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LIPOPOLYSACCHARIDE (LPS) AND MURAMYL DIPEPTIDE (MDP) INDUCED CHRONIC LIVER INJURY IN HIGH FAT DIET FED RAT–A POSSIBLE MODEL FOR NON ALCOHOLIC FATTY LIVER DISEASE

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ABSTRACT: Liver defend the PAMPs or DAMPs via regulation of innate immunity through modulation of "TOLL LIKE RECEPTORS and NOD LIKE RECEPTORS" A breakdown of tolerance may induce in appropriate immune response leads to acute and chronic liver diseases like NASH/NAFLD thus considered as disorders of immune response. HFD is itself a basic component for generating metabolic disorders and major source for formation of endotoxins in body through leaky gut and sensitization of the TLR4. NAFLD is one of the groups of disease which links the innate immunity through the metabolic disorder. Agonist of TLR4 - LPS is the primary and NLRP3 agonist MDP is secondary signal transducer for the sensitization of the Caspase activation cascade and TNFα activation serves as the proinflammatory cytokines in disease like NAFLD. The present study was elucidated to develop combinations of Lipopolysaccharide and Muramyl dipeptide induced experimental model of NAFLD in HDF fed rats. Various parameters such as body weight, SGOT, SGPT, ALP, Direct Billirubin, serum triglyceride, weight of liver, and ratio of liver weight to body weight were done at the end of study. The established model was statistically evaluated by measuring significant changes in these parameters. The outcome of the study was found that the MDP alone is also a trigger for the development of NAFLD.

INTRODUCTION: Non alcoholic fatty liver disease (NAFLD) is a chronic liver disease with wide spectrum of hepatic abnormalities similar to alcoholic liver disease but without history of alcohol consumption [1]. NAFLD is characterized by fatty infiltration of liver ranging from steatosis, Steatohepatitis, fibrosis, cirrhosis, and leads to hepatocellular carcinoma [2]. Moreover it is also associated with insulin resistance, diabetes, obesity, hyperlipedemia, hypercholesterolemia [1, 3]. Therefore, NAFLD is also considered as metabolic disorder.

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However, the individual role of various metabolic aberrations in pathogenesis and natural history of NAFLD are still incompletely understood. The closest and common denominator could be insulin resistance [1, 4]. The involvement of immunological component and dietary imbalance (pattern of diet) has also been highlighted in patient with uncomplicated non alcoholic steatohepatitis, mainly high intake of lipid and carbohydrate [5]. Above both factors are also reported as major causes for the disease progression.

Further liver is the lymphoid organ with an overwhelming innate immune system, therefore various types of liver cells appear to be preferred source and target of cytokines signaling. Dysregulation of various inflammatory pathways play a key role in pathogenesis of liver diseases [6]. Immunological components involve modulation of

pattern recognition receptors (PPRs) such as Toll Like Receptors 4 (TLR 4) and Nod Like receptors (NLRs) both types of receptors are key components of innate immune system to recognize the pathogen associated molecular patterns (PAMPs) of micro organisms as well as they also sense danger signal released from damaged cells[7, 2].

The strong association of dietary imbalance (High Fat Diet intake), change in intestinal microbiota and alteration of gut permeability with liver damage/ injury has been documented [8, 9]. Feeding of HFD for 28 days induces colonization of gram negative bacteria and therefore increase gut permeability. Moreover, colonization of gram negative bacteria increases Lipopolysaccharide concentration two to three times, known as metabolic endotoxemia. Importantly, high fat diet also increased the proportion of an LPS containing microbiota in gut[10].

NAFLD and its complications are metabolic disorders characterized by low grade inflammation with decrease immunological components. Seeking an immune inflammatory involvement of factor causative of onset liver injury, we have identified bacterial Lipopolysaccharide (LPS) as a triggering factor for metabolic components; we have identified high fat diet (a rich source of free fatty acids). In addition to these two causative factors, we have also identified Muramyl Dipeptide (MDP), as a causative factor as a part of immune inflammation. MDP an agonist to NOD like receptor, specifically NLRP3, acts as inflammasomes and contributes in the metabolic endotoxemia and release pro inflammatory cytokines and synergies the liver disease.

