6. ACTIVITY OF ENCAPSULATED ATRA AGAINST INFLAMMATION AND ITS IMMUNOMODULATORY EFFECT

6.1 Introduction

Inflammation is a physiological process in response to tissue damage resulting from microbial pathogen infection, chemical irritation and wound (Philip et al., 2004). This leads to the activation of a complex array of inflammatory mediators such as pro- and anti-inflammatory mediators or cytokines and restoring tissue structure and function. Chronic inflammation is a long lasting type changes that may persist for weeks, months or even years and brought on by acute inflammation and may leads to the cancer by DNA mutation as well as cell membrane damage. It is a pathological condition characterized by concurrent active inflammation, tissue destruction and attempts at repair. During these processes, acute inflammatory cells are replaced by a chronic type such as macrophages under appropriate activation, infiltrating into the inflamed tissue and existing macrophages exit the tissue via lymphatic system (Copstead and Banaski, 2000).

Inflammation has long been associated with the development of cancer but cancer also can cause inflammation. Pre-malignant tumors are “wound-like” (Coussens et al., 1999). Such tumors are similar to healing or desmoplastic tissue in many ways, such as the presence of activated platelets (Mueller and Fusenig, 2004). As described by Coussens and Hanahan, tumor growth may be “biphasic” (Coussens et al., 1999). In the first phase, the body treats early tumors as wounds. This phase is characterized by tumor growth mediated by the actions of the stroma “indirect control” as occurs in physiologic tissue repair. For example, in murine models of skin and pancreatic carcinogenesis, bone-marrow derived cells, including mast cells, are responsible for
providing matrix metalloproteases which convert VEGF into a biological active form to stimulate the pro-tumorigenic angiogenic switch (Coussens et al., 1999; Coussens et al., 2000; Bergers et al., 2000). However, during later tumor growth, it appears pro-inflammatory factors, such as MMPs, come under direct control by the tumors themselves (Coussens et al., 1999). A similar transition in the regulation of inflammation by early vs. late tumors may be at hand in spontaneous intestinal tumorigenesis in both mice and humans. COX-2 is expressed by stromal cells in early tumors (Sonoshita et al., 2002), perhaps as part of a response to early tumor associated wounding; in larger tumors, COX-2 is expressed by the dysplastic epithelium itself (Sheehan et al., 1999). One intriguing hypothesis as to why this phenomenon occurs is that there are intact regulatory mechanisms present in tumor associated stromal cells, limiting their expression of tissue repair factors. This may lead to the selective emergence of tumor cells that autonomously can maintain these ancillary processes and are not dependent on the “wound-like stroma.” Eventually, however, the tumor associated stroma may undergo selective pressure, as there have been recent reports of genetic changes in tumor associated stroma (Moinfar et al., 2000) and even loss of p53 in tumor-associated fibroblasts (Hill et al., 2005). In addition to playing a critical role in tumor growth, such as by mediating angiogenesis, the inflammatory response may have a role in other aspects of progression, such as tissue invasion and metastasis (Seth, 2006).

ATRA play a major role in a wide range of physiological pathways such as cell proliferation, embryogenesis, differentiation, morphogenesis, and inflammation (Mark et al., 2004). ATRA exert its function on inflammatory factors through binding to the retinoic acid receptor (RAR), and each receptor is divided into three subtypes, which are referred as RAR-α, -β, or -γ, which are encoded by separate genes (Lefebvre, 2001). After binding of retinoids, RAR form a homodimer or a heterodimer and activate the cellular machinery for an increased or decreased transcription rate. But RAR can
alternatively induce gene trans-repression by sequestering transcription factors such as activator protein-1 (AP-1) or nuclear factor-interleukin-6 (NF-IL-6) without binding to DNA (Lefebvre, 2001). Based on the regulatory role of these transcription factors in the control of many inflammatory mediators, RAR-ligand complexes can repress a broad spectrum of genes, including inflammatory proteins, cytokines, or matrix metalloproteases (MMPs) (DiSepio et al., 1997). ATRA also has anti-inflammatory effects and inhibits the expression of proinflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and IL-6, in various cell types (Montrone et al., 2009). Retinoic acid has been described as a potent modulator of inflammatory diseases (Perez de Lema et al., 2004; Adams et al., 2005). ATRA treatment significantly reduces the cellular infiltrates in the renal interstitium in in vivo models (Moreno-Manzano et al., 2003; Suzuki et al., 2003; Perez de Lema et al., 2004). Retinoid-mediated inhibition of IL-12 production in macrophages showed the biological effects of retinoids and suggest that it can be useful in treatment of Th1-mediated immunological disorders such as rheumatoid arthritis, and inflammatory bowel diseases (Kang et al., 2000). The aim of this chapter is to study the anti-inflammatory activity of ATRA as well as encapsulated forms in an induced inflammatory condition in vivo.

ATRA is a cancer chemo preventive agent and a pluripotent morphogen. It besides being inducers of differentiation and growth inhibitors its principal effects, exert immunomodulatory and anti inflammatory functions (Brinckerhoff et al., 1983; Orfanos and Bauer, 1983; Judy and Raghubir, 2000). One of the advantages of vitamin A is that it can enhance immunity for health. The relation between Vitamin A deficiency and infectious diseases have been known for a long period. The immunologic effects of vitamin A appear to be primarily mediated through its major metabolites, ATRA and 9-cis-retinoic acid, which are important ligands to retinoid receptors (Napoli, 1996).
The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of many diseases. Immunology is thus probably one of the most rapidly developing areas of biomedical research and has great promises with regard to prevention and treatment of wide range of disorders, inflammatory diseases of skin, gut, respiratory tract, joints and central organs (Vikas et al., 2010). Immune dysfunction may result in infectious diseases and cancer. Synthetic drugs are used as immunosuppressive and immunostimulating agents, but there are major limitations and increased risk to the general use of these agents (Anita et al., 2011). Immunomodulation is required when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders (Papiya et al., 2012). Host immunosuppression by the carcinogenic agent is one of the hallmarks of cancer establishment as noted in several cancer cases (Seth, 2006; Smyth et al., 2006).

