ORIGINAL ARTICLE

A Laboratory-Based Experimental Study on Ameliorative Effects of Vitamin D on Chronic Hepatitis Induced by Prolonged Use of Norethisterone in Female Rats

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ABSTRACT

Objective: To assess the ameliorative effects of vitamin D on chronic hepatitis, which was induced by prolonged use of norethisterone in female rats.

Study Design: A laboratory-based experimental animal study.

Place and Duration of Study: The study was primarily conducted in the Department of Anatomy at the Army Medical College (AMC), Rawalpindi in association with the National Institute of Health (NIH) in Islamabad, Pakistan from August 2021 to November 2021.

Methods: A total of thirty female Sprague-Dawley rats weighing between 250 and 300 grams were allocated into three groups randomly by the lottery method. There were ten animals in each group (N = 10 per group). Group A was a control group in which no intervention was carried out. For 8 weeks, Norethisterone at a dose of 4.55 mg/kg per day was administered orally via gavage to group B. Group C received the same dose of norethisterone for eight weeks, coupled with an intraperitoneal injection of vitamin D in a dose of 1000 IU/kg per day for five days a week. At the completion of the experiment, every animal was euthanized. H & E stains were used to stain the liver specimens after they had been processed. All the specimens were evaluated histologically for focal, portal, and periportal hepatic inflammation.

Results: Intergroup comparison produced the results. Significant focal, portal, and periportal hepatic inflammation was observed in experimental group B, with conclusions of 0.001, 0.001, and 0.033 (*P*-values). In experimental group C, there was significant improvement in hepatic inflammation, as indicated by *P*-values of 0.001, 0.001, and 0.029.

Conclusion: Vitamin D has ameliorative effects on chronic hepatitis induced by prolonged use of norethisterone in female rats.

Keywords: *Hepatitis, Liver, Norethisterone, Vitamin D.*

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Introduction

Globally, liver disease is a major contributor to various public health problems.[1] Pakistan has the second-highest hepatitis rate after Egypt. Viral hepatitis, drug-induced hepatitis, and autoimmune hepatitis are the most prevalent pathophysiological disorders of the liver.[2] Currently, there are over a hundred medications known to impair liver function, including synthetic steroid sex hormones.[3] Although liver transplantation and the use of immunosuppressant medications are already available as treatments for hepatitis, they are not regarded as the best options.[4] These results emphasize the fundamental requirement for understanding pathophysiological changes in hepatic injury, as well as the need to develop an effective therapy for liver impairments produced by hepatotoxic substances.

Norethisterone is a first-generation synthetic

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progestin that is approved for the treatment of many gynecological and endocrine conditions, such as contraception, menorrhagia, menstrual postponement, dysfunctional uterine bleeding, breast cancer, and ovarian cysts, according to the Joint Formulary Committee, 2018. Prolonged use of norethisterone in these disorders has been associated with impaired immunity, leading to increased susceptibility to multisystem disorders, including liver impairments.[5] Hargreaves (1969) stated several years ago that the liver plays a major role in the metabolism of almost all steroid sex hormones, including norethisterone.[6] These hormones mainly affect the liver and produce many physiological and pathological effects on the body, such as jaundice, liver damage, and hepatic abnormalities. There are many studies which show that progestin, including norethisterone, has been associated with many adverse effects on the liver, causing hepatitis. Animal models suggest that progestin can cause hepatotoxicity by influencing Kupffer cells, which leads to the development of pro-inflammatory cytokines. These pro-inflammatory cytokines stimulate infiltration of lymphocytes, neutrophils, and macrophages into the parenchymal and portal areas of the liver, thereby facilitating an inflammatory response.[7]

