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MODIFICATION OF EXPRESSION OF TNF-α AND IL-10 RECEPTORS BY ANDROGRAPHOLIDE LOADED CHITOSAN NANOPARTICLES: A PROMISING APPROACH IN MANAGEMENT OF RHEUMATOID ARTHRITIS

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ABSTRACT

The present study evaluates the immunomodulatory efficacy of a natural molecule known as andrographolide (AG) in management of rheumatoid arthritis (RA). AG is a diterpenoid lactone located inside the aerial portions of *Andrographis paniculata*. It has been reported to be used in traditional medicine system in India and China. RA is a debilitating autoimmune disorder manifested by the inflammation of synovial membranes, bones, and cartilages. These symptoms are often triggered by disparity in the levels of various cytokines leading to an inflammatory immune response. AG had been reported to be a potent anti-inflammatory agent but poor water solubility and physiological instability in gastro-intestinal tract restrict its use as an oral therapeutic agent. To overcome these limitations, AG was encapsulated in chitosan nanoparticles and this particulate carrier system was explored for its immunomodulatory effects on the expression of Tumor Necrosis factor- α (TNF-α) and Interleukin-10 (IL-10) in a mouse model of RA. Immunohistochemistry of spleen of arthritic mice treated with AG elucidated significant decrease and increase in TNF-α and IL-10 immunopositive cells, respectively, suggesting AG as a good candidate for management of the RA safely and effectively.

KEYWORDS: Andrographolide, Chitosan nanoparticles, Rheumatoid arthritis, Immunohistochemistry, TNF-α, IL-10.

INTRODUCTION

Rheumatoid Arthritis (RA), a destructive auto-immune disorder, that leads to chronic inflammation of joints depicted by synovial inflammation and hyperplasia. T-Cells along with interleukins are reported to play major role in manifestation and development of inflammatory response in the disease. Elevated tumor necrosis factor (TNF)-α level plays key role in development of inflammation hence molecular inhibition of TNF-α is one of the key therapies used for the management of RA.[1-2] TNF-α has also been reported to have a significant effect on the function of T-regulatory cells (T_{reg}). The upregulation of TNF-α might interfere with the mechanism controlling the suppressive function of T_{reg} in RA patients.[3] Modulation of expression of key Cytokines by natural anti-inflammatory agents may prove to be a promising approach for management of RA.

Andrographolide (AG) is one of the naturally occurring bioactive compounds with anti-inflammatory properties. It is reported to downregulate production of inflammatory mediators such as Nitric Oxide[4] (NO), TNF-α, IL-6 in peritoneal macrophages in dose dependent manner. AG downregulates the production of free radicals and pro inflammatory cytokines thereby reducing the progression of neuro-degenerative disorder induced by antigenic challenge.[5] It is also reported to inhibit Cyclooxygenase (COX) by directly targeting NFκβ signaling pathway. Complete Freund's adjuvant induced paw edema was reduced significantly when treated with AG.[6] AG is reported to be an anti-Arthritic agent manifested by suppression of matrix metalloproteinases and inducible nitric oxide synthase (iNOS) in human osteoarthritic chondrocytes.[7] AG suppress the production of inflammatory mediators in Freund's complete adjuvant induced arthritis.[8]

Full therapeutic potential of AG has not been achieved due to its poor bioavailability, rapid metabolism, and clearance from the body.[9] Nano formulations of AG has been observed to improve stability and bioavailability along with an advantage of targeted tissue distribution.[10-11] Chitosan, a naturally occurring biopolymer has gained considerable attention as a carrier system with improved bioavailability and no toxicity at active physiological conditions.[12] Hence, in present research AG was encapsulated in chitosan nanoparticles to enhance its bioavailability thereby enhancing its cytokine modulation efficacy in adjuvant induced arthritic mouse model.

Materials: All the reagents used were of analytical grade and stored according to the instructions given by the manufacturer. AG and Freund's complete adjuvant were purchased

from Sigma Aldrich, Germany, Chitosan was procured from Himedia labs. Primary Goat polyclonal antibody to IL-10 was procured from Santa Cruz biotech and is used at a dilution ratio of 1:100 whereas secondary anti goat antibody was purchased from Sigma Aldrich and used at a dilution ratio of 1:400. For the evaluation of TNF-α positive cells in spleen tissue primary goat polyclonal antibody and secondary anti goat antibody was procured from Santa Cruz biotech and are used at a dilution rate of 1:2000 and 1:800 respectively. All the experiments were performed in triplicates. The results are represented as mean ± standard deviation with level of significance ($p < 0.05$).

