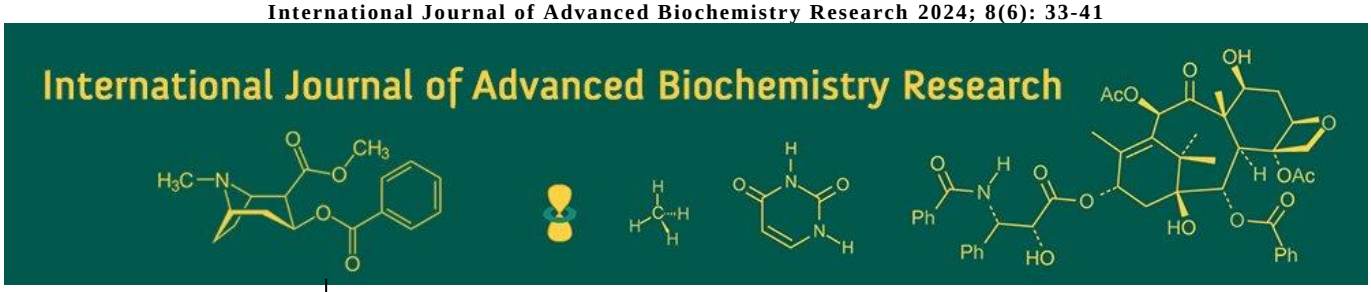

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Biochemical effects of simvastatin on autoimmune arthritis in a mouse induced model

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Abstract
Background: Autoimmune arthritis is a type of arthritis in which the immune system attacks the body itself. The link between cell death, inflammation and autoimmune diseases has been established, and dysregulation of autophagy has been reported in several autoimmune diseases such as autoimmune arthritis. Simvastatin is a lipid-lowering drug with pleotropic anti-inflammatory and immunomodulatory effects.
Aim of Study: The aim of the present study was to explore the cross-talk between autophagy, necroptosis and metabolic changes in pristane-induced arthritis with highlighting the effect of simvastatin.
Material and Methods: The study was conducted on 50 female mice, which were divided into 5 groups. Group I served as a control; group II was given simvastatin only; group III was given pristane to induce arthritis; group IV was treated with simvastatin from the first day with pristane injection; and group V was treated by simvastatin with the appearance of arthritic manifestations induced by pristane. Joint tissue samples were collected for histopathological examination and biochemical assessment of cholesterol, triacylglycerol and lactate levels using colorimetric techniques, as well as the levels of RIPK3 and Akt using ELISA technique.
Results: Administration of simvastatin from the first day corrected the biochemical changes, induced autophagy, suppressed necroptosis and protected against the arthritic changes. However, the delay in its administration after the appearance of arthritic manifestations resulted in partial improvement. The histopathological findings confirmed the laboratory results.
Conclusion: Simvastatin administration markedly alleviated joint inflammation and damage induced by pristane through inhibition of inflammation, induction of autophagy and suppression of necroptosis. The use of simvastatin as a protective drug against progression of autoimmune arthritis seems to be promising.

Keywords: Mice, arthritis, autophagy, necroptosis, pristine, simvastatin

Introduction
Arthritis is a global health problem that affects millions of people, with billions of dollars spent annually for their treatment. Prevalence of this disease is higher among females and those living in developed countries. Arthritis includes several types having various manifestations, complications and outcomes. One category of this disease is autoimmune arthritis (AA) in which the immune system attacks the body itself. The most common types of AA include rheumatoid arthritis (RA) and psoriatic arthritis (Silman and Pearson, 2002; Rudan *et al*., 2015) [35, 34].
Autoimmune arthritis shows important pathological features such as synovial hyperplasia, tissue hypoxia, inflammation and increased infiltration of immune cells with increased production of inflammatory cytokines, chemokines, adhesion molecules and matrix metalloproteinase. These pathological changes may end with cartilage damage, bone erosion and deformity (Garside *et al*., 2011; Tuncel *et al*., 2016) [46, 38].
Animal models of AA, such as the model of pristane-induced arthritis, have been designed to study the pathogenesis of that disease and to estimate efficacy of anti-arthritic drugs for clinical use. The most important criteria in model selection are the morphological similarity of the model to human disease and its capacity to predict efficacy in humans (McInnes and Schett, 2011; Krishnamurthy *et al*., 2016) [25, 18].

The links between different types of cell deaths, inflammation and autoimmune diseases have been established. Under normal circumstances, the intracellular process of autophagy is tightly controlled. However, in auto-immune and degenerative joint diseases, such as rheumatoid arthritis, dysregulation of autophagy can occur, and this contributes substantially to the joint pathology (Rubinsztein *et al*., 2012; Hu and Dai, 2015) [33, 14].

