



# *In vivo* anti-psoriatic activity, biodistribution, sub-acute and sub-chronic toxicity studies of orally administered methotrexate loaded chitin nanogel in comparison with methotrexate tablet

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## ABSTRACT

The anti-psoriatic efficacy of orally administered methotrexate loaded chitin nanogel (MCNG) was evaluated (two doses- 2.715 mg/kg and 5.143 mg/kg) and compared against orally administered methotrexate tablet MTX (5.143 mg/kg). MCNG at both dose levels of 2.715 mg/kg and 5.143 mg/kg exhibited significant anti-psoriatic activity which is very much comparable with MTX, caused normalization of histological features and inflammatory score associated with induced psoriasis. Biodistribution studies revealed the presence of drug in serum and in vital organs at all the three cases with highest amount in MCNG at 5.143 mg/kg dose, followed by MTX tablet and are lowest in MCNG at 2.715 mg/kg dose. MCNG at the highest dose of 5.143 mg/kg caused liver, lung and kidney toxicities on sub acute toxicity studies and MTX tablet was found to be toxic on liver and lung on sub chronic toxicity studies. MCNG 2.715 mg/kg was found to be safe on both sub acute and sub chronic administrations, suggesting that it can provide sufficient serum and tissue level of methotrexate necessary to clear psoriatic lesions, without inducing systemic toxicity and expected to be a better alternative for orally administered conventional methotrexate tablet for patients who need systemic medications for psoriasis.

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## 1. Introduction

Psoriasis is a disease which is characterized by the hyper proliferation of keratinocytes along with their incomplete differentiation. Available literatures reveal a worldwide prevalence rate of 2–3% with 0.7% in Indians [1]. The disease is autoimmune in origin, which starts with the activation of Antigen Presenting Cells (APC) located in the stratum spinosum layer of the skin by an unknown antigen. Activated APC migrated to regional lymph node, present the antigen to dendritic cells leading to the clonal proliferation and differentiation of T cells as occurs in immune stimulated condition. Once T cells get activated they will be the major players involved in disease pathogenesis, causing disruption of normal cell division and differentiation processes of keratinocytes through the release of cytokines and chemokines [2]. Even though the major phenotypic changes in psoriasis are reflected on the skin, due to

the auto-immune nature, it demands systemic therapy especially in moderate to severe cases [3,4].

Out of the systemic therapies for psoriasis, three drugs approved by FDA for oral use are methotrexate, cyclosporine and acitretin. Cyclosporine is an immunosuppressant drug used in psoriasis therapy. Due to the high cost, nephrotoxicity and hypertensive potential, it is often reserved for patients who cannot tolerate methotrexate. Acitretin is the only synthetic retinoid approved by FDA for oral use in psoriasis therapy. Long time needed for exerting an effect and delayed clearance from the body limits the use of acitretin in psoriasis. So ultimately methotrexate becomes the favorite choice of practitioners for oral use in psoriatic patients who need systemic medications [5]. The clinical effects produced by methotrexate in psoriasis are by multiple mechanisms. Early reports mainly focused the cytotoxic activity of the drug towards the proliferated keratinocytes by its anti-metabolite actions. But recent reports indicate that the toxicity of the drug towards the stimulated T cells than keratinocytes is mainly responsible for the clinical efficacy of methotrexate in psoriasis therapy. In addition anti-inflammatory effects mediated by increased level of cellular

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adenosine also contribute for its efficacy in inflammatory condition associated with psoriasis [6].

Even though the use of methotrexate is unavoidable in psoriasis, systemic toxicities make its use limited [7]. The vital organs particularly affected by the toxic reactions of methotrexate include kidney, liver, lung, spleen, bone marrow and hemopoietic system [7–10]. So routine monitoring and frequent repetition of tests for vital functions including blood, liver, lung and kidney are recommended by FDA for safer use of drug in psoriasis therapy [11]. This makes the therapy unaffordable for economically weak patients.

Recently nano drug carrier systems have been reported to exhibit improved efficacy at low dose level without inducing toxic effects in diverse kind of diseases including inflammatory disorders. Methotrexate nano lipid vesicles are reported to exhibit improved efficacy in inflammatory condition associated with active rheumatoid arthritis in a rat model of arthritis [12]. Similarly methotrexate nanoparticles exhibited greater efficacy to treat Inflammatory Bowel Disease (IBD) in pediatric population [13]. Our research team previously reported the improved efficacy of topically applied methotrexate loaded chitin nanogel in comparison with conventional carbopol gel formulation of methotrexate [14].

Oral route remains as the most convenient route of drug administration because of the lack of need for medical assistance for administration, non-invasiveness and probability of drug absorption throughout the GIT [15]. In the formulation of MCNG we used chitin as the polymer which is reported to be biocompatible and is relatively safe for oral use at low dose levels [16,17]. Along with chitin, lot of chitin derivatives have also been tried for various drug delivery applications. Tanvi Jain et al. reported the development of 5-FU loaded dibutyryl chitin nanoparticles using butyric unhydride and perchloric acid as catalyst. The developed system exhibited enhanced drug release at acidic conditions and was hemocompatible, and hence expected to be a promising approach for the delivery of the anti-cancer drug 5-FU [18]. Even though chitin is insoluble in water, its 50% deacetylated derivative is soluble in water and this water soluble derivative can be used as starting materials through smooth modifications in solution phase for the development of useful systems for various drug delivery applications [19]. Using these beneficial effects of chitin as drug delivery vehicles, we expected that methotrexate loaded chitin nanogel will be a suitable delivery system for oral delivery of methotrexate in psoriasis. In addition the anti-psoriatic activity of methotrexate is mainly mediated through a systemic mechanism, a systemic delivery which can provide significant anti-psoriatic activity without inducing toxic effects is seemed to be beneficial. So it is expected that orally administered methotrexate loaded chitin nanogel can provide sufficient anti-psoriatic activity at lower doses without inducing systemic toxicities by using the advantages offered by nano systems in comparison with conventional drug delivery systems. Various Nano drug delivery systems are reported to control the drug release at the site of action, are famous for alter the pharmaco-kinetic profile of loaded drugs, can escape from Reticulo Endothelial System (RES) uptake and hence offer increased circulation time and improved bioavailability [20]. As the particle size of MCNG is below 200 nm, it may escape from RES uptake as it often targets the bigger sized particles. So there may be chance of enhanced bioavailability of the active drug from MCNG. In addition the biocompatibility offered by chitin also seemed to make the system suitable for oral delivery in psoriasis. Moreover MCNG followed a slow release profile that also expected to provide additional advantages by preventing the rapid drug loss resulting from faster drug release and systemic wash out. Hence the present works aims to evaluate the anti-psoriatic efficacy, biodistribution and toxicity of methotrexate loaded chitin nanogel (MCNG) administered orally, in comparison with methotrexate tablet (MTX).