Preparation of AG encapsulated chitosan nanoparticles: AG encapsulated chitosan nanoparticles were prepared by ionic gelation technique.[13] Blank chitosan nanoparticles were synthesized by similar process without incorporating AG in chitosan solution. The drug encapsulation efficiency (EE) and Loading Capacity (LC) was evaluated.[14] The size and Zeta potential of AG loaded chitosan nanoparticles were estimated by standard protocol using Zeta sizer (Malvern Co. UK). Swelling Index and Mucoadhesivity index on Pig intestinal mucosa was studied to evaluate the adhesion potential of the AG loaded chitosan nanoparticles.[15-16]

Animals

Maintenance of Swiss albino mice in animal house

4 weeks old Swiss albino mice were procured from a local breeder and kept at Central animal house facility, Punjabi University, Patiala, Punjab, 147002 (India). The animals were acclimatized to laboratory conditions for a period of one week. They had free access to food and clean water ad libitum. They were provided with standard diurnal cycle of 12 h light and 12 h darkness period with the temperature maintained at 23°C. The floor husk in cages was changed every third day. The hygiene and sanitation were maintained throughout the experiment. All the experiments were performed according to guidelines of Institutional Animal Ethical Committee (IAEC) under *Reg. No. 107/99/CPSCEA/2013-04.*

***Induction of rheumatoid arthritis (RA)*[17]**

150µg of Ovalbumin prepared in Freund's complete adjuvant (FCA) was administered twice to animals through intra-peritoneal route with an inter-immunization gap of seven days. The third immunization was done by injecting 20 µl of OVA in FCA to right paw after one week of second immunization to induce RA. The day after successful confirmation of RA was considered as day Zero.

Confirmation of RA in mice models

Besides various physical markers, the progression of RA was also confirmed by measuring the levels of total serum nitrite[18-19] serum alkaline phosphatase (ALP) levels as markers of RA.[20-21] Standard histopathological analysis of bone and cartilage was also done to ensure successful induction of RA.

***Animal groups*:** The mice were allotted into eight groups with 6 animals in each group as follows:

1. RA Control (Diseased and untreated)
2. RA + CSNP's (Blank Chitosan nanoparticles)
3. RA +AGCSNP's (AG loaded Chitosan nanoparticles)
4. RA + Free AG
5. Normal model (Untreated and non-diseased)
6. Normal + CSNP's
7. Normal + AGCSNP's
8. Normal + Free AG

Dosage: *AG* was orally administered to Swiss albino mice at a concentration of 1mg/Kg body weight. 30µg of AG was administered orally to normal and RA Groups in free and encapsulated form with blank chitosan nanoparticles as control.

Experimental Setup

Various formulations of AG (Free and Encapsulated in nanoparticles) were administered orally to the respective groups on zero day, 3rd day and 5th day. Total serum nitrite levels of all the groups were also analyzed on 9th day. The animals were euthanized on 18th day and serum samples were collected to study total serum nitrite and alkaline phosphatase levels for evaluation of the therapeutic effects of AG in different formulations. The spleen samples were collected, fixed, and analyzed for expression of IL-10 and TNF-α by immunohistochemistry (IHC).

The therapeutic efficacy of AG was evaluated by comparing total serum nitrites in the serum samples of various groups collected on 9th and 18th day (Grisham *et al.,* 1996. The serum samples collected on 18th day were also evaluated for Alkaline Phosphatase (ALP).[20-21] The thickness of foot pad of normal and Arthritic mice was also recorded on 4th and 8th day.