NOD like receptors (NLRs) as specially NLRP3 an intracellular receptors present on various cells and tissue of liver. NLRP 3 is involved in sensing danger signals and promotes the cleavage and maturation of pro inflammatory cytokines to promote inflammation[11]. The inflammasomes activation is a two step process, in which the first step usually occurs by LPS induced upregulation of pro inflammatory cytokines via TLR 4. Second signal activation of inflammation via modulation of NLRP 3 by PAMP, DAMP, and MAMPs[12, 13]. Till date only the bacterial wall component Muramyl Dipeptide (MDP) and bacillus anthracis lethal toxin has been identified to activate NLRP3[12, 14]. The activation of NLRP3 is highly regulated at transcriptional level via NF-κB activation such as TLR 4 ligand (LPS) [15, 16]. In another set of study, loss of TLR4 attenuated hepatic lipid accumulation and levels of fibrinogenic marker (such as collagen α 1 and TGF-β 1) in MCD diet induced steatohepatitis, indicating the importance of TLR 4 in NASH[17].

On the basis of above survey we have identified three hits, High Fat Diet (rich source of FFA), Lipopolysaccharide (LPS) and Muramyl Dipeptide (MDP), as triggering factors for NAFLD, which links the inflammation with TLR4 and NLRP3. Therefore, we made An attempt to develop Lipopolysaccharide and Muramyl Dipeptide induces model of NAFLD and its complications in HFD-fed rats.

MATERIALS AND METHODS:

Experimental Animals: Healthy male Sprague Dawley rats, weighing 180-220gm were used in study the protocol (KBIPER/2013/445) was approved by CPCSEA/IAEC committee before carrying out experiment. The animals were housed in polypropylene cage at 25^0 C; 12:12 hrs dark-light cycle, with free access to standard chow diet and water *ad libitum* before dietary manipulation. The animals were acclimatized to surrounding for one week prior to experiment.

The animals were divided in following groups-

Group I (NPD+ LPS + LPS) LPS 10µg/animal i.p.+ LPS 10µg/animal

Group II (HFD+LPS+LPS) LPS 10µg/animal i.p. + LPS 10µg/animal i.p.

Group III (HFD+MDP+ MDP) MDP (10µg/animal, i.p.)+ MDP (10µg/animal, i.p.)

Group IV (HFD+LPS+MDP) LPS 10µg/animal i.p. MDP +(0.5µg/animal, i. p.)

Group V (HFD+LPS+MDP) LPS 10µg/animal i.p. MDP +(1µg/animal, i. p.)

Group VI (HFD+LPS+MDP) LPS 10µg/animal i.p. MDP+ (10µg/animal, i. p.)

TABLE 1: COMPOSITION OF HIGH FAT DIET-HFD [17, 18]

Ingredient	Weight(Gm)	Appox. Calories (Kcal)	Significance Of The Cmponent
Normal Pellet Diet	200	500	Normal Food Diet
Casein	125	500	Source Of Protein Also Elevate Tg Level
Lard	300	2700	Main Source Of Fat
Sucrose	213	861.37	For The Sweetness
Corn Starch	150	600	Elevate Insulin Resistance
Vitamine	6	---	For Normal Health Conditions
Salt	1	---	Taste And For Homeostasis
Soya Bean Oil	5	45	Fat Content
Total	1000	5206.37	Total Fat=48.93%

The animals were divided in to six groups as mentioned above. All animals were fed with high fat diet composed according to **Table 1**, except group I animals fed with Normal pellet diet for entire study period.

After 28 days of dietary manipulation (4 weeks) all animals (except for group III) were sensitized with lipopolysaccharide (10 µg / animal, i.p.) for 14 days. Group III animals were sensitized with muramyl di peptide (10 µg / animal, i.p.).Group I and II animals previously sensitized with LPS (10 µg / animal, i.p.) were challenged with LPS (10 µg/ animal, i.p.) on day 21 of first dose of sensitization animals of group IV , V and VI were challenged with different doses of MDP(0.5, 1 10 µg / animal, i.p.), respectively on day 21 of first dose of sensitization. While animals of group III were challenged with highest dose of MDP (10 µg).

Above process for challenge was repeated on day 28, 35, and 42 of first dose sensitization, later on the animals were kept on observation for another 14 days. Therefore the entire study duration was 84 days. Blood samples were collected from retro-orbital plexus under light ether anesthesia in to anti coagulant free Eppendorf, The blood samples were allowed to settle for 30 minutes. The serum was separated by centrifugation under cooling (0-4^0 C) at 5000 RPM for 10 – 15 minutes and analyzed for various biochemical parameters.