## 2. Materials and methods

### 2.1. Methods

Preparation and characterization of MCNG are already reported [14]. Methotrexate tablet (15 mg) was purchased from local medical shop and dissolved in Phosphate Buffered Saline (PBS) at a final concentration of 0.5 mg/ml. MCNG dispersed in water has a strength of 1.36 mg/ml. However the dose of active drug methotrexate tested for animal studies was same for both, that is 5.143 mg/kg which is the mouse dose equivalent to clinical dose of 25 mg tablet administered to an average 60Kg human [21]. MCNG was tested at half dose also (2.5715 mg/kg). Volume of tablet solution and MCNG administered were adjusted to give the selected doses to animals. Administration of samples to animals was done with the help of oral sample administration tubes. The anti-psoriatic efficacy of the formulation were evaluated using imiquimod (IMQ) induced psoriatic animal model in Balb/C mice [21–24]. As Balb/C mice were used for evaluating anti-psoriatic efficacy, the same animal species was selected for toxicity and bio-distribution studies.

### 2.2. Animals

All the experimental procedures were started after got approval from Institutional Animal Ethical Committee (IAEC/2013/3/2/amendment). Balb/C mice were received from the animal house, Amrita Institute of Medical Sciences. Animals were housed in mice cages, supplying standard animal food and water. Animals of 8–11 weeks age were used in the present study. The animals were acclimatized with the experimental conditions before initiating the study.

### 2.3. Anti-psoriatic activity studies

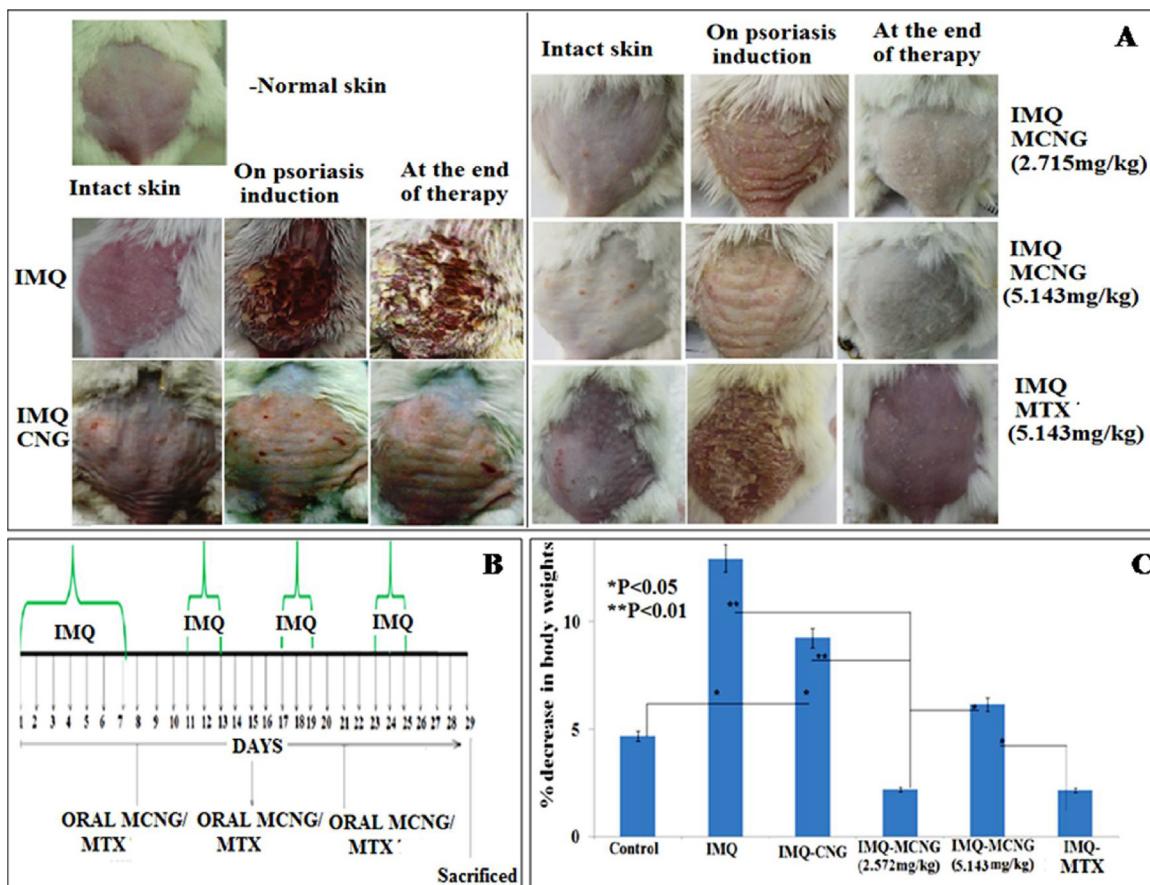
Imiquimod (IMQ) induced psoriatic animal model is reported to be a suitable animal model for evaluating the anti-psoriatic efficacy of the formulations and was used in the present study. This model was optimized and used for anti-psoriatic research in our laboratory previously. Commercially available 5% IMQ cream was topically applied (62.5 mg) on the shaved back of animals for 7 consecutive days to develop psoriatic skin lesions. IMQ application was continued for further 3 weeks intermittently to retain the developed lesions. MCNG samples and MTX tablet were administered orally after one week after the commencement of the experiment when the animals expressed psoriatic skin changes [22–25]. The test samples were administered once in a week for 3 weeks, so that animals received three doses in a three weeks period.

#### 2.3.1. Assessment of anti-psoriatic efficacy

The severity of inflammation was scored by using an objective scoring system; in which erythema, thickness and scaling of psoriatic skin was scored individually from scale of 0–4; with no sign of inflammation-0, slight-1, moderate 2, marked 3 and severe 4. Cumulated Psoriatic Area Severity Index (PASI) was calculated for every day. Percentage reduction in PASI at the end of treatment in comparison with that on the 7th day (before starting formulation treatment) was calculated [22–25].

#### 2.3.2. Body weight and spleen weight changes

Body weights of animals were noted initially and at the end of the experiment. Changes in body weights were determined and% changes in body weights were plotted against different treatment groups. At the end of therapy all animals were euthanized, blood samples were collected by cardiac puncture for biochemical and



**Fig. 1.** (A) Macroscopic appearance of animals, (B) Experimental paradigm and (C) body weight changes of animals in anti-psoriatic activity studies.

hematological analysis. Spleen weights were noted and spleen weight to body weight ratio (SW: BW Ratio) was calculated [26,27].

#### 2.3.3. Histological analysis

Skin and other organs were collected for histological analysis at the time of euthanasia. Collected organs were fixed in neutral buffered formalin, processed using isopropanol and xylene, embedded in paraffin, 5  $\mu$  thick sections were prepared using microtome, transferred to glass slides, stained with eosin and hematoxylline and mounted using DPX and observed under microscope. All samples were analyzed with the help of pathologist and inflammatory signs of skin samples were scored histologically. Acanthosis and presence of inflammatory cells were scored on a scale of 0–4 with no inflammation-0, mild acanthosis with low level of inflammatory cells infiltration-1, moderate acanthosis with mononuclear cells infiltration-2, marked acanthosis with high degree of inflammatory cells-3, severe acanthosis with microabcess and pustules-4. Presence of granular layer (presence 0, focal presence 1, absence 2) and parakerotosis(presence 2,focal presence 1,absent 0) was scored. Cumulative score was calculated and plotted. Epidermal thickness was measured using image J software and plotted [22,28].