Protein kinase B, also known as Akt, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes. Akt phosphorylates and regulates many proteins involved in metabolism, survival/apoptosis, differentiation and proliferation. Studying the regulatory pathways and the *in vivo* functions of Akt can potentially improve treatment of diseases in which Akt signaling is dysregulated, such as diabetes and autoimmune arthritis (Fan *et al*., 2010; Mobasheri *et al*., 2017; Chu *et al*., 2018) [1, 26, 4].

Apoptosis is one of several types of regulated of cell death that may play an essential role in autoimmune disease pathogenesis. Necroptosis is another type of regulated of cell death, which is believed to be a more potent inducer of inflammation than apoptosis. Necroptosis is mediated by immune ligands including tumor necrosis factor alpha (TNF-α) leading to activation of receptor-interacting serine/threonine-protein kinases (RIPK), RIPK1/RIPK3. RIPK3 phosphorylates and activates mixed-lineage kinase domain-like protein (MLKL), which then translocates into the inner leaflet of the plasma membrane and disturbs the integrity of the cell with membrane ruptures and more inflammation (Kanduc *et al*., 2002; Sun *et al*., 2012; Newton *et al*., 2014; Dhuriya and Sharma, 2018) [15, 37, 28, 6].

Statins, such as simvastatin, are a group of lipid-lowering drugs that have been successfully used to control hyperlipidemia and reduce cardiovascular diseases risk and their associated mortality in high risk individuals. Recently, statins are reported to have other important pleotropic roles including anti-inflammatory action and immune system modulation (Dai *et al*., 2016; Tuuminen *et al*., 2016) [5, 39].

The aim of this study was to explore the cross-talk between autophagy, necroptosis and metabolic changes in autoimmune arthritis and the alteration in disease course with statin as an inducer of autophagy.

Material and Methods

Ethical considerations

Care of the animals as well as the experimental procedures were performed in Medical Biochemistry Department, Faculty of Medicine, Tanta University, Egypt in accordance with guidelines of the Ethics Committee of the Faculty (Approval code 30977/5/16).

Animals

This study was conducted on fifty female mice, weighing 20-30g. The mice were housed in galvanized metallic cages at room temperature with free access to drinking water and food. They were acclimatized to the laboratory environment for one week before initiation of the experiment.

Study design

The mice were randomly divided into the following 5 groups (10 mice each):

Group I (Control): The mice were given saline intradermal 100 ul at the dorsal side of the tail.

Group II: Each mouse was treated with simvastatin at a dose of 10 mg/kg/day once daily for 55 days (Palmer *et al*., 2004) [30].

Group III (Pristane-induced arthritis): Arthritis was induced by an intradermal injection of 100 µl pristane (2, 6, 10, 14 tetramethylpentadecane) at the dorsal side of the tail base. The severity of arthritis was graded visually by assessing the level of swelling in each paw, including the tarsus (Ankle) or carpus (Wrist) joints (Leung *et al*., 2003; Patten *et al*., 2004) [20, 31].

Group IV: Each mouse was treated with simvastatin at a dose of 10 mg/kg/day once daily for 55 days starting from first day with pristane injection (Palmer *et al*., 2004) [30].

Group V: Each mouse was treated with simvastatin at a dose of 10 mg/kg/day once daily for 55 days with appearance of clinical manifestations (Palmer *et al*., 2004) [30].

Sampling and data collection

At the end of the treatment period, mice were sacrificed, and the study samples were taken. Joints' tissues were extracted, divided in to pieces for histopathological and biochemical assessments. Samples for histopathological examination were preserved in 10% formal-saline to be stained with hematoxylin and eosin for light microscopy. Samples for biochemical parameters' assessment were stored at –20 °C to be used later for tissue homogenization and assessment of cholesterol, triacylglycerol (TAG) and lactate levels (For detection of Warburg effect) using colorimetric techniques, as well as the levels of RIPK3 (For assessment of necroptosis) and Akt (For assessment of autophagy) using ELISA according to the manufacturers' instructions.

Statistical analysis

Data analysis was carried out using SPSS version 22 for windows. Numerical variables were checked for normality by Shapiro Wilk test. Normally distributed data were summarized as mean ± standard deviation, and differences between groups were tested by one-way ANOVA and a suitable post hoc test. Data that did not follow normal distribution were summarized as median and interquartile range, and comparison between groups was achieved by Kruskal-Wallis test and Dunn-Bonferroni post hoc test. A p-value of < 0.05 was considered statistically significant.