Statistical Analysis:

All values were expressed as mean ±SEM of six observations. The statistical analysis for determining significant difference was performed using student unpaired t- Test and Two way ANOVA followed by-Values of less than 5% ($p < 0.05$) were considered to be statistical significant.

Effect of HFD, LPS AND MDP ON SGOT/AST activity:

SGOT is an important marker for the liver disease. Here we found significant increase in SGOT activity in HFD fed and lipopolysaccharide (10 µg/animal i.p.) sensitized rats when challenged with different dose of MDP (0.5, 1, 10 µg/animal i.p.) as compared to rats challenged with lipopolysaccharide (10 µg/animal i.p.) at day 56 and after. Rise was significant in MDP (10 µg/animal i.p.) injected animal according to **Table 2**. However HFD- fed rats sensitized and challenged with MDP (10 µg/animal i.p.) showed significant rise compared to HFD-fed rats presensitized with LPS (10µg/animal i.p.) and challenged with MDP (0.5, 1, 10 µg/animal i.p.) on day 84. This result was quite contradicted as compared to other results.

TABLE2: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF SGOT ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV	V	VI
0 DAY	41.33±2.06	32.09±2.92	23.18±3.97	20.63±2.45	28.48±4.36	33.4±4.81
28 DAY	62±3.15	43.58±3.49	45.77±0.93	48.13±1.86	46.56±1	45.97±3.81
42 DAY	76.67±2.41	46.69±2.16	51.27±1.73	47.74±1	48.91±1.6	49.5±1.94
56 DAY	76.5±2.83	66.67±3.77	105.69±4.15	99.6±4.66	95.67±3.06	99.4±4.58
70 DAY	75.04±3.95	87.41±2.22	65.81±2.87	57.95±3.66	56.58±2.97	84.47±3.49
84 DAY	74.53±2.77	84.85±2.37	109.62±4.75	88.08±3.32	90.33±2.71	99.2±4.5

Effect of HFD, LPS AND MDP on SGPT/ALT activity:

Significant increase in SGPT activity was observed in rats fed with high fat diet as compared to rats fed with normal pellet diet on day 28 and after. The rise was gradual, further rise in SGPT activity was observed in HFD-fed rats sensitized with LPS 10 (μg/rat) for 14 days as compared to HFD-fed rats as mentioned in **Table 3**. Challenged with different doses with MDP (.5, 1, 10 μg/rat) in LPS pre sensitized rats(group IV,V,VI) shows significant increase in SGPT activity as compared to rat fed with HFD on day 56, rats with NPD and injected with LPS (group I). HFD fed rats when sensitized with LPS and challenged with LPS shows significant increase in SGOT as compare to IV, V, VI, SGPT level was gradual. Highest SGPT activity was found in HFD- fed and LPS sensitized rats challenged with MDP (10 μg/rat).

TABLE3: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF SGPT ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV	V	VI
DAY 0	40.33±1.76	32.09±3.92	21.22±1.9	26.72±5.35	22.98±3.32	25.73±7.09
DAY 28	72.83±3.64	43.58±2.49	47.93±4.24	39.49±2.21	50.29±3.4	47.54±2.39
DAY 42	24.83±2.43	46.69±3.16	55.4±2.51	55.4±3.48	50.88±0.56	50.49±0.56
DAY 56	22.5±2.86	66.67±2.77	59.92±5.37	55.2±2.1	54.22±2.87	52.65±2.19
DAY 70	35±3.79	87.41±5.22	69.15±4.54	63.06±3.37	70.13±2.04	64.43±3.29
DAY 84	31.5±1.55	122.85±2.37	71.31±4.28	63.84±2.93	71.9±3.78	66.4±3.62

Effect of HFD, LPS AND MDP on Alkaline Phosphatase Activity: Feeding of high fat diet to rats significantly increased alkaline phosphatase activity as compared to rats fed with NPD on day 42 and on subsequent days. High fat diet fed pre sensitized with MDP (10 μg/animal i.p.) showed significant increase in ALP activity as compared to HFD- Fed rats presensitized with LPS (10 μg/animal i.p.) as per the data given in **Table 4**. However, subsequently challenged with different doses of MDP (0.5, 1, 10 μg/animal i.p.) showed significant increased in ALP activity as compared to HFD + MDP treated rats challenged with MDP (10 μg/animal i.p.) on day 70 and 84.Moreover more prominent effect was observed with highest dose of MDP (10 μg/animal i.p.).