#### 2.4. Biodistribution studies

Animals were grouped into four. First group animals received PBS, second group received a single dose (5.143 mg/kg) of MTX tablet orally, third group received MCNG at 2.5715 mg/kg and fourth group received MCNG at 5.143 mg/kg dose orally. Blood samples were withdrawn after 24 h of sample administration and after 1 week at the time of euthanasia and were processed for HPLC analysis. Organs were collected at the time of euthanasia, washed

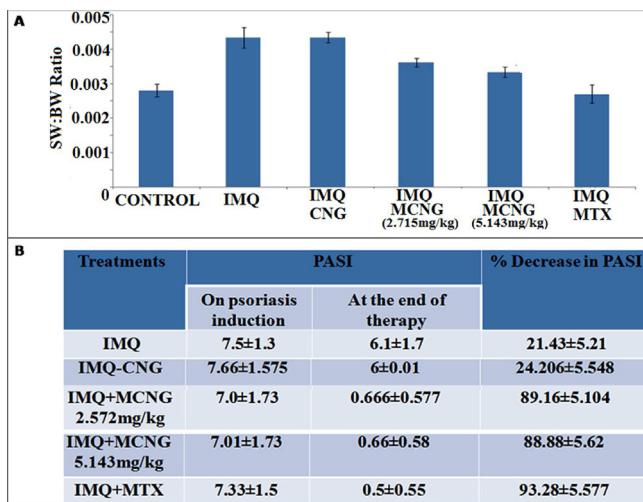
properly, weighed and 10% homogenate was prepared in phosphate buffer of pH6 and centrifuged at 4000 rpm for 10 min. Collected supernatant were processed for HPLC analysis [29,30].

#### 2.4.1. HPLC analysis

Collected blood samples were centrifuged at 3000 rpm for 10 min and plasma was collected. Supernatant collected from tissue homogenates and plasma samples were treated with di-ethyl ether and make up to the specified volume with mobile phase mixture (Acetonitrile: Phosphate buffer at a ratio of 10:90). The upper layer was collected and introduced into HPLC for analysis. Running time used was 5 min, Volume injected was 20  $\mu$ l, flow rate adjusted was 1 ml/minute and retention time observed was 2.8 min. Drug quantification was done using regression equation obtained from standard drug calibration curve plotted in plasma of normal animals [31–33].

#### 2.5. Toxicity studies

As most of the toxic effects of methotrexate are reported to be occurred due to the drug accumulation resulting from long term use of the drug, we conducted sub acute and sub chronic toxicity studies by administering the drug and formulation once in a week for 3 [1,14] and 12 weeks [34,35] respectively. The doses used were same as in anti-psoriatic activity studies. Animals were observed for any toxic signs daily. Body weights and percentage body weight changes were noted. At the end of therapy, animals were euthanized, blood samples were collected for biochemical and toxicological analysis; all organs were collected and subjected to histological analysis [22,23].



**Fig. 2.** (A) SW: BW ratio of animals and (B) PASI scoring of animals in anti-psoriatic activity studies.

## 2.6. Statistics

Animal number used in each group is 6; average values were taken and was expressed as  $\pm$  standard deviation. Student's *t*-test was used to evaluate the statistical significance between different groups and *p* value  $<0.05$  was considered significant.

## 3. Results and discussion

### 3.1. Anti-psoriatic activity studies

We used imiquimod (IMQ) induced psoriatic animal model for evaluating the anti-psoriatic efficacy of our formulations. IMQ is an immune stimulator and its topical application is reported to induce psoriatic type skin lesions. The model was developed and optimized by us in our laboratory [14,22]. The macroscopic appearance of animals that received different treatment strategies are shown in Fig. 1A and experimental paradigm is shown in Fig. 1B. IMQ is reported to act on Toll receptor 7 and stimulate immune system cascade to induce psoriatic type skin lesions [36].

#### 3.1.1. Body weight changes

Decrease in body weight of animals was noted. In case of control animals an average decrease of 4.9% is noted. This weight decrease is due to the reduced food intake associated with regular handling of these small animals during the treatment period [37]. IMQ treatment resulted in significant increase in weight reduction (average 12.8%) indicating the reduced food intake associated with the unhealthy condition due to the developed psoriasis (Fig. 1C). IMQ caused weight reduction in animals is reported previously [38,39]. Treatment with control chitin nanogel (CNG) could not able to provide significant effect on weight reduction associated with the IMQ treatment. Treatment with MCNG at 2.713 mg/kg and MTX tablet normalized the reduced weight reduction indicated the improved food intake and health status resulting from the clearance of developed psoriasis. MCNG at 5.143 mg/kg can improve the developed psoriasis but this dose could not normalize the weight reduction. This may be because of the decreased food intake due to the unhealthy condition of animals associated with the toxic effects of this dose at the vital systems as demonstrated in sub acute toxicity studies.

#### 3.1.2. Spleen weight changes

Spleen weight to body weight ratio (SW: BW ratio) of animals indicated that control animals have an average SW: BW ratio of 0.0028. IMQ treatment increased this value to 0.0042 (Fig. 2A). The increase in SW: BW ratio is due to the increased spleen weight associated with immune stimulator treatment [36]. MCNG at 2.715 mg/kg and 5.143 mg/kg dose levels reduced the SW: BW ratio to 0.0037 and 0.0035 respectively. In case of MTX tablet treated group animals the average value of SW:BW ratio observed was 0.003. This reduction in SW: BW ratio is the result of methotrexate treatment, as it is well known immune suppressant drug that antagonizes the stimulatory effects of IMQ on immune system. None of these changes were statistically significant.