RESULTS AND DISCUSSION

Preparation and characterization of Chitosan Nanoparticles

The AG loaded chitosan nanoparticles (AGCSNP's) were prepared by ionic gelation technique. Dynamic light scattering elucidated the mean diameter of AG loaded nanoparticles to be 252 nm as depicted in Fig 1 and the zeta potential was found to be +24mVas shown in Fig 2. The polydispersity index (PDI) of 0.3 indicates narrow size distribution of the AGCSNP's. These nanoparticles were harvested and characterized for their drug release profile, swelling index and mucoadhesivity index. The drug release profile of AGCSNP's was illustrated by calorimetric estimation of AG released by them in aqueous suspension at 37 ºC under constant stirring after definite time intervals.[22] An initial burst release of AG was observed followed by its sustained release for 48 hours as shown in Fig.3. Similar drug release profile of CSNP's has been also reported earlier.[13]

The half-life and bioavalibility of AG under physiological conditions is very limited and it is cleared from the body very quickly.[11] Hence, the sustained release of AG observed might enhance its residence time thereby increasing its therapeutic potential in biological systems. The encapsulation efficiency of AGCSNP's was observed to be 55% and the loading capacity was observed to be 6.76 µg per mg of CSNP's. These findings were also supported by previous research reports.[23] Swelling index is the indirect measure of drug holding capacity of the nanoparticles. The swelling index was calculated to be 800%. Similar results with a swelling index of 756% were also been reported earlier.[24] The mucoadhesivity index of AGCSNP's was estimated to be 70 %. Similar mucoadhesivity index was also reported by independent workers.[16] High mucoadhesivity index ensures better interaction of nanoparticles with intestinal mucosal that is vital in improving the pharmacokinetics of the drug.[25] It facilitated release of AG in vicinity of intestinal tissue ensuring better absorption and safe delivery of AG in harsh physiological conditions.[26]

Induction of rheumatoid arthritis (RA) in Swiss albino mice

The RA was induced successfully in Swiss albino mice by delivering OVA in FCA at a concentration of 5mg/kg body weight.

Confirmation of rheumatoid arthritis in animal models

The arthritic models were feeling uncomfortable and stressed when the foot paw joints were touched indicating joint inflammation. Elevated serum nitrite levels were also reported as potent biomarkers of RA.[19] Elevated serum ALP level due to osteoblastic activity of bone

cells was considered as biomarker in RA patients.[27-28] The average total serum nitrite levels in RA mice were observed to be very high (9.27µg/ml) as compared to that of Normal mice (2.54µg/ml). Elevated ALP activity of 303.7 U/L was observed in RA mice whereas in the normal mice the ALP activity was found to be only 117.8 U/L. The present results corroborated with the findings reported earlier.[29-30] The edema in challenged foot pad joint was more pronounced as compared to control footpad The abnormally higher levels of total serum nitrite, ALP and foot pad edema indicated joint damage.

The serum total nitrite levels of normal and RA on 9th day and 18th day were shown in Fig 4. Chitosan was found to be slight immuno-stimulatory whereas AG suppressed the production of nitrites. This might be attributed to the negative regulation of inducible Nitric oxide synthase enzyme by macrophages and other immunological cells.[31] It had also been reported to regulate nitric oxide production by inhibiting the iNOS enzyme at transcriptional level involving NFκβ.[6]

Free AG and AGCSNP's lowered the levels of serum nitrite but AGCSNP's were found to be more effective in counteracting the immunological situation in RA mice. About 55% reduction of total serum nitrite was observed on 18th day in RA mice administered orally with AGCSNP's whereas free AG could downregulate the serum nitrite levels by 20% only as compared to their respective control. This might be attributed to longer residence time of AG when delivered in CSNP's. Similar findings had also been reported earlier.[32]

In a clinical study, elevated serum nitrite levels in RA patients were reported earlier[33,19] The similar results were also reported earlier[31] that elucidated the downregulating effect of AG on iNOS.

Serum alkaline phosphatase (ALP) activity after administration of free AG and AGCSNP's in normal and RA mice

Serum ALP level of different mice groups on 18th day was estimated as shown in Table 1. Elevated serum ALP levels were observed in RA mice as compared to normal mice. The rise in ALP might be attributed to osteoblastic activity of bone cells in RA models. The similar results were also reported.[34] AGCSNP's were capable of normalization of serum ALP level in RA mice to 154.8U/L whereas the AG did not show any significant effect on ALP levels of RA mice. Besides, it significantly reduced edema and inflammation of foot pad and joints respectively on 8th day.