TABLE4: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF ALP ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV	V	VI
DAY 0	34.75±2.55	32.71±3.05	62.38±1.81	65.99±3.04	64.79±3.93	45.2±2.62
DAY 28	38.99±2.81	53.75±1.62	71.72±3.39	72.02±1.05	61.77±2.79	83.77±1.06
DAY 42	52.6±3.73	68.79±3.19	109.99±2.49	113.6±2.35	108.18±3.44	83.77±3.9
DAY 56	54.07±4.21	72.05±1.07	139.52±3.91	141.63±3.36	141.33±3.88	143.74±2.98
DAY 70	56.04±3.41	88.76±1.07	152.78±2.32	153.68±2.62	152.78±2.34	155.19±2.78
DAY 84	66.05±2.73	125.96±1.06	158.8±1.22	154.89±3.9	155.19±1.27	176.34±2.59

Effect of HFD, LPS AND MDP on Total Protein Activity: Total protein is one of the indicators of the disease progression in any inflammatory disease condition. In our study, group I at II shows no significant change in the total protein but in the groups III, IV, V and VI the total protein was increased gradually and constant rate on day 42,56,70 and 84. There was no significant difference between group III, IV, V, and VI at all days according to the data of **Table 5.**

TABLE5: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF TOTAL PROTEIN ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV	V	VI
DAY 0	5.48±0.32	5.63±0.29	12.37±1.11	6±0.91	6.52±1.44	6.97±0.89
DAY 28	3.72±0.14	6.26±0.25	14.85±2.46	19.57±3.14	12.6±0.77	15.07±1.56
DAY 42	6.35±0.51	6.39±0.16	19.35±0.73	21.15±1.48	20.55±1.02	19.27±0.44
DAY 56	6.42±0.5	6.65±0.15	28.8±3.7	22.42±3.51	19.72±1.27	20.55±1.29
DAY 70	4.74±0.47	6.84±0.07	30.58±2.98	38.42±3.48	38.31±2.76	34.85±2.79
DAY 84	4.85±0.17	4.86±0.24	24.9±3.06	24.37±2.23	25.72±1.92	3.33±2.93

Effect of HFD, LPS AND MDP on albumin activity:

Albumin is also one of the indicators of the NAFLD and in liver injury, normal ranges (2.8-5.4 gm/dl) of albumin increases in the inflammation and liver damage. In comparison with group I and II (NPD +LPS and HFD +LPS) albumin level was significantly increased in the group III, IV, V and VI. However, between group III (MDP 10μg/rat i.p. + MDP10μg/rat i.p.) and group IV (LPS10μg/rat + MDP 1μg/rat), no significant difference was observed on all days except day 84. HFD- fed rats and presensitized with LPS showed significant increase in albumin level when challenged with different doses of MDP (0.5, 1, μg/rat i.p.). Moreover, MDP 1 μg and 10 μg showed more prominent effect as compared to MDP (0.5 μg/rat i.p.) but statistically significant result was observed as mentioned in **Table 6.**

TABLE6: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF ALBUMIN ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV	V	VI
DAY 0	3.24±0.22	2.5±0.08	3.16±0.04	3.4±0.03	3.03±0.01	3.43±0.04
DAY 28	2.35±0.1	2.6±0.1	4.73±0.12	4.15±0.11	4.1±0.13	3.93±0.26
DAY 42	3±0.21	3.53±0.11	5.13±0.18	6.16±0.25	6.08±0.31	5.52±0.12
DAY 56	3.42±0.24	3.83±0.17	7.83±0.36	8.39±0.35	8.48±0.31	8.66±0.43
DAY 70	2.66±0.2	3.96±0.19	7.83±0.36	8.39±0.35	8.48±0.31	8.66±0.43
DAY 84	2.12±0.24	4.86±0.24	9.84±0.2	9.09±0.21	9.82±0.2	9.69±0.16

Effect of HFD, LPS AND MDP on triglyceride activity:

As all the groups are fed HFD in entire period of study, the triglyceride levels are measured to check the effect of dietary manipulation which is linked with the metabolic endotoxemia. In all groups there is a gradual increase in the TG levels on day 0, 28,42,56,70 and 84. No significant difference was observed between all groups on all days as mentioned in **Table 7**.

