Figure 3.2E). There was a marked distortion of the hepatic parenchyma with loss of the hepatic cords and apparent loss of the sinusoidal space. These changes were most apparent at week 6 of exposure to the high dose of vitamin A (Figure 3.2E). Other changes seen were infiltration of inflammatory cells especially around the portal areas. When vitamin A was discontinued, there was reversal of the parenchymal damage as evidenced by the restoration of the hepatic cords and the sinusoidal space (Figure 3.2F, Figure 3.3 D and F). However, the collagen deposition seemed to persist relative to the controls albeit at lower levels compared to what was seen during vitamin A administration (Figure 3.2F).

Figure 3.2 – General Stromal Changes and Collagen Fibre Deposition (Masson’s Trichrome stain). Scale: 150 µm.

A: Photomicrograph of a control rat liver. Hepatocytes are arranged in cords. There is minimal collagen fiber deposition along the peri-sinusoidal space.

B: Photomicrograph of a rat liver (periportal) at 2 weeks of exposure to a high dose of vitamin A. There is some collagen fiber deposition around the sinusoids.

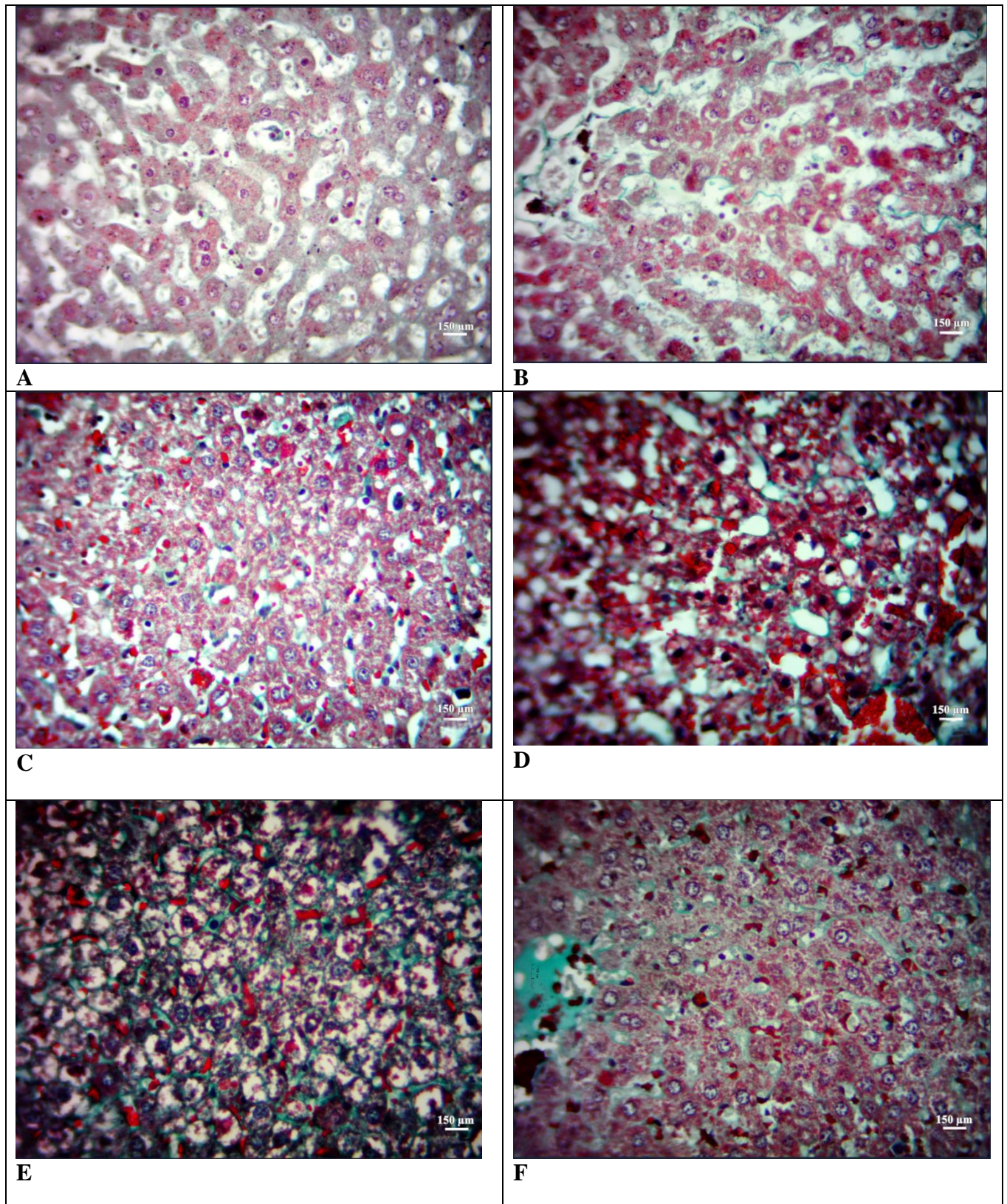
C: Photomicrograph of a rat liver (pericentral) at 2 weeks after acute exposure to a high dose of vitamin A. Note that the collagen fiber deposition is slightly reducing relative to week 2 of exposure. (Masson’s Trichrome stain). Scale: 150 µm.

D: Photomicrograph of a rat liver 2 weeks after acute exposure to a high dose of vitamin A (Pericentral). Note the disruption of hepatocyte cords. Collagen fiber deposition along the sinusoids is still evident although to a lesser extent as compared to at 4 weeks of exposure to the high dose vitamin A.

E: Photomicrograph of a rat liver at 6 weeks of exposure to a high dose of vitamin A. There is evidence of increased collagen fiber deposition along the peri-sinusoidal space. Note the accompanying distortion of hepatic cords. (Masson’s Trichrome stain). Scale: 150 µm.

F: Photomicrograph of a rat liver at 8 weeks of exposure to a high dose of vitamin A showing increased fibrosis. The cells are also of a smaller caliber relative to preceding weeks and the disruption of the hepatic cords is still evident.

Figure 3.2: General Stromal Changes and Collagen Fibre Deposition



3.5 Hepatocyte Density

The hepatocyte density for the control group remained relatively constant over the entire study period (low of 49.8891 ± 12.6405 and high of 51.6191 ± 6.3031). The hepatocytes were heterogeneously arranged in cords separated by sinusoids. There was no notable difference in the hepatocyte distribution between the periportal and pericentral regions of the hepatic parenchyma. Majority of the hepatocytes appeared euchromatic while the rest were heterochromatic. While a majority of the hepatocytes were mononucleated, a few were binucleated. The hepatocytes appeared to be relatively uniform in their size and some had prominent nucleoli (Figure 3.3 A and Figure 3.3B).

Following acute exposure to high dose vitamin A the hepatocyte density rose up progressively with maximum numbers recorded in the 4th week ($p < 0.001$). The hepatocytes nearer the central veins were more vacuolated than those in the periportal areas. Their vacuoles were also larger (Figure 3.7B & C). Conversely, the periportal areas appeared to have a higher concentration of hepatocytes relative to the pericentral areas, their cells were also more deeply staining were of varied sizes (Figure 3.3C). Binucleation of the hepatocytes showed a temporal increase with a predominance of smaller hepatocytes being seen. The periportal nuclei were also more compact compared to the pericentral ones (Figure 3.3D). Discontinuing the acute exposure resulted in a decline of the hepatocyte density both at week 6 ($p = 0.205$) and at week 8 ($p = 0.901$) (Table 3.2, Panels 3.4E & F). However, the pericentral hepatocytes still remained vacuolated with concurrent distortion of the hepatic cords.