To neutralize the hepatotoxic effects of these synthetic steroid sex hormones, a safe and effective natural supplement needs to be identified. One such supplement is vitamin D, which has non-classical properties, including immunological regulation and anti-inflammatory effects across several human organs.[8] There is proof that vitamin D, particularly its active metabolite, influences the immune system significantly through regulating its physiological activity. One possible explanation for the protective effect of vitamin D is its ability to inhibit the accumulation of highly harmful oxidative stress mediators. Furthermore, the hepatoprotective effects of vitamin D supplementation in drug-induced hepatitis are demonstrated by boosting endogenous levels of anti-inflammatory and antioxidant molecules.[9]

With an annual incidence of 10–15 per 100,000 people and a considerable mortality rate in Pakistan, drug-induced liver damage (DILI) continues to be a serious global health concern.[1] Despite being widely used, synthetic steroid sex hormones, such as norethisterone, have been linked to uncommon but dangerous hepatitis cases. This study is to assess the therapeutic potential of vitamin D in reducing hepatic inflammation in light of Pakistan's high prevalence of liver disease and the paucity of regional data on hormone-induced hepatotoxicity. The use of vitamin D against exogenous hormone-induced hepatitis has a solid biological basis due to its immunomodulatory and antifibrotic properties, which may provide a unique clinical management adjunct.

Methods

It was a controlled animal study. The sample was collected by a nonprobability convenience sampling technique. The study was mainly conducted in the Department of Anatomy of the Army Medical College (AMC), Rawalpindi under the supervision of the National University of Medical Sciences (NUMS), Rawalpindi, Pakistan. Laboratories of the Armed Forces Institute of Pathology and the Military Hospital, Rawalpindi were also utilized. From August 2021 to November 2021, the study was conducted in association with the National Institute of Health (NIH), Islamabad, Pakistan. Army Medical College Rawalpindi ethical committee has approved its clearance before any procedures involving care and handling of animals may be carried out vide letter no: ERC/ID/130, dated: 07th December 2020.

Inclusion Criteria: The study comprised Sprague - Dawley rats (female, non-pregnant), thirty in number, weighing 250–300g and 2-3months old, which were purchased from NIH Islamabad.

Exclusion Criteria: Female rats with any obvious deformity.

Rats were placed at the animal house of NIH under standard laboratory conditions, including a 12-hour light and dark cycle and a temperature of 21 ± 2° C. There were 5 rats per cage, which were of standard size. Animals were fed on standard laboratory rat chow and water ad libitum. Three randomly selected groups of ten rats each were formed (N=10) by lottery method. Each rat in the corresponding group had its tail permanently marked with numbers ranging from 1 to 10 for identification. The study design and categorization specified in Table 1 were used.

Table 1: Grouping of animals

Groups	No of animals	Intervention
Group A (Control)	10	Rats in this group received no intervention. They were given only standard laboratory rat chow and water ad libitum for eight weeks
Group B (Experimental)	10	In rats of this group, norethisterone in a dose of 4.55 mg/kg per day was administered orally through oral gavage for eight weeks.[7]
Group C (Experimental)	10	Rats in this group received the same dose of norethisterone of 4.55 mg/kg per day for eight weeks, coupled with a vitamin D intraperitoneal injection in a dose of 1000 IU/kg per day for five consecutive days in a week

When the experiment was completed (8 weeks), we euthanized all animals. The liver was removed from the abdominal cavity through dissection. Then it was placed in a 10% formalin solution for 24 hours of fixation. After that, tissue samples were processed for 17 hours in (Leica TP 1020 automatic) tissue processor. Using the LEICA EG 1160 embedding center, these tissue samples were infiltrated and embedded in paraffin at 58°C to create paraffin blocks. After complete processing of section blocks, a rotary microtome (Leica RM 255) was used to create transverse liver sections that were 5 micrometers thick. After that, sections were stained using Hematoxylin &Eosin (H&E) staining to assess focal, portal, and periportal inflammation in each rat's liver specimen.