Results

Table (1) shows statistically significant differences in esterified cholesterol levels among the studied groups ($F = 28.662$, $p<0.001$). Group II showed significantly lower levels than all other groups. Group III had the highest level, with a statistically significant difference from groups I and II. The free cholesterol levels show statistically significant differences among the studied groups ($F = 73.824$, $p<0.001$). Group II showed the lowest level with statistically significant difference from all other groups. Group I had a statistically significant lower level than groups III, IV and V. Group III had the highest level, with a statistically significant difference from all other groups

(Table 2). Table (3) shows statistically significant differences in TAG levels among the studied groups (F = 53.549, $p<0.001$). Group II showed the lowest level with a statistically significant difference from groups III, IV and V. Group I had a significantly lower level than groups III, IV and V. Group III and V had the highest mean level, with a statistically significant difference from groups I and II. Lactate levels show statistically significant differences among the studied groups (F = 8.117, $p<0.001$). Group III showed the highest level with statistically significant differences from groups I, II, IV and V. Group IV showed a statistically significant decrease compared with group III (Table 4). Statistically significant differences in nuclear pAkt levels among the studied groups are shown in Table (5) (Z = 24.867, $p<0.001$). Group III showed statistically significant increase in nuclear pAkt level compared to control group I and group IV. Table (6) shows statistically significant differences in nuclear RIPK3 levels among the studied groups (Z = 11.057, P = 0.026). Group V showed statistically significant increase in RIPK3 level compared to control group I.

Both control (Group I) and simvastatin-treated (group II) groups show normal joint spaces and synovial linings with no remarkable pathological changes (Figures 1 & 2). Induction of arthritis with pristane in group III mice was associated with synovial hyperplasia and inflammatory cellular infiltration (Figure 3). Figure 4 shows that the administration of simvastatin from the first day of the experiment to group IV mice had markedly decreased their arthritic changes. However, delaying the administration of simvastatin after appearance of arthritic manifestations in group V resulted only in partial improvement, and the histopathologic changes in these mice were midway between the arthritic group and the simvastatin-protected group (Figure 5).

Discussion

The current study shows that the induction of arthritis was associated with increases in cholesterol, TAG, Akt and RIPK3 levels in the tissue homogenates of arthritic joints, as well as synovial hyperplasia and inflammatory cellular infiltration, indicating the occurrence of joint inflammation, inhibition of autophagy and induction of necroptosis. Administration of simvastatin from the first day of the experiment markedly decreased the biochemical changes, induced autophagy, suppressed necroptosis and protected against the arthritic changes. However, delaying the administration of simvastatin after appearance of arthritic manifestations resulted only in partial improvement, where both biochemical and histopathologic changes were midway between the arthritic group and the simvastatin-treated group.

The present study revealed increased esterified and free cholesterol levels in pristane-induced arthritic group when compared to the control and simvastatin-treated groups. Elevated cholesterol level in the arthritic group may be due to impaired HDLc efflux resulting from down-regulation of apolipoprotein A-I responsible for promoting reverse cholesterol transport. This ends with impaired cholesterol efflux and intracellular lipid deposition (Arkill and Winlove, 2006; Charles-Schoeman *et al*. 2012) [1, 3]. Elevated cholesterol levels in arthritic group is comparable to an earlier study that reported increased cholesterol and lipoprotein content in RA synovial fluid. In inflammatory conditions, increased intracellular free cholesterol concentration in macrophages is accompanied by enhanced inflammatory response of these cells (Oliviero *et al*., 2009) [29]. In the present study, administration of simvastatin to mice in which arthritis was induced by pristane caused a greater decrease in cholesterol levels in the group receiving simvastatin from the first day than those receiving it after appearance of manifestations. This indicates that simvastatin might have a better protective rather than a curative effect.

The TAG levels were significantly elevated in pristane-induced arthritic group compared to the control and simvastatin-treated groups. However, TAG levels in groups treated by both simvastatin and pristane were nearly similar to that of pristane-induced arthritis group. Elevation of TAG levels in the inflamed joints may be due to inflammation-induced triggering of articular adipocytes for the production of cytokines that inhibit lipolysis with subsequent increased level of lipid components in joint tissue (Xu *et al*., 2016) [43]. These findings are in accordance with Srivastava *et al*. (2018) [36] who reported a positive correlation between the levels of several cytokines during joint inflammation and TAG in joint tissue in CIA rat model. The current study did not show a difference in TAG levels in arthritic mice with simvastatin treatment, although statin-induced depression of fatty acid metabolism and TAG synthesis has been reported and suggested to be due to HMG-CoA reductase-mediated inhibition of acetyl coenzyme A carboxylase and fatty acid synthase, the two important regulatory enzymes in fatty acid biosynthesis (Liu and Chiu, 2019) [23].