Figure 3.3 – Hepatocyte and HSC Cellular Changes Following Acute Exposure (H&E stain). Scale: 150 μ m.

A: Photomicrograph of a control rat liver taken near a central vein (CV) showing hepatocytes arranged in cords while hepatic stellate cells (arrow heads) are in the peri-sinusoidal spaces. The hepatocytes are of a near uniform size.

B: Photomicrograph of the same liver taken near a portal vein (PV) showing hepatocytes (arrows) arranged in cords and HSC (arrow heads).

C: Photomicrograph of a rat liver after week two of high dose vitamin A administration taken around a central vein (CV). There is increased binucleation of the hepatocytes (arrows) which are predominantly euchromatic, with prominent nucleolus and more closely packed. The HSC (arrow heads) are more pronounced with increased vacuolation.

D: Photomicrograph of a rat liver at week two of high dose vitamin A administration near a portal vein (PV). Note the increase in number of hepatocytes and HSC with accompanying vacuolation.

E: Photomicrograph of a rat liver at week four of high dose vitamin A administration around a central vein (CV). Note the hyperplasia of the hepatocytes and the HSC and increased fatty liver changes.

F: Photomicrograph of a rat liver at week 4 of high dose vitamin A administration from around a portal area. The hepatocytes are smaller in size and portray a euchromatic and heterochromatic mixture.

Figure 3.3: Hepatocyte and HSC Cellular Changes Following Acute Exposure

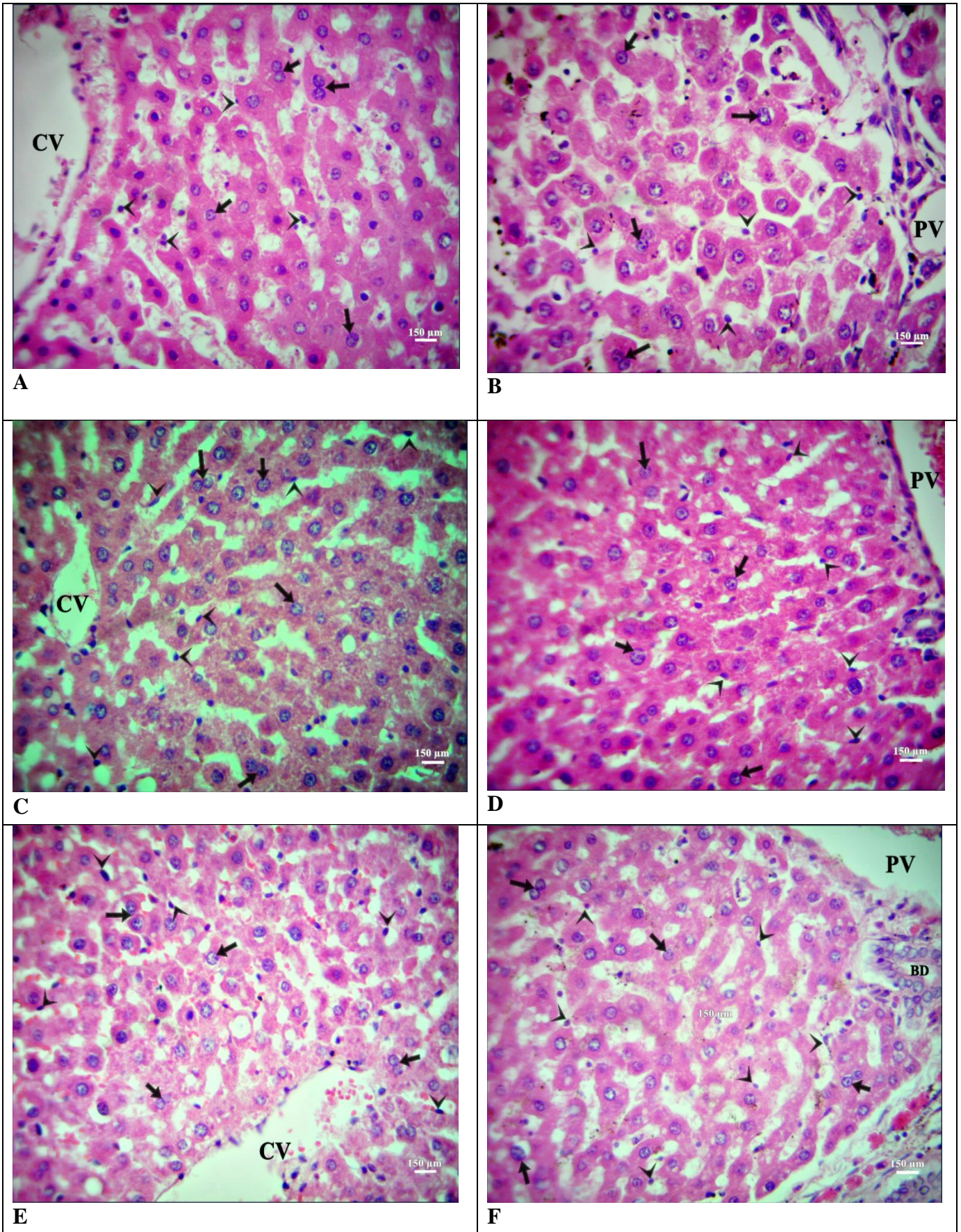


Figure 3.4 – Reversal of Hepatocyte and HSC Cellular Changes Following Acute Exposure. (H&E stain). Scale: 150 μ m.

A: Photomicrograph of a rat liver at week four of high dose vitamin A administration around a central vein (CV). Note the hyperplasia of the hepatocytes and the HSC and increased fatty liver changes.

B: Photomicrograph of a rat liver at week 4 of high dose vitamin A administration from around a portal area. The hepatocytes are smaller in size and portray a euchromatic and heterochromatic mixture.

C: Photomicrograph of a rat liver 2 weeks after acute exposure to a high dose of vitamin A taken from around a central vein (CV). Note the presence of vacuolation of both the hepatocytes and HSC albeit at reduced levels.

D: Photomicrograph of a rat liver 2 weeks after acute exposure to a high dose of vitamin A taken from around a portal vein (PV). Notice the vacuolation of the hepatocytes and HSC.

E: Photomicrograph of a rat liver 4 weeks after acute exposure to a high dose of vitamin A taken from around a central vein (CV). There is an improvement on the arrangement of the hepatic cords. The hepatocytes are still of small caliber but mainly heterochromatic and multinucleated. Some are euchromatic. There is also an accompanying hyperplasia of the HSC.

F: Photomicrograph of a rat liver 2 weeks after acute exposure to a high dose of vitamin A taken from around a portal area. The hepatocytes are now mainly heterochromatic and there is restoration of the hepatic cords. Evidence of vacuolation is however present.