Effects of free AG and AGCSNP's on bone histology of RA mice

The joints of arthritic animal models were analyzed by haematoxylin and eosin staining. A considerable infiltration of immune cells (neutrophils and macrophages) was observed in the inflamed areas. The normal control was free from any infiltration of immune cells as depicted by the histopathological examination of the bone given in Fig. 5(A). The anatomy of the normal bone was found to be normal. Vehicle was not found to cause any significantly effect on the histology of RA mice as shown in Fig. 5(D). The RA control was highly affected due to mass infiltration of neutrophils. Considerable damage to synovium was noticed. The endothelial hyperplasia (Arteritis) was also seen in arteries, characteristic feature of development of RA as shown in Fig. 5 (E) and (F).

The bones were significantly affected, and synovitis was also investigated. Free AG was capable of reduction of neutrophil infiltration to small extent only as shown in Fig.5 (B). It failed to ameliorate the pathological condition significantly. This may be due to early clearance of AG from the body before the therapeutic effect was realized. AGCSNP's were quite potent in healing the synovial membrane evident by reduced infiltration of immune cells in nearby areas as shown in Fig.5(C). This can be attributed to slow and sustained release of AG by AGCSNP's for longer duration maintaining its constant physiological concentration. The unloaded CSNP's were unable to reduce the neutrophil infiltration as compared to untreated control as shown in Fig. 5 (C).

In vivo cytokine expression modification by free AG and AGCSNP's in arthritic mouse

The present study demonstrated the effect of AGCSNP's and free AG on the expression of cytokines (TNF-α and IL-10) in RA mice. TNF-α and IL-10 expression in spleen of RA mice was investigated by IHC technique.

TNF-α is a key pro inflammatory cytokine which was reported earlier as indirect regulator of regulatory-T cells which are involved in maintaining self-tolerance.[35] In current study the spleen sections of normal and Arthritic mice were stained using immunohistochemical (IHC) technique to localize TNF-α immunoreactive cells. The therapeutic effects of various formulations of current bioactive compound on expression of TNF-α^{+}cells in spleen sections of various mice groups are shown in Figure 6.

IHC elucidated a significant increase in population of TNF-α positive cells in spleen sections of RA control group compared to normal control as shown in Fig. 7(B) and (A) respectively.

The vehicle was also observed to increase the number of TNF-α positive cells compared to normal control, but the increase was observed to be 10% as compared to RA control group as shown in Fig. 7 (C).

Both AG free and AGCSNP's elucidated downregulation of expression of TNF-α positive cells. A 33% decline in population of TNF-α positive cells were observed in AG Free group as depicted in Fig. 7 (D) as compared to RA control whereas AGCSNP's demonstrated a significant decrease of TNF-α immunoreactive cells by 68% as shown in Fig. 7(E). AGCSNP's were found to be 52 % more efficient in downregulating the expression of TNF-α positive cells when compared to AG free treated group.

The spleen section of normal control showed reduced expression of TNF-α positive cells. This might be attributed to lack of antigenic challenge and infiltration of macrophages in normal group of mice. The higher efficiency of downregulation of expression of TNF-α immunoreactive cells as compared to free AG treated group might be attributed to enhanced bioavailability and stability of AG under *in vivo* conditions when administered encapsulated in CSNP's.

AG free was also reducing the density of TNF-α positive cells as compared to RA control but the effect was not as pronounced as that of AGCSNP's that showed 68% decline in population of TNF-α positive cells. Similar results were also observed earlier.[36-37] It clearly indicated that the CSNP's encapsulated delivery of AG enhances its therapeutic efficiency as compared to when it was delivered orally in free form. The inhibitory effect of AG on TNF-α[38-39] was also found to ameliorate RA by upregulation of the development of T_{Reg} cells.[40-41]

$CD4^+$ cells and macrophages are believed to be actively involved in pathogenesis of RA. They are the main effectors of the inflammatory response. There is initial burst of proinflammatory cytokines like TNF- α, IL-1, granulocyte monocyte colony stimulating factors (GM-CSF). The immune system responds to this situation by producing certain endogenously inhibiting molecules like IL-1 receptor antagonists (IL-1ra), IL-10, TNF-receptors (TNFR) and various other soluble factors. IL-10 is reported to be an anti-inflammatory cytokine that work in antagonism with IL-12. IL-10 was also reported to decrease production of various proinflammatory cytokines like GMCSF and TNF-α.[42]