Immunomodulation is a process of an immune response and it can do any change in the immune response and may involve induction, expression, amplification of any part or phase in the immune response. Modulation may be very specific and limited to a given antigen/agent or non-specific, with a great effect on immune response. Stimulation of the immune response is preferred for certain people such as immunocompromised patient, whereas, suppression of immune response is considered for others such as transplant recipient or inflammatory diseases (Maushmi et al., 2012). Vitamin A has a significant role in maintenance of mucosal surfaces, generation of antibody responses, haematopoiesis and function of T and B lymphocytes (Semba, 1999). Feeding a vitamin A-deficient diet to chicks has shown decreased thymus weight (Davis and Sell, 1983). Vitamin A deficiency has been associated with immunosuppression and reduced resistance to infection (Bowman et al., 1990; Friedman et al., 1991; Akbari et al., 2008). ATRA can stimulate the immunomodulation activity through RAR receptors (Gullu and Francine, 2002). A
number of *in vitro* and *in vivo* test systems are available for screening immunomodulatory activity. Delayed type Hypersensitivity (DTH) reactions are manifestations of T cell effectors arm of the immune system and are used for studying preliminary inflammatory processes (Aditya *et al.*, 2009). The other aim of this chapter is thus to study the immunomodulatory efficiency of encapsulated ATRA as it is associated with cancer and inflammation reactions.

The present study has thus designed to check the anti-inflammatory activity of encapsulated ATRA over free ATRA treatment in acute and chronic inflammation mice model induced by carrageenan and formaldehyde respectively. The present study also attempts to study the immunomodulatory potencies of ATRA by analyzing the bone marrow cellularity, α-esterage activity and DTH. Immunomodulation using encapsulated ATRA can provide an alternative approach to conventional chemotherapy for a variety of diseases including cancer, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immune-suppression is desired.

### 6.2 Rationale

Research reports stated that ATRA is having the activity against inflammation. Retinoids are also required for the maintenance of immune system, as they are important immunomodulators. ATRA is used to treat a number of haematological malignancies as it causes haematopoietic cells to differentiate and prolong the lifespan of cells. More over inflammation and immunomodulation are certain symptoms of cancer development as well as later stage of cancer growth. These associations and potency of ATRA on inflammation and immunomodulation made us to study the effects of encapsulated ATRA on these processes.
6.3 Objectives

To evaluate the encapsulated ATRA activity against an induced inflammation. Also to study the immunomodulatory effect of encapsulated ATRA in SRBC challenged mice.

6.4 Hypothesis to be analyzed

ATRA exert its function through binding to the retinoic acid receptor (RAR) and can alternatively induce gene by activating the transcription factors such as activator protein-1 (AP-1) or nuclear factor-interleukin-6 (NF-IL-6). Based on the regulatory role of these transcription factors in the control of many inflammatory mediators, RAR-ligand complexes can repress a broad spectrum of genes, including inflammatory proteins, cytokines, or matrix metalloproteases (MMPs). The immune system is a complex system consisting of a network of interacting cells, tissues, and organs. ATRA can stimulate the immunomodulation activity through RAR and RXR receptors. All these may contribute to the anticancer activity exerted by ATRA.
6.5 Materials and Methods

6.5.1 Animals

C57BL/6 male mice and BALB/c male mice (4-6 weeks old) were purchased from National Institute of Nutrition (Hyderabad, Andhra Pradesh, India). C57BL/6 male mice were used for inflammation study and BALB/c male mice were used for immunomodulation study. The animals were kept in well-ventilated cages in a facility maintained at 22 (± 1) °C, with a 40 (±10) % relative humidity, and a 12-hr light-dark during the experimental period. Mice were provided access to normal mouse chow (Sai Feeds, Bangalore, India) and filtered water *ad libitum*. All the animal experiments were carried out according to the rules and regulation of the Institutional Animal Ethics Committee.

6.5.2 Chemicals

ATRA was purchased from Sigma Chem. Co. (St Louis, MO). All other chemicals used were of analytical grade.

6.5.3 Study of Anti-inflammatory Activity of Encapsulated ATRA

**Experimental Groups for Carrageenan Model**

- Group I: Carrageenan alone injected
- Group II: Carrageenan + Olive oil
- Group III: Carrageenan + Free liposome
- Group IV: Carrageenan + Standard drug (Diclofenac)
- Group V: Carrageenan + Free ATRA
- Group VI: Carrageenan + Encapsulated ATRA
6.5.3.1 Study in Carrageenan Induced Acute Inflammation

Acute inflammation was studied utilizing carrageenan-induced mice paw oedema model as previously described (Winter et al., 1962). C57BL/6 mice were divided into six groups and each group contains six mice. Group II to group VI were received 0.1ml of olive oil, free liposome, standard drug Diclofenac (1mg/kg body weight), free ATRA (0.60 mg/kg body weight) and encapsulated ATRA (0.60 mg ATRA encapsulated in liposome/kg body weight) respectively and group I was received 0.1ml saline through IP for 10 doses. After 60 minutes of last dose given to the all mice from group I to group VI 0.1ml of 1% carrageenan was injected subcutaneously into the planter region of the right hind paw to induce inflammatory oedema. The paw size was measured initially (before carragenan injection) and then after carrageenan injection at 1hr intervals for 5hrs and on 24th hr using Vernier Caliper. The relative potency of the ATRA under investigations was calculated based upon the percentage inhibition of the inflammation at end of the experiment (24th hr). Paw tissue was dissected out on 24th hr and kept in formaldehyde (10%) for histopathology analysis. The percentage of inhibition of paw thickness was calculated using the formula:

\[
\text{Percentage of inhibition (\%)} = \left( \frac{(C_n - C_0) - (T_n - T_0)}{C_n - C_0} \right) \times 100
\]