TABLE 7: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF TRIGLYCERIDE ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	III	IV	V	VI
DAY 0	63.27±3.69	63.75±2.39	66.02±4.2	67.56±5.29
DAY 28	85.98±3.93	93.1±5.99	88.15±2.08	84.25±4.4
DAY 42	108.04±3.93	116.86±3.61	121.94±2.09	114.68±2.29
DAY 56	126.62±4.49	133.27±2.93	138.88±2.52	129.64±2.25
DAY 70	143.86±3	148.85±3.19	160.8±2.48	152.77±2.87
DAY 84	177.53±3.79	192.88±4.03	196.93±5.5	207.04±4.27

Effect of HFD, LPS AND MDP on direct billirubin activity: Direct Billirubin is also helpful parameter in diagnosis of liver dysfunction. Direct Billirubin was increased in high fat diet fed rats, when sensitized with lipopolysaccharide (10 μg/rat i.p.) and MDP (10 μg/rat i.p.) as compared to rats fed with NPD and sensitized with LPS. However HFD - fed / LPS sensitized rat challenged with different dose of MDP (0.5, 1, 10 μg/rat i.p.) showed significant increase in direct Billirubin levels as compared to HFD – fed / MDP sensitized rats challenged with MDP (10 μg/rat i.p.) on day 70 and 84. However, no significant difference was observed between groups IV, V, VI as per **Table 8.**

TABLE8: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF DIRECT BILLIRUBIN ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV	V	VI
DAY 0	0.69±0.04	0.33±0.05	12.37±1.11	6±0.91	6.52±1.44	6.97±0.89
DAY 28	0.61±0.06	0.54±0.02	14.85±2.46	19.57±3.14	12.6±0.77	15.07±1.56
DAY 42	0.78±0.04	0.69±0.03	19.35±0.73	21.15±1.48	20.55±1.02	19.27±0.44
DAY 56	0.89±0.07	0.72±0.07	28.8±3.7	22.42±3.51	19.72±1.27	20.55±1.29
DAY 70	0.74±0.14	0.89±0.07	30.58±2.98	38.42±3.48	38.31±2.76	34.85±2.79
DAY 84	0.86±0.1	1.26±0.06	24.9±3.06	24.37±2.23	25.72±1.92	3.33±2.93

Effect of HFD, LPS AND MDP on change in AST/ALT:

AST/ALT ratio is important factor for the development for the liver injuries. The different stages of the liver injury can be defined with the help of AST/ALT ratio. As disease progressed the ratio of AST/ALT increases in initial stages of liver injury AST/ALT ratio is less than 1 according to data given in **Table 9.**

TABLE 9: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF AST/ALT ON DIFFERENT DAYS OF MODEL DEVELOPMENT

	I	II	III	IV
DAY 0	0.39±0.08	0.3±0.11	0.3±0.08	0.39±0.08
DAY 28	0.53±0.14	0.64±0.1	0.79±0.06	0.79±0.05
DAY 42	0.85±0.05	0.77±0.02	0.83±0.04	0.78±0.03
DAY 56	0.92±0.04	0.67±0.03	1.21±0.14	1.18±0.09
DAY 70	0.96±0.06	1.04±0.08	1.13±0.13	1.33±0.12
DAY 84	1.57±0.23	1.37±0.22	1.01±0.05	1.69±0.14

Effect of HFD, LPS AND MDP on liver weight/body weight ratio:

Liver to body weight ratio was found to be increased in group II and III which were sensitized with LPS 10 μg/rat i.p and challenged with different doses of MDP (0.5, 1 μg/rat i.p.) as per **Table 10**.

TABLE10: EFFECTS OF LPS AND MDP ON BIOCHEMICAL RANGES OF AST/ALT ON DIFFERENT DAYS OF MODEL DEVELOPMENT

HFD+MDP(10)+ MDP(10)	HFD+LPS(10)+ MDP(0.5)	HFD+LPS(10)+ MDP(1)	HFD+LPS(10)+ MDP(10)
45±3.95	58.43±2.42	55.47±6.46	50.4±3.02

DISCUSSION: In the present study we demonstrated several novel findings that support the substantial role of innate immunity in the pathogenesis of NAFLD. Liver injury involves many cell types present in liver includes Kupffer cells, hepatocytes, hepatic stellate cells and epithelial cells [19, 20]. Liver injury leads to recruitment of immune cells at the site of inflammation which further promote the fibrotic process via inflammatory mediators such as cytokines, chemokines and co-stimulatory molecule. NAFLD is one of the metabolic disorders which involved the fat deposition in the liver and insulin resistance is also one of the reasons of progression of the disease. The five components that compose the metabolic syndrome are central (truncal) obesity, hyperglycemia, hypertension, hypertriglyceridemia, and low levels of HDL-cholesterol.

