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Chitosan encapsulated meloxicam nanoparticles: *In vitro* and *in vivo* safety evaluation

Muralidhar Yegireddy, Prakash Nadoor, Suguna Rao, Pavithra BH, Rashmi R, Santhosh CR, Girish MH, Sravanthi M, Prasad TNVKV, Shivaprasad GR, Vaibhavi K, Patel TGV and Reddy CV

Abstract
Rising interest in the development of therapies based on nanomaterials (NM) and their size-dependent effects has increased significantly in recent times, but the safety of NMs remains a concern due to the limited animal studies. In the current study the safety of synthesized chitosan encapsulated meloxicam nanoparticles (CEMNPs) was evaluated by both *in vitro* and *in vivo* methods. The cytotoxicity assay was carried out by using murine spleenocytes revealed that both CEMNPs and MLX did not alter the cell viability even at the highest concentration (100 ppm) applied. *In vivo* safety evaluation was carried out in male Wistar rats by randomly dividing into five groups of six rats each. Group-I served as untreated control, while Group-II meloxicam (MLX) (1 $mg.kg^{-1}$ b. wt.; *p. o*) for a period of 21 days. Experimental rats in Group-III and Group-IV received 1 $mg.kg^{-1}$ b.wt. and 0.2 $mg.kg^{-1}$ b.wt. of CEMNPs, respectively for a similar duration. Experimental rats in Group-V received chitosan nanoparticles (CNPs) @ 1 $mg.kg^{-1}$ b.wt (P.o) alone for a similar duration. There was no significant change in the enzymatic - and non-enzymatic antioxidant status or peroxidative injury in liver, spleen, testis and kidney of different experimental groups of rats. The histopathological examination of these organs revealed apparently normal architecture and comparable to that of control group, thus there was no treatment specific effect. To conclude the synthesized CEMNPs compounds did not show toxicity *in vitro* or *in vivo* and appears safe. However, long-term toxicity studies are necessary before therapeutic exploitation.

Keywords: Meloxicam, chitosan, nanoparticles, safety evaluation, wistar rats

1. Introduction

In the field of drug delivery and therapies, nanoparticles have proven to be a flexible tool that may be used as scaffolds for tissue engineering as well as effective platforms for the treatment of a wide variety of disorders (Bellich *et al*., 2019; Rizeq *et al*., 2019) [22, 17]. By virtue of cationic functionality and solubility in aqueous media, chitosan is one of the most popular natural polymers for developing drug and gene delivery systems in recent times (Kato *et al*., 2005; Kritchenkov *et al*., 2017; Panzarasa *et al*., 2018) [5-6, 14]. The possible use of chitosan as carrier matrix for drug delivery warrants to evaluate the biological safety of chitosan. This is especially important when chitosan is included in a formulation at the nano level, which raises questions about nanotoxicity (Sonin *et al*., 2020) [20]. Chitosan-based NPs are widely explored as carriers for gene/drug delivery, but the toxicity of chitosan NPs is not yet fully studied and understood to a large extent. However, reports available on various studies on chitosan NPs has revealed that chitosan exhibits low toxicity in various study models via different routes of administration (Mohammed *et al*., 2017) [11]. Chitosan encapsulated meloxicam nanoparticles (CEMNPs) were developed to investigate their potential anti-inflammatory activity through oral administration. In the current study, we investigated the safety of synthesized chitosan encapsulated meloxicam nanoparticles.

2. Material and Methods

2.1 Synthesis and characterization

The chitosan encapsulated meloxicam nanoparticles (CEMNPs) synthesized and characterized for size, shape and surface charge, UV-Vis spectroscopy and FT-IR analysis as reported in our previous publication (Yegireddy *et al*., 2022) [21] was employed in the current study.

2.2 *In vitro* safety evaluation

The *in-vitro* safety evaluation was carried out as per the cytotoxicity MTT assay described by

Corresponding Author:
Muralidhar Yegireddy
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Mosmann (1983) [12]. The *in vitro* cytotoxicity of the CEMNPs was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay on mouse splenocytes. Briefly, to 100 µl of serially twofold diluted compound in Roswell Park Memorial Institute (RPMI) medium in a 96-well plate 100 µl of mouse splenocytes (1.0×10^6 cells/well; >90% viability) were added and incubated at 37 °C for 20 h in a CO_2 incubator. Four hours prior to the completion of incubation, 10 µl of MTT was added to each dilution. Later, the plate was centrifuged at 1200g for 10 min and the supernatant was discarded. The formazan formed was dissolved in 100 µl of DMSO and the absorbance was read at 530 nm after 10 min.