Where,

- \(C_n\) = Paw thickness at particular time point of control mice.
- \(C_0\) = Paw thickness at before induction.
- \(T_n\) = Paw thickness at particular time point of treated mice.
- \(T_0\) = Paw thickness at before induction.
6.5.3.2 Study in Formaldehyde Induced Chronic Inflammation

Experimental Groups for Formaldehyde Model

Group I: Formaldehyde alone injected
Group II: Formaldehyde + Olive oil
Group III: Formaldehyde + Free liposome
Group IV: Formaldehyde + Standard drug (Diclofenac)
Group V: Formaldehyde + Free ATRA
Group VI: Formaldehyde + Encapsulated ATRA

Formaldehyde induced sub chronic inflammatory state was carried out as previously described (Jain, 1981). C57BL/6 mice were divided into six groups and each group contains six mice. Group II to group VI were received 0.1ml of olive oil, free liposome, standard drug Diclofenac (1mg/kg body weight), free ATRA (0.60 mg/kg body weight) and encapsulated ATRA (0.60 mg ATRA encapsulated in liposome/kg body weight) respectively and group I was received 0.1ml saline through IP for 10 days. After 60 minutes of last dose given to the all mice from group I to group VI 0.1ml of 1% formaldehyde was injected subcutaneously into the planter region of the right hind paw to induce inflammatory oedema. The paw size was measured initially (before formaldehyde injection) and then after formaldehyde injection at 24 hr intervals for 5 days using Vernier Caliper. The relative potency of the drugs under investigations was calculated based upon the percentage inhibition of the inflammation at the end of the experiment (5th day). Paw tissue was dissected on 5th day and kept in formaldehyde (10%) for histopathology analysis. The percentage of inhibition of paw thickness was calculated using the formula:

\[
\text{Percentage of inhibition (\%) of paw thickness} = \frac{(C_n - C_0) - (T_n - T_0)}{C_n - C_0} \times 100
\]
Where,
\[ C_n = \text{Paw thickness at particular time point of control mice.} \]
\[ C_0 = \text{Paw thickness at before induction.} \]
\[ T_n = \text{Paw thickness at particular time point of treated mice.} \]
\[ T_0 = \text{Paw thickness at before induction.} \]

6.5.3.3 Histopathology Analysis of Paw Tissues for Inflammatory Changes

A portion of paw tissue from above mentioned carrageenan model on 24\textsuperscript{th} hr and formaldehyde model on 5\textsuperscript{th} day were blocked in paraffin after processing. Sections (4-5 \( \mu \text{m} \) thickness) were taken and subjected to histopathological analysis using Hematoxylin and Eosin (H and E) stain as per the standard protocol (Booran et al., 1990). Histology changes were observed under light microscope; all results were verified and certified by a chief histopathologist.

6.5.4 Immunomodulation Study of Encapsulated ATRA

6.5.4.1 \textit{In vivo} ATRA Toxicity Study in BALB/c Mice

A group of six male BALB/c mice was given graded doses of ATRA (i.e., 0.5-1.0 mg/kg body weight [BW]) via intraperitoneal (IP) injection and continuously monitored for 5 hr to note any changes in various autonomic and behavioral responses. Thereafter, the mice were examined daily for 30 days to allow for extended observations of any ATRA-induced mortality.

6.5.4.2 Preparation of Sheep Red Blood Cells (SRBC) and Host Sensitization

Healthy sheep blood was collected from a local butcher house. The material was mixed with sterile Alsever’s solution (1:1) and then centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the SRBC pellets washed with sterilized PBS (pH
7.2) three times. The SRBC were re-suspended in PBS and adjusted the desired cell concentration \((1 \times 10^8 \text{ cells})\).

6.5.4.3 Study Design for Immunomodulation

ATRA was suspended in olive oil to the desired concentration \((0.60 \text{ mg/kg body weight [BW]} \cdot \text{day})\) and liposome encapsulated ATRA was suspended in PBS to the desired concentration \((0.60 \text{ mg/kg body weight [BW]} \cdot \text{day})\). The drugs and vehicles were administered via IP injection starting from 0th day to the end of experiment day (14th day). Mice were primed on 7th day and challenged on 14th day with SRBC \((1 \times 10^8 \text{ cells})\). Following total leukocyte count, body weight and relative immune organ weight, bone marrowcellularity and \(\alpha\)-esterage activity were analyzed by using following experimental groups.

**Experimental Groups for Immunomodulatory Study**

The male BALB/c mice were randomly divided into the following groups \((n = 6 \text{ per group})\):

- **Group I**: Normal mice received normal saline \(0.1 \text{ ml}\)
- **Group II**: SRBC alone injected \((1 \times 10^8 \text{ cells, IP})\)
- **Group III**: SRBC injected + olive oil
- **Group IV**: SRBC injected + free liposome
- **Group V**: SRBC injected + free ATRA
- **Group VI**: SRBC injected + encapsulated ATRA

6.5.4.4 Determination of the Effect of ATRA on Total WBC Count

Blood was collected from the tail vein and total WBC count (Benjamin 1985) was recorded on the 14th day. The total WBC count was carried out based on the method described by Benjamin, 1985. Briefly, 0.38 ml of diluting fluid was mixed with 0.02 ml of blood and loaded in to the hemocytometer counting chamber and the cells
were allowed to settle at the bottom of the chamber for 2 minutes. Four large squares were counted under a microscope with 10 x objective.

6.5.4.5 Determination of the Relative Organ Weight

Body weight was taken at the end of experiment to calculate relative organ weight. Six mice from each group were sacrificed at the end of the experiment (on 14\textsuperscript{th} day) by cervical dislocation and lymphoid organs such as thymus and spleen were excised out. The organs of each mouse were weighed and were expressed as relative organ weight by using the formula:

Relative organ weight = organ weight / body weight x 100

6.5.4.6 Determination of the Bone Marrow Cellularity (BMC)

Bone marrow cellularity was determined according to the method of Sredni et al. 1992. Bone marrow was collected at the end of the experiment from the femur into the medium containing 2% serum to make single cell suspension. The number of cells was determined using a haemocytometer and is expressed as total cells determined by tryphan blue (1% in saline) exclusion method per femur.