Increased lactate levels in pristane-induced arthritis compared to the control group were reported in the present study. This could be attributed to changes in glucose metabolism and increased nitric oxide (NO) production, which inhibits cytochrome C oxidase enzyme and mitochondrial respiration. Switching of metabolic processes from oxidative phosphorylation to anaerobic glycolysis results in increased lactate production. This process is known as "Warburg effect" and was described in tumors and autoimmune diseases like RA (Kim *et al*., 2002; Garcia-Carbonell *et al*., 2016) [16, 13]. Fujii *et al*. (2015) [11] reported that the activity of lactate dehydrogenase (LDH) enzyme and the rate of glycolytic metabolism were increased in synovial tissues of RA patients. Peng *et al*. (2016) [32] found that LDH play an important role in promoting inflammation and T-cell effector functions via increasing IFN-γ transcription. Arthritic mice treated with simvastatin showed significant reductions in their elevated lactate levels. Statins suppress inducible nitric oxide synthase expression, with subsequent decrease in lactate levels in simvastatin-treated groups. These current results are consistent with Moncada and Erusalimsky (2002) [27] who reported that lactate production was increased with joint inflammation. On the contrary, Dibble *et al*. (2015) [8] reported a statin-induced increase in lactate production. This might result from decreased production of coenzyme Q10, which is an essential carrier in the mitochondrial respiratory chain that participates in oxidative phosphorylation. Consequently, there is decreased activity of mitochondrial complex 1 and reduced electron carrier transport.

The present study revealed that pAkt level was significantly increased in pristane-induced arthritic mice. Comparable results were reported by Wang *et al*. (2012) [41] in RA patients. Di Lorenzo *et al*. (2009) [7] revealed that pAkt may account for the anti-apoptotic response of synovial

fibroblasts to TNF-α and transforming growth factor-β, suggesting that high Akt levels may contribute to the synovial hyperplasia in RA patients. Also, pAkt may increase during acute inflammation as it affects vascular permeability, leading to edema and leukocyte extravasation (Bellacosa *et al*., 2004) [2]. This study reported that statin significantly reduced the pAkt levels in arthritic mice treated with simvastatin from the start of induction, and to lesser degree in those who were treated after the appearance of manifestations. It was reported that statins inhibited Akt survival pathway in RA synovial fibroblasts. This was explained by the effect of statins as autophagy inducers via interference with mevalonic acid synthesis and subsequently biosynthesis of geranylgeranyl diphosphate that activates AMPK and inactivates Akt/mTOR pathway activity. Therefore, Akt levels are decreased when autophagy is induced (Lee *et al*., 2007; Dillon *et al*., 2014) [19, 9]. However, Yang *et al*. (2004) [45] showed that statins can enhance the expression of PI3K/Akt after experimental intracerebral hemorrhage, which might propose that statin effects depend on the tissue and cell types.

Although statistically non-significant, RIPK3 levels in the arthritic group were higher than the control group, but the levels in the group treated with simvastatin after the appearance of manifestations were significantly higher than all other groups. This could be explained by activation of necroptosis pathway during joint inflammation and liberation of cytokines from the inflammatory cells, particularly TNF-α that interacts with RIPK1 and RIPK3 (Lee *et al*., 2007; Dillon *et al*., 2014) [19, 9]. Necroptosis plays an important role in inflammatory diseases. Dying cells could directly trigger inflammation by releasing DAMPs, including interleukin-1 family cytokines, S100 proteins, as well as a range of pro-inflammatory cytokines into the intracellular space, thereby exposing them to the immune system and creating an inflammatory reaction (Dhuriya and Sharma, 2018) [6]. This result agrees with Lee *et al*. (2017) [19] who performed histological analysis of joint tissue from CIA mice model. They showed that CIA stimulate immune cell infiltration and cartilage damage and MLK/ RIPK1 and RIPK3 levels were increased significantly in CIA mice.