2.3 *In vivo* safety evaluation

2.3.1 Experimental animals

Male Wistar rats (N = 30) of 8 weeks of age and weighing 185.68 ± 0.48 g were procured from the authorized vendor (Biogen® Laboratory Animal Facility, Bengaluru-562107, KS, India). All the rats were housed in Small Animal Facility of Veterinary College, Bengaluru and maintained as per the standard guidelines of Committee for the Control and Supervision of Experiments in Animals (CCSEA, New Delhi, India). Prior to imitation of the study institutional animal ethics committee approval was obtained (No. VCH/IAEC/2021/123; dated 27.07.2021).

Wistar rats were randomly divided into five groups of six rats each. Group-I served as untreated control, while Group-II meloxicam (MLX) (1 $mg.kg^{-1}$ b.wt.; *p.o*) for a period of 21 days. Experimental rats in Group-III and Group-IV received 1 $mg.kg^{-1}$ b.wt. and 0.2 $mg.kg^{-1}$ b.wt. of CEMNPs, respectively for a similar duration, while rats in Group-V received 1 $mg.kg^{-1}$ b.wt of chitosan nanoparticles (CNPs) alone through oral route. At the end of the experimental study all the animals were sacrificed and vital organs *viz*., liver, spleen, kidney and testis were collected and stored at -20°C till further analysis.

2.3.2 Preparation of tissue homogenate

All the organs collected from each of the experimental rats were minced with scissors, transferred into a centrifuge tube and homogenized in a homogenizer by using 1:10 ratio of extraction buffer (ice-cold 50 mM potassium phosphate buffer, pH 7.4) at 3-5 ºC. The homogenate was then centrifuged at 10,000 x*g* at for 10 min and the supernatant was stored at -80ºC until further assays.

2.3.3 Evaluation of biochemical parameters

The protein content (Lowry *et al*., 1951) [8], superoxide dismutase (SOD) activity (Madesh and Balasubramanian, 1998) [9], catalase (CAT) activity (Aebi, 1983) [1], glutathione reductase (GR) activity (Goldberg and Spooner, 1983) [4], Reduced glutathione (GSH) content (Sedlak and Lindsay, 1968) [18] and lipid peroxidation (Paula *et al*., 2005) [15] was estimated.

2.3.4 Histopathology

The representative portions of different tissues were fixed in 10% neutral buffered formalin and dehydrated in ascending grades of alcohol. Later, the tissues were embedded in paraffin and sections of 4-7 µm were cut with a microtome and stained with haematoxylin and eosin.

3. Results and Discussion

3.1 *In vitro* cytotoxic assay

The survivability of mouse spleenocytes assessed by MTT assay after exposure to CEMNPs and MLX was presented in Fig. 1. The spleenocytes maintained a survivability of over 95% at all lower concentrations and survivability was maintained over 90% even at highest concentration used (100 ppm). Further, MLX also maintained the murine spleenocytes survivability more than 89% even at highest concentration applied.

In vitro cytotoxicity assay revealed that the synthesized CEMNPs and MLX at all the concentrations used did not modified cell viability of murine spleenocytes. These results are in agreement with the previous reports (Arantes-Rodrigues *et al*., 2013; Nishihira *et al*., 2018; Poradowski *et al*., 2021) [2, 13, 16]. In a recent study cytotoxic potential of PEGylated meloxicam-loaded nano capsules as well as free MLX was examined using human lymphocytes and they observed that neither of the tested compounds shown deleterious effects on cell viability (Nishihira *et al.,* 2018) [13].

3.2 *In vivo* safety evaluation

3.2.1 Total protein

The mean (±SE) protein content of liver, kidney, spleen and testis tissues in various experimental groups of rats is presented in Table 1. There was no significant ($p \geq 0.05$) change in protein content of liver, kidney, spleen and testes tissues in different experimental groups of rats

3.2.2 Enzymatic antioxidants

The enzymatic antioxidants *viz*., SOD, CAT, GR activities of liver, kidney, spleen and testis tissues in different experimental groups of rats were presented hereunder.