Hepatic inflammation was observed in 4 randomly selected fields in each slide under a 10X objective. Hepatic inflammations were scored on a semi-quantitative score based on the histology activity index (HAI), which was adopted from the modified Ishak grading score system. According to this, grading of focal hepatic inflammation was done as follows: 0 = absent inflammation, 1 = mild (one focus per field), 2 = moderate (less than five foci per field), 3 = marked (five to ten foci per field), and 4 = severe (more than ten foci per field).

Portal hepatic inflammation was graded as: 0 = None (no inflammatory cells identified at HPF), 1 = mild (20%–40% portal areas have inflammatory cells), 2 = moderate (50%–70% portal areas have inflammatory cells), 3 = marked (80% portal areas have inflammatory cells), 4 = Severe (all portal areas have inflammatory cells), and grading of periportal hepatic inflammation was done as follows: 0 = absent, I = mild (20% - 40% portal areas involved), 2 = moderate (50% - 100% portal areas involved), 3 = marked (less than 50% of tracts involved), 4 = severe (more than 50% of tracts involved).

The data was analyzed using SPSS 22. Frequency and percentage were used to present variables. To compare variables, the chi-square test was applied for frequency and percentages. For intergroup comparison, Fisher's exact test was used. A value of ≤ 0.05 was considered a significant *P*-value.

Results

Focal inflammation was assessed histologically (Figure 1). Control group A showed no focal inflammation. Only 10% of liver specimens in experimental group B were normal, showing no focal inflammation. The remaining 50% of liver specimens exhibited grade-1 focal inflammation, and 40% presented with grade-2 focal inflammation. In contrast, liver specimens in experimental group C, were 60% normal and 40% had grade-1 focal inflammation, as shown by Table 2. Chi-Square test showed highly significant results (19.843, 0.0001). For intergroup comparison, Fisher's Exact test showed a highly significant difference (16.157, 0.0001). When comparing group B with groups A and C. However, when group A was compared with C, the result was not significant, with a *P*-value of 0.087.

Portal inflammation was also observed histologically and graded accordingly (Figure 2). There was absence of portal inflammation in control group A. In experimental group B, 50% of liver specimens had mild (grade 1) portal inflammation, and 50% had moderate (grade 2) portal inflammation. While in experimental group C, 70% of liver specimens were normal, and only mild (grade 1) portal inflammation

Table 2: Intergroup comparison between control group A and experimental groups B and C for focal inflammation

Parameter	Histological findings	Group A (N =10)	Group B (N =10)	Group C (N =10)
	Grade 0	10 (100%)	1 (10%)	6 (60%)
Focal inflammation	Grade 1	-	5 (50%)	4 (40%)
	Grade 2	-	4 (40%)	-
Parameter	-	A vs C	B vs C	A vs B
Focal inflammation	-	0.087	0.001	0.001

Table 3: Intergroup comparison between control group A and experimental groups B and C for portal inflammation in the liver

Parameter	Histological findings	Group A (N =10)	Group B (N =10)	Group C (N =10)
	Grade 0	10 (100%)	-	7 (70%)
Portal inflammation	Grade 1	-	5 (50%)	3 (30%)
	Grade 2	-	5 (50%)	-
Parameter	-	A vs C	B vs C	A vs B
Portal inflammation	-	0.211	0.001	0.001

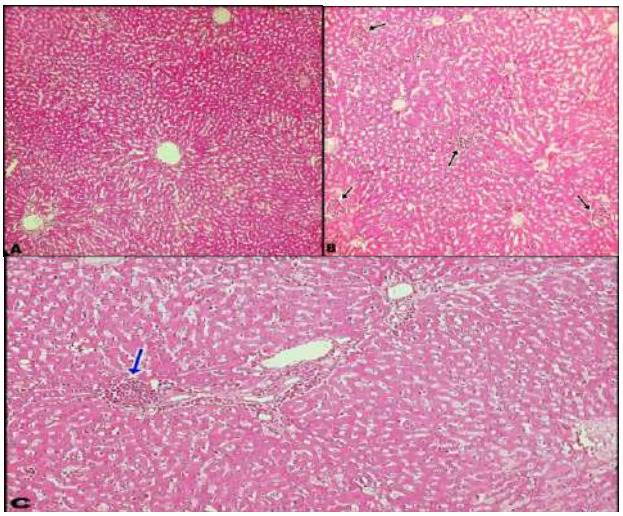

Fig.1: Photomicrograph showing comparison between liver specimens of group A, B, and C for focal inflammation Hematoxylin and Eosin stain. X100