4.8.2 Expression of IL-10$^+$ in spleen sections of RA mouse model

Spleen sections were stained using IHC technique to localize IL-10$^+$ cells. Fig 8 illustrated the effect of various AG formulations on the expression of IL-10 immunopositive cells in spleen sections of mice groups. IHC elucidated a significant increase in population of IL-10$^+$ cells in spleen sections of RA control group compared to the control as shown in Fig. 9 (B) and (A) respectively. The vehicle was also observed to increase the number of IL-10 cells compared to normal control, but the elevation was not significant compared to RA control group as shown in Fig.9 (C). AG in all the test formulations (AG free and AGCSNP's) showed upregulation of IL-10$^+$ cell expression. A four-fold increase in IL-10+ cells were observed in AGCSNP's treated group as compared to RA control as shown in Fig. 9 (E) whereas free AG demonstrated only 2-fold rise in expression of IL-10$^+$ cells as depicted in Fig.9 (D). AGCSNP's were found to be 50% more efficient in upregulating the expression of IL-10$^+$ cells when compared to AG free treated group. Similar findings have been reported by previous research.[43]

The spleen section of normal control showed reduced expression of IL-10$^+$cells. This might be attributed to lack of infiltration of macrophages in absence of antigenic challenge. AG free group also show IL-10$^+$macrophages indicating the stimulatory effect of AG on production of IL-10. Similar results were also reported earlier[44-45] by Wang *et al.*, 2010. The findings of the author illustrated a decline in ratio of IL-12/ IL-10 by AG due to macrophage phenotypic polarization to M2. (Alternatively activated macrophage) phenotype rather that M1 (classically activated macrophages). M1 phenotypes mainly produce inflammatory cytokines like IL-12 whereas M2 type macrophages are more inclined towards the production of anti-inflammatory cytokines like IL-10. The ratio of cytokines produced by M1 and M2 macrophages ensure a balance in the body.

Increased expression of IL-10 immunoreactive cells compared to free AG treated group might be attributed to the enhanced bioavailability and stability of AG under *in vivo* conditions when it was delivered encapsulated in CSNP's.

List of Figures

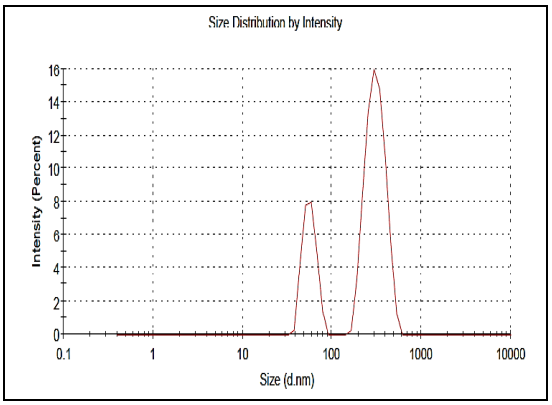


Fig. 1: Mean diameter of the AGCSNP's

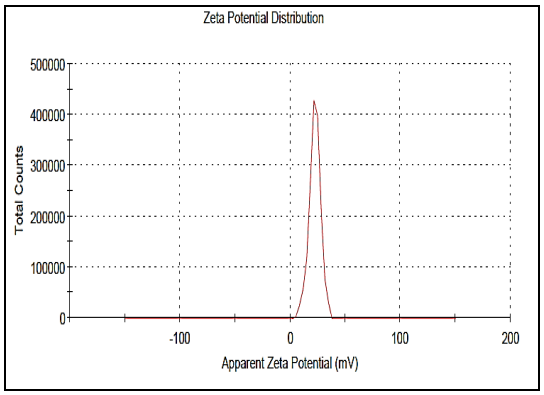


Fig 2 : Zeta potential of the AGCSNP's

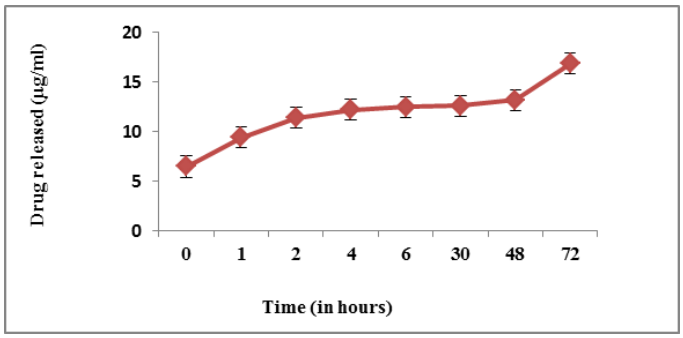


Fig.3: AG release profile of AGCSNP's.