The mean (±SE) SOD activity in liver, kidney, spleen and testes tissues of various experimental groups of rats is presented in Table 2. There was no significant ($p \geq 0.05$) change in SOD activity of liver, kidney, spleen and testis tissues in different experimental groups of rats when measured at term of experimental study and were statistically similar ($p \geq 0.05$) with each other, respectively (Table 2).

The mean (±SE) CAT activity in liver, kidney, spleen and testes tissues of various experimental groups of rats is presented in Table 3. There was no significant ($p \geq 0.05$) change in CAT activity of liver, kidney, spleen and testis tissues in different experimental groups of rats when measured at term of experimental study and were statistically similar ($p \geq 0.05$) with each other, respectively (Table 3).

The mean (±SE) GR activity in liver, kidney, spleen and testes tissues of various experimental groups of rats is presented in Table 4. There was no significant ($p \geq 0.05$) change in GR activity of liver, kidney, spleen and testis tissues in different experimental groups of rats when measured at term of experimental study and were statistically similar ($p \geq 0.05$) with each other, respectively (Table 4).

The enzymatic antioxidant system has been considered as the first line of defense against oxidative stress (Kurutas, 2016). In the current study, experimental rats receiving different compounds does not induce oxidative stress status in various tissues as evidenced by non-significant change in the SOD, CAT, and GR activities.

3.2.3 Non-enzymatic antioxidants

The non-enzymatic antioxidant reduced glutathione (GSH)

content of liver, kidney, spleen and testis tissues in different experimental groups of rats was presented hereunder.

The mean (±SE) GSH content in liver, kidney, spleen and testis tissues of various experimental groups of rats is presented in Table 5. There was no significant ($p \geq 0.05$) change in GSH content in liver, kidney, spleen and testis tissues of different experimental groups of rats when measured at term of experimental study. The GSH content in liver, kidney, spleen and testis tissues of all the experimental groups were statistically similar ($p \geq 0.05$) with each other.

Reduced glutathione (GSH), an -SH containing antioxidant protects the membrane-polyunsaturated fatty acids from free radical-mediated lipid peroxidation to maintain cellular redox homeostasis (Sies, 1999) [19]. Further, it can act as a co-factor for several detoxifying enzymes. Thus, the tissue level of GSH is considered a critical determinant for the threshold of tissue injury caused by chemicals (Meister and Tate, 1976). In the current experimental study, the test compounds received by rats of different experimental groups did not induce any change in GSH content of different tissues indicated the safety of compounds even at the highest dose employed.

3.2.4 Lipid peroxidation (LPO)

The LPO in various experimental groups of rats measured in terms of MDA formed in liver, kidney, spleen and testis tissues is shown in Table 5. There was no significant ($p \geq 0.05$) change in MDA levels in liver, kidney, spleen and testis tissues of different experimental groups of rats when measured at term of experimental study. The MDA levels in liver, kidney, spleen and testis tissues of all the experimental groups were statistically similar ($p \geq 0.05$) with each other.

Free radicals are produced as a result of chemically induced cell damage, attack huge quantities of polyunsaturated fatty acids in tissues, and cause lipid peroxidation. The peroxidation of membrane lipids can be extremely harmful and results in changes to the biological characteristics of the membrane, such as the degree of fluidity, membrane integrity, and permeability. It can also result in the inactivation of membrane-bound receptors or enzymes, which may then compromise regular cellular function or result in cell death (Kurutas, 2016) [7]. In the current study, peroxidative damage in different tissues was measured as thiobarbituric acid reactive substance called malondialdehyde (MDA) formed *per* 'g' of tissue. The experimental rats in different groups receiving test compounds did not induce significant change in LPO as measured as MDA formed *per* 'g' of tissue indicated the safety of compounds even at the highest dose employed.