6.5.4.7 Determination of α-esterase Activity

Alpha-esterase (α-esterase) activity was measured by using the azodye coupling method (Bancroft and Cook, 1984). Bone marrow from both femurs of mice was collected in PBS at the end of the experiment, washed thrice in PBS and smeared over the slides. Air dried slides were fixed in freshly prepared fixative (25 ml of 37% formaldehyde, 45 ml of Acetone, 20 mg of Na\textsubscript{2}HPO\textsubscript{4}, 100 mg of KH\textsubscript{2}PO\textsubscript{4} and 30 ml of distilled water and pH 6.6) for 30 seconds at 4°C and is dipped in double distilled water thrice. Air dried slides were incubated at room temperature with the following freshly prepared filtered solutions: 1.2mL solution A (rosanilic acid 1gm in 20 ml distilled
water and add 5 ml of HCl) and 1.2mL solution B (4% NaNO₃) was mixed well and allowed to react for one minute after which solution C (50 mg of 1-Naphthylacetate in 2-5ml of ethylene glycol) was added and made up to 50mL solution by phosphate buffer (pH 7.4).

Slides were incubated for 45 minutes at 37°C. After incubation slides were washed in double distilled water for 10 minutes and counter stained with Haematoxylin for one minute. After staining, slides were washed in tap water for long time and were observed under microscope (100X, oil immersion) for scoring α-esterase positive and negative cells out of 4000 cells.

6.5.5 Determination of Effect of ATRA on Delayed Type Hypersensitivity (DTH)

Experimental Design for DTH

The male BALB/c mice were randomly divided into the following groups (n = 6 per group)
Group I: SRBC alone injected (1 × 10⁸ cells, IP).
Group II: SRBC injected + olive oil
Group III: SRBC injected + free liposome
Group IV: SRBC injected + free ATRA
Group V: SRBC injected + encapsulated ATRA

Five groups (6 mice/group) of BALB/c mice were used in all the experiments. Group IV and V mice were treated intraperitoneally with free ATRA and encapsulated ATRA at concentration of 0.60 mg/kg body wt/dose/mice respectively from 0th day to the end of the experiment (14th day). Group II and III were treated with vehicles (olive oil and free liposome respectively) and group I was kept as SRBC control.
The method described by Doherty, 1981 and Puri et al. (Doherty, 1981; Puri et al., 1994) was used for the delayed type hypersensitivity (DTH) study. All the treatments were administered on day 0 and continued till the day of challenge (14th day). The mice were primed with 0.1 ml of SRBC suspension containing \(1 \times 10^8\) cells through i.p., on day 7 from the drug treatment and then challenged on the day 14 with SRBC (\(1 \times 10^8\) cells in 50 μl) into their left hind paw after 14 days of drug treatment. The extent of host DTH reaction was then assessed by using Vernier Calipers [Yong-kang Chite import/Export Co., Zhejiang, China] to measure the thickness of each footpad 24 hr after the challenge. From these values, the degree of DTH response, and consequently, the percentage inhibition of DTH associated with the host treatments, was determined by using following formula:

\[
\text{Percentage inhibition of DTH} = \frac{(C_f - C_i) - (T_f - T_i)}{(C_f - C_i)} \times 100
\]

Where, 
- \(C_f\) = Final paw volume of SRBC control mice
- \(C_i\) = Initial paw volume of SRBC control mice
- \(T_f\) = Final paw volume of treated mice
- \(T_i\) = Initial paw volume of treated mice

6.5.6 Statistical analysis

All data were expressed as mean (± SD). The statistical analysis was done using one-way analysis of variance (ANOVA) followed by a Dunnett’s test (using Graphpad InStat version 3.00 for Windows XP; GraphPad Software, Inc., La Jolla, CA). P-value \(\leq 0.05\) was considered significant.
6.6 Results

6.6.1 Effect of Encapsulated ATRA on Carrageenan Induced Paw Thickness and Histology Changes

The intraplantar injection of 1% carrageenan into the mice right hind paw produced an intense oedema. The paw oedema induced by carrageenan in mice was significantly (p ≤ 0.01) inhibited by the free ATRA (Gr-V) and more efficiently by encapsulated ATRA (Gr-VI) when compared with carrageenan alone induced control mice (Gr-I). This result was similar to the inhibition by the standard drug Diclofenac (1mg/kg, p.o; Gr-IV) (Figure 6.1). As shown in Table 6.1, the free ATRA and encapsulated ATRA have reduced the paw oedema by 55.56±0.10 % and 66.67±0.40 % respectively as compared with carrageenan control group and is closer with 77.78±0.80 percentage (%) reduction produced by Diclofenac.

The carrageenan induced mice paw histology was characterized by epithelial and conjunctive tissue blisters and infiltrates of inflammatory PMN cells, mainly neutrophils (Figure 6.2). In the pretreatment with free ATRA and encapsulated ATRA (0.60 mg/kg, i.p.) significant decreases in paw oedema were observed in which the encapsulated ATRA treatment showed better reduction in paw oedema when compared with free ATRA treatment. These free ATRA and encapsulated ATRA pretreatment have also shown decreases in infiltration of inflammatory cells in the paw tissue when compared with carrageenan control mice (Figure 6.2). However the standard drug Diclofenac (1mg/kg, i.p.) showed highest reduction in paw edema and cell infiltration in paw tissue as compared with free ATRA and encapsulated ATRA treatments (Figure 6.2).
6.6.2 Effect of Encapsulated ATRA on Formaldehyde Induced Paw Oedema

The intraplantar injection of 1% formaldehyde into the mice right hind paw produced an intense oedema. The paw oedema produced by formaldehyde was significantly ($p \leq 0.01$) reduced by the treatments by free ATRA, encapsulated ATRA and by Diclofenac (1mg/kg, p.o) (Figure 6.3). The free ATRA and encapsulated ATRA reduced the paw oedema by 60.87±0.30 % and 69.57±0.25% respectively and 86.96±0.20% reduction was produced by standard drug Diclofenac (Table 6.2).