Figure 3.4: Reversal of Hepatocyte and HSC Cellular Changes Following Acute Exposure

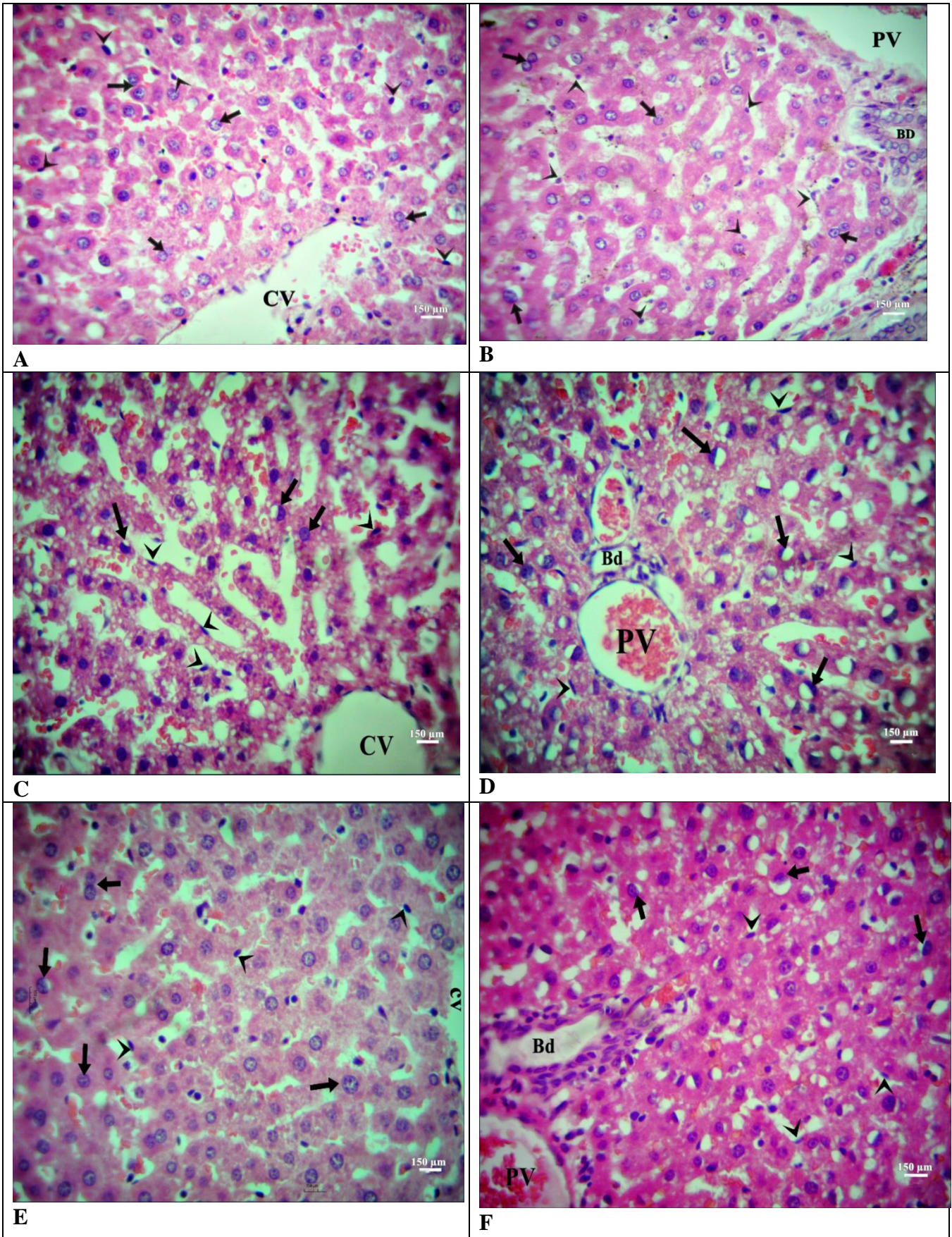


Table 3.2: Comparisons of Means for Hepatocyte Density for the Various Study Groups Acutely Exposed to High Dose Vitamin A (* statistically significant)

Week	Mean (Hepatocyte density/mm ²)	Median (Hepatocyte density/mm ²)	p-value	
			Against Control	Against Immediate Preceding Group
Control	50.3553+/-7.6841	54.2019		
Week 2	65.4288+/-15.9712	66.4411	0.058	0.058
Week 4	78.6413+/-15.8087	80.4286	<0.001*	0.007*
Week 6 Reversal	69.9244+/-11.4795	71.4721	0.002*	0.205
Week 8 Reversal	65.1977+/-19.9515	62.9442	0.055	0.901

Persistent exposure to vitamin A beyond 4 weeks resulted in a significant decline in the hepatocyte density at week 6 ($p < 0.001$) followed by a significant rebound at week 8 ($p < 0.001$) (Table 1.3). Week 6 of high dose vitamin A exposure was further characterized by increased vacuolation of the hepatocytes which now extended to the periportal areas (Figure 3.7D). The hepatocytes were predominantly heterochromatic with most having features of necrosis as evidenced by loss of the sharp border demarcating the hepatocytes from the surrounding stroma. The nucleoli were hardly visible. These changes were accompanied by distortion and apparent loss of the sinusoidal space. Even though these changes were seen across the hepatic parenchyma, the severest damage was found in the pericentral areas (Figures 3.5 C & D). Week 8 of persistent exposure to vitamin A was characterized by marked hepatocyte hyperplasia cutting across the liver parenchyma but most prominent in the periportal area compared to the pericentral areas. This was characterized by an increase in multinucleated cells and variant sizes of the hepatocytes with a preponderance of the small sized hepatocytes. These hepatocytes were mainly devoid of vacuoles (Figures 3.5 E & F). The

distortion and apparent loss of sinusoidal space seen in week 6 also continued significantly into week 8.

Following cessation of exposure to high dose vitamin A at week 8, the hepatocyte density had a significant decline at week 10 relative to the high numbers recorded at week 8 of exposure ($p < 0.001$) (Table 3.3; Figure 3.1.3). During this period, features of hepatocyte hyperplasia were still evident but a rebound of vacuolation, relative to week 8 was seen. The hepatocytes still occurred in various sizes, however, with large ones predominating (Figures 3.6 C & D).

Table 3.3 Comparisons of Means of Hepatocyte Density for the Various Study Groups Exposed to Persistent High Dose Vitamin A (* statistically significant)

Week	Mean (Hepatocyte density/mm ²)	Median (Hepatocyte density/mm ²)	p-value	
			Against Control	Against Immediate Preceding Group
Control	50.3553+/-7.6841	54.2019		
Week 2	65.4288+/-15.9712	66.4411	0.058	0.058
Week 4	78.6413+/-15.8087	80.4286	<0.001*	0.007*
Week 6 Intervention	58.3199+/-12.1527	57.9752	0.772	<0.001*
Week 8 Intervention	78.1751+/-26.882	76.9318	<0.001*	<0.001*
Week 10 Reversal	62.1385+/-12/9319	60.9814	0.259	<0.001*
Week 12 Reversal	59.4867+/-8.281	59.4473	0.614	0.997

Figure 3.1.3: General Trend of the Hepatocyte Density over Time

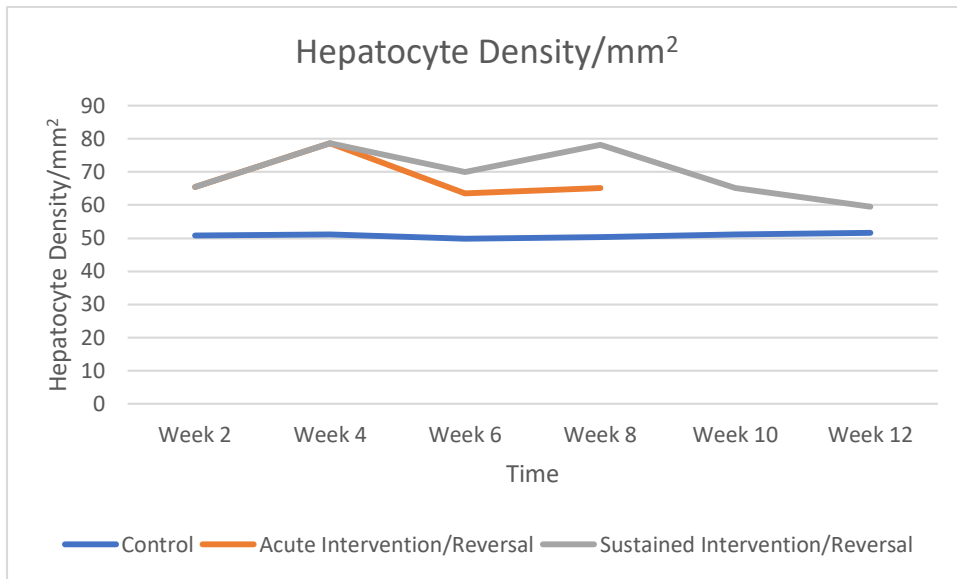


Figure 3.5 – Cellular Changes Following Persistent Exposure (H&E stain). Scale: 150 μ m.