A. Liver of animal A2 exhibited normal architecture and showed no inflammatory foci (group A)
B. Liver of animal B1 exhibited moderate focal inflammation shown by the black arrow (group B)
C. Liver of animal C7 exhibited mild focal inflammation shown by the blue arrow (group C)

was found in 30% of the specimens (Table 3). Chi-Square test showed highly significant results (24.044[a],0.001). For intergroup comparison, Fisher's Exact test showed a highly significant difference (20.825, 0.001). When Comparison of group B with groups A and C was done. However, when group A was compared with C, the result was not significant (*P* value of 0.211).

Periportal hepatic inflammation was also established in histological sections (Figure 3). No periportal inflammation was seen in control group A. In group B, 50% of specimens showed no inflammation, and

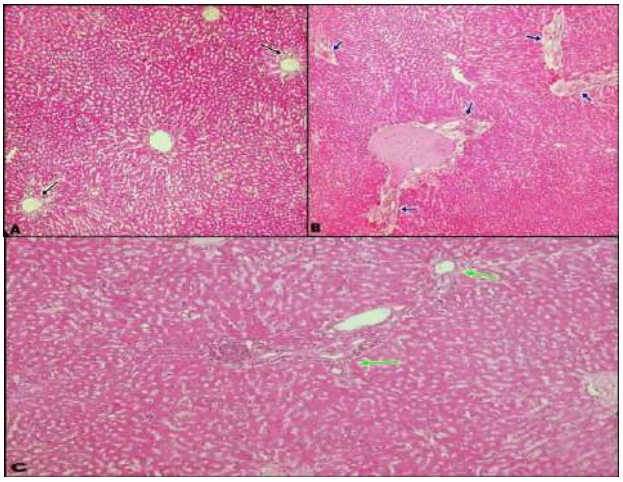

Fig.2: Photomicrograph showing comparison of portal inflammation in liver specimens of groups A, B, and C. Hematoxylin and Eosin stain X100

A. Specimen of animal A2 with normal portal areas shown by the black arrow (group A)
B. Specimen of animal B5 exhibited increased portal inflammation shown by the blue arrow (group B)
C. Specimen of animal C7 with reduced portal inflammation shown by green arrow (group C)

50% showed mild (grade 1) periportal inflammation. On the other hand, in experimental group C, 80% of specimens were normal, and only 20% showed mild (grade 1) periportal inflammation (Table 4). Chi-Square test showed highly significant results (7.081[a], 0.001). By using Fisher's Exact test intergroup comparison of groups A and C with group B showed significant results (6.667, 0.033 and 0.029). However, the comparison between groups A and C was insignificant with a *P*-value of 0.474.

Table 4: Intergroup comparison for periportal inflammation of liver in control group A and experimental groups B and C

Parameter	Histological findings	Group A (N =10)	Group B (N =10)	Group C (N =10)
Periportal inflammation	Grade 0	10 (100%)	5 (50%)	8 (80%)
	Grade 1	-	5 (50%)	2 (20%)
	Grade 2	-	-	
Parameter	-	A vs C	B vs C	A vs B
Periportal inflammation	-	0.474	0.029	0.033