The condition ranges from simple hepatic fat accumulation (steatosis) to nonalcoholic steatohepatitis, where fat is accompanied by hepatocyte injury, and necroinflammation. This condition poses an increased risk of cirrhosis and hepatocellular carcinoma[21].

Pattern recognition receptors are the basic protein molecules which link the innate immunity in the cell[21]. Extracellular receptors TLRs are first recognize the DAMP, PAMP and MAMP in the cells then through the signal transduction via intracellular receptors like NLRs the innate immunity pathway is passed in the cell[22]. TLRs are associated with liver diseases including alcoholic liver injury, ischemia/reperfusion liver injury, liver fibrosis, and liver cancer. Among 13 TLRs identified in mammals, TLR2, TLR4, and TLR9 play a role in the development of NAFLD[23].

Moreover, LPS is a major cell wall component of gram negative bacteria with reported ligand of TLR4 and found to be strong inducer of inflammation which causes liver damage by increase the secretion of proinflammatory cytokines including IL-1, IL-6, IL-12 and TNF-α from kupffer cells which are detrimental mediator for liver injury [6]. The liver is comprised of both parenchymal (hepatocytes) and immune cells (macrophages, dendritic cells, T-cells, NK/NKT-cells), where hepatocytes represent the majority of the cell populations[25]. The role of inflammasomes has been mostly studied in immune cells, but there

is increasing evidence that NLRs exist in non-immune cells also, caspase activation and the release of pro inflammatory cytokines are the major factor for the liver diseases. In the inflammation the role of inflammasomes is stabilized due to the stimulation of the sensory pathways of the cell signaling activated through the PAMPs, DAMPs and other stimulators which activate the TLR and NLR pathways in the cell signal transduction[22].

MDP is peptidoglycan derivative of the bacterial cell wall content which activates innate immunity via the host sensor NOD2. N- acetylmuramyl-L-alanyl-D-isoglutamine PGN moiety usually referred to as N- acetyl muramyl dipeptide (MDP). It was discovered to be the minimal structure required for the efficacy of Freund's Complete Adjuvant (FCA), one of the most potent and widely used adjuvants in animal experimental models. Moreover, MDP and other muropeptides (tripeptides and disaccharide tri- and tetrapeptides) induce immune responses by increasing IFN-γ and other cytokine production, stimulating the differentiation and proliferation of lymphocytes, a subset of white blood cells that play and integral role in the body's defense against foreign intruders[14, 23].

To date several studies shows that LPS is one of the causes for the liver injury and GUT endotoxemia. In our study we reveal that the NLRPs are also involved in the development of the histopathological changes of the liver injury.

Subjects with specified values for at least three of these components are considered to have metabolic syndrome, in this model, high-fat diet caused an accentuated increase in body weight when compared to a Standard diet[24]. After overnight fasting, serum levels of TG and cholesterol in the HF group remained significantly elevated, compared to the NC group. These results indicated that the rat model successfully recapitulated several key features of human metabolic syndrome. HFD is used in development of model which primarily produced the symptoms of the metabolic disorders in the animals like obesity, endotoxemia and FFA production in the duration of initial 28 days (4weeks). Moreover the HFD for four week increased in plasma LPS concentration two to three times and the LPS recognition complex (TLR4 and MD-2) activates NADPH in liver steatosis and induces fibrosis in a NASH model of mice [25]. After overnight fasting, serum levels of TG and cholesterol in the HF group remained significantly elevated, compared to the NC group. These results indicated that the rat model successfully recapitulated several key features of human metabolic syndrome. HFD is used in development of model which primarily produced the symptoms of the metabolic disorders in the animals like obesity, endotoxemia and FFA production in the duration of initial 28 days (4weeks). Moreover the HFD for four week increased plasma LPS concentration two to three times and the LPS recognition complex (TLR4 and MD-2) activates NADPH in liver steatosis and induces fibrosis in a NASH model of mice [25, 26]. These data support the role of these receptors in the development of steatosis, inflammation and fibrosis in NASH.

According to previously done study and present study of LPS and MDP in HFD-fed rats ,above mentioned parameters examined and analyzed on day 28, 42, 56, 70, 84 shows significant damage to liver as compared to NPD + LPS, HFD + LPS , combination of the LPS + MDP shows momentous damage to liver.