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3.2.5 Histopathology

3.2.5.1 Liver

Histopathological examination of sections of liver in control group (Group-I) rats showed apparently normal architecture with normal cords of hepatocytes arranged radially around central vein and normal portal triad (Fig. 2A). Photomicrographic sections of liver tissues belonging to all other experimental groups showed normal architecture of liver comparable to vehicle treated control indicated that there was no treatment specific effect on liver (Fig. 2B, 2C, 2D & 2E).

3.2.5.3 Spleen

Histopathological examination of rat spleen sections belonging to vehicle control group (Group-I) showed apparent normal architecture with both red pulp and white pulp compartments with globular arrangement (Fig. 2F). The spleen section from all other experimental groups showed also showed apparently normal architecture comparable to that of control group indicated that there was no treatment specific effect on spleen (Fig. 2G, 2H, 2I & 2J).

3.2.5.4 Testis

Histopathological examination of sections of testes belonging to vehicle treated control group revealed apparently normal architecture of seminiferous tubules consisting of uniformly arranged spermatogonia close to the basal membrane, primary and secondary spermatids and sertoli cells (Fig. 2K). Similar architecture of seminiferous tubules was also observed in Group-II, Group-III, Group-IV and Group-V rats indicated that there was no treatment specific effect on testis (Fig. 2L, 2M, 2N & 2O).

3.2.5.2 Kidney

Light microscopic examination of the sections of kidney belonging to vehicle control group (Group-I) rats showed apparent normal architecture of glomeruli and tubules a (Fig. 2P). The kidney section from all other experimental groups showed also showed apparently normal architecture comparable to that of control group indicated that there was no treatment specific effect on kidney (Fig. 2Q, 2R, 2S & 2T).

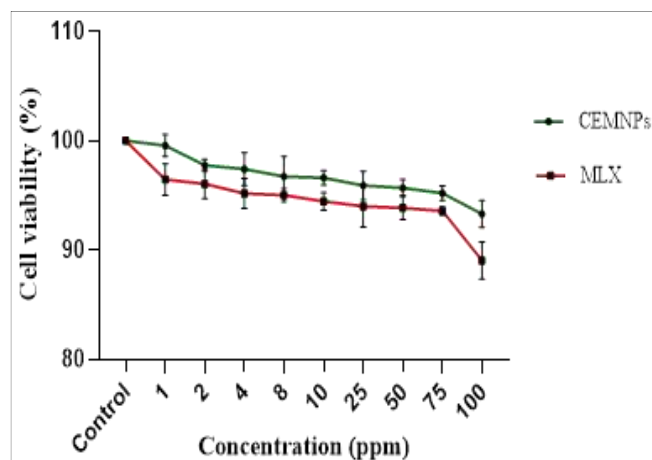

Fig 1: Assessment of cell viability (%) of murine spleenocytes exposed to meloxicam (MLX) or chitosan encapsulated meloxicam nanoparticles (CEMNPs)

Table 1: Mean (±SE) protein content (mg.g^{-1}) in tissues of different experimental groups of rats (n=6)

Experimental group (s)	Protein (mg.g^{-1})			
	Liver	Kidney	Spleen	Testes
C	8.74±0.40	5.26±0.15	8.57±0.14	5.08±0.28
MLX	8.68±0.22	5.26±0.13	8.49±0.27	5.01±0.29
High dose CEMNPs	8.73±0.29	5.27±0.22	8.57±0.27	5.02±0.17
Low dose CEMNPs	8.79±0.26	5.27±0.24	8.58±0.28	5.10±0.14
CNPs	8.72±0.20	5.27±0.17	8.51±0.31	5.06±0.24

Note: Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison *post hoc* test; Values bearing dissimilar alphabets within the column vary significantly; $p \leq 0.05$; [C = Vehicle control; MLX = Meloxicam; High dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticles]

Table 2: Mean (±SE) superoxide dismutase (SOD) activity (U.mg^{-1} protein) in tissues of different experimental groups of rats (n=6)

Experimental group (s)	SOD activity (U.mg^{-1} protein)			
	Liver	Kidney	Spleen	Testes
C	0.42±0.04	0.271±0.009	0.264±0.005	0.147±0.005
MLX	0.42±0.01	0.270±0.006	0.265±0.004	0.146±0.001
High dose CEMNPs	0.43±0.01	0.273±0.015	0.262±0.008	0.147±0.002
Low dose CEMNPs	0.42±0.01	0.270±0.006	0.261±0.015	0.146±0.007
CNPs	0.42±0.01	0.269±0.007	0.261±0.003	0.147±0.008