Histopathologic examination of joint tissue from the studied groups proved the biochemical findings discussed above. Joint tissue histological study from pristane arthritic group showed hyperplastic synovial lining with underlying moderate inflammatory reaction that was composed of lymphocytes, macrophages and few plasma cells, with mild fibrosis. These changes are consistent with those reported in various animal models of induced arthritis (Yamagata *et al*., 2007) [44]. Hyperplasia of synovial cells results in more infiltration of inflammatory cells into the synovial membrane that release pro-inflammatory cytokines, which mediated the destruction of hyaline cartilage and bone by stimulating the synthesis and release of collagenase, metalloproteinase and eicosanoids in addition to, phagocytic activity that was displayed by synoviocytes thus aggravating the lesion (Liu *et al*., 2009; Xie *et al*., 2016; Tuuminen *et al*., 2016) [23, 42, 39]. Administration of simvastatin simultaneously with pristane resulted in marked improvement in histopathological features with no inflammation and no remarkable pathological changes. Meanwhile, sections from group that received simvastatin after the appearance of arthritic manifestations exhibited histopathological features that were nearly similar to untreated arthritic group, but with less severe inflammation. The present study results were consistent with Funk *et al*. (2008) [12] who found that simvastatin prevented synovial hyperplasia and joint inflammation when given prophylactically in a rat model of experimental arthritis, but resulted in much less improvement when given to manifested arthritic rats.

The ability of simvastatin to ameliorate or protect against arthritis is achieved through various mechanisms. Statins inhibit the production of inflammatory mediators, such as IL-6 and TNF production, and stimulate the release of anti-inflammatory cytokines, such as IL-10; this explains the reduced leucocytic infiltration after simvastatin administration as cytokines attract, activate and facilitate the influx of leukocytes (Van Roon *et al*., 2001; Liew and McInnes, 2002; Yang *et al*., 2004) [40, 22, 45]. In addition, simvastatin decreases the number of synovial fibroblasts, most probably due to induction of autophagy through the inhibition of Akt (Kok *et al*., 2011) [17].

In conclusion, treatment with simvastatin markedly alleviated pristane-induced joint inflammation and damage in experimental mice as evidenced by alterations in the biochemical indices and joint histopathological changes. The present study demonstrated that simvastatin has a protective effect against arthritic changes induced by pristane. The effect that may occur through modulation of metabolic switch, alteration of tissue lipidomic status, induction of autophagy and suppression of necroptosis, in addition to the decreased joint tissue infiltration with inflammatory cells. Thus, the use of simvastatin for autoimmune arthritis seems to be promising. However, evaluation of its efficacy and safety as well as assessment of the underlying mechanisms of actions deserve further studies.

Table 1: Statistical comparison of esterified cholesterol (mg/g tissue) among the studied groups using one-way ANOVA test.

	Esterified Cholesterol level (mg/g tissue)						**One Way ANOVA test**	
	Range			**Mean ± SD**			**F**	**P-value**
Group I (Control)	0.056	-	0.091	0.075	±	0.015	28.662	<0.001*
Group II (Statin only)	0.011	-	0.029	0.022	±	0.007		
Group III (Pristane only)	0.109	-	0.390	0.233	±	0.112		
Group IV (Statin with pristane from first day)	0.050	-	0.180	0.104	±	0.050		
Group V (Statin with appearance of manifestations)	0.070	-	0.250	0.129	±	0.064		

Games-Howell post hoc test					
	I	II	III	IV	V
I	1.000	0.001*	0.029*	0.509	0.180
II	0.001*	1.000	0.007*	0.007*	0.006*
III	0.029*	0.007*	1.000	0.080	0.211

IV	0.509	0.007*	0.080	1.000	0.874
V	0.180	0.006*	0.211	0.874	1.000

SD: standard deviation; * significant at $p<0.05$.

Table 2: Statistical comparison of free cholesterol level (mg/g tissue) among the studied groups using one-way ANOVA test.

	Free Cholesterol level (mg/g tissue)						One Way ANOVA test	
	Range			Mean ± SD			F	P-value
Group I (Control)	0.071	-	0.091	0.084	±	0.008	73.824	<0.001*
Group II (Statin only)	0.019	-	0.052	0.040	±	0.011		
Group III (Pristane only)	0.410	-	0.960	0.587	±	0.167		
Group IV (Statin with pristane from first day)	0.119	-	0.394	0.247	±	0.090		
Group V (Statin with appearance of manifestations)	0.233	-	0.462	0.360	±	0.073		
Games-Howell post hoc test								
	I		II	III		IV	V	
I	1.000		<0.001*	<0.001*		0.004*	<0.001*	
II	<0.001*		1.000	<0.001*		0.001*	<0.001*	
III	<0.001*		<0.001*	1.000		0.003*	0.036*	
IV	0.004*		0.001*	0.003*		1.000	0.066	
V	<0.001*		<0.001*	0.036*		0.066	1.000	

SD: standard deviation; * significant at $p<0.05$.

Table 3: Statistical comparison of TAG (mg/g tissue) among the studied groups using one-way ANOVA test.