After 28 days study animals were sensitized with the LPS and MDP in different groups with the dose 10µg/animal i.p., dosing of these primary endotoxins combined with the endotoxemia produced by the HFD and activates the sensitivity of the TLR4 receptors, as LPS is agonist of TLR 4 receptor.

Challenge doses of the different strength of MDP (0.5, 1, 10µg/animal i.p) on day 21 of first sensitization aggravate liver damage. MDP is an NLRP3 agonist, works in a synergy with TLR4 for liver damage. To evaluate various changes, various parameters such as SGOT (AST), SGPT (ALT), ALP, total triglyceride, total cholesterol, direct Billirubin level were examined before sensitization on day 0, 14, 28 and after on day 42, 56,70 and 84. Histopathology of liver was studied after completion of study.

Commonly available liver function tests includes enzyme estimation, like alanine Transaminase

(AST) and Asparted Transaminase (ALT), Alkaline phosphatase (ALP), Gamma glutamyl transfareses, Serum bilirubin, Serum albumin, and Total protein; Ratio of AST/ALT and the liver weight to body weight ratio are another measures in the liver disease progression. The enzymes tested are most commonly raised in liver diseases.

SGOT (AST) is a major marker for the liver diseases, as disease worsens the SGOT levels were also increases. AST is present in cytosolic and mitochondrial isoenzymes and is found in the liver, cardiac muscle skeletal muscle, kidneys, brain, pancreas, lungs, leucocytes, and red cells. It is less sensitive and specific for the liver. In the study, NPD and HFD (sensitized and challenged with LPS) there is small rise in the levels of the SGOT but in the groups pre sensitized with MDP and sensitized with MDP The levels of SGOT are increasing gradually as the days of study are increases from day 42 the increase in SGOT is seen with the graph but in group which is combination of LPS + MDP on day 70 the SGOT levels are seems low after giving the challenged dose of MDP in LPS Presensitized rats. Group which is pre sensitized with LPS 10 µg and challenged with MDP 10 µg shows gradual increase in SGOT levels.

SGPT (ALT), a cytosolic enzyme is found in its highest concentrations in the liver and is more specific to the liver. Hepatocellular injury and not necessarily cell death is the trigger for release of these enzymes into the circulation. When faced with an abnormality, the first step should be to assess the degree of abnormality. The tests should probably be repeated if the abnormality is mild. Further investigation is warranted if repeated tests confirm abnormality. Very high levels should prompt further evaluation without delay. Common causes are non-alcoholic fatty liver disease, alcoholic liver disease, chronic hepatitis B and C, autoimmune liver disease, haemochromatosis, Wilson's disease, α1- antitrypsin deficiency, and coeliac disease. In completed study highest SGPT activity was found in HFD-fed and LPS sensitized rats challenged with MDP10 µg/rat.

ALP originates mainly from two sources: liver and bone. The enzymes may be present in a variety of other tissues namely intestine, kidney placenta, and leucocytes. The elevation may be physiological or pathological. The physiological role of these enzymes is not entirely clear but production increases in tissues undergoing metabolic stimulation[26]. Elevations seen. ALP is also one of the markers for the liver injury which shows increased in levels in the study in all model development groups from day 42 onwards. In group I (NPD +LPS 10 µg/rat) there is no change in activities of ALP but a little increase on day 56,70 and 84 in group II (HFD +10µg/rat).when the group III,IV,V,VI were compared with group II there is a significant change in ALP activity in all the groups on day 42,56,70 and 84.group III(MDP10 µg/rat+ MDP10 µg/rat) shows high activity on day 70 and 84 according to the statistics which may be due to the metabolic dysfunction in the animals fed with HFD.

Total protein is one of the indicators of the disease progression in any inflammatory disease condition. In our study groups kept on NPD and HFD shows no increase in the total protein but in the groups III, IV, V and VI the total protein is increased in the gradual and constant rate on day 42, 56, 70 and 84.The total protein increase is similar on days 56, 70, 84 in group III IV V and VI.