Note: Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison *post hoc* test; Values bearing dissimilar alphabets within the column vary significantly; $p \leq 0.05$; [C = Vehicle control; MLX = Meloxicam; High dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticles]

Table 3: Mean (±SE) catalase (CAT) activity (mmol of H_2O_2 utilized.min^{-1}.mg^{-1} protein) in tissues of different experimental groups of rats (n=6)

Experimental group(s)	CAT activity (mmol of H_2O_2 utilized.min^{-1}.mg^{-1} protein)			
	Liver	Kidney	Spleen	Testes
C	628.89±19.43	590.73±25.80	366.47±11.32	636.06±23.57
MLX	627.58±15.85	572.04±19.95	360.46±26.38	626.08±10.89
High dose CEMNPs	649.35±25.35	598.92±30.49	368.74±18.22	633.92±22.59
Low dose CEMNPs	633.93±18.43	590.11±23.60	353.83±14.67	624.94±19.53
CNPs	610.78±20.10	575.77±45.46	359.70±28.92	624.13±22.09

Note: Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison *post hoc* test; Values bearing dissimilar alphabets within the column vary significantly; $p \leq 0.05$; [C = Vehicle control; MLX = Meloxicam; High dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticles]

Table 4: Mean (±SE) glutathione reductase (GR) activity (mmol of NADPH oxidized. min^{-1}.mg^{-1} protein) in tissues of different experimental groups of rats (n=6)

Experimental group(s)	GR activity (mmol of NADPH oxidized.min^{-1}.mg^{-1} protein)			
	Liver	Kidney	Spleen	Testes
C	6.85±0.14	11.23±0.22	6.64±0.15	10.99±0.58
MLX	6.86±0.13	11.04±0.29	6.62±0.95	11.22±0.27
High dose CEMNPs	6.89±0.14	11.21±0.06	6.65±0.17	11.45±0.27
Low dose CEMNPs	6.73±0.10	11.12±0.19	6.70±0.15	10.97±0.07
CNPs	6.80±0.92	11.18±0.54	6.57±0.35	10.88±1.38

Note: Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison *post hoc* test; Values bearing dissimilar alphabets within the column vary significantly; $p \leq 0.05$; [C = Vehicle control; MLX = Meloxicam; High dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticles]

Table 5: Mean (±SE) reduced glutathione (GSH) content (mmol.g^{-1}) in tissues of different experimental groups of rats (n=6)

Experimental group (s)	GSH content (mmol.g^{-1})			
	Liver	Kidney	Spleen	Testes
C	0.44±0.07	0.60±0.12	0.79±0.10	0.41±0.09
MLX	0.43±0.07	0.59±0.09	0.76±0.03	0.40±0.08
High dose CEMNPs	0.44±0.07	0.59±0.11	0.79±0.09	0.42±0.06
Low dose CEMNPs	0.44±0.07	0.56±0.08	0.77±0.09	0.41±0.09
CNPs	0.44±0.06	0.57±0.12	0.77±0.10	0.42±0.10

Note: Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison *post hoc* test; Values bearing dissimilar alphabets within the column vary significantly; $p \leq 0.05$; [C = Vehicle control; MLX = Meloxicam; High dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticles]

Table 6: Mean (±SE) MDA formed (nmol.g^{-1}) levels in tissues of different groups of experimental rats (n=6)

Experimental group (s)	MDA formed (nmol.g^{-1})			
	Liver	Kidney	Spleen	Testes
C	22.07±2.59	24.86±1.60	32.85±2.35	26.60±2.12
MLX	26.78±1.94	25.72±1.95	33.81±3.09	27.28±1.51
High dose CEMNPs	25.18±1.66	24.89±2.33	33.08±1.33	26.20±1.24
Low dose CEMNPs	25.39±2.98	24.68±1.45	34.16±5.55	26.27±1.38
CNPs	25.86±4.74	26.40±2.75	33.25±5.03	27.33±2.42