#### 3.1.3. PASI reduction

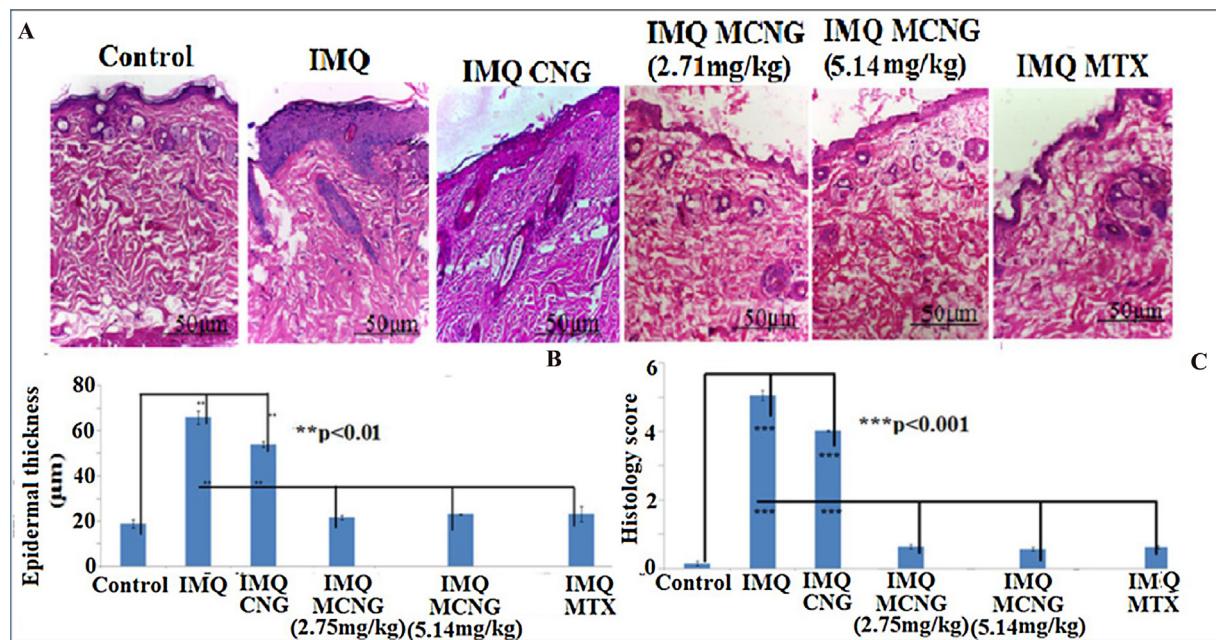
Reduction in Psoriatic Area Severity Index (PASI) was measured for each group of animals and compared. Reduction in PASI is used to access the efficacy of anti-psoriatic therapy and ideally a reduction of 75% (PASI 75) is needed to consider an anti-psoriatic therapy clinically effective [45,46]. In the IMQ alone treated group there is psoriatic inflammation and reduction in PASI at the end of experiment is only 21% (PASI 21), which may be due to the self healing nature of the model. CNG treatment failed to provide significant reduction in PASI (average reduction achieved is 24%). MCNG at 2.715 mg/kg and 5.143 mg/kg dose levels provided an average PASI reduction of 88% (PASI 88) and 89% (PASI 89) respectively. MTX tablet treatment provided an average 93% reduction in PASI (PASI 93) indicating improvement in disease condition of animals (Fig. 2B). Significant reduction in PASI indicated that methotrexate will act by different mechanisms to provide beneficial effects in psoriasis. Cytotoxicity towards the proliferated keratinocytes and T cells, immuno suppressant activities along with reduced cytokine production, anti-inflammatory activity due to enhanced cellular adenosine level together constitute its anti-psoriatic efficacy clinically [6]. MCNG at both dose levels provides significant reduction in PASI which is very much comparable with clinically used MTX tablet.

#### 3.1.4. Skin histology

Skin histology analysis revealed control animals skin with orthokeratotic stratum corneum, intact granular layer and organized thin epidermis (Fig. 3A). IMQ treatment resulted in lining of epidermis with regular acanthosis and bulbous rete pegs. Histology is further characterized by focal parakeratosis and absence or thinning of granular layer of epidermis. Intra epidermal and dermal neutrophilic infiltrates as microabcess are also observed at some areas of skin. So histological analysis clearly indicate that IMQ treatment resulted in skin lesions which exactly mimic psoriasis histologically [22–26]. CNG treated skin histology is very much similar to that of IMQ treated one except that there is slight reduction in IMQ induced acanthosis. This reduction may be the result of anti-oxidant and anti-inflammatory activity associated with chitin [40,41]. However this beneficial effects exhibited by chitin is not sufficient to bring down the inflammatory reactions associated with developed psoriasis. MCNG treatment at both dose levels and MTX normalized the histological features to that of control animals, due to the multiple mechanisms of action exhibited by methotrexate as explained previously [6].

#### 3.1.5. Epidermal thickness

Quantification of epidermal thickness revealed an average thickness of 18  $\mu\text{m}$  for control animals and IMQ treatment increased this to 65  $\mu\text{m}$  due to the severe acanthosis associated with induced inflammatory reactions (Fig. 3B). CNG reduced the epidermal thickness to 57  $\mu\text{m}$  may be due to the beneficial effects exhibited by chitin. MCNG treatment at both dose levels and MTX normalized



**Fig. 3.** (A) Skin histology, (B) Epidermal thickness and (C) histological score of animals in anti-psoriatic studies.

the increased epidermal thickness to almost that of the control animals, due to the alleviated inflammatory reactions resulting from the use of methotrexate [22].

### 3.1.6. Histological scoring of inflammation

Histology inflammatory score further proves the beneficial effects exhibited by MCNG and MTX on IMQ model of psoriasis. IMQ catalyzed inflammations with acanthosis, parakeratosis, absence or thinning of granular layer and intra dermal inflammatory cells infiltration altogether resulting in a total histological score of 5 in this group (Fig. 3C). CNG treatment reduces this value to around 4.3 due to the reduction in acanthosis caused by chitin. Normalized epidermal thickness and keratinocyte differentiation by MCNG and MTX treatments is reflected as decreased histological score of inflammation in these animal groups, which is very much comparable with that of control animals [22,26,27].

### 3.2. Biodistribution studies

In order to find out the distribution of methotrexate from MCNG and MTX to serum and vital tissues we conducted bio-distribution studies. Serum measurement indicated that after 24 h an average of 8.5, 5.2 and 19.2  $\mu\text{g}/\text{ml}$  of methotrexate is available in serum from MTX, MCNG 2.715 mg/kg and MCNG 5.143 mg/kg dose treatments respectively (Fig. 4A). Greater serum availability from MCNG is due to the fact that the drug is within the chitin nanogel network and it follows a slow release profile [14]. This will help to prevent the rapid drug loss resulting from systemic washout, which may happen with a burst release system and with conventional tablet MTX [42]. In addition having a size below 200 nm, chances for phagocytosis by monocytes is comparatively less as they often target large sized particles [14,43]. Drug is not detected in serum after a period of one week from any of the system may be because of the process of drug disposition and tissue distribution happened at this period.

Tissue distribution studies revealed an average amount of 18.3, 8.8, 16.4, 10.2 and 3.7  $\mu\text{g}/\text{g}$  methotrexate accumulation in liver, kidney, spleen, lung and stomach respectively after a period of 1 week from single dose administration of MCNG 5.143 mg/kg. From MTX tablet an average amount of 13.8, 4.8, 13.2, 4.8 and 4.3  $\mu\text{g}/\text{g}$  of methotrexate is accumulated in liver, kidney, spleen, lung and

stomach respectively. Amount of methotrexate accumulated from MCNG at 2.715 mg/kg dose level in liver, kidney, spleen, lung and stomach is 4.98, 3.4, 7.1, 3.29 and 4.1  $\mu\text{g}/\text{g}$  respectively (Fig. 4B). Drug is not detected in any of the heart samples collected from animals treated with MCNG and MTX. Methotrexate is a drug which is reported to accumulate in tissues and released from there to exhibit clinical efficacy [44]. Greater serum availability is the reason for greater tissue accumulation of drug from MCNG 5.143 mg/kg and MTX. In case of MCNG 2.715 mg/kg, the administered dose is half only, providing less serum level and hence less tissue accumulation [45].