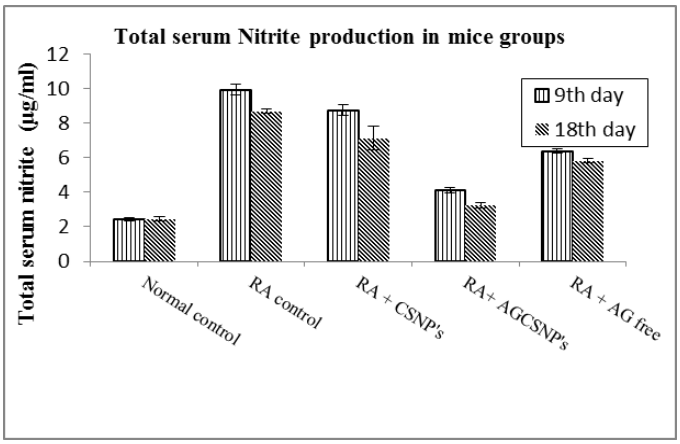


Fig 4: Total serum nitrite in normal and RA mice on 9th and 18th day.

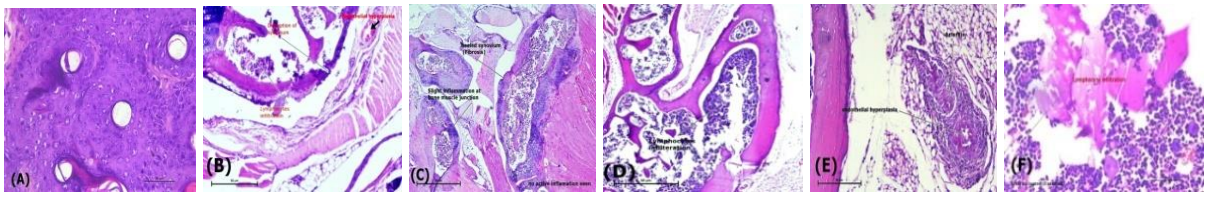


Fig. 5(A-F): Histopathological condition of the joints IN RA and normal mice models

(A) Normal control (B) Free AG treated group (C) AGCSNP's Treated group (D) CSNP's

treated group (E) Untreated control with endothelial hyperplasia (F) Untreated control with neutrophil infiltration.

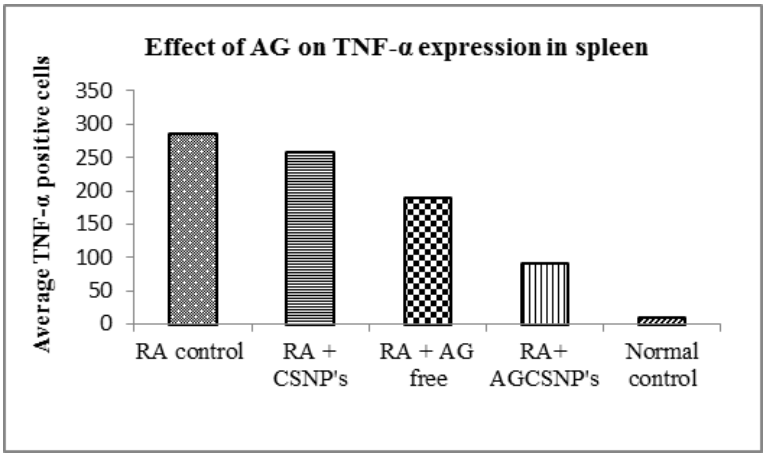
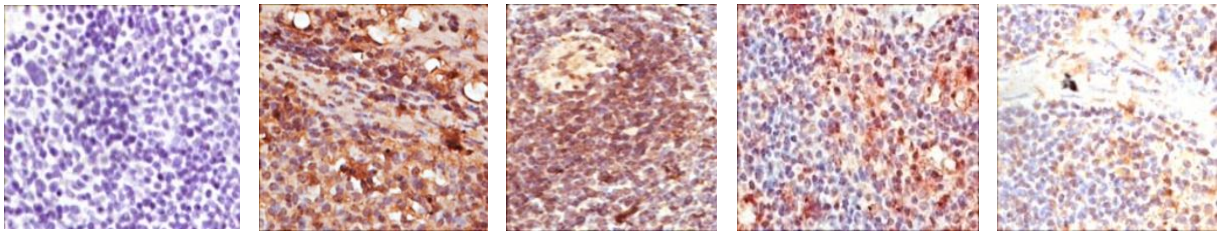


Fig.6: Expression of TNF-α^{+}cells in spleen sections of RA mice.