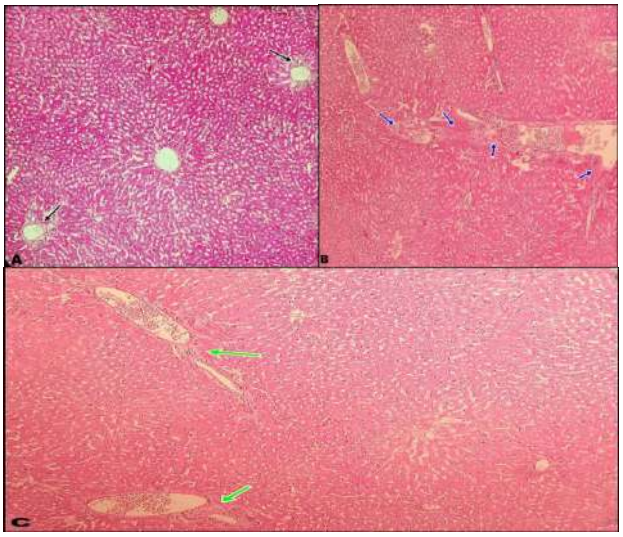

Fig.3: Photomicrograph showing comparison between groups A, B, and C for periportal inflammation in liver specimen. Hematoxylin and Eosin stain. X100

A. Liver of animal A2 with normal portal areas shown by the black arrow (group A)
B. Liver of animal B8 exhibited increased areas of periportal inflammation, shown by the blue arrow (group B)
C. Liver of animal C6 exhibited fewer areas of periportal inflammation shown by the green arrow (group C)

Discussion

Over the past 30 years, synthetic steroid sex hormones have advanced greatly. Today, first-generation progestin norethisterone is widely prescribed for various medical conditions.[10] Even though the use of these steroid sex hormones has drastically lowered the incidence of health-related issues like unsafe abortions and maternal mortality, research is still needed to determine the long-term consequences of these hormones on vital human body organs, such as the liver.[11,12] There are now rumors that a lot of women have given up on these synthetic sex hormones, such as norethisterone, because of the negative impact they have on their liver, body weight regulation, glucose metabolism, and general homeostatic regulation.[13] It has become a source of concern for all medical professionals who use these drugs extensively in their prescriptions.

Therefore, in order to counteract the hepatotoxic effects of these synthetic steroid sex hormones, it is necessary to find an effective and secure natural compound that can be used in combination with them. One of these supplements can be vitamin D, a pre-prohormone with anti-proliferative and anti-inflammatory properties that affect several body organs.[14] Vitamin D, vital for bone mineralization, also shows pleiotropic effects. With receptors present in most body cells, vitamin D may influence nearly all tissues.[15] Several studies have shown associations between vitamin D effects and various chronic diseases.[16] Therefore, those with chronic liver illnesses may also benefit from improving their vitamin D status.[17] Vitamin D has been used as a protective agent against drug toxicity in many recent studies, including those involving the liver.[18] Therefore, vitamin D could be effectively used as a therapy for chronic hepatitis caused by prolonged

use of sex hormones as well. From this point of view, this study was conducted to investigate possible protective effects of vitamin D against chronic hepatitis caused by norethisterone.

According to this study, long-term use of norethisterone showed disrupted architecture with highly significant focal and portal inflammation and significant periportal inflammation in the liver specimens of female rats. Similar significant changes in liver architecture leading to chronic hepatitis were also demonstrated previously by a study, in which there was long-term (three weeks) use of norethisterone as an oral contraceptive in female rabbits. As observed by Ahn S et al. focal hepatic inflammation is defined as small clusters of lymphocytes seen within liver parenchyma.[19,20] In our investigation, long-term norethisterone usage resulted in chronic hepatitis, with periportal inflammation or interface hepatitis caused by portal inflammatory infiltrates that disrupted the limiting plate and extended into zone 1.[21] This outcome aligns with many prior researches indicating liver complications associated with progesterone use.[3,22]

In the current study, portal inflammation was dominant in chronic hepatitis with foci of lymphocyte collection, which was predominant in the centrilobular area (zone 3) near the central vein. While a few areas of periportal inflammation were also found in this chronic hepatitis. The same effects of prolonged use of norethisterone were also observed in a study by Wang and his colleagues.[7] This study was conducted on zebrafish and marked hepatic cellular damage, and inflammation was observed after 72 days of usage of norethindrone. In the current study chronic hepatitis was also associated with hepatic congestion. This effect of progesterone-based sex hormone was also observed by Alassaf FA et al. in their study on female rats.[23] In which they exposed the female rats to these hormones for 8 weeks, and marked hepatic sinusoidal congestion was observed.