### 3.3. Sub acute toxicity studies

Sub acute toxicity studies were performed to detect the toxic effects of methotrexate on application for a short period of 3 weeks. No detectable behavioral changes are there in any of the treatment group.

#### 3.3.1. Body weight changes and spleen weight changes

A 4.9% of weight reduction is observed with control animals and the change in weight of all treatment groups except MCNG 5.143 mg/kg is more or less comparable with that of control animals. In case of MCNG at 5.143 mg/kg, a significant weight reduction (8.2%) is observed (Fig. 5A). This weight reduction indicated the decreased food intake associated with unhealthy condition of animals with this dose level as explained in histological reports. Spleen weight to body weight ratio (SW: BW ratio) of animals revealed that there is a slight increase in SW: BW ratio in MCNG and MTX treated groups may be due to the hyper cellularity of spleen. As methotrexate is an immune suppressant drug, body try to counteract its effects through increased cellularity in spleen leading to increased spleen weights [22]. However none of these changes is significant when compared with control animals (Fig. 5B).

#### 3.3.2. Biochemical parameters

Renal function parameters analysis indicates that Blood Urea Nitrogen (BUN) of control animals is 40 mg/dl and in all other groups, BUN is very much close to this value. At 5.143 mg/kg dose of MCNG, an increased BUN of 60 mg/dl is observed, however this

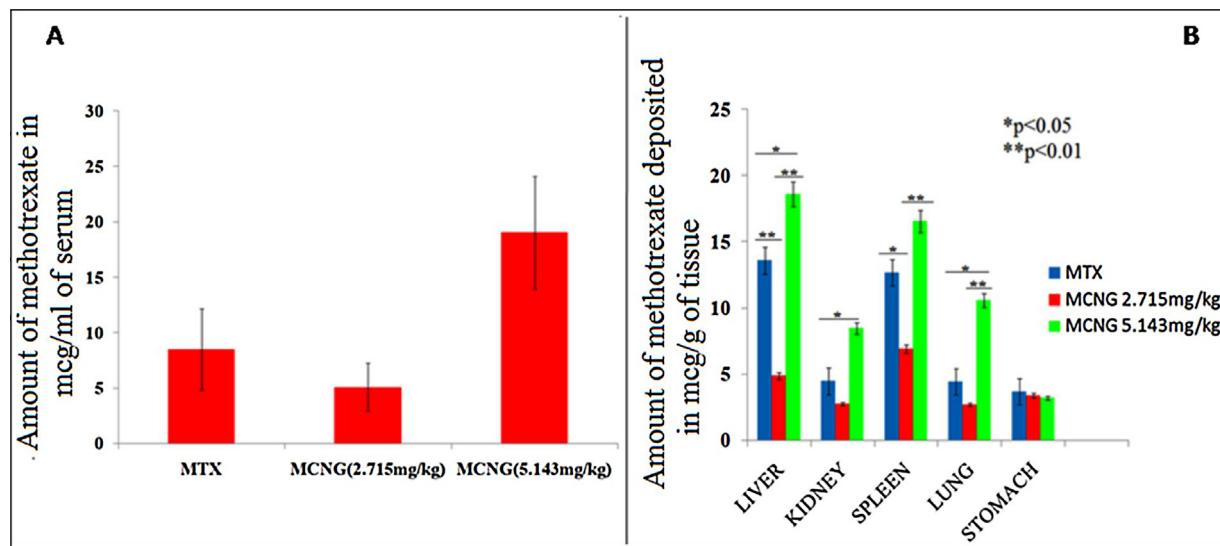


Fig. 4. (A) Serum level of methotrexate and (B) tissue distribution of methotrexate in bio distribution studies.

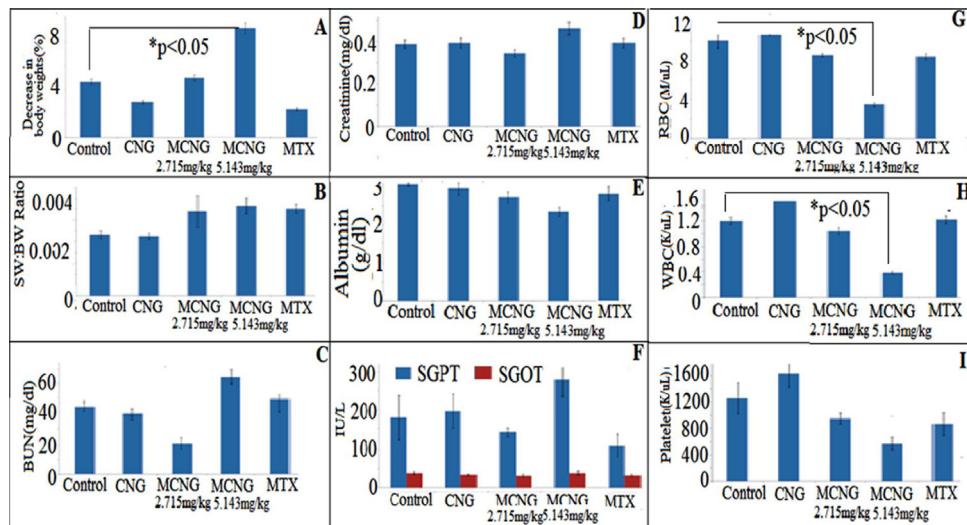


Fig. 5. (A) Body weight changes, (B) SW:BW Ratio, (C) BUN (mg/dl), (D) creatinine, (E) albumin, (F) SGOT and SGPT, (G) RBC, (H) WBC and (I) Platelet of animals in sub acute toxicity studies.

change is not significant. The creatinine level of control animals is 0.4 mg/dl and the change in this parameter is not significant in any of the treatment group when compared with control animals. Even though it is not significant there is a slight increase in serum creatinine of MCNG 5.143 mg/kg treated animal with an average value of 0.45 mg/dl (Fig. 5C & D). Liver function analysis indicate an albumin level of 3.1 g/dl, SGPT level of 180 IU/L and SGOT level of 30 IU/L for control animals. There is slight decrease in albumin (2.49 g/dl) with increase in SGPT (280IU/L) and SGOT level (35 IU/L) in MCNG 5.143 mg/kg dose received animals. In all other groups, there are no much changes in these parameters (Fig. 5E & F). The changes in renal and liver function parameters may be associated with the pathological changes in kidney and liver, associated with MCNG 5.143 mg/kg as explained in histological analysis. There are literatures supporting the fact that methotrexate toxicity is associated with altered renal and liver function parameters [10,11].