The formaldehyde induced mice paw histology was characterized by the epithelial and conjunctive tissue blisters and infiltrates of inflammatory PMN cells (Figure 6.4). The mice paw oedema was significantly decreased in free ATRA pretreatment (0.60 mg/kg, i.p.) whereas it was even better significantly decreased with encapsulated ATRA pretreatment (0.60 mg/kg, i.p.). In compared with formaldehyde control inflammatory cells infiltration, free and encapsulated ATRA pretreatments have shown decreases in inflammatory cells infiltration in paw tissue (Figure 6.4). However the highest reduction in paw eodema and cell infiltration in paw tissue was found in standard drug Diclofenac (1mg/kg, i.p.) pretreatment when compared with free and encapsulated ATRA pretreatments (Figure 6.4).

6.6.3 In-vivo Drug Toxicity in BALB/c Mice

ATRA did not induce any notable changes in autonomic/behavioral responses of the mice. All mice lived healthy, even up to 30 days after a dose of 1.0 mg ATRA/kg.

6.6.4 Effect of ATRA on Total WBC Count

Administration of the free ATRA and encapsulated ATRA decreased the total WBC count which was raised during SRBC challenging in BALB/c mice (Figure 6.5). The WBC counts in SRBC induced mice was found to be $12.63 \times 10^3 \pm 0.15$ cells/mm$^3$
and with treatment with free ATRA and encapsulated ATRA were $11.15 \times 10^3 \pm 0.12$ cells/mm$^3$ and $10.35 \times 10^3 \pm 0.15$ cells/mm$^3$ respectively at the end of the experiment.

6.6.5 Effect of ATRA on Relative Organ Weight for Immunomodulatory Activity

The effect of free ATRA and encapsulated ATRA on body weight and organ weight was measured on 14th day. The relative weight of spleen was decreased in free ATRA-treated mice ($0.68 \pm 0.01$ g/100 g BW) and encapsulated ATRA treated mice ($0.65 \pm 0.01$ g/100 g BW) compared to SRBC induced mice ($0.75 \pm 0.01$ g/100 g BW). The relative weight of the thymus was also significantly decreased in free ATRA treated ($0.45 \pm 0.01$ g/100 g BW) and encapsulated ATRA treated ($0.43 \pm 0.01$ g/100 g BW) mice respectively when compared to SRBC induced mice ($0.54 \pm 0.01$ g/100 g BW) (Figure 6.6).

6.6.6 Effect of ATRA on Bone Marrow Cellularity and α-esterase Positive Cells

The effect of free ATRA and encapsulated ATRA on the bone marrow cellularity and α-esterase positive cells is given in Figure 6.7 and 6.8 respectively. Administration of the free ATRA and encapsulated ATRA showed a significant ($p \leq 0.01$) suppression in the bone marrow cellularity ($14.85\times10^6 \pm 0.20$ cells/femur and $14.10\times10^6 \pm 0.10$ cells/femur respectively) compared to the SRBC induced mice ($17.34\times10^6 \pm 0.19$ cells /femur) (Figure 6.7).

Moreover the number of α-esterase positive cells was also found to be suppressed significantly ($p \leq 0.01$) in the free ATRA and encapsulated ATRA treated mice ($1420 \pm 17.0$ cells/4000 cells and $1310 \pm 15.0$ cells/4000 cells respectively) compared to the SRBC induced mice ($1985 \pm 11.0$ cells/4000 cells) (Figure 6.8).
6.6.7 Effect of ATRA on Delayed Type Hypersensitivity (DTH) Reaction

The effect of ATRA on delayed type hypersensitivity is given in Table 6.3. Free ATRA and encapsulated ATRA were found to inhibit the delayed type hypersensitivity reaction remarkably. The maximum inhibition of DTH reaction was observed in free ATRA treated (60.0±1.10%) and encapsulated ATRA treated (75.0±1.50%) mice.
Figure 6.1: Effect of encapsulated ATRA on carrageenan induced paw oedema

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

Carrageenan alone vs. Free ATRA treatment: **p≤0.01 and ns (non-significant)

Carrageenan alone vs. Encapsulated ATRA treatment: **p≤0.01 and ns (non-significant)

Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01, *p≤0.05 and ns (non-significant)
Table 6.1: Effect of encapsulated ATRA on carrageenan injected mice model on percentage inhibition of inflammation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Differences in paw size (mm) on 24th hr</th>
<th>Percentage (%) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr-I (Carrageenan alone)</td>
<td>0.9±0.02</td>
<td>_</td>
</tr>
<tr>
<td>Gr-II (Carrageenan + Olive oil)</td>
<td>0.9±0.01</td>
<td>00.00</td>
</tr>
<tr>
<td>Gr-III (Carrageenan + Free Liposome)</td>
<td>1.0±0.01</td>
<td>-11.11±0.20</td>
</tr>
<tr>
<td>Gr-IV (Carrageenan + standard drug)</td>
<td>0.2±0.02</td>
<td>77.78±0.80</td>
</tr>
<tr>
<td>Gr-V (Carrageenan + Free ATRA)</td>
<td>0.4±0.04**</td>
<td>55.56±0.10</td>
</tr>
<tr>
<td>Gr-VI (Carrageenan + Encapsulated ATRA)</td>
<td>0.3±0.02**,<strong>,</strong></td>
<td>66.67±0.40**</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