Note: Data were analyzed by One-way ANOVA followed by Tukey's multiple comparison *post hoc* test; Values bearing dissimilar alphabets within the column vary significantly; $p \leq 0.05$; [C = Vehicle control; MLX = Meloxicam; High dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low dose CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticles]

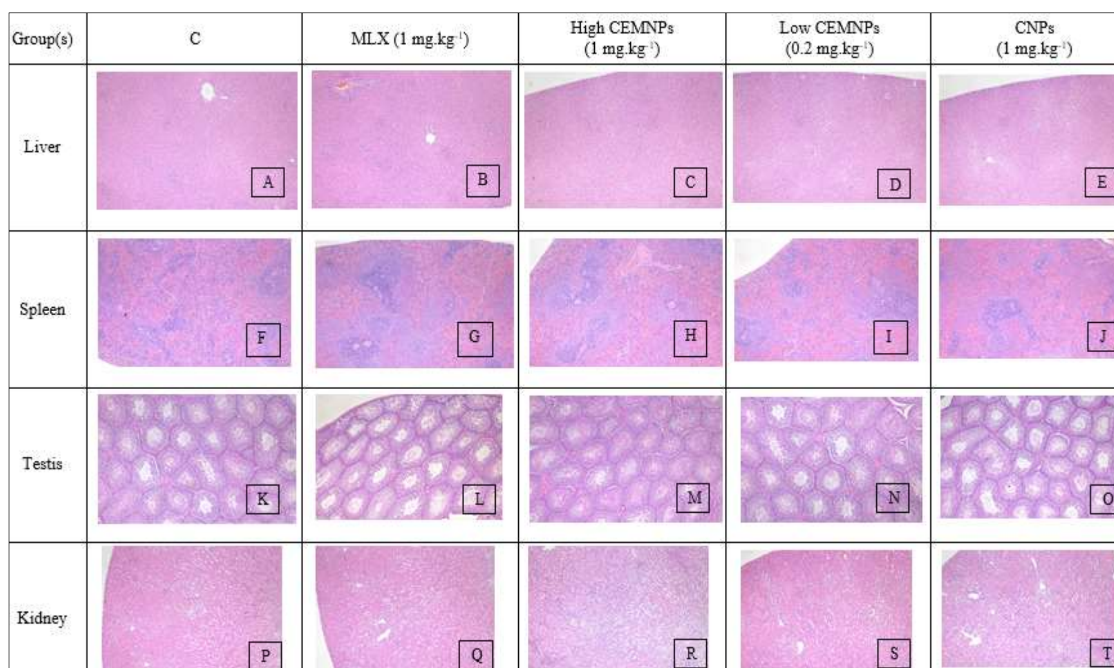

Fig 2: Histopathologic sections of liver (A-E), spleen (F-J), Testis (K-O) and Kidney (P-T) from different experimental groups of rats

Note: C = Vehicle control; MLX = Meloxicam; High CEMNPs = Chitosan encapsulated meloxicam nanoparticles high dose; Low CEMNPs = Chitosan encapsulated meloxicam nanoparticles low dose; CNPs = Chitosan nanoparticle

4. Conclusion

The synthesized chitosan encapsulated meloxicam nanoparticles (CEMNPs) did not show toxicity *either in-vitro* or as *in vivo*. The synthesized polymeric nanoparticles were found safe in Wistar rats when following *per os* administration for a duration of 21 days. Further, additional investigations are warranted including long-term toxicity studies and before exploring for possible antiarthritic therapeutics.

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Author's Details

Muralidhar Yegireddy
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Prakash Nadoor
(1) Veterinary College, Vinobanagar, Shivamogga, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Suguna Rao
(1) Department of Veterinary Pathology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Pavithra BH
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Rashmi R
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Santhosh CR
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Gadag, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Girish MH
(1) Department of Veterinary Anatomy, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Sravanthi M
State Level Diagnostic Laboratory, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Prasad TNVKV
Nanotechnology Laboratory, Regional Agricultural Research Station, ANGRAU, Tirupati, Andhra Pradesh, India

Shivaprasad GR
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Vaibhavi K
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Patel TGV
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India

Reddy CV
(1) Department of Veterinary Pharmacology and Toxicology, Veterinary College, Hebbal, Bengaluru, Karnataka, India
(2) Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka, India