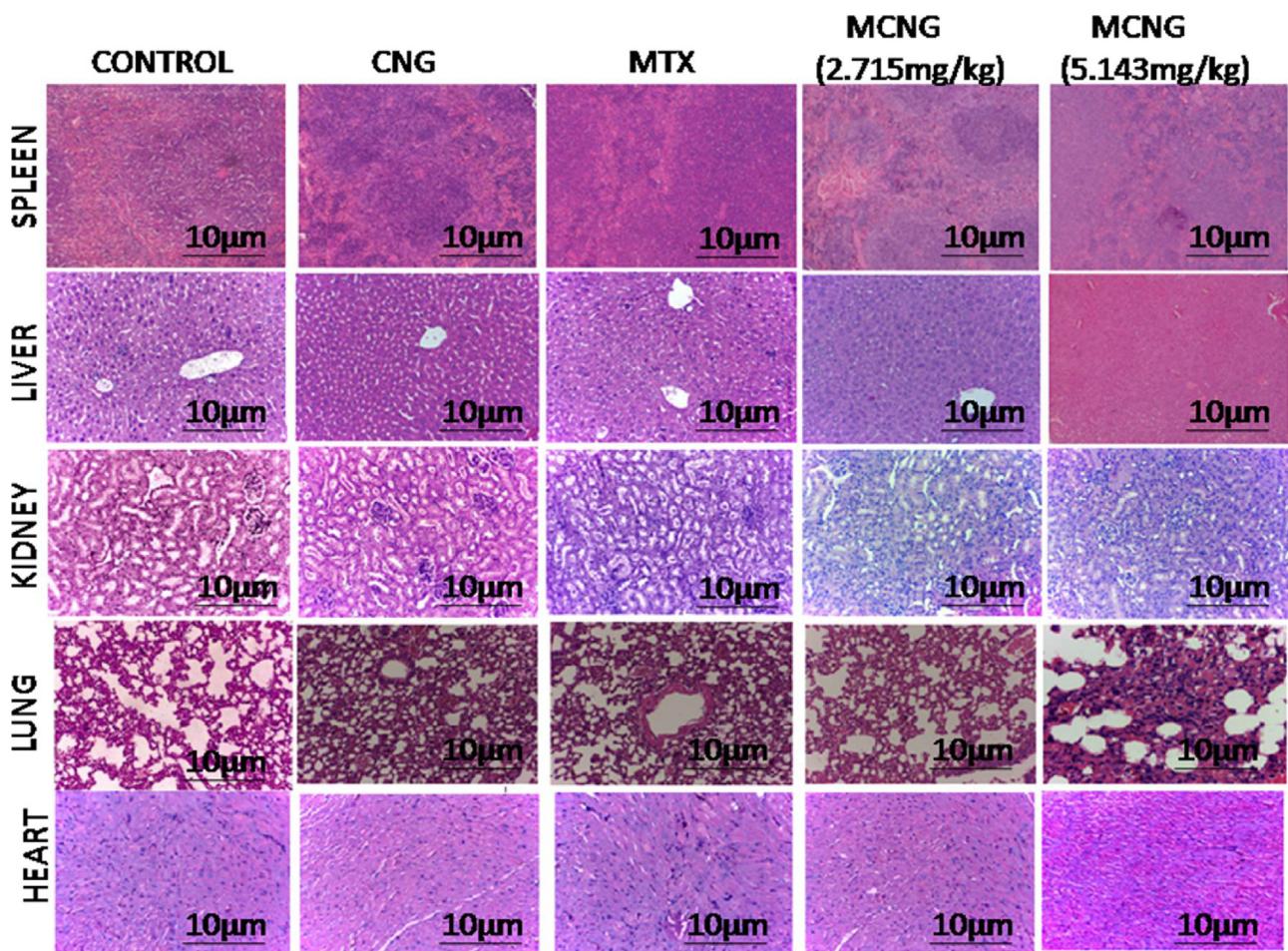
### 3.3.3. Hematological analysis

Hematology analysis revealed an RBC level of 10 M/uL, WBC level of 1.2 K/uL and a platelet level of 1280 K/uL for control ani-

mals (Fig. 5G–I). MCNG at 2.715 mg/kg decreased RBC level to 8.9 M/uL, WBC to 1.18 K/uL and platelet to 1000 K/uL. Significant reduction with an average RBC level of 4 M/uL, WBC level of 0.5 K/uL and platelet level of 600 K/uL is observed with MCNG at 5.143 mg/kg dose level. In case of MTX the observed level for RBC, WBC and platelet is 8.7 M/uL, 1.2 K/uL and 980 K/uL respectively. The decrease in hematological parameters is due to the bone marrow suppressant effects exhibited by the drug. Since methotrexate is a drug which is more sensitive to highly proliferating tissues like bone marrow, which is the centre for hemopoiesis, has got significant effects on blood cells number [46]. Significant effects observed with MCNG at 5.143 mg/kg treatment indicated greater availability of the drug at this dose level from the nano formulation when compared with corresponding dose applied as tablet MTX. However with MCNG at 2.715 mg/kg, the drug dose available is comparatively low, so net effects on blood cells is not significant.

### 3.3.4. Histopathological analysis

Histological analysis of tissue samples gives more detailed view of toxic effects of formulations. Control spleen histology revealed