A: Photomicrograph of a control rat liver taken near a central vein (CV) showing hepatocytes arranged in cords while hepatic stellate cells (arrow heads) are in the peri-sinusoidal spaces. The hepatocytes are of a near uniform size.

B: Photomicrograph of the same liver taken near a portal vein (PV) showing hepatocytes (arrows) arranged in cords and HSC (arrow heads).

C: Photomicrograph of a rat liver at 6 weeks of exposure to a high dose of vitamin A taken from around a central vein (CV). Notice the increased vacuolation with signet ring shaped HSC. The hepatocytes seem fewer than in preceding weeks and separated from each other by large lipid vacuoles. Massive distortion of the hepatic cords and apparent loss of sinusoidal space is also evident.

Panel D: Photomicrograph of a rat liver at 6 weeks of exposure to a high dose of vitamin A taken from around a portal area. The vacuolation involves both the deeply basophilic HSC and the hepatocytes and is accompanied by extensive distortion of the hepatic parenchyma.

Panel E: Photomicrograph of a rat liver at 8 weeks of exposure to a high dose of vitamin A taken from around a central vein (CV). There is extensive vacuolation of both the hepatocytes and HSC. Marked distortion of the hepatic parenchyma is also seen.

Panel F: Photomicrograph of a rat liver at 8 weeks of exposure to a high dose of vitamin A taken from around a portal vein (PV). There is cellular vacuolation with signet ring shaped HSC. The asterisk shows a bile ductile.

Figure 3.5: Cellular Changes Following Persistent Exposure

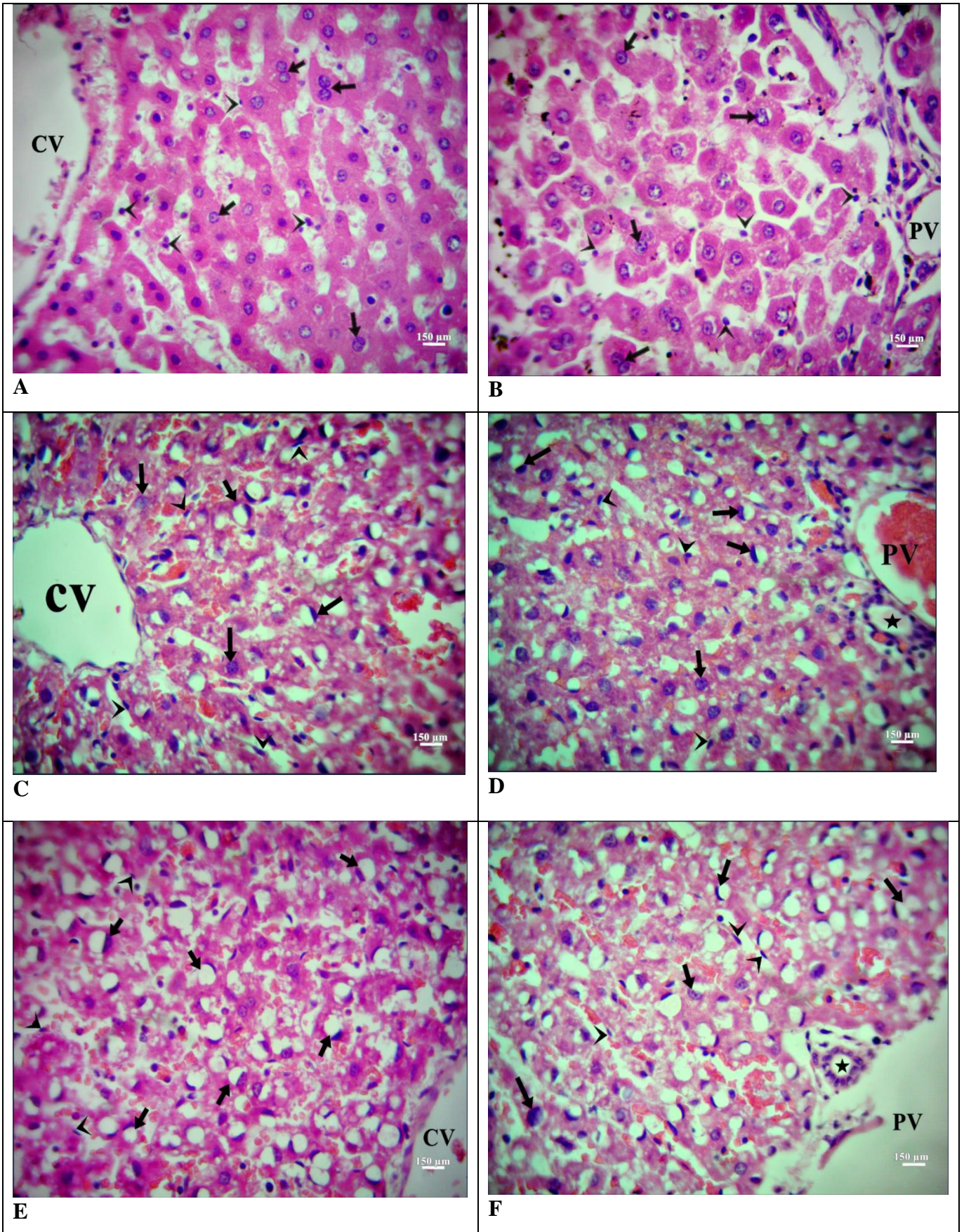


Figure 3.6 – Reversal of Cellular Changes Following Persistent Exposure (H&E stain).

Scale: 150 μm .

A: Photomicrograph of a rat liver at 8 weeks of exposure to a high dose of vitamin A taken from around a central vein (CV). There is extensive vacuolation of both the hepatocytes and HSC. Marked distortion of the hepatic parenchyma is also seen.

B: Photomicrograph of a rat liver at 6 weeks of exposure to a high dose of vitamin A taken from around a portal vein (PV). There is cellular vacuolation with signet ring shaped HSC. The asterisk shows a bile ductile.