In those female rats in which intraperitoneal injection of vitamin D along with norethisterone was given showed significant protection against chronic hepatitis as evidenced by preservation of liver parenchymal architecture observed in their liver specimens. There was highly significant improvement in focal and portal inflammation, with a marked reduction in hepatic congestion with vitamin D. These results were previous recorded in another study in which anti-inflammatory response of vit D was found against chronic hepatitis caused by high fat diet in mouse.[24] But in our current research, when vitamin D was given, it was also found that the rats were significantly protected from periportal inflammation as well, which was more effectively assessed and revealed by histological specimens. This protective role of vitamin D was also evidenced by a study by El-Boshy and his colleagues, which demonstrated that vitamin D ameliorates the acute hepatic inflammation caused by acute toxicity of paracetamol by activating VDR on Kupffer cells in the liver and inhibiting the release of proinflammatory cytokines to reduce inflammation.[25] These effects of vitamin D were also recorded by many other studies investigating its protective role in drug toxic effects on the liver.[26,27] These studies showed that vitamin D reduced hepatotoxicity induced by agents such as pioglitazone and CPF, though histological evidence was limited. In our study, histological improvements were clearer against norethisterone-induced chronic hepatitis, yet further research is needed to assess cellular-level effects on hepatocytes.

In our present study, vitamin D very effectively prevents the establishment of chronic hepatitis, and its effects on liver architecture, which were caused by prolonged use of norethisterone, appeared in the form of reduced inflammatory infiltrates within liver parenchyma, with reduced congestion revealed very clearly by histological specimens. These results were also parallel with results of some previous studies, in which El-Magd NFA et al. studied the molecular mechanism of ameliorative effects of vitamin D on acute liver injury caused by paracetamol.[28] Same anti-inflammatory and anti-apoptotic properties of vitamin D were also assessed in another study, in which its ameliorative effects were revealed against iodine uptake. But in the present study, the protective effects of vitamin D were observed in norethisterone-induced chronic hepatitis, which has not been studied before.[29] Thus, supplementing vitamin D has been considered a safe and inexpensive approach to generate better health outcomes, especially in women taking prolonged therapy of norethisterone, to undo its chronic

inflammatory response in the liver, as proved by the results of this study. However, this study still has some holes because it has limits of its tiny experimental model, does not explain the molecular processes of vitamin D's protective effect, or does not assess whether the advantages are transient or long-lasting. Although the results are encouraging, the therapeutic relevance remains unknown, and human trials are needed to validate safety, efficacy, and dosing.

Conclusion

The current research led to the conclusion that prolonged use of the synthetic steroid sex hormone norethisterone has a significant inflammatory effect on the liver. Vitamin D supplementation had ameliorative effects on this chronic hepatic inflammation. This study thus supports the hepatoprotective effect of vitamin D against chronic hepatitis produced by norethisterone.

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Conflict of Interest: The authors declare no conflict of interest

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AT: Conception, design of the work, and approval for final submission
MRBK: Revising, editing, supervising for intellectual content, and approval for final submission
AZ: Data acquisition, curation, statistical analysis, and approval for final submission
RK: Manuscript writing for methodology design, investigation, and approval for final submission
SB: Writing the original draft, proofreading, and approval for final submission
SI: Validation of data, interpretation, write-up of results, and approval for final submission
AT is the nominated guarantor and takes full responsibility for the overall content and integrity of the work