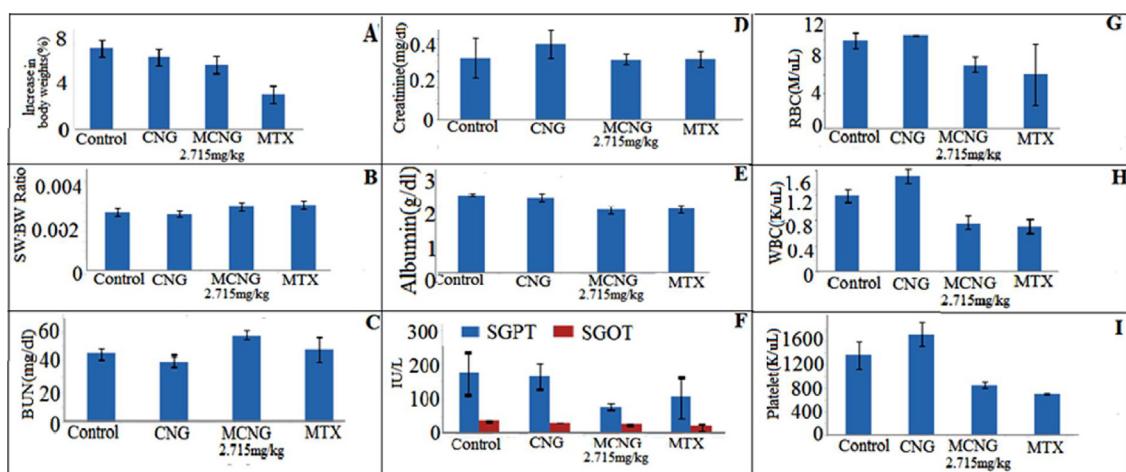


**Fig. 6.** Histology of different organs in sub acute toxicity studies.

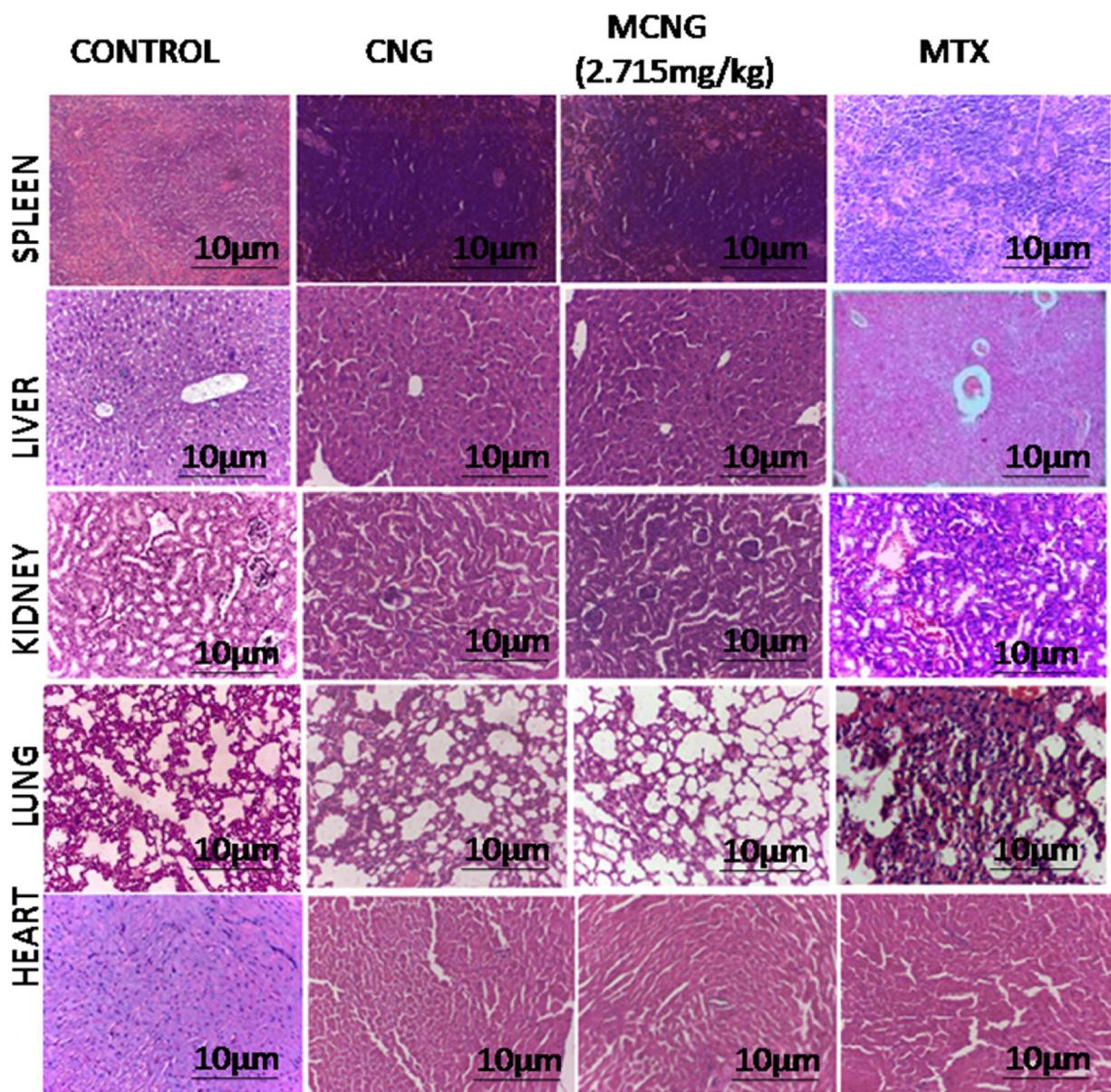
normal splenic parenchyma composed of white pulp and red pulp covered by capsule composed of connective tissue [46]. White pulp is predominantly composed of lymphocytes and red pulp is the reticular tissue containing reticulocytes and fibers, which is the centre for extramedullary hemopoiesis. In case of MCNG at 2.715 mg/kg and MTX group, white pulp hypercellularity with lymphoid hyperplasia is observed (Fig. 6). At MCNG 5.143 mg/kg spleen histology revealed splenic parenchyma composed of white pulp with hyper plastic lymphoid follicles and red pulp with congested sinusoids and hematopoietic elements with erythroid, myeloid and megakaryocytic origin. Hyper cellularity in splenic components indicate the compensatory mechanisms to counteract the immune suppressant effects caused by methotrexate, as spleen is a major site for extra medullary hemopoiesis. The effect is more prominent in MCNG 5.143 mg/kg due to greater availability of methotrexate at this dose level. Liver histology revealed central vein was surrounded by hepatocytes arranged as lacunae interspersed with sinusoids [46]. Histology of liver samples is very much comparable with that of control animals for all treatment groups except MCNG 5.143 mg/kg. In this case histology revealed, cytoplasmic basophilia with mild degree of hydropic degeneration. Cytoplasmic basophilia is a condition where an abnormally elevated basophils are noted and often occurs with allergic and inflammatory diseases. In this case methotrexate induced toxicity is suggested to cause cytoplasmic basophilia in liver. Hydropic degeneration is one of the indications of early cellular damage characterized by cellular cloudy swelling, which is often considered as nonlethal, since the condition is reversible when the root cause is removed, which is reported to be associated with methotrexate induced liver damage

[47,48]. Renal histology revealed renal capsule composed of specialized renal tubules along with glomerulai and renal blood vessels in all groups except MCNG at 5.143 mg/kg group. Here congested glomerulai along with congested renal blood vessels are observed. Otherwise the histology is comparable with that of control group. A congested renal blood vessels and glomerulai suggested the interaction of methotrexate with renal tissue and subsequent cellular damage [46]. These toxic effects on histology may be the reason behind the altered biochemical parameters occurs at this dose level. Lung histology revealed lung parenchyma with alveoli, bronchi and bronchioles in all groups. At MCNG 5.143 mg/kg dose level lung histology revealed alveoli with congestion of septal capillaries along with intra alveolar hemorrhage. There are areas with dense inflammatory cells infiltrate and eosinophilic fibrous exudates, along with alveolar wall. Greater toxic effects on lung parenchyma indicate high sensitivity of methotrexate towards the lung tissue [46,48]. Although most of the toxic effects of drug occurs by cumulated effects on long term use, there are reports supporting the fact that methotrexate induced lung toxicity occur even with the use of small dose for short period of 4–6 weeks [49]. Histology of heart samples revealed myocardium with organized cardiac muscle fibers interconnected by intercalated disc in all groups. Maintenance of normal histology of heart supported the results of bio-distribution studies, as we could not detect methotrexate in this tissue.