	TAG (mg/g tissue)						One Way ANOVA test	
	Range			Mean ± SD			F	P-value
Group I (Control)	0.022	-	0.078	0.041	±	0.024	53.549	<0.001*
Group II (Statin only)	0.011	-	0.038	0.022	±	0.011		
Group III (Pristane only)	0.072	-	0.099	0.084	±	0.008		
Group IV (Statin with pristane from first day)	0.073	-	0.095	0.083	±	0.008		
Group V (Statin with appearance of manifestations)	0.073	-	0.098	0.084	±	0.008		
Games-Howell post hoc test								
	I		II	III		IV	V	
I	1.000		0.410	0.030*		0.034*	0.031*	
II	0.410		1.000	<0.001*		<0.001*	<0.001*	
III	0.030*		<0.001*	1.000		1.000	1.000	
IV	0.034*		<0.001*	1.000		1.000	0.999	
V	0.031*		<0.001*	1.000		0.999	1.000	

SD: standard deviation; * significant at $p<0.05$.

Table 4: Statistical comparison of lactate (mg/g tissue) level among the studied groups using one-way ANOVA test.

	Lactate mg/g tissue						One Way ANOVA test	
	Range			Mean ± SD			F	P-value
Group I (Control)	0.045	-	0.090	0.071	±	0.017	8.117	<0.001*
Group II (Statin only)	0.062	-	0.150	0.089	±	0.028		
Group III (Pristane only)	0.099	-	0.199	0.148	±	0.038		
Group IV (Statin with pristane from first day)	0.023	-	0.106	0.078	±	0.026		
Group V (Statin with appearance of manifestations)	0.021	-	0.145	0.069	±	0.043		
Tukey post hoc test								
	I		II	**III**		**IV**	**V**	
I	1.000		0.827	0.001*		0.990	1.000	
II	0.827		1.000	0.007*		0.961	0.723	
III	0.001*		0.007*	1.000		0.001*	<0.001*	
IV	0.990		0.961	0.001*		1.000	0.975	
V	1.000		0.723	<0.001*		0.975	1.000	

SD: standard deviation; * significant at $p<0.05$.

Table 5: Statistical comparison of nuclear pAkt (pg/mg nuclear protein) among the studied groups using Kruskal-Wallis test.

	Nuclear pAkt pg/mg nuclear protein				Kruskal-Wallis test	
	Range	Median	IQR	Mean ranks	Z	P-value
Group I (Control)	2.810 - 19.180	8.705	3.760 - 15.150	5.8	24.867	<0.001*
Group II (Statin only)	15.750 - 80.030	34.970	21.865 - 51.945	22.9		
Group III (Pristane only)	37.040 - 967.730	95.170	60.335 - 194.800	34.5		
Group IV (Statin with pristane from first day)	8.300 - 49.840	16.830	11.860 - 23.850	13.4		
Group V (Statin with appearance of manifestations)	18.090 - 62.140	33.370	20.470 - 58.660	22.8		
Dunn-Bonferroni post hoc test						
	I	**II**	**III**	**IV**	**V**	

I	1.000	0.070	<0.001*	1.000	0.060
II	0.070	1.000	0.467	0.969	1.000
III	<0.001*	0.467	1.000	0.002*	0.391
IV	1.000	0.969	0.002*	1.000	0.903
V	0.060	1.000	0.391	0.903	1.000

IQR: interquartile range; * significant at $p<0.05$.

Table 6: Statistical comparison of nuclear RIPK3 level (pg/mg nuclear protein) among the studied groups using Kruskal-Wallis test.

	Nuclear RIPK3 level pg/mg nuclear protein				Kruskal-Wallis test	
	Range	**Median**	**IQR**	**Mean ranks**	**Z**	**P-value**
Group I (Control)	0.007 - 0.200	0.015	0.008-0.030	7.3	11.057	0.026*
Group II (Statin only)	0.040 - 0.350	0.119	0.067-0.170	22.1		
Group III (Pristane only)	0.036 - 0.480	0.108	0.072 -0.233	22.6		
Group IV (Statin with pristane from first day)	0.026 - 0.390	0.052	0.048 -0.290	19.3		
Group IV (Statin with appearance of manifestations)	0.044 - 0.423	0.170	0.102 -0.360	27.2		
Dunn-Bonferroni post hoc test						
	I	II	III	IV	V	
I	1.000	0.191	0.154	0.525	0.013*	
II	0.191	1.000	1.000	1.000	1.000	
III	0.154	1.000	1.000	1.000	1.000	
IV	0.525	1.000	1.000	1.000	1.000	
V	0.013*	1.000	1.000	1.000	1.000	

IQR: interquartile range; * significant at $p<0.05$.