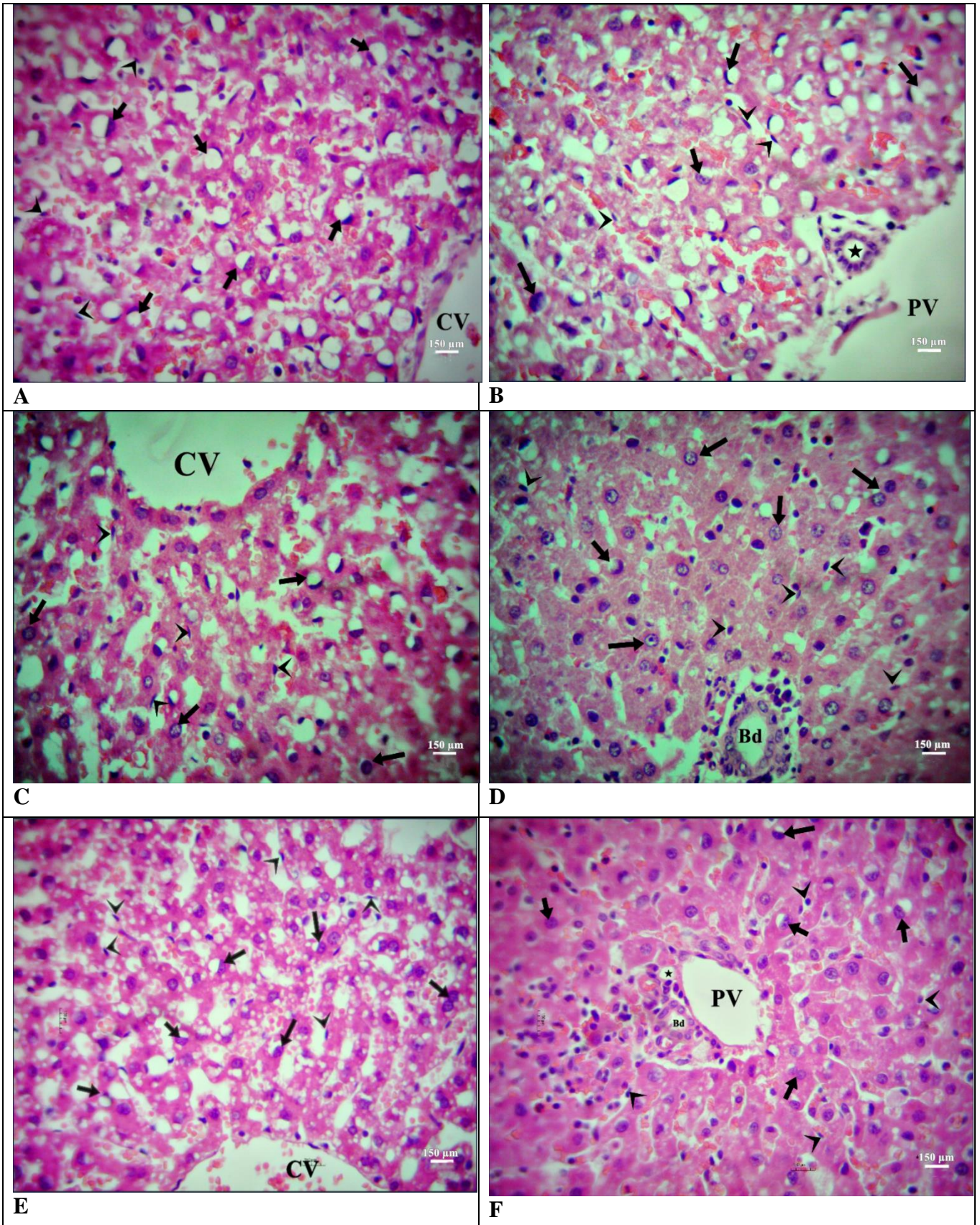
C: Photomicrograph of a rat liver 2 weeks after persistent exposure to a high dose of vitamin A taken from around a central vein (CV). The hepatocytes are arranged in cords albeit in a discontinuous manner. Cellular vacuolation is still evident.

D: Photomicrograph of a rat liver 2 weeks after persistent exposure to a high dose of vitamin A taken from around a portal vein (PV). The hepatocytes are euchromatic and close to each other. There is evidence of restoration of hepatic cords. Fatty change is however still present.

E: Photomicrograph of a rat liver 4 weeks after persistent exposure to a high dose of vitamin A taken from around a central vein (CV). The hepatocytes are arranged in cords radiating towards the central vein (CV) with the HSC in the peri-sinusoidal space. There is a notable decline in cellular vacuolation.

F: Photomicrograph of a rat liver 4 weeks after persistent exposure to a high dose of vitamin A taken from around a central vein. Note that while some hepatocytes have a sharp border, others do not have. Hyperplasia of the hepatocytes and HSC is however evident.

Figure 3.6: Reversal of Cellular Changes Following Persistent Exposure



3.6 Hepatic Stellate Cell Density

The HSC density for the control group, unlike in the intervention group, remained relatively constant for the entire period of the study. These cells were located in the peri-sinusoidal space and appeared deeply eosinophilic. There was no notable difference in their distribution between the periportal and pericentral areas. For the intervention group, high dose vitamin A caused a time dependent increase in the HSC density that reversed considerably with discontinued administration of the vitamin. Relative to the control group, the greatest change in the HSC density occurred at week 6 ($p=0.05$). The HSC density differences within the subgroups of the acutely exposed rats were however not statistically significant (Table 3.4). The increase in the HSC density was more marked in the periportal zones compared to the pericentral ones. These cells became increasingly vacuolated with time with accompanying displacement of the nucleus to the periphery and the assumption of a signet ring shape by week 6 (Figures 3.5D and 3.5F). The hyperplasia of the HSC seemed to occur in clusters and cut across the hepatic parenchyma even though it was more marked in the periportal area.

Table 3.4: Comparisons of Means of HSC Densities for the Various Study Groups Exposed to Persistent High Dose Vitamin A (* statistically significant)

Week	Mean density/mm ² (HSC)	p-value	
		Against Control	Against Immediate Preceding Group
Control	10.024+/-4.74		
Week 2	15.55+/-8.59	0.262	0.262
Week 4	17.17+/-7.88	0.063	0.924
Week 6 Reversal	17.41+/-11.37	0.05*	1
Week 8 Reversal	14.53+/-8.66	0.447	0.552

Persistent exposure to high dose vitamin A resulted in a minimal decline of the HSC density at week 6 which then slightly rebounded at week 8. These changes were not statistically significant ($p>0.05$). Stopping the administration of high dose of vitamin A after week 8

however, resulted in a significant decline in the HSC density by week 12 ($p=0.013$) (Figure 3.1.4) accompanied by a considerable reduction in their vacuolation.

Figure 3.1.4: General Trend of the Hepatic Stellate Cell Density over Time

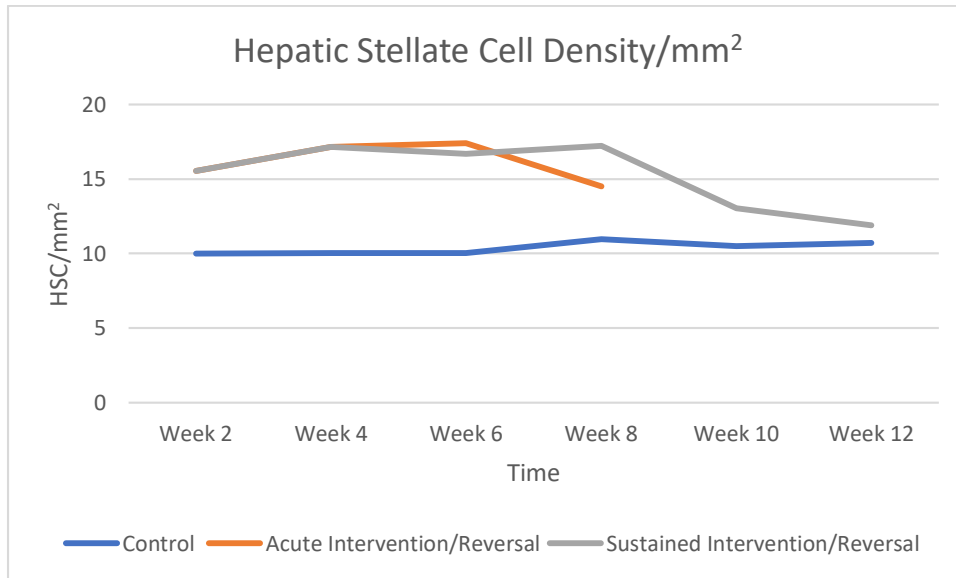


Figure 3.7 Cellular Vacuolation and Fatty Liver Change over Time. (PAS stain). Scale: 600 μm .