Carrageenan alone vs. Free ATRA treatment: **p≤0.01
Carrageenan alone vs. Encapsulated ATRA treatment: **p≤0.01
Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01
(I) Carrageenan control showing intense inflammation, characterized by epithelial and conjunctive tissue blisters and infiltrates of inflammatory PMN cells. (II & III) Vehicle groups showing same characteristics as group I. (IV) Diclofenac treated (standard drug) shows drastic reduction in conjunctive tissue blisters and less infiltrates of inflammatory PMN cells were found compared with group I. (V) Free ATRA treated paw shows very less conjunctive tissue blisters and less infiltrates of inflammatory PMN cells compared with group I. (VI) Encapsulated ATRA treated paw showing faster restoration of normal histology with very few conjunctive tissue blisters and infiltrates of inflammatory PMN cells compared with group I and V.
Figure 6.3: Effect of encapsulated ATRA on formaldehyde induced paw oedema

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

Formaldehyde alone vs. Free ATRA treatment: **p≤0.01 and ns (non-significant)
Formaldehyde alone vs. Encapsulated ATRA treatment: **p≤0.01 and ns (non-significant)
Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01, *p≤0.05 and ns (non-significant)
<table>
<thead>
<tr>
<th>Groups</th>
<th>Differences in paw size (mm) on 5th day</th>
<th>Percentage (%) inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr-I (Formaldehyde alone)</td>
<td>2.3±0.02</td>
<td>—</td>
</tr>
<tr>
<td>Gr-II (Formaldehyde + Olive oil)</td>
<td>2.2±0.05</td>
<td>04.35±0.10</td>
</tr>
<tr>
<td>Gr-III (Formaldehyde + Free Liposome)</td>
<td>2.2±0.04</td>
<td>04.35±0.10</td>
</tr>
<tr>
<td>Gr-IV (Formaldehyde + standard drug)</td>
<td>0.3±0.01</td>
<td>86.96±0.20</td>
</tr>
<tr>
<td>Gr-V (Formaldehyde + Free ATRA)</td>
<td>0.9±0.05**</td>
<td>60.87±0.30</td>
</tr>
<tr>
<td>Gr-VI (Formaldehyde + Encapsulated ATRA)</td>
<td>0.7±0.03**,**</td>
<td>69.57±0.25**</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

Formaldehyde alone vs. Free ATRA treatment: **p≤0.01

Formaldehyde alone vs. Encapsulated ATRA treatment: **p≤0.01

Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01
Figure 6.4: Histopathology of effect of encapsulated ATRA on formaldehyde induced paw tissue on 5th day

(I) Formaldehyde control showing intense inflammation, characterized by epithelial and conjunctive tissue blisters and infiltrates of inflammatory PMN cells. (II & III) Vehicle groups showing same characteristics as group I. (IV) Diclofenac treated (standard drug) shows drastic reduction in conjunctive tissue blisters and less infiltrates of inflammatory PMN cells were found compared with group I. (V) Free ATRA treated paw shows very less conjunctive tissue blisters and less infiltrates of inflammatory PMN cells compared with group I. (VI) Encapsulated ATRA treated paw showing faster restoration of normal histology with very few conjunctive tissue blisters and infiltrates of inflammatory PMN cells compared with group I and V.
Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

SRBC Sensitized alone vs. Free ATRA treatment: **p≤0.01
SRBC Sensitized alone vs. Encapsulated ATRA treatment: **p≤0.01
Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01
Figure 6.6: Effect of ATRA on organ weight and relative organ weight

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

SRBC Sensitized alone vs. Free ATRA treatment: ** *p*≤0.01 and ns (non-significant)

SRBC Sensitized alone vs. Encapsulated ATRA treatment: ** *p*≤0.01 and * *p*≤0.05

Free ATRA treatment vs. Encapsulated ATRA treatment: ns (non-significant)
Figure 6.7: Effect of ATRA on bone marrow cellularity

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

SRBC Sensitized alone vs. Free ATRA treatment: **p≤0.01
SRBC Sensitized alone vs. Encapsulated ATRA treatment: **p≤0.01
Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01
Figure 6.8: Effect of ATRA on α-esterase positive cells

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

SRBC Sensitized alone vs. Free ATRA treatment: **p≤0.01
SRBC Sensitized alone vs. Encapsulated ATRA treatment: **p≤0.01
Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01
Table 6.3: Effect of ATRA on delayed type hypersensitivity reaction (DTH)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial paw thickness (cm)</th>
<th>Paw thickness after 24 hours (cm)</th>
<th>Percentage (%) of inhibition compared to SRBC Antigen challenged alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr-I: SRBC sensitized alone</td>
<td>0.23 ± 0.02</td>
<td>0.43 ± 0.03</td>
<td>-</td>
</tr>
<tr>
<td>Gr-II: SRBC + Olive oil</td>
<td>0.23 ± 0.03</td>
<td>0.43 ± 0.02</td>
<td>00.00</td>
</tr>
<tr>
<td>Gr-III: SRBC + free liposome</td>
<td>0.22 ± 0.04</td>
<td>0.44 ± 0.01</td>
<td>-10.00±0.05</td>
</tr>
<tr>
<td>Gr-IV: SRBC + Free ATRA treatment</td>
<td>0.22 ± 0.02</td>
<td>0.30 ± 0.01 **</td>
<td>60.00 ± 1.10</td>
</tr>
<tr>
<td>Gr-V: SRBC + Encapsulated ATRA treatment</td>
<td>0.22 ± 0.02</td>
<td>0.27 ± 0.01 ** **</td>
<td>75.00 ± 1.50 **</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SD (n = 6). The significant difference was determined between:

SRBC Sensitized alone vs. Free ATRA treatment: **p≤0.01

SRBC Sensitized alone vs. Encapsulated ATRA treatment: **p≤0.01

Free ATRA treatment vs. Encapsulated ATRA treatment: **p≤0.01
6.7 Discussion

Generally, inflammatory processes mainly involve three phases associated with acute inflammation, immune response and chronic inflammation. The acute inflammation is the initial response to tissue injury and is related to the release of autacoids, as histamine, serotonin, bradykinin, prostaglandins and leukotrienes. Acute inflammation presents a relatively short duration, and its main characteristics are the exudation of fluid and plasma proteins and the migration of leukocyte, predominantly neutrophils, to the site of injury. This acute process may be followed by an immune response characterized by the activation of immune cells and by chronic inflammation (Liz et al., 2011).