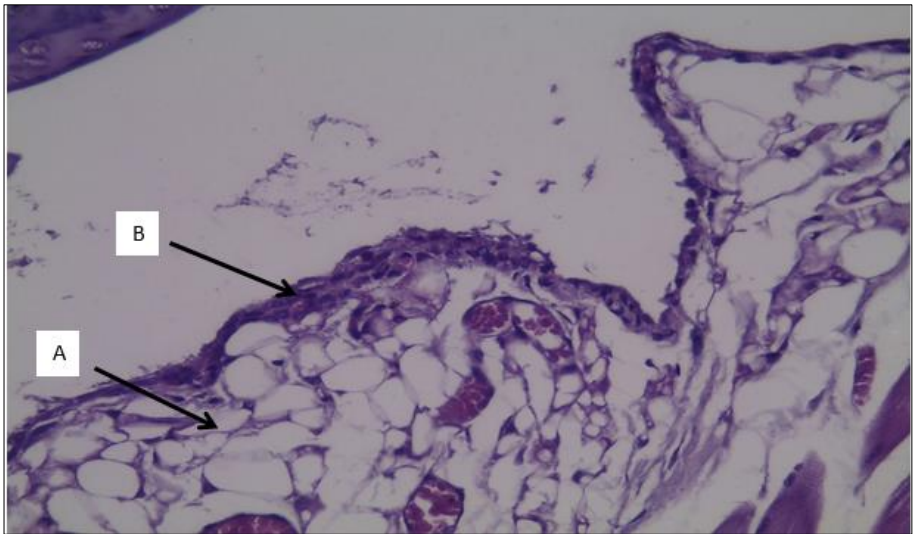

Fig 1: Section from joint space of Group I mice showing synovial lining with underlying fat, no inflammation and no remarkable pathological changes., A: Fat cells B: Synovial lining

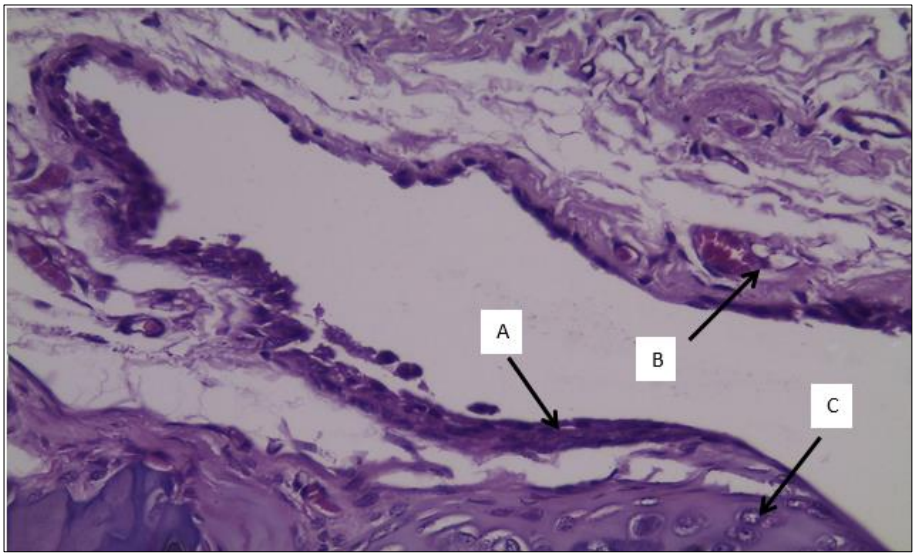

Fig 2: Section from joint space of Group II mice showing synovial lining with underlying fibrohyaline tissue, no inflammation and nonremarkable pathological changes, A: Synovial lining B: Blood vessel C: Bone