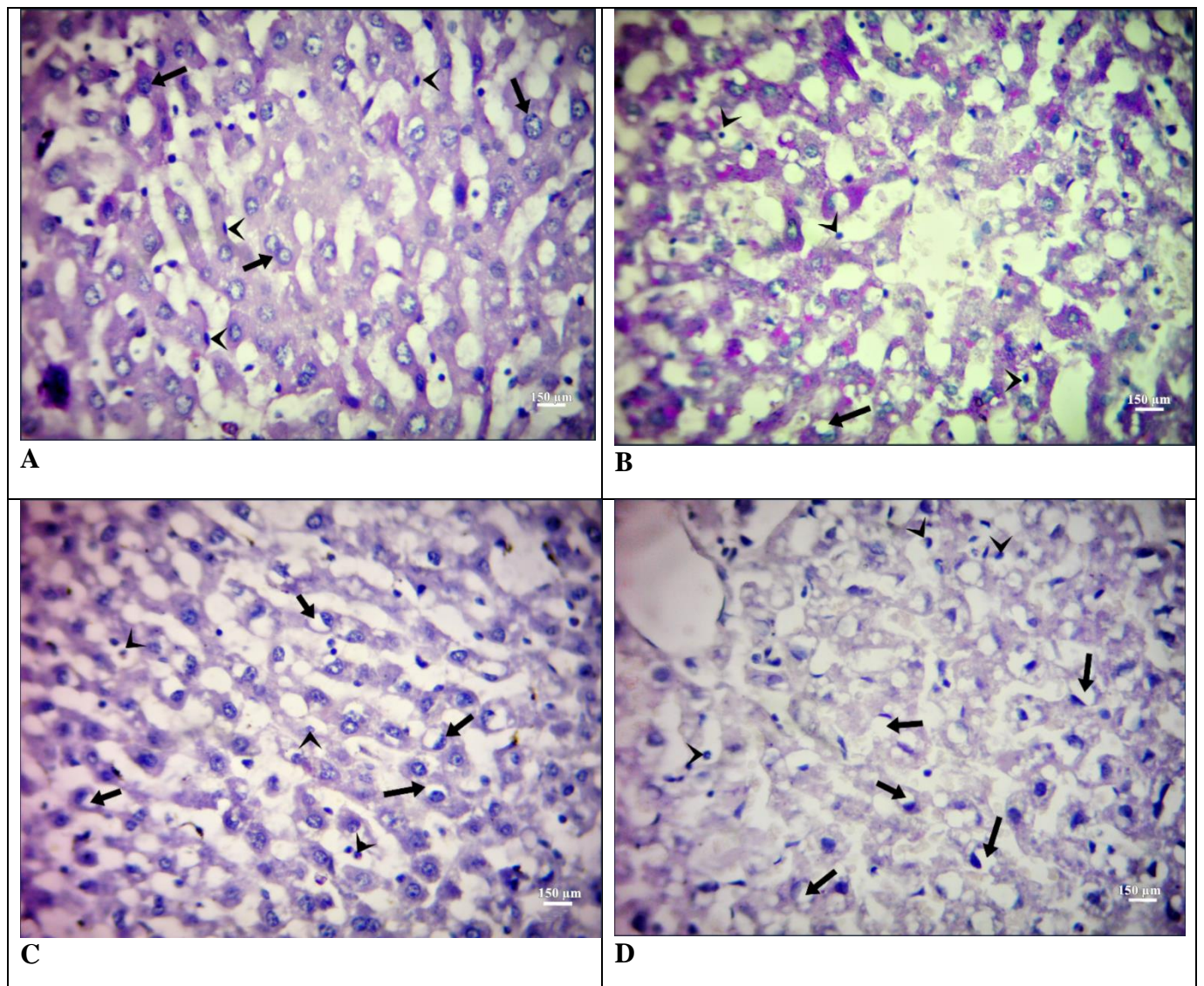
A: Photomicrograph of a control rat liver showing arrangement of hepatocytes in cords (arrows) and perisinusoidal HSC (arrow heads).

B: Photomicrograph of a rat liver at week 2 on high dose vitamin A showing increased fatty changes with predominant vacuolation of HSC (arrow heads) and onset of fatty liver changes.

C: Photomicrograph of a rat liver showing at week 4 of injection showing increased cellular vacuolation now even involving the hepatocytes (arrows).

D: Photomicrograph of a rat liver at week 6 of injection showing extensive cellular vacuolation, increased fatty liver change and massive disruption of hepatic cords.

Figure 3.7 Cellular Vacuolation and Fatty Liver Change over Time



CHAPTER 4

4. DISCUSSION

Complications emanating from chronic hypervitaminosis A include portal hypertension with reported mortalities ensuing from the resultant esophageal varices (Geubel et al., 1991). The negative effects to the liver following enhanced retinoic intake can therefore not be overlooked.

4.1 General Health of the Animals

Exposure to high dose vitamin A did not alter the study animals' behavior nor their general wellbeing. Similar observations have been documented previously in laboratory animals where despite the presence of organ specific damage being established, no clinical features of hypervitaminosis A were reported (Hidiroglou and Batra, 1996; Johansson et al., 2002; Raoofi et al., 2010). This has also been reported in human patients who had tissue diagnosis of vitamin A induced hepatic damage in the absence of clinical features (Forouhar et al., 1984; Levine et al., 2003).

4.2 Absolute Liver Volumes

The figures of the absolute liver volumes for the control group were like those reported by Piao et al., 2013. These near constant figures over time could be attributed to the fact that the animals were recruited into the study as young adults and thus had attained optimal adult liver size. The increase in liver volumes with time in animals exposed to high dose vitamin A is in keeping with previous reports that have documented hepatomegaly in patients with chronic vitamin A intoxication (Geubel et al., 1991; Muentner et al., 1971). Such increase has further been known to precede gross morphological changes in animals subjected to toxicological studies (Piao et al., 2013). Furthermore, feeding rats on a high fat diet has also been shown to increase the hepatic volume independent of tissue shrinkage secondary to histological processing (Altunkaynak and Altunkaynak, 2007). This has been attributed to the liver's paramount role

in energy metabolism. Increase in the liver volume therefore seems to be an important early sign of hepatic injury secondary to hepatotoxins and more so in hypervitaminosis A.

Hepatomegaly seen in liver disease has partly been attributed to the resultant fatty liver changes and hepatic fibrosis which directly tend to increase the liver span (Shrestha, 2015). The latter has also been associated with portal hypertension which, with the resultant blood stasis and tissue deformation, may further cause hepatomegaly (Le Bail et al., 1990; Soon and Yee, 2008). Unfortunately, the increase in the liver size secondary to chronic hypervitaminosis A has been known to persist way after cessation of the excess intake of the vitamin (Muentner et al., 1971) which might explain the continued rise of the liver volumes in our study even after cessation of vitamin A injection for both the acute and persistently exposed rats.