Albumin synthesis is an important function of the liver. Approximately 10 g is synthesized and secreted daily. With progressive liver disease serum albumin levels fall, reflecting decreased synthesis. Albumin levels are dependent on a number of other factors such as the nutritional status, catabolism, hormonal factors, and urinary and gastrointestinal losses. Albumin concentration does correlate with the prognosis in chronic liver disease [27]. Albumin is one of the indicators of the NAFLD and in liver injury normal ranges of albumin increases in the inflammation and liver damage. In comparison with groups on NPD +LPS+ LPS and HFD +LPS + LPS albumin levels are increased in the group and in between the groups the groups MDP 10µg/rat + MDP10µg/rat and group on LPS10µg/rat + MDP 1µg/rat sensitized with MDP shows high levels of albumin on day 84 compared to the days 42,56 and 70.

Triglyceride increases when the FFA levels in the body increases, NAFLD is disease related to the accumulation of the fats in the liver. As all the groups fed HFD in entire period of study, the triglyceride levels are measured to see the effect of dietary manipulation which is linked with the metabolic endotoxemia [27]. In all groups there is a gradual increase in the TG levels on day 0, 28, 42, 56, 70 and 84.The presensitized and challenged with MDP group are shows significant increase on day 84 and 70 in TG levels compared to the group fed with NPD HFD, pre sensitized and challenged with LPS 10 µg.

Bilirubin is formed from the lysis of red cells (the haem component) within the reticuloendothelial system. Unconjugated bilirubin is transported to the liver loosely bound to albumin. It is water insoluble and therefore cannot be excreted in urine. Conjugated bilirubin is water soluble and appears in urine. Within the liver it is conjugated to bilirubin glucoronide and subsequently secreted into bile and the gut respectively. Intestinal flora breaks it down into urobilinogen, some of which is reabsorbed and either excreted via the kidney into urine or excreted by the liver into the gastrointestinal tract. Billirubin is helpful in measuring the severity of liver diseases. Serum bilirubin is normally mainly in an unconjugated form reflecting a balance between production and hepatobiliary excretion. Bilirubin production increases in haemolysis, ineffective erythropoiesis, resorption of a haematoma, and rarely in muscle injury[28].

Direct Billirubin is an marker for the unconjugated Billirubin levels, in our study group kept on NPD and HFD presensitized and challenged with LPS have no increase in Billirubin levels as compare to the group pre sensitized with MDP and challenged with MDP and major difference was seen in the combination which is pre sensitized with LPS and challenged with MDP as the gut microflora increases the Billirubin level due to breakdown of it. On day 70 and 84 the levels of Billirubin are high in combination groups but there is a rise on day 56 which is decreased on the day 70 and 84.

The AST: ALT ratio (De Ritis ratio) may assist in differentiating the site of biliary obstruction. When associated with a cholestatic picture, an AST: ALT ratio of <1.5 suggests an extrahepatic obstruction. In such circumstances the ALT titer is frequently considerably higher than AST.

AST: ALT ratio of >1.5 indicates intrahepatic (mechanical or medical) cholestasis is more likely.AST: ALT ratio is found <1 on day 84 in groups kept on HFD + LPS + MDP combination compare to other groups [29].

In the histopathological sections the images shows the damage to liver cells in the different groups the damage is associated with the severity as compared to the normal group. Histological changes showed the confirmation to the liver damage in the study.

Liver weight to body weight is one of the criteria for diagnosis of the liver injury, as HFD is rich source for the FFAs causes' fatty liver or steatosis [27]. As the weight of animal increases the weight of liver also increases. In study performed, the liver weight to body weight was found to be increased in group II and III which were sensitized with LPS 10 µg/rat i.p. and challenged with different doses of MDP (0.5, 1, 10 µg/rat i.p.).

Furthermore, available models for liver injury cannot describe the stages of liver diseases. The advantage of our model of liver injury is to describe NAFLD and various stages occurring during liver injury.

CONCLUSION: From results and discussion, it can be concluded that Combination of LPS and MDP produced prominent liver damage as compared to MDP and MDP combination. These results shows the potential of TLR4 and NLRP3 in pathogenesis of chronic liver injury, however effect of combination of MDP with HFD on progression of liver injury is remaining portion of discovery. The proposed model of combination of LPS + MDP might be valuable tool for pre-clinical studies to develop therapy for chronic liver diseases.

Future expects:

- Being a chronic model, it can be further developed to study the NAFLD and liver injury in animals.

- Model can be used in the screening of drugs available for the treatment of NAFLD.
- Possibilities of variety of modification in the models.
- Combination of LPS AND MDP in HFD-fed rats can be used for other metabolic diseases.
- MDP+LPS combination can also be evaluated in the treatment aspects of the other Immuno-Inflammatory diseases.

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