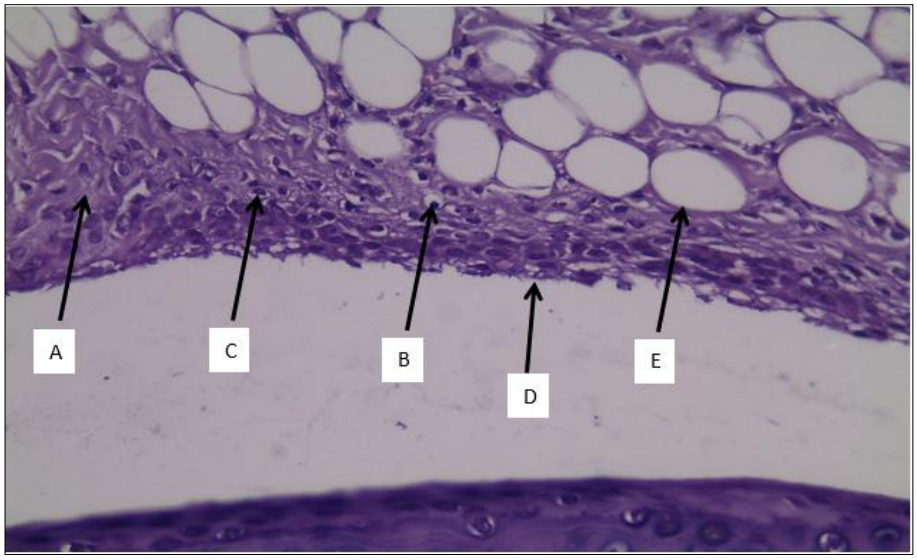

Fig 3: Section from joint space of Group III mice showing slightly hyperplastic synovial lining with underlying moderate inflammatory reaction composed of lymphocytes, macrophages and few plasma cells with mild fibrosis, A: Fibrosis B: Lymphocyte C: Plasma cell D: Synovial lining E: Fat cell

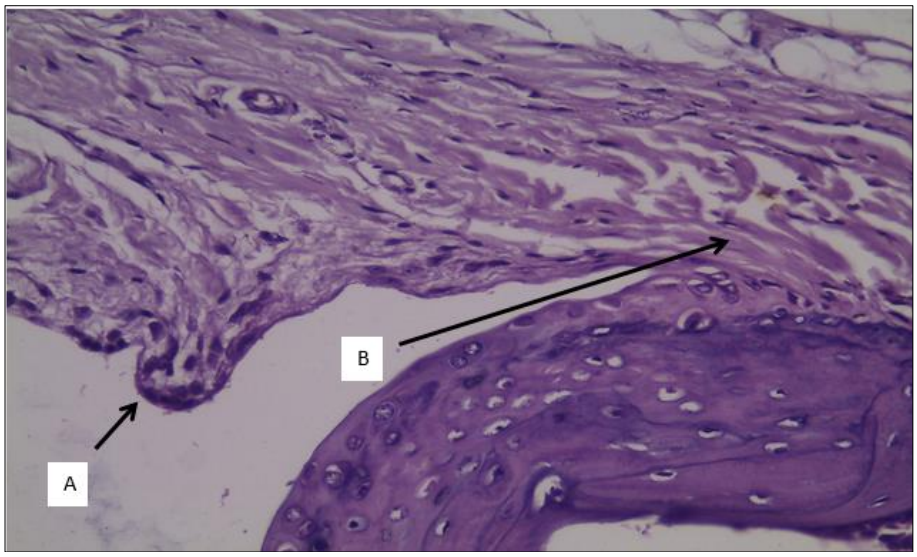

Fig 4: Section from joint space of Group IV mice showing synovial lining with underlying fibrohyaline tissue, no inflammation and no remarkable pathological changes, A: Unremarkable synovial lining B: Fibrosis

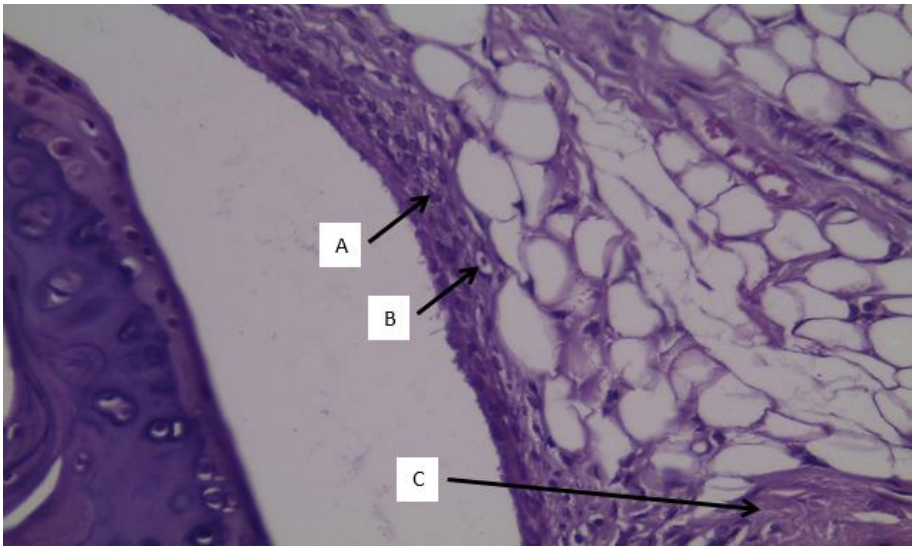

Fig 5: Section from joint space of Group V mice showing synovial lining with mild inflammatory reaction composed of lymphocyte, macrophage and few plasma cells with mild fibrosis, A: Plasma cell B: Lymphocyte C: Fibrosis

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