4.3 Body Mass -Normalized Liver Volume (BM-NLV)

From the current study findings, there was a reversible decline in the BM-NLV with exposure to hypervitaminosis A for both interventional groups and which was unlike in the control group. This decline closely paralleled the significant decline in the hepatocyte cell population and since these cells account for approximately 80% of the total liver cell population (Taub, 2004; Wiśniewski et al., 2016), a significant decline in their numbers would foreseeably have a direct impact on the apparent liver volume. This results mainly from the hepatotoxic effects of excessive serum vitamin A levels (Kowalski et al., 1994; Minuk et al., 1988; Sheth et al., 2008) since the liver is the main organ involved in the storage, metabolism and distribution of vitamin A in the body (Paula et al., 2006; Puche et al., 2013). This would therefore explain the observed decrease in the BM-NLV for the intervention group without marked changes in body morphology.

Previous studies have similarly reported results showing alterations in enteric organ size and weight in rodents independent of the body mass index (BMI) (Song and Wang, 2006; Zhu et al., 2012). Such findings have been attributed to changes the blood flow to the said organs

servicing to either increase or reduce their metabolic activity. For instance, a high-fat diet in rats resulted in hepatomegaly (Altunkaynak and Ozbek, 2009) while protein-energy malnutrition resulted in a significantly decreased liver mass (Parra et al., 1995). Therefore, dietary alterations and variations in the ambient environment are among the physiological conditions known to cause isolated hepatic size while initially sparing the body weight.

With discontinuation of exposure to high dose vitamin A however, the BM-NLV was seen to normalize and this closely mirrored the recovery of hepatocyte numbers. Such recovery of the liver following chemotoxin and drug induced injury has also been documented (de Menezes et al., 1984; Kaplowitz, 2004; Ramachandran and Kakar, 2009; Saad et al., 2013). However, it has also been noted that even though resolution of chemical induced liver injury may occur with discontinuation of exposure to the inducing agent, full recovery may take much longer and progression of the disease despite withdrawal of the said agent may still occur (Ramachandran and Kakar, 2009). Close follow up of affected patients would therefore be paramount.

4.4 Pattern of Vitamin A Storage

For the control group in our study, we found that the HSC in the albino rat were generally distributed uniformly within the hepatic parenchyma with no preponderance to any particular hepatic segment. This is unlike in Sprague- Dawley rats where in optimal nutritional levels, vitamin A storing HSCs are only limited to the peripheral zone (Kenjiro Wake, 1974). Different hepatic vitamin A storage patterns have also been described for other animals like the arctic foxes, polar bears and pigs (Higashi et al., 2004; Wake and Sato, 1993; Zou et al., 1998) reaffirming further its genetic predetermination.

Exposure to high dose vitamin A in Sprague- Dawley rats has been shown to significantly affect the distribution of HSC where they were found not only in the peripheral zone but also in the intermediate and central zones (Higashi et al., 2004; Wake, 1971; Kenjiro Wake, 1974). The

hyperplasia of these cells has been thought to originate from the peripheral zone and spread towards the central vein (Higashi et al., 2005; Raoofi et al., 2010). This may explain the findings in the current study where hepatocyte vacuolation and fatty changes were worse around the central veins while there was initial relative sparing of the periportal zone because of the rebound hyperplasia of HSC. However, with persistent exposure to excess of the vitamin the vacuolation and fatty changes extended into the periportal zone highlighting the progressive detrimental effects of chronic hypervitaminosis A and the liver's ultimate inability to withstand the prolonged exposure.

4.5 General Changes Seen in Hepatic Parenchyma

The increased liver fibrosis evident with exposure to high dose vitamin A has been established as the hallmark of chronic vitamin A toxicity. This fibrosis results from an imbalance between the production and dissolution of extracellular matrix (Jiao et al., 2009) and has been known to range from patchy areas of perisinusoidal fibrosis (Croquet et al., 2000; Forouhar et al., 1984; Levine et al., 2003) to a full blown diffuse liver cirrhosis (Geubel et al., 1991; Nollevaux et al., 2006). As was evident from the present study findings, the increased collagen deposition and fibrogenesis has been positively associated with the hyperplasia and transformation of HSC (Lemoinne et al., 2013; Senoo, 2004). In later stages of chronic hypervitaminosis A, central vein, periportal and bridging fibrosis are also known to occur (Davis et al., 1990; Forouhar et al., 1984; Geubel et al., 1991). These fibrotic changes eventually result in narrowing of the sinusoids which ultimately lead to portal hypertension. The contractile nature of the myofibroblasts is also another established cause of vascular distortion further leading to increased vascular resistance (Soon and Yee, 2008).

Other than fibrosis, expansion of the space of Disse has been attributed to the proliferation and expansion of the HSC (Senoo, 2004; Wells, 2008). This was evident in the current study findings and coupled with the hyperplasia of hepatocytes could have led to the distortion of the

hepatic parenchyma and loss of hepatic cords. These hypertrophied cells also bulge into the sinusoids narrowing them further. Moreover, activated HSC besides collagen deposition, show an increased synthesis of a basement membrane like substance along the sinusoids which clogs their fenestrations (Iwaisako et al., 2014; Lemoine et al., 2013; Mederacke et al., 2013; Senoo, 2004). The resultant changes act as a barrier that deprives hepatocytes of much needed oxygen and nutrients eventually resulting in necrosis (Hruban et al., 1974). Attempts at healing results in formation of regenerative nodules that are routinely surrounded by fibrous septa, marking the onset of liver cirrhosis (Wells, 2008). The propagation of this vicious cycle may negatively affect the liver's ability to heal culminating in hepatic failure.

The infiltration of the hepatic parenchyma by inflammatory cells, as seen in the current study findings, is an established response by the liver to hypervitaminosis A (Schon and Weiskirchen, 2014). These cells are known to release various chemokines and immunomodulators vital for the activation of HSC (Li et al., 2008; Tsuchida and Friedman, 2017). The inflammatory cells include Kupffer and Th1 and 2 cells and release various factors including TNF- α , interferons and interleukins (Shi et al., 1997; Sugimoto et al., 2005; Wynn, 2004). Their greatest concentration has been known to occur in regions of focal peri-sinusoidal fibrosis (Nolleaux et al., 2006). These immune cells coupled with the contractile nature of activated HSC have also been proposed to negatively regulate sinusoidal blood flow within the hepatic parenchyma further exacerbating the injury due to hypervitaminosis A (Soon and Yee, 2008).

4.6 Hepatocyte Density and Vacuolation

The rebound reversible hyperplasia with hypervitaminosis A as seen in this study reflects the liver's remarkable ability to regenerate itself following injury. These findings are similar to previous reports documenting the liver's regenerative capacity following partial hepatectomy or administration of hepatotoxic chemicals (Li et al., 2009; Matsuo et al., 2003; Michalopoulos, 2007; Miyaoka and Miyajima, 2013). Such a recovery is essential for the continued pivotal

homeostatic functions carried out by its highly specialized cells (Taub, 2004). Such functions include regulation of body glucose and lipid levels and the metabolism of amino acids, drugs and xenobiotic compounds (Wiśniewski et al., 2016). Any marked derangements in these functions would be detrimental to the survival of an organism.