Carrageenan induced acute inflammation is commonly used in animal models and is believed to be a biphasic event (Vinegar et al., 1969). The acute inflammation condition induced by carrageenan involves step-wise release of vasoactive substances such as histamine, bradykinin and serotonin in the early phase and prostaglandins in the acute late phase (Dirosa et al., 1971; Heller et al., 1998). The early phase (1-2 h) is attributed to the release of histamine and serotonin followed by a later phase of oedema due to production of bradykinin and prostaglandins. The second phase has been reported to be sensitive to both steroidal and non-steroidal anti-inflammatory agents (Vinegar et al., 1969). These chemical substances produce increase in vascular permeability, thereby promoting accumulation of fluid in tissues that accounts for the oedema (Williams and Morley, 1973; White, 1999; Umukoro and Ashorobi, 2006).

In this study, the ATRA did not show a significant anti-inflammatory effect in the early phase but showed significant effect at the later phases after 3-4 h. The results suggest that the ATRA acts at the later phase involving arachidonic acid metabolites possibly by the inhibition of cyclooxygenases (Ayoola et al., 2009). The subcutaneous injection of carrageenan into the C57BL/6 mice paw produces inflammation, resulting
from plasma extravasation, increased tissue water and plasma protein exudation, along with neutrophil extravasation, all due to the metabolism of the arachidonic acid. While the first phase begins immediately after the injection of carrageenan, and diminishes in two hours, the second one begins at the end of the first phase, and remains up to 5 h. It was found that encapsulated ATRA showed the better inhibitory result in compared with free ATRA treatment in carrageenan induced inflammation.

In chronic inflammatory model, inhibition of formalin-induced pedal edema in rats is considered as one of the most suitable studies to screen chronic anti-inflammatory agents as it closely resembles human arthritis (Greenwald, 1991). Although the inflammatory process is a localized protective response vital for the survival of all complex organisms, the sustained production of inflammatory mediators can lead to severe pathological conditions (Carrillo et al., 2005; Naza et al., 2010). Formalin induction causes the changes in connective tissue metabolism, is one of the major biochemical events during the process of inflammation. These changes are effected in the alteration of relative composition of various constituents of connective tissue such as muco polysaccharides, glyco protein, hexosamine, hydroxy proline and sialic acid (Houck and Jacob 1969). Similarly, formaldehyde, a potent inflammatory agent, produced inflammation through the release of several inflammatory mediators including prostaglandins (Tjolsen et al., 1992; Umukoro and Ashorobi, 2006). Our study found that the encapsulated ATRA has the better inhibition of paw inflammation in compared with free ATRA treatment.

According to research study, administration of ATRA does not show any abnormal behavior and the animals did not show any behaviour compatible with pain or discomfort, suggesting that ATRA on its own does not induce any proalgesic action or pain (Alique et al., 2006). Research report showed that one possible mechanism for the anti-inflammatory effect of ATRA is its ability to regulate Toll-like receptor-2
(TLR2) expression and activation. Therefore, the ability of ATRA to inhibit TLR expression and activation provides a novel therapeutic approach to manage diseases in which TLR-induced inflammation contributes to tissue injury (Philip et al., 2005). Retinoic acid has been extensively used as an anti-inflammatory agent in many inflammatory diseases including nephropathies, due to its inhibitory effect on the expression of pro-inflammatory mediators (Moreno-Manzano et al., 2003; Vicente-Manzanares et al., 2003, 2005; Perez de Lema et al., 2004; Adams et al., 2005; Escribese et al., 2007).

Histopathology report of carrageenan induced mice paw showed the infiltration of cells and accumulation of fluid in paw tissue which results in thickness of paw. The formaldehyde injection in mice paw also showed the same effects and hypertrophy of the subcutaneous regions which are used as signs of chronic inflammation after a local injection of formalin (Dumka et al., 1996; Kenjo et al., 2002). Infiltration of inflammatory cells also results in a marked increase in the thickness of the skin (including hypodermis) (Kim et al., 2006; Hyeong et al., 2010). However, in the present study, these histopathological changes were markedly inhibited after treatment with ATRA. In this study encapsulated ATRA treatment showed better result in compared with free ATRA treatment for resorting the paw tissue architecture and reducing or killing the infiltration inflammatory cells. These inhibitions were considered as direct evidence that ATRA has a relatively favorable effect on reducing the acute and chronic inflammatory responses.

Nanoparticles can target a specific lesion and are less likely to burst at an early stage of administration, therefore enabling them to gradually release the agent at the site of the lesion over a prolonged period. A targeted intravenous drug delivery approach using the liposome encapsulated ATRA could be a highly attractive alternative to currently available treatment strategies. Research study showed that the
poly (D, L-lactic/glycolic acid)-nanosteroids (PLGA) provide an opportunity to achieve high concentrations of glucocorticoid delivered selectively to all inflamed arthritic joints by a simple intravenous injection and can lead to a rapid, complete, and durable resolution for joint inflammation owing to the enhanced and preferential localisation of betamethasone sodium phosphate (BSP) in the inflamed joints (Higaki et al., 2005). In our study we have found that encapsulated ATRA showed better inhibition for acute and chronic inflammation in C57BL/6 mice model because the liposome encapsulation formula helped to achieve more ATRA concentration and slow release in the paw tissue in compared with free ATRA treatment. As a result encapsulated ATRA showed better inhibition of inflammation and reduced the paw thickness.