(A) (B) (C) (D) (E)

Fig. 7(A-E): Immunohistochemical staining for localization of TNF-α immunoreactive cells in spleen of RA mice group*(A) TNF-α positive cells in spleen of normal control mice (B) TNF-α positive cells infilteration in RA control (C) Infilteration of TNF-α immunoreactive cells in RA+ CSNP's group (D) TNF-α immune reactive cell infiltration in RA+ AG Free group (E) TNF-α immunoreactive cells in RA+AGCSNP's group.*

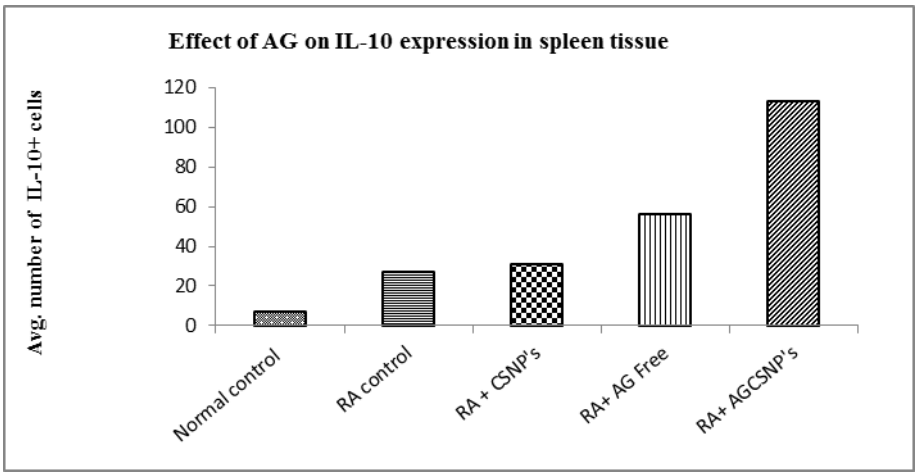
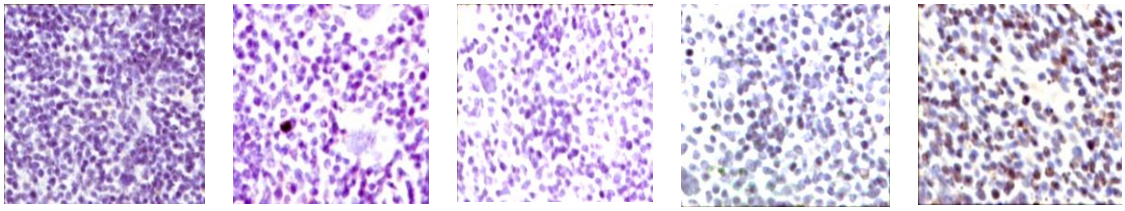


Fig. 8: Effect of AG on IL-10 expression in spleen tissue.



(A) (B) (C) **(D)** **(E)**

Fig. 9(A-E): Immunohistochemical staining for localization of IL-10 $^{+}$ cells in spleen of rheumatoid arthritic (RA) mice. *(A) IL-10 $^{+}$ cells in spleen of normal control mice (B) IL-10 $^{+}$ cells in RA control (C) IL-10 $^{+}$ cells in RA+ CSNP's group (D) IL-10 $^{+}$ in RA+ AG Free group (E) IL-10 $^{+}$ cells in RA+AGCSNP's group.*

CONCLUSIONS

Chitosan nanoparticle can protect physiologically labile drugs such as AG in the GI tract of the body. Hence, the limited therapeutic activity of AG can be enhanced to manage RA by modifying the expression of TNF-α, a chief pro inflammatory cytokine related to disease development and progression. Chitosan encapsulated AG can also upregulate the expression of IL-10 receptors to better combat the disease. This modification of the expression of interleukins may serve as a therapeutic option in the management of RA and should be explored in future studies.

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