The process of liver regeneration, although predominantly carried out by dividing hepatocytes (Schaub et al., 2014; Yanger et al., 2014), can occur by all mature liver cells. This is informed by reports documenting that hepatocytes and biliary cells can function as facultative stem cells for each other (Michalopoulos, 2007; Tarlow et al., 2014). This observation was reflected in this study's findings as the rebound hyperplasia of the hepatocytes most markedly seen in week 8 of exposure to high dose vitamin A and which was accompanied by the profound enlargement and hyperplasia of the biliary duct cells. Other events contributing to the regeneration of the liver include hepatocyte hypertrophy, as seen from our study findings, and activation of the oval cell compartment (Erker and Grompe, 2008; Fausto, 2004; Fausto and Campbell, 2003; Miyajima et al., 2014; Miyaoka et al., 2012; Sakamoto et al., 1999).

Hepatocyte hyperplasia seen in liver regeneration is surprisingly a fast process. For instance, it takes 5 – 7 days in rats to restore the original hepatic mass following partial hepatectomy involving up to 2/3 of the liver (Fausto and Campbell, 2003; Grisham, 1962). Initially, up to 60 – 95% of all available hepatocytes, the first cell line to respond to hepatic injury, are recruited to undergo mitosis (Michalopoulos, 2007; Taub, 2004). This occurs in waves of increasingly less recruited hepatocytes until the hepatic mass is restored. However, even though two waves of mitotic division of the hepatocytes has been reported to restore 70% of the hepatic mass (Fausto, 2004; Miyaoka et al., 2012), this largely depends on whether the hepatic trauma occurs as a continuum. These 'waves of mitosis' involving hepatocytes begins in the periportal area of a lobule and then progress towards the pericentral area (Grisham, 1962; Rabes, 1977). This was evident in our findings as the increased cellularity of the periportal area accompanied by

more multinucleated hepatocytes. An overshoot of this proliferative response is corrected by inducing a small wave of apoptosis of hepatocytes at the end of DNA synthesis (Sakamoto et al., 1999). This might explain the finding in the current study of the occurrence of apoptotic cells interspersed in the periportal areas.

Vacuolation of periportal hepatocytes in hypervitaminosis A, as seen in the present study findings, has previously been described (Cheruvattath et al., 2006; Forouhar et al., 1984). The vacuolation seen, and most conspicuous at week 6 of exposure to vitamin A, paralleled hypertrophy of the hepatocytes and an apparent reduction in their density. This might explain the dip in total hepatocyte numbers at week 6 but which went on to recover by week 8 of exposure owing to the increased hyperplastic response.

The extensive hepatocyte vacuolation seen in acute exposure to high dose vitamin A, although considered as an adaptation of the cells to resist further damage and hence beneficial to the host (Nayak et al., 1996), may result in their inability to carry out their functions normally in the short term. This is because vacuolation is considered a marker of senescence and with it comes reduced functionality (Aravinthan et al., 2012). It often leads to eventual hepatocellular dysfunction and synthetic failure. A consequent reduction in the secretion of retinoic binding protein further dents the body's ability to handle the excess vitamin resulting into a vicious cycle of vitamin A induced hepatotoxicity (Bui et al., 2007; Domingos et al., 2016; Noy, 2000; Paula et al., 2006).

4.7 Hepatic Stellate Cell Density

Hepatic stellate cells, normally found in a non-proliferative and quiescent phenotype, are the main cells involved in the storage of vitamin A in the body (Higashi et al., 2005; Senoo, 2004; Wake, 1980). This was reflected in the current study by the rapid increase in the HSC density by week two of exposure to high dose vitamin A. Previous reports have also documented the time and dose dependent hyperplasia of these cells following exposure to hypervitaminosis A

(Davis et al., 1990; Geubel et al., 1991; Nagy et al., 1997; Wake, 1971). Furthermore in humans, the pattern of hyperplasia is mostly time dependent with recent intake being associated with lobular hyperplasia while chronic intake led to a localized hyperplasia limited mainly in the portal spaces and/or within the fibrous septa (Geubel et al., 1991). The hyperplasia of these cells in hypervitaminosis A has been known to occur even in extrahepatic tissues further emphasizing the significance of stellate cells in the metabolism of vitamin A (Nagy et al., 1997).

Discontinuing exposure to vitamin A in both the acute and persistently exposed animals resulted in a decline in the HSC numbers albeit not immediately. This can be attributed to the body's inability to effectively dispose excess retinol (Graham-Maar et al., 2006; Penniston and Tanumihardjo, 2006) and hence the continued elevated HSC density relative to the controls. Apoptosis of the HSC has however been credited with the eventual decline of the activated stellate cells during resolution from hepatic injury (Friedman, 2010; Issa et al., 2001; Wright et al., 2001). This is mediated partly by the resolution of the inflammatory response and the recovery of hepatocytes which are the key sources of cytokines responsible for the activation of HSC.

Other than hyperplasia, HSC respond to acute states of hypervitaminosis A by having an increase in the number and size of their cytoplasmic vacuoles (Nagy et al., 1997; Kenjiro Wake, 1974). This response was similarly observed in our study. It was however reversible with stopping administration of vitamin A for both the acutely and persistently exposed groups. We also observed that there was markedly reduced vacuolation of HSC by week 8 of exposure possibly signifying the duration over which these cells potentially converted in to myofibroblasts in the current study. This conversion however is known to not only be time dependent but also dose dependent (Davis et al., 1990; Geubel et al., 1991) and hence our

findings may be inconclusive in establishing duration over which HSC transformation into myofibroblasts occurs.

5. CONCLUSION

Hypervitaminosis A causes significant but reversible spatial and temporal changes in both the gross and histological aspects of the albino rat liver. This is made possible by the enormous regenerative capacity of the liver. Withdrawal of exposure to vitamin A in cases of suspected toxicity would therefore be paramount in the management of both acute and chronic hypervitaminosis A.

6. STUDY RECOMMENDATIONS

We recommend that future studies should:

1. Have histological and stereological analysis done on tissues obtained from serial liver biopsies of the same animal in order to monitor the progression of hepatic changes. This is because recorded changes did not seem to have a regional differences. This would avoid the bias of documenting hepatic changes on different cohorts of animals.
2. Involve the terminal deoxynucleotidyl transferase nick end labelling test (TUNEL) test to monitor the temporal changes in apoptosis of hepatocytes and HSC with hypervitaminosis A.
3. Include biochemical studies that monitor the levels of RBP and serum vitamin A levels and then correlate them with the accompanying hepatic changes.
4. Include electron microscopy to document ultra-structural changes in progression and resolution of hepatic changes with hypervitaminosis A.

7. Appendices

Ethical approval form



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Dr Jeremiah Munguti
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REF:FVM BAUEC/2017/129

Dear Dr Munguti

19/06/2017

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Structural changes induced by hypervitaminosis A in the liver of Albino rats (*Rattus norvegicus*)

By Jeremiah K. Munguti (H56/81074/2015)

We refer to the above proposal that you re-submitted to our committee for review and approval. We have now reviewed the proposal and note that you have addressed satisfactorily, the issues that had been raised in our letter to you dated 24/05/17. Among these were, inclusion of details on restraint and handling of animals, euthanasia and waste disposal. It is the duty of the principal investigator to ensure adequate measures are taken, for occupational health purposes, when handling tissue processing chemicals. We hereby approve your study as detailed in your revised proposal.

Rodi O. Ojoo BVM MSc PhD
Chairman,
Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

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