Immunomodulation is a procedure which can alter the immune system of an organism and when the immune system is enhanced, it’s called as an immunostimulative drug which primarily implies stimulation of specific and non specific system, i.e. granulocytes, macrophages, complement certain T-lymphocytes and different effectors substances. Immunosuppressant mainly acts opposite to the immunostimulation. The immunomodulant thus involves both immunosuppressive as well as immunostimulating effects depending on the need. The uses of immunomodulators have an important place in the current development of immunotherapy (Raphael and Kuttan, 2000). In this study, effects on blood leukocyte count and relative organ weight especially for spleen and thymus were determined to investigate immunomodulatory effect of ATRA. Among different organs of immune system, spleen represents a major secondary lymphoid organ involved in elicitation of immune response. Unlike lymph nodes, which are specialized to trap-localized antigen from regional tissue space, the spleen is adapted to filter blood and trapping blood-borne antigens and thus can respond to systemic infections (Anamika et al., 2010; Papiya et al., 2012). Results obtained from the present study revealed a significant decrease in the blood leukocytes count (P ≤ 0.01) and weight of spleen and thymus (P ≤
0.01) in the free ATRA and encapsulated ATRA treatment groups compared to SRBC alone induced control group, suggesting the suppression action of treatment on the abnormally enhanced immune cell production during SRBC challenge by the spleen and thymus. In compared with free ATRA treatment encapsulated ATRA showed better suppressive activity in total WBC count and relative organ weight.

The hemopoietic stem cells of bone marrow are the source of major cell types involved in the immune system. For the antigen independent differentiation of B-cells requires microenvironment which generally provide by bone marrow. The present study shows significant decreased in the total WBC count and bone marrow cellularity. Stem cells possess nonspecific esterase activity but the differentiated monocytes have α-specific esterase activity. The decrease in the number of bone marrow cells and less differentiating stem cells with less α-esterase activity in ATRA treated mice also shows the effect of ATRA on suppressing the immunological response. The increased relative organ weights of lymphoid organs like thymus and spleen in SRBC induced mice compared to normal mice indicated the activated immune response by lymphocytes and ATRA treatment showed the suppressing in level of WBC, bone marrow cellularity and α-esterase positive cells. Study also showed that administration of the ATRA was found to decrease the total WBC count, indicating that the ATRA suppresses the bone marrow activity. In compared with free ATRA treatment encapsulated ATRA showed better suppressive activity in bone marrow and α-esterase activity.

DTH is divided into two phases, an initial sensitization phase after the primary contact with SRBC antigen (Maushmi et al., 2012). Cell-mediated immunity (CMI) involves effectors mechanisms carried out by T lymphocytes and their products (lymphokines). DTH requires the specific recognition of a given antigen by activated T lymphocytes which subsequently proliferate and release cytokines. These in turn increase the vascular permeability, induce vasodilatation, macrophage accumulation,
and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated TH1 cells encounter certain antigens, viz. SRBCs and they secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity (Dashputre et al., 2010; Jain and Mishra, 2011). A number of studies indicate a role for vitamin A in the regulation of IL-10 secretion. IL-10 produced by Th2-helper T cells inhibits the synthesis of proinflammatory Th1-type cytokines, including IFN-γ and IL-2, in both T and NK cells. This mechanism is important in limiting inflammatory responses (Eduardo and Wafaie, 2005). In compared with free ATRA treatment encapsulated ATRA showed better inhibition in DTH.

ATRA is a cancer chemo preventive agent and it belongs to the class of retinoids family. Besides its differentiation and growth inhibitors activity, it exerts immunomodulatory and anti-inflammatory functions. Retinoids, including ATRA, are known to bind to two distinct nuclear receptors, retinoic acid receptors (RARs) and retinoid x receptors (RXRs) (Jonat et al., 1990) and to form transcription factors. Heterodimers of RAR and RXR or homodimers of RXRs bind to retinoid receptor-responsive elements present in the promoters of the retinoid target genes to regulate their expression (Mangelsdorf et al., 1993; Zhang et al., 1992; Nozaki et al., 2005). Retinoid receptors may modulate gene expression directly by binding to response elements within gene promoters or indirectly by binding to other transcription factors. The promoter gene encoding IL-1β and TNF-α contain sequences for binding several nuclear transcriptional factors including activator protein-1 (AP-1) and nuclear factor-kB (NF-kB) (Siebenlist et al., 1994). These transcriptional factors participate to various extents in inducing genes encoding cytokines. This RAR is known to bind directly to the transcription factor AP-1 to antagonize AP-1-dependent transcription (Shultz et al., 1991; Schule et al., 1991; Nozaki et al., 2005). Retinoic acid receptors can interact with AP-1 and inhibit its activity (Salbert et al., 1993; Schule et al., 1991; Yang et al., 1991;
Judy and Raghbir, 2000) and due to above said mechanisms ATRA can exhibit their multiple actions such as anti-inflammation, immunosuppression and inhibition of cell growth.

Some activities of retinoids on cellular and humoral immunity have been reported for 13-cis-retinoic acid (13-cRA) which can suppress T cell-mediated immunity in rats. The immunosuppressive activity of 13-cRA included suppression of interleukin-2 whose production was inhibited in splenocytes (Luca et al., 1991). Research study stated that retinoid concentration ranging from $10^{-4}$ M to $10^{-5}$ M can suppress some functions of total T-cell receptors (Moriguchi et al., 1985). Previous studies highlighted the fact that vitamin A or its metabolites can regulate immunity. Retinoid treatment can enhance delayed-type hypersensitivity responses in both animal models and humans (Micksche et al., 1977; Colizzi and Malkovsky, 1985; Mohamad et al., 2003). In a study stated that additional supplementation of diet with vitamin A may influence the immune function by altering the proportion of different WBCs in the blood (Akbari et al., 2008). Overall this study showed that encapsulated ATRA treatment gives better suppression in immune system in SRBC induced C57BL/6 mice in compared free ATRA treatment. It may be the due to the liposome encapsulation which helps to maintain more concentration of ATRA during the experimental condition compared with free ATRA treatment. More concentration of ATRA helps alters the immune system and thereby in this study more suppressive activity was observed in our experimental condition. So, ATRA play as an immune suppressive agent when SRBC induced the immune system of the C57BL/6 mice.