The results of the anti-psoriatic activity studies along with biodistribution and sub acute toxicity studies revealed that, even though MCNG at 5.143 mg/kg exhibited anti-psoriatic activity, it is showing toxic effects on vital systems like kidney, liver and



**Fig. 7.** (A) Body weight changes, (B) SW:BW Ratio, (C) BUN(mg/dl), (D) Creatinine, (E) albumin, (F) SGOT and SGPT, (G) RBC, (H) WBC and (I) Platelet of animals in sub chronic toxicity studies.



**Fig. 8.** Histology of different organs in sub chronic toxicity studies.

lung on 3 weeks application. But we achieved a significant level of anti-psoriatic efficacy with better tissue distribution without toxic effects on use of its half dose. So MCNG 5.143 mg/kg dose level is omitted from the further studies.

### 3.4. Sub chronic toxicity studies

In order to detect the toxic effects associated with tissue accumulation on long term use, we performed sub-chronic toxicity evaluations of MCNG 2.715 mg/kg in comparison with MTX tablet.

#### 3.4.1. Body weight changes and spleen weight changes

Unlike in anti-psoriatic and sub acute toxicity studies, here we observed an increase in body weights for all groups, with an average 7.5% in control animals (Fig. 7A). The selected animals were 8–11 weeks old, it means that animals were in a growing stage and not aged, adjusted well with experimental condition during the 12 week period (unlike short period of 3 weeks in sub acute toxicity). So the experimental procedure not interfere with the normal food and water intake of animals, caused an increase in body weight even though we handled the animals during this period. However the weight gain attained in MTX group is 3.7%, which is considerably less when compared with 7.5% observed in control animals. It may be due to the decreased food intake associated with a disturbed vital functions caused by accumulated methotrexate in this group. Change in SW: BW ratio is not significant for any of the treatment groups when compared with control animals. Slight increase (Fig. 7B) observed with MCNG and MTX may be due to the result of hyper plastic splenic microstructures as revealed by histological analysis results.

#### 3.4.2. Biochemical parameters

There is no much change in measured biochemical parameters like BUN, Serum creatinin, Albumin, SGOT and SGPT of animals in any of the tested groups (Fig. 7C–F). Although the unaltered biochemical parameters indicate normal vital functions, it needs to confirm with histological analysis. There are reports stating the fact that at least in some cases, the bio chemical parameters remains normal, until a profound toxic effect on vital system happened [50].

#### 3.4.3. Hematological analysis

MCNG treatment resulted an RBC, WBC and platelet count of 6.8 M/uL, 1 K/uL, 840 K/uL respectively when compared with 10 M/uL (RBC), 1.2 K/uL (WBC) and 1280 K/uL (Platelet) observed for control animals. MTX treatment further reduces the values to 5.9 M/uL, 0.99 K/uL, 610 K/uL for RBC, WBC and platelet respectively (Fig. 7G–I). The profound decrease in hematological parameters is due the result of bone marrow suppression caused by methotrexate. Bone marrow suppression is an inherent effect of the selected drug and it cannot be avoided. However the effect is less with MCNG 2.75 mg/kg dose when compared with MTX [46].

#### 3.4.4. Histopathological analysis

Spleen histology of MCNG treated animals revealed white pulp hyper cellularity with hyperplastic lymphoid follicles (Fig. 8). In case of MTX treatment group, spleen histology revealed elements showing extra medullary hemopoiesis. Presence of mega karyocytes confirmed extramedullary hemopoiesis happened to counteract the bone marrow suppression caused by methotrexate [46]. Liver histology is very much comparable with that of control animals in all groups except for MTX group. In this case liver sections showed hepatic lobules with congested central hepatic vein surrounded by cords of hepatocytes separated by dilated sinusoids, hepatocytes adjuscent to central vein is atropic [46,50]. However it seemed that these changes were not sufficient to bring a significant influence on liver function parameters. Normal

microscopy is maintained in case of renal structures, indicated the unaltered renal function in all tested groups. Even though some amount of drug is cumulated in kidney as per bio-distribution studies, this amount is not sufficient to cause toxicity in the tested period of 12 weeks. Histology of lung in case of MTX treated animals revealed intra alveolar hemorrhage with congested septal vessels and dense inflammatory cells infiltrate formed predominantly of neutrophils along with lymphocytes and histiocytes. In all other cases the lung histology is comparable with that of control animals [46,48,49]. It seemed that even though the amount of drug cumulated in lungs is comparatively low (compared with spleen and liver) as per bio-distribution studies, lung tissue is extremely sensitive to the effects of methotrexate resulting in profound histological changes. Also there are case report indicate that lung toxicity can happen even with short term use of the drug. Histology of heart is very much normal in all tested group indicated the maintained vital structure, confirmed the absence of accumulation of methotrexate in this tissue as revealed by the bio-distribution studies.

Methotrexate loaded chitin nanogel showed a slow release pattern of methotrexate, will help to prevent the drug loss through wash out by circulatory fluid. In addition being a nano sized system it has got additional advantages of enhanced permeation and retention effects. Here due to the inflammatory condition associated with the developed diseases, the nano-carrier has got higher affinity towards the inflamed skin, provides an additional passive targeted effect to retain more MCNG to this area. In addition the inflamed area is acidic in nature and chitin nanogel is reported to swell more at acidic condition and hence more drug release. This may be the reason for anti-psoriatic efficacy achieved with half the dose of MCNG when compared with clinically used dose of MTX tablet. In addition bio distribution studies revealed MCNG at half dose can provide sufficient blood level of methotrexate necessary to exhibit systemic actions without much accumulation in tissues in comparison with MTX and MCNG 5.143 mg/kg dose. Because of the less tissue distribution it is not showing toxicity even on sub-chronic administration when compared with MTX where it is showing liver and lung toxicities.

## 4. Conclusion

Through the orally administered methotrexate loaded chitin nanogel (MCNG) formulation, it is possible to achieve the anti-psoriatic efficacy even with the half of clinically used dose of methotrexate tablet. The biodistribution studies revealed that MCNG and MTX tablet at a dose of 5.143 mg/kg showed greater serum level with significant accumulation in vital organs. Greater tissue accumulation leads to liver and lung toxicity on sub acute application in case of MCNG 5.143 mg/kg. In case of MTX tablet the lung and liver toxicity was observed on sub chronic administration. Low serum level and tissue distribution achieved with MCNG 2.715 mg/kg dose limits its systemic toxicities, while providing significant anti-psoriatic activity, when compared with conventional methotrexate tablet. So orally administered MCNG at 2.715 mg/kg dose is seemed to be better alternative for MTX tablet, to whom a systemic anti-psoriatic therapy is necessary, as there is a chance for systemic toxicities on long term use